The influence of iron and zinc nutrition on the composition of the gut microbiota

A thesis submitted for the degree of Doctor of Philosophy

by

Kang Shen OOI
Division of Microbiology
School of Biological Science
The University of Reading

Supervisors: Prof Simon Andrews, Dr Gemma Walton

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Abstract

Iron and zinc deficiency present global issues affecting populations in both developed and developing countries. Efforts have been made to tackle these problems either through diet intervention, fortification or supplementation. Although these strategies had been beneficial to host nutrition, the impact of the unabsorbed nutrient on the gut microbiota has yet to be fully understood and this was investigated here using various *in vitro* approaches. The impact of iron on the gut microbiota was initially considered using Hungate tube and single vessel batch cultures approaches inoculated with human faecal slurries. Growth was monitored by measuring total bacterial number using Flow-FISH and the microbiota composition was analysed by Next Generation Sequencing (NGS). The total bacterial numbers and composition were similar between the control and iron regime in the Hungate tube system. This was considered to be due to the limited growth exhibited and the unregulated pH of the Hungate tube culture would obscure any influence of iron, although the presence of iron (haem/$FeSO_4$) caused a substantial increase in *Roseburia faecis.* Inclusion of strong buffers in Hungate tubes was explored and 300 mM 2-(N-morpholino)ethanesulfonic acid was found to restrict pH fluctuation within a desirable range (pH range 5.5-6.7) and the bacterial counts were higher in the presence of buffer. Single vessel batch cultures containing gut model medium and 0.1% (w/v) faecal slurry from four different healthy donors were set up to investigate the impact of different forms of iron (haem and $FeSO_4$) on the gut microbiota. The presence of haem caused a slower growth rate but all regimes showed similar total bacterial counts by 48 h. Haem increased the growth of *Bacteroides, Parabacteroides, Clostridium, Lactobacillus* and Rikenellaceae. *Dorea formicigenerans,* Ruminococcaceae and *Veillonella dispar* showed an increase in the presence of $FeSO_4$ but not haem. *Sutterella,* Enterobacteriaceae, *Bifidobacterium*, *Ruminococcus* and *Faecalibacterium prausnitzii* showed a decrease in growth in the presence of haem with $FeSO_4$. The effect of zinc on the gut microbiota was investigated using single vessel batch cultures and three-stage gut models containing 'modified' gut model medium supplemented with Zn at 77 (low), 192 (medium) and 770 μM (high) concentration. In the batch

cultures, the presence of 77 or 192 μM zinc increased total bacterial counts while 770 μM zinc caused a lower growth. The presence of zinc increased the growth of *Streptococcus* and *Sutterella* but reduced the growth of *Odoribacteria,* Rikenellaceae, *Roseburia faecis* and Enterobacteriaceae. In the gut model, *Faecalibacterium prausnitzii* and *Roseburia faecis* showed a significant increase ($p<0.05$) in the presence of 770 μM Zn while *Lachnospira* displayed a significant decrease ($p<0.05$) in both the absence of zinc and presence of 77 μM Zn, indicating it favours a moderate zinc level. The ability of bacteria to degrade phytic acid, a major dietary inhibitor of zinc absorption, and use it as a phosphate and/or carbon source was investigated using an *Escherichia coli* triple mutant whereby all three known phytases were knocked out. The wild-type and mutant were grown in phosphate limited or carbon free M9 minimum medium, supplemented with 2.5, 5 and 10 mM phytic acid. Phytic acid was found to act as a good phosphate source but a poor carbon source for the growth of *E. coli*. However, the mutant showed comparable growth to the wild-type, indicating the phytases previously identified have no essential role in utilisation of phytate, suggesting a yet to be discovered phytate utilisation pathway. The impact of phytic acid on the gut microbiota was also investigated using single vessel batch cultures, containing phosphate-limited or carbon-free basal medium. Similar results were obtained whereby the gut microbiota were able to utilise phytic acid as a phosphate source but not a carbon source.

Contents

List of abbreviations

ABC- ATP-binding cassette

ATP- Adenosine triphosphate

BPP- β-propeller phytases

CM- Cytoplasmic membrane

CD- Crohn's disease

CDF- Cation diffusion facilitators

CPT1b- Carnitine palmitoyltransferase 1B

DCYTB/Dcytb- Duodenal cytochrome B

DHPPP- Dihydropterin pyrophosphate

DMT1- Divalent metal transporter 1

DSS- Dextran sulphate sodium

FAD- Flavin adenine dinucleotide

Fbp- Ferric binding protein

Feo- Ferrous iron transport

Flow-FISH- Fluorescence in situ hybridisation coupled with flow cytometry

FMN- Flavin mononucleotide

FOS- Fructo-oligosaccharides

Fpn1- Ferroportin

G1P- Glucose-1-phosphate

GI- Gastrointestinal tract

GMM- Gut model medium

mGMM- Modified gut model medium

GOS- Galacto-oligosaccharides

GTP- Guanosine triphosphate

HAP- Histidine acid phosphatases

HDAC- Histone deacetylase

HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hp- Hephaestin

ICP-OES- Inductively coupled plasma - optical emission spectrometry

IBD- Inflammatory bowel disease

L-broth/L-agar- Lysogeny broth/agar

Lf- Lactoferrin

LfR- Lactoferrin receptor

MES- 2-(N-morpholino)ethanesulfonic acid

MFRN- Mitoferrin 1

NGS- Next generation sequencing

OM- Outer membrane

PA- phytic acid

PABA- *p*-aminobenzoate

PAP- Purple acid phosphatases

PBP- Periplasmic binding proteins

PCR- Polymerase chain reaction

qPCR- Quantitative polymerase chain reaction

PCG-1α- Peroxisome proliferator–activated receptor gamma coactivator-1 alpha

pmf- Proton motive force

PpIX- Protoporphyrin IX

PPAR-γ- Peroxisome proliferator- activated receptor gamma

PTP- Protein tyrosine phosphatase

RBB+C- Repeated bead beating and column-based purification

SCFA- Short chain fatty acids

SLC transporter- Solute carrier transporter

SOC- Super optimal broth with catabolite repression

SS- Steady state

STEAP3- Six-Transmembrane Epithelial Antigen of Prostate 3

TAPS- [tris(hydroxymethyl)methylamino]propanesulfonic acid

Tf- Transferrin

TfR1- Transferrin receptor protein 1

UC- Ulcerative colitis

XOS- Xylo-oligosaccharides

ZIP- Zrt- and Irt-like protein

ZnT- Zinc transporter

Chapter 1 Introduction

1.1 Chemistry of Iron

Iron is the fourth most abundant metal in the Earth's crust (Andrews et al, 2003). It has an atomic weight of 55.854 and an atomic number 26 (Wieser et al, 2013). Iron is a *d*-block transition element, residing in group 8 in the periodic table (Figure 1.1). Like the other elements in group 8, iron exist in multiple oxidation states, ranging from -2 to +6 but the +2 (ferrous), +3 (ferric) and +4 (ferryl) states are most commonly found in biological systems (Beard, 2001). The ability of iron to convert between oxidation states enables iron to participate in electron transfer and more importantly it has the ability to bind ligands, namely oxygen, sulphur and nitrogen atoms which are the preferred ligands in biological systems. Iron also has the tendency to change its electronic spin state and biological redox potential according to the ligand which it binds. Because of the exploitation of its various oxidation states, electron spin states and redox potentials, iron is involved in a considerable number of biochemical reactions (Beard, 2001).

Iron is an essential nutrient to almost all living organisms as it participates in numerous crucial biochemical reactions which maintain the wellbeing of the organisms. The unique properties that iron possesses enable it to be incorporated into proteins, facilitating various biochemical reactions. There are four major classes of iron-containing proteins:

1) iron-containing nonenzymatic proteins (haemoglobin and myoglobin),

2) haem-containing enzymes,

3) iron-sulphur enzymes and

4) non-iron-sulphur, nonhaem iron-containing enzymes (Beard, 2001).

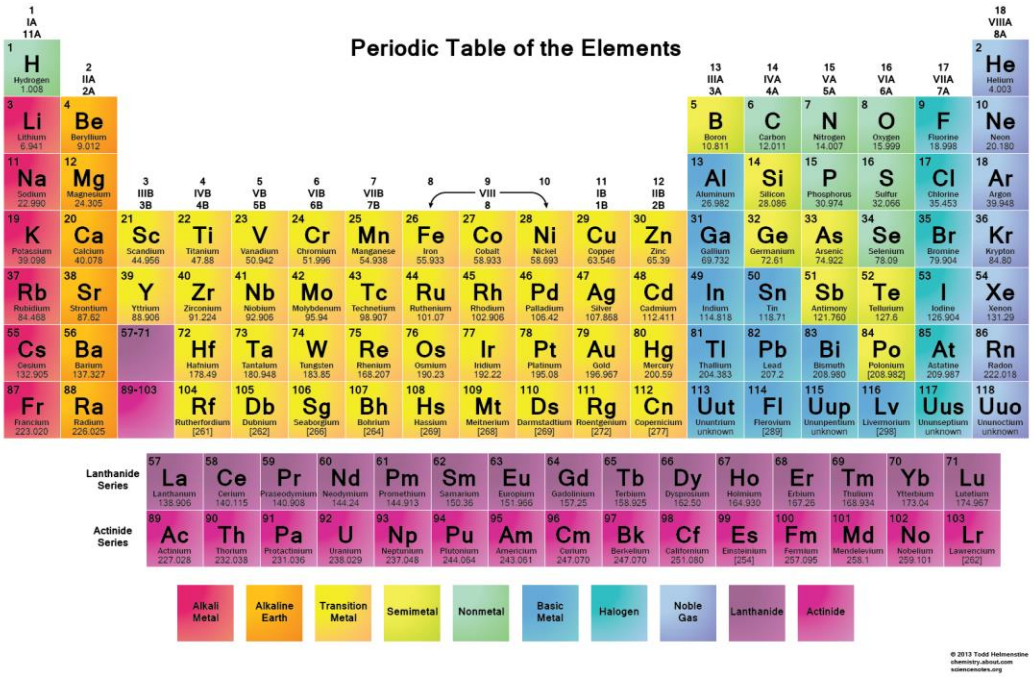


Figure 1.1. **Periodic Table of Elements**

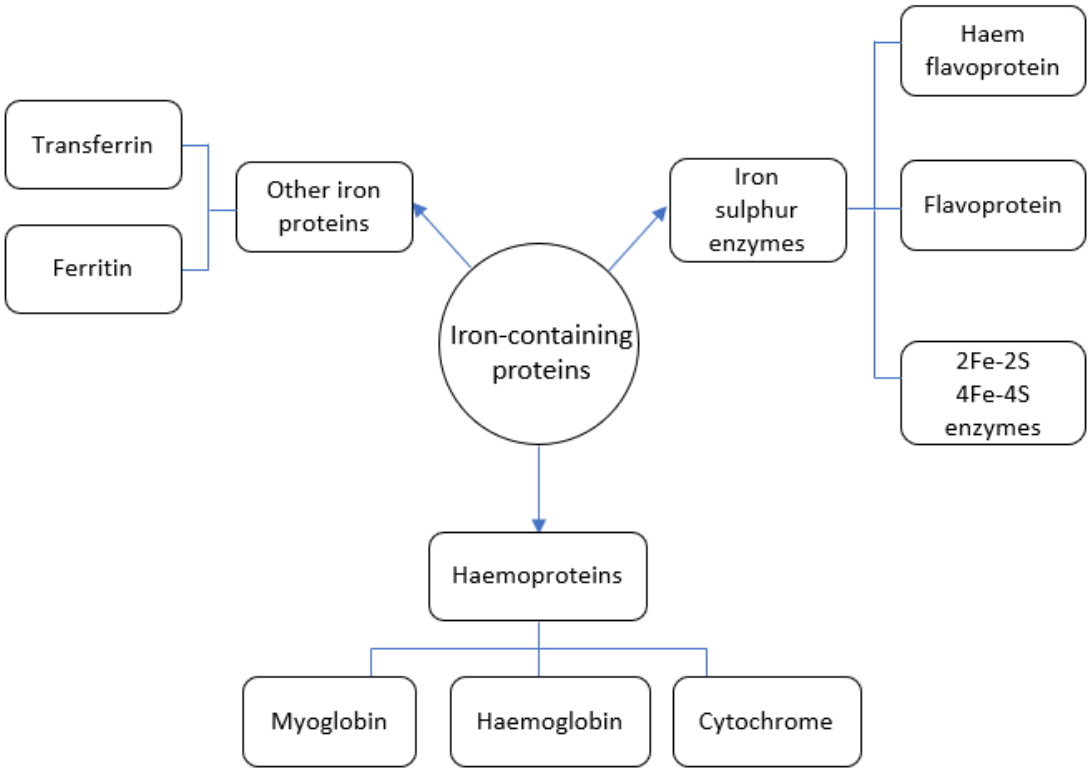


Figure 1.2. **Classification of major iron-containing proteins**. These proteins are present in the human body and have a wide range of biological function. From Beard, 2001.

1.2 Dietary iron and requirement

Table 1.1. Dietary reference values for iron. (SACN, 2010). Values are in mg/day (µmol/day).

Age	Lower reference nutrient intake (LRNI)	Estimated average requirement (EAR)	Reference nutrient intake (RNI)
0-3 months	0.9 (15)	1.3 (20)	1.7 (30)
4-6 months	2.3 (40)	3.3 (60)	4.3 (80)
7-9 months	4.2 (75)	6.0 (110)	7.8 (140)
10-12 months	4.2 (75)	6.0 (110)	7.8 (140)
1-3 years	3.7 (65)	5.3 (95)	6.9 (1120)
4-6 years	3.3 (60)	4.7 (80)	6.1 (110)
7-10 years	4.7 (80)	6.7 (120)	8.7 (160)
11-14 years (males)	6.1 (110)	8.7 (160)	11.3 (200)
11-14 years (females)	8.0 (140)	11.4 (200)	14.8 (260)
15-18 years (males)	6.71(110)	8.7 (160)	11.3 (200)
15-18 years (females)	8.0 (140)	11.4 (200)	14.8 (260)
19-50 years (males)	4.7 (80)	6.7 (120)	8.7 (160)
19-50 years (females)	8.0 (140)	11.4 (200)	14.8 (260)
50+ years	4.7 (80)	6.7 (120)	8.7 (160)

***1µmol = 55.9µg**

Since iron plays important roles in biological systems due to its involvement in a variety of biochemical reactions, the levels of iron must be maintained within certain ranges to ensure an adequate amount is present for normal function. The current Reference Nutrient Intake (RNI) for iron is 8.7 or 14.8mg/day for adult males or women in their reproductive years respectively (SACN, 2010). The iron requirement of 97.5% of the population will be met for iron intakes levels above the RNI, thus a sufficient amount of iron must be consumed either from dietary sources or supplementation. The amount and bioavailability of iron from dietary sources varies, depending on various factors such as the form of iron, the presence of enhancers or inhibitors of iron uptake from the diet as well as type of food. Dietary iron can be classified simply into haem and nonhaem iron. (Hurell and Egli, 2010). Haem iron usually comes from animal sources where iron is tightly bound to the porphyrin ring of haemoglobin and myoglobin (Han, 2011) while nonhaem iron can be found in both plant and animal tissues (Hurell and Egli, 2010), where it can be associated with ferritin and the

vacuoles of plant cells (Fuqua et al, 2012). Between the two types of dietary iron, haem iron is more readily absorbed compared to nonhaem iron and can contribute nearly 40% of total absorbed iron (Hurell and Egli, 2010). The mechanism of iron uptake from the diet will be discussed in later part.

1.2.1 Dietary factors affecting the uptake of iron

1.2.1.1 Dietary factors that enhance the uptake of iron

The absorption of iron can be enhanced or inhibited through interaction with other dietary components in the diet (Han, 2011), leading to an increased or decreased iron uptake from the gut. Citric acid, ascorbic acid, amino acids (Han, 2011) and muscle protein (Hurrell and Egli, 2010) are enhancers of iron absorption. Dietary iron is usually in the oxidised Fe(III) form which has low solubility and bioavailability (Fuqua et al, 2012). The ability of ascorbic acid to reduce the less soluble ferric to the more soluble ferrous iron and the potential to chelate iron greatly enhance the absorption of both native and fortified iron (Hurrell and Egli, 2010). Surprisingly, muscle tissues (meat, poultry and fish) show enhancing effects on iron uptake but animal proteins (milk proteins and egg albumin) show no such effect and indeed exhibit inhibitory effects on iron absorption (Hurrell and Egli, 2010). Numerous studies have shown an increased iron absorption in diet containing muscle tissues. The presence of a high number of cysteine-containing peptides in myofibrillar protein might explain the enhancing effect of muscle tissues on iron absorption (Hurrell and Egli, 2010). This might be due to the formation of soluble complexes between cysteine residues preventing iron binding to phytates and phenolic compounds (Hurrell et al, 2008) or to the ascorbic acid-like property of cysteine which reduces and chelates iron (Hurrell and Egli, 2010). Such effects would be expected to increase iron absorption. Glycosaminoglycans and L-α-glycerophosphocholine might also enhance the absorption of iron in meat but the results are not consistent in human studies, possibly due to the presence of ascorbic acid which masks the effects of glycosaminoglycans and L-α-glycerophosphocholine (Hurrell and Egli, 2010).

1.2.1.2 Dietary factors that inhibit the uptake of iron

Some naturally occurring food components such as polyphenols, phytates, calcium, tannic acid (Kim et al, 2008) and animal proteins (Hurrell and Egli, 2010) are inhibitors which reduce uptake of iron in

the gut. Polyphenols are usually found in plant foods and beverages such as legumes, tea and coffee. The inhibitory effect of polyphenols is affected by the amount and type present in food (Kim et al, 2008). Polyphenols are able to chelate iron and form complexes with iron, which prevent them from exiting the basolateral membrane (Kim et al, 2008). Tannic acid on the other hand binds with high affinity (Glahn et al, 2002) and chelates iron, reducing iron access to the apical surface of enterocytes (Kim et al, 2008). The effects of calcium intake and the absorption of iron are well reviewed by Lynch, involving numerous studies, from small scale human studies to large scale cohort (Lynch, 2000). Evidence linking calcium and iron absorption showed varied results, but has either little or no effect, suggesting that intake of calcium has minimal effects on iron absorption. The inconsistency in finding may be due to experimental design, consumption of single or mixed meals as well as the presence of other enhancer or inhibitor (Lynch, 2000). Phytates, which bind iron and form insoluble complexes (Gibson et al, 2006) are the main inhibitors of iron uptake in plant based diets (Hurrell and Egli, 2010), and thus have greater impact in vegetarian than omnivorous diets. Haem iron is less susceptible to the inhibitory effect of phytate due to the presence of the porphyrin ring structure which protects haem iron from phytates (Glahn et al, 2002). However, the negative effect of phytate on iron absorption is dose dependent and can be easily reversed. For instance, germination or malting of cereals increases phytase activity while milling reduces phytate contained in the outer aleurone layer or in the germ. Microbial fermentation produces microbial phytase enzymes which hydrolyse higher inositol phosphates to lower inositol phosphates. These microbes can either be found naturally on the surface of cereals and legumes or introduced via inoculation (Gibson et al, 2006).

1.3 Distribution of iron in the body

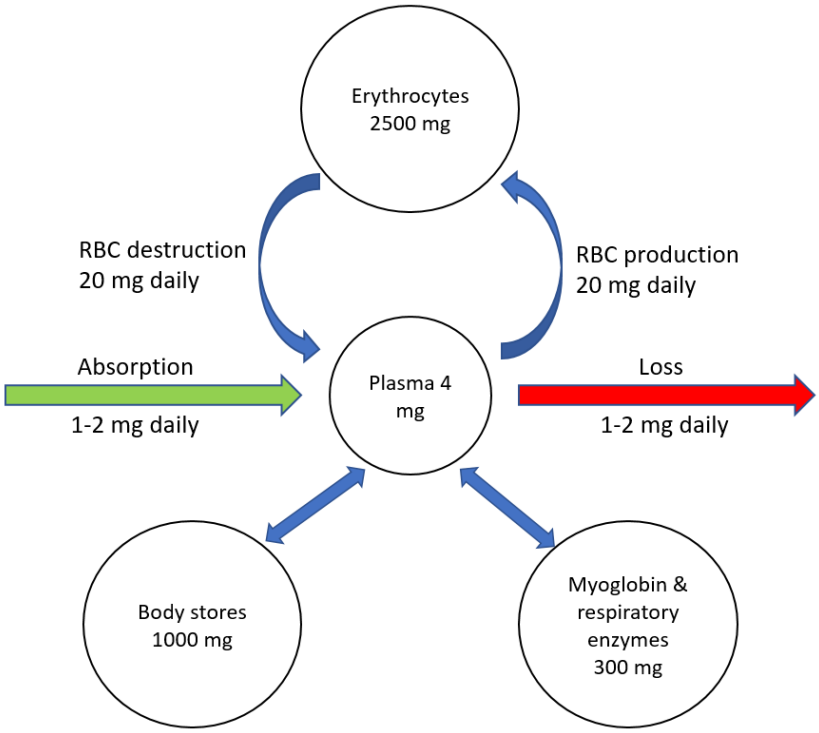


Figure 1.3 Distribution of iron in the body. Iron is absorbed from the gut which will then be distributed throughout the body for the formation of erythrocytes and enzymes or being stored in storage proteins. Iron can be lost through shedding of cells or in urine and sweat.

Concentration of body iron is roughly 30-40mg/kg (Beard, 2001) and a healthy adult has a total body iron content of about 4 g (Conrad and Umbreit, 2002). However, the total iron content varies between humans, depending on age, gender and the possible presence of diseases. The majority (60-70%) of iron is utilised in red blood cells, contained within haemoglobin which accounts ~90% of the protein content of the erythrocyte (Papanikolaou and Pantopoulos, 2005). The body has an iron store of 15mg/kg or approximately 20-30% of total body iron (Papanikolaou and Pantopoulos, 2005, Beard, 2001). Iron is usually stored within ferritin or its degradation product haemosiderin found in hepatocytes and reticulonendothelial macrophages (Papanikolaou and Pantopoulos, 2005) with 60% stored in the liver and 40% in the reticuloendothelial system (Beard, 2001) . The remaining body iron is localised in myoglobin, cytochromes and iron-containing enzymes. Around 1-3mg of iron is absorbed from the diet daily (Figure 3.1) to replace iron losses in urine, sweat and cell desquamation from the skin and intestine (Anderson et al, 2012). A woman of reproductive years may lose more

than 42mg per cycle, depending on how heavily she menstruates (Killip et al, 2007). Thus, it is important that the body absorbs enough iron to compensate for such losses to prevent any clinical symptoms from occurring.

1.3.1 Iron uptake from gut lumen

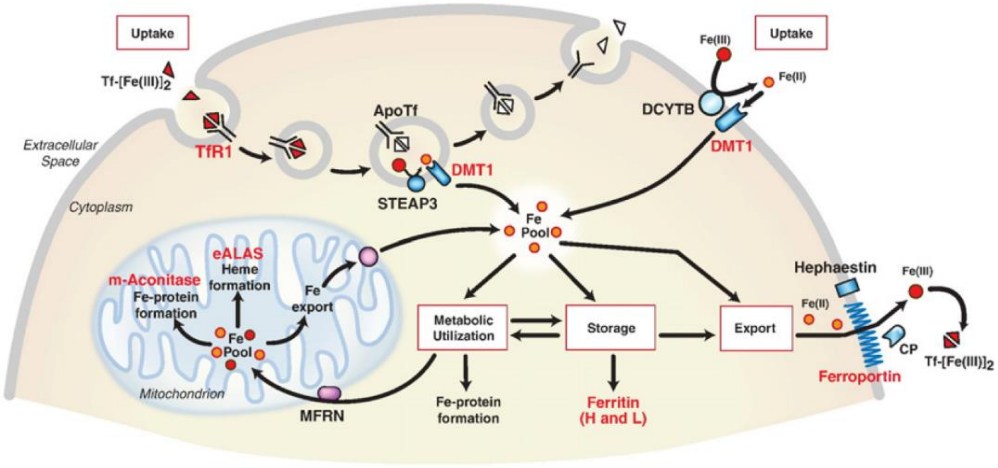


Figure 1.4. **The cellular pathway of handling iron, from uptake to storage and to export into circulation**. Iron-bound transferrin binds to TfR1 and subsequently endocytosed. Fe (III) is released from transferrin through acidification and reduced to Fe (II) by STEAP3 which then exported in the cytosol by DMT1 for storage or formation of proteins. From Anderson et al, 2012.

Iron is absorbed in the duodenum and upper portion of the jejunum (Gkouvatsos et al, 2012). Both haem and non-haem iron enter the systemic circulation by crossing the apical and basolateral membranes of enterocytes via specific transporters (Gkouvatsos et al, 2012). The low pH of the stomach, together with proteolytic enzymes in the stomach and small intestine facilitates the release of haem from haemoproteins. Although haem has limited solubility, it is able to interact with other components in the gut, forming soluble complexes (Fuqua et al, 2012). Haem is more readily absorbed than non-haem iron through a yet to be defined mechanism. It is most likely to involve a specialised haem carrier but the carrier has yet to be found. The proposed carrier, haem carrier protein 1 (HCP1) was later identified as a folate transporter (Gkouvatsos et al, 2012; West and Oates, 2008). Haem may also be taken up via direct transport or through receptor-mediated endocytosis (Gkouvatsos et al, 2012). The haem-binding protein is found on microvillus membrane of the upper small intestine of both pigs and humans, as well as the membrane of erythroleukemia cells. Haem

uptake is affected by temperature and ATP, and the combined evidence shows the ability of cells to endocytose haem actively (West and Oates, 2008). The absorbed haem then undergoes degradation, releasing Fe(II), which follows the same fate as absorbed non-haem iron (Gkouvatsos et al, 2012). Non-haem iron predominates as the insoluble Fe(III) form in the intestinal lumen; this form has low bioavailability. Prior to its uptake into the cell, it needs to be reduced to Fe(II). This is done by the brush border ferrireductase (the duodenal cytochrome *b*, Dcytb) which utilises ascorbate to facilitate ferrireduction (Han, 2011). Iron deficient and hypoxic conditions increase the expression of Dcytb, stimulating iron absorption (Han, 2011). However, experimental results show that Dcytb may not be the only ferrireductase present at the brush border as the disruption of Dcytb has no effect on normal iron metabolism in knockout mice (Hentze et al, 2010). The Fe(II) is subsequently transported through the apical membrane by divalent metal transporter 1 (DMT1), an energy dependent symporter which also imports a proton (Dunn et al, 2006).

1.3.2 Fate of internalised iron

1.3.2.1 Export into systemic circulation

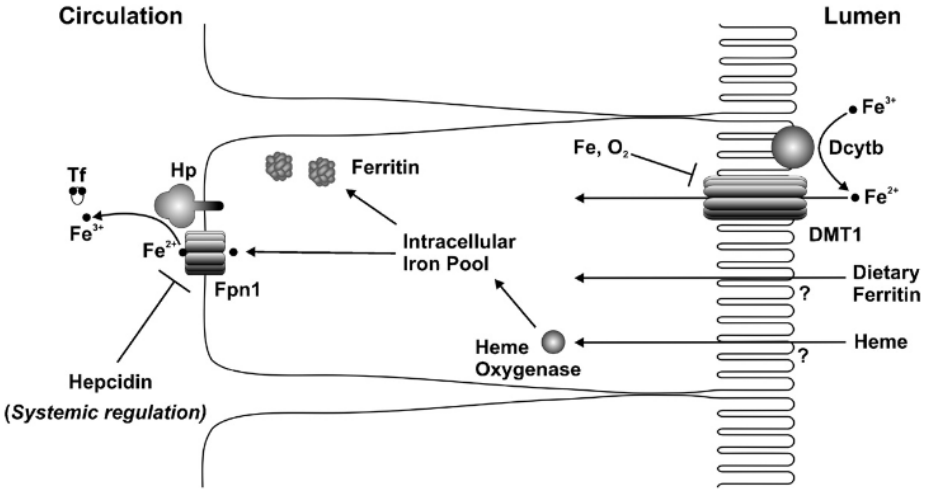


Figure 1.5 Passage of iron across the enterocyte into the systemic circulation. Fe (III) is reduced by Dcytb to Fe (II) prior to being transported into the cell via DMT1. Iron will then enter the circulation via Fpn1 and oxidised to Fe (III) before binding to transferrin for transport. Dietary ferritin and haem enter the cell via an unknown mechanisms and will join the intracellular iron pool. From Fuqua et al, 2012.

The absorbed iron then enters the systemic circulation to be transported around the body for cell utilisation and distribution of the absorbed iron is dependent on the body's iron requirement (Han, 2011). Located at the basolateral membrane, ferroportin (FPN1) which is part of the SLC transporter family (SLC40A1), exports ferrous iron into the circulation (Gkouvatsos et al, 2012). Experimental mouse models showed the role of ferroportin as an iron exporter. Donovan and colleagues showed that $Fpn^{null/null}$ mice die prematurely due to an impaired iron transfer from the mother. Inactivation of duodenal ferroportin caused a marked accumulation of iron in enterocytes but not in the liver or macrophages, indicates a failure to export the absorbed iron. However, iron is not retained in hepatocytes or macrophages as part of response to iron deficiency (Donovan et al, 2005). Global inactivation of ferroportin causes an increased iron retention in macrophages of liver and spleen due to the lack of mechanisms in exporting recovered iron from phagocytosed erythrocytes (Donovan et al, 2005). Thus, the evidence shows an important role for ferroportin as an iron exporter in several tissues. Ferroportin exports iron in its ferrous form, which needs to be oxidised to the ferric form before loading to apo-transferrin (apo-Tf) to allow iron to be carried around the body (Gkouvatsos et al, 2012). The oxidation process is carried out by the membrane bound copper dependent ferroxidase, hephaestin (Petrak and Vyoral, 2005). Hephaestin shares amino acid sequence similarity with ceruloplasmin, a well-known serum multi-copper ferroxidase, but it contains an additional 86 amino acids at its C-terminus (Petrak and Vyoral, 2005). In terms of structure, both have the same β-fold, cysteine residues for forming disulphide bridges and a negatively charged aspartate tract near the iron binding site; these similarities indicate that hephaestin is also an important ferroxidase, like ceruloplasmin (Petrak and Vyoral, 2005). Although hephaestin and ceruloplasmin possess many similarities, they are found in different parts of the body. Hephaestin is highly expressed in the small intestine while ceruloplasmin is found in storage organs such as the liver and cells from the reticuloendothelial system (Petrak and Vyoral, 2005). Sex-linked anaemia (sla) mice, where hephaestin is absent, results in impaired iron export but normal iron uptake from the intestinal lumen into enterocytes. On the other hand, patients with aceruloplasminaemia and ceruloplasmin-

null mice have accumulation of iron in liver and pancreas but normal intestinal absorption of iron (Petrak and Vyoral, 2005). Both observation prove that hephaestin and ceruloplasmin are important components for iron transport and they act at different sites. Once loaded on to transferrin (Tf), the absorbed iron is transported around the body or used by the various tissues.

1.3.2.1.1 Cellular uptake of iron from systemic circulation

The bilobal transferrin can reversibly binds two Fe(III) ions and deliver them to various cells where the receptor-mediated endocytosis uptake process is mediated by the transferrin receptor (Crichton et al, 2002). Erythroid precursors and other rapidly dividing cells have a high demand for iron, thus they require an efficient uptake of transferrin to this need. The iron-loaded transferrin (Tf-Fe_2) interacts with cell surface transferrin receptor 1 (TfR1) which forms a disulphide-bonded homodimer, binding one transferrin molecule at each of its subunit (Gkouvatsos et al, 2012). Following the binding of diferric transferrin, the Tf-Fe_2/TfR1 complex is internalised via clathrin-coated vesicles. The vesicles then lose their coat, resulting in smooth vesicles that fuse with the endosome (Crichton et al, 2002). A proton pump ATPase lowers the pH to 5.5, triggering a conformational change in Tf that releases Fe(III). The released ferric iron is reduced by the six-transmembrane epithelial antigen of the prostate-3 (STEAP3) and is subsequently transported out of the endosome via DMT-1 (Gkouvatsos et al, 2012). After delivering the iron, apotransferrin, still bound to transferrin receptor, returns to the cell membrane with the aid of the trafficking protein Sec1511. Once released back to the bloodstream, transferrin is able to recapture iron and the cycle is repeated (Gkouvatsos et al, 2012).

Macrophages acquire iron via a different route than that of most other cells, involving phagocytosis of senescent erythrocytes resulting in uptake of large amounts of iron (Gkouvatsos et al, 2012). Following haemolysis, natural resistance-associated macrophage protein 1 (NRAMP 1), a divalent metal transporter homologous to DMT1, exports the released iron from phagocytic vesicles. The ferrous iron is subsequently exported to the circulation via ferroportin (Hentze et al, 2010). Free haem or haemoglobin may be released from damaged erythrocytes, which exhibit oxidative activity,

causing damage to other components in the body. Haptoglobin and haemopexin are proteins that bind to free haemoglobin and free haem respectively, forming a complex that will be endocytosed by macrophages (Gkouvatsos et al, 2012), rapidly clearing these iron-containing molecules from the circulation.

1.3.2.2 Iron storage

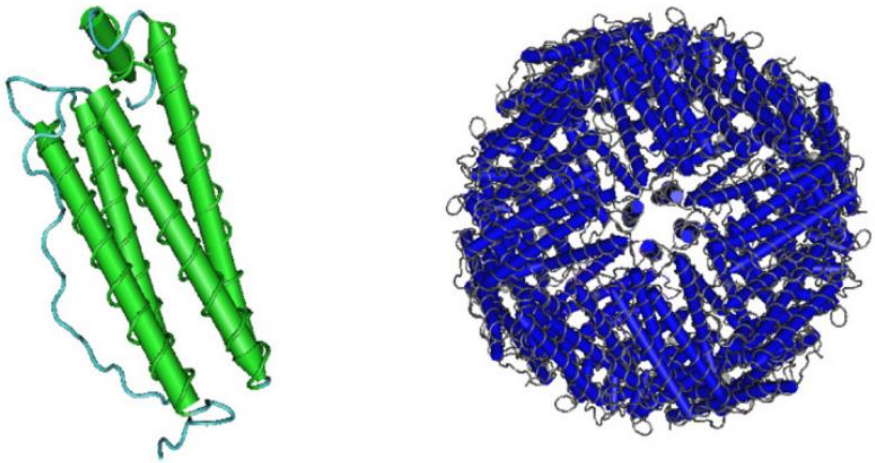


Figure 1.6 Structural aspects of the ferritins. The subunits typically fold in a 4-helical bundle (left), which can pack in shells made of 24 subunits like human ferritins (right). From Ariso et al, 2009.

Cellular iron that is not used immediately for cellular function is stored in ferritin. Ferritins are basically composed of 24 subunits, folding in a 4-helical bundle and form an almost spherical protein shell with 4,3,2 point symmetry (Ariso et al, 2009). This arrangement give ferritin a hollow shell with an 80 Å diameter cavity and a storage capacity up to 4500 Fe(III) ions (Harrison and Arosio, 1996). Eukaryotes have two major ferritin genes, encoding the H (heavy) and L (light) subunits which co-assemble to form heteropolymers (Ariso et al, 2009). H subunit contains ferroxidase activity which is required for iron deposition while L subunits are involved in iron nucleation and increased turnover of the ferroxidase site (Dunn et al, 2010). L-rich ferritins are usually found in iron-storage organs such as liver and spleen whereas H-rich ferritins are present in other tissues such as the heart and brain (Harrison and Arosio, 1996). Mammalian tissues contain a mixture of isoferritins, each with a different ratios of H and L subunit (Harrison and Arosio, 1996). Storage of iron in ferritin is

considered biologically important due to the high storage capacity acting as a reservoir, concentrating the iron in a compact form which can readily be used when needed. On the other hand, storing of iron in ferritin prevents the participation of iron in Fenton chemistry, potentiating oxygen toxicity through the production of various free radical species. These free radicals react with amino acids, purine and pyrimidine bases of DNA as well as initiating lipid peroxidation (Dunn et al, 2010), and are thus potentially highly damaging.

1.4 Bacterial iron uptake

Microorganisms, like higher organisms require iron to sustain themselves. With the exception of lactobacilli which have the ability to utilise cobalt and manganese to replace iron as biocatalysts (Guerinot, 1994), virtually all other microorganisms require iron to perform various biochemical functions, namely respiration, gene regulation and DNA biosynthesis. Bacterial cells contain 10^5 to 10^6 iron ions per cell which are required for these biochemical reactions (Wandersman and Stojiljkovic, 2000). Due to its reactivity, iron is usually sequestered to host protein such as ferritin, transferrin and lactoferrin or is incorporated into protoporphyrin in haemoproteins for specialised activity (Wandersman and Delepelaire, 2004). Under aerobic conditions, ferrous ion is unstable as it undergoes oxidation to form ferric ion (Krewulak and Vogel, 2008). In the presence of hydrogen peroxide (which accumulates during aerobic metabolism), ferrous iron can partake in Fenton reactivity:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$$

Ferric ions are redox stable and insoluble at neutral pH, such that free ferric ions are limited to an equilibrium concentration of approximately 10^{-17}M (pH 7) which is well below the optimal value for microbial growth (10^{-8} to 10^{-6} M) (Guerinot, 1994; Andrews et al, 2003). In order to overcome the iron shortage and limitation, most bacteria develop several mechanisms to acquire iron from different sources (Wandersman and Delepelaire, 2004). They can either obtain iron directly from

host proteins or secrete ferric ion chelators to salvage insoluble Fe(III) from the environment. Iron acquisition by bacteria is receptor mediated with various specific receptors located on the outer surface to recognise a wide range of substrates. The difference between obtaining iron using iron chelators (siderophores) and the acquisition of iron from host protein is that siderophores are taken up as intact molecules whereas iron is first extracted from transferrin and lactoferrin before subsequent transport into the cell (Krewulak and Vogel, 2008).

Iron uptake has been identified in both Gram-negative and Gram-positive bacteria. The porins present on the outer membrane (OM) only enables small solutes less than ~600Da to diffuse through. Ferric-siderophores, haemoglobin, lactoferrin and transferrin exceed this molecular weight cutoff and so require specific OM receptors for transport across the outer membrane. In Gram-negative bacteria, iron uptake involves the translocation of substrate via an outer membrane receptor using energy dependent mechanism provided by a 3 protein TonB complex, composed of TonB, ExbB and ExbD anchored to the cytoplasmic membrane. Periplasmic binding protein (PBP) then conveys the substrate to an inner membrane ATP-binding cassette (ABC) which channels it to the cytoplasm for cellular function. Gram-positive bacteria lacking the outer membrane uptake iron in a different but similar mechanism. It involves a membrane-anchored binding protein, resembling the PBP and an ABC transporter (Krewulak and Vogel, 2008).

1.4.1 Bacterial Iron Sources

1.4.1.1 Iron ions

Iron is present in its oxidised ferric form in aerobic environments. It is insoluble at neutral pH, so bacteria produce siderophores to solubilise iron prior to uptake. Iron exist as the reduced or ferrous iron under anaerobic or low pH environments (Cartron et al, 2006). It is the preferred form of iron utilisation due to its solubility and can diffuse freely through the porins of the OM of Gram-negative bacteria (Wandersman and Delepelaire, 2004). Bacteria use the Feo transport system instead of siderophore dependent system in acquiring ferrous iron (Andrews et al, 2003).

1.4.1.2 Lactoferrin/Transferrin

Lactoferrin(Lf) and transferrin(Tf) are closely related glycoproteins which exhibit high level of sequence and structural conservation (Gray-Owen and Schryvers, 1996). They are two lobed 80kDa glycoproteins which have a high affinity constant for Fe(III) ion and are able to bind two Fe(III) per molecule (Gray-Owen and Schryvers, 1996). With the ability to sequester iron, Lf and Tf are part of innate immunity in preventing the establishment and propagation of bacterial infection (Gray-Owen and Schryvers, 1996). Transferrin is found in serum and lymph while lactoferrin is usually found in milk and secretions (Perkins-Balding et al, 2004). Lf and Tf both have protective function but the latter has an additional iron transport role.

1.4.1.3 Haem and Haem-containing proteins

The abundance of haem-containing proteins in the host makes them a valuable source of iron. Haem refers to Fe(II) iron protoporphyrin IX while haemin is the Fe(III) form of the molecule (Genco and Dixon, 2000). Due to its toxicity, haem is bound to proteins and rarely found free. Besides iron sources for bacteria, protoporphyrin is also a source for bacterial species such as *Enterococcus feacalis* (Wandersman and Delepelaire, 2004). Haem is usually found in haemoglobin, which is the main component of red blood cells. It is penta-coordinated to four nitrogens in the porphyrin ring and to imidazole of a histidine residue. The sixth coordination can be either free or bound to oxygen or carbon dioxide (Wandersman and Delepelaire, 2004). Another haem-related protein is haptoglobin which binds haemoglobin when it is released during haemolysis. The haptoglobin-haemoglobin complex is considered an iron source for several bacteria (Wandersman and Delepelaire, 2004). While haptoglobin binds haemoglobin, haemopexin binds haem released into plasma. Haem-haemopexin can also be utilised by bacteria (Wandersman and Delepelaire, 2004).

1.4.2 Bacterial iron uptake

1.4.2.1 Siderophores

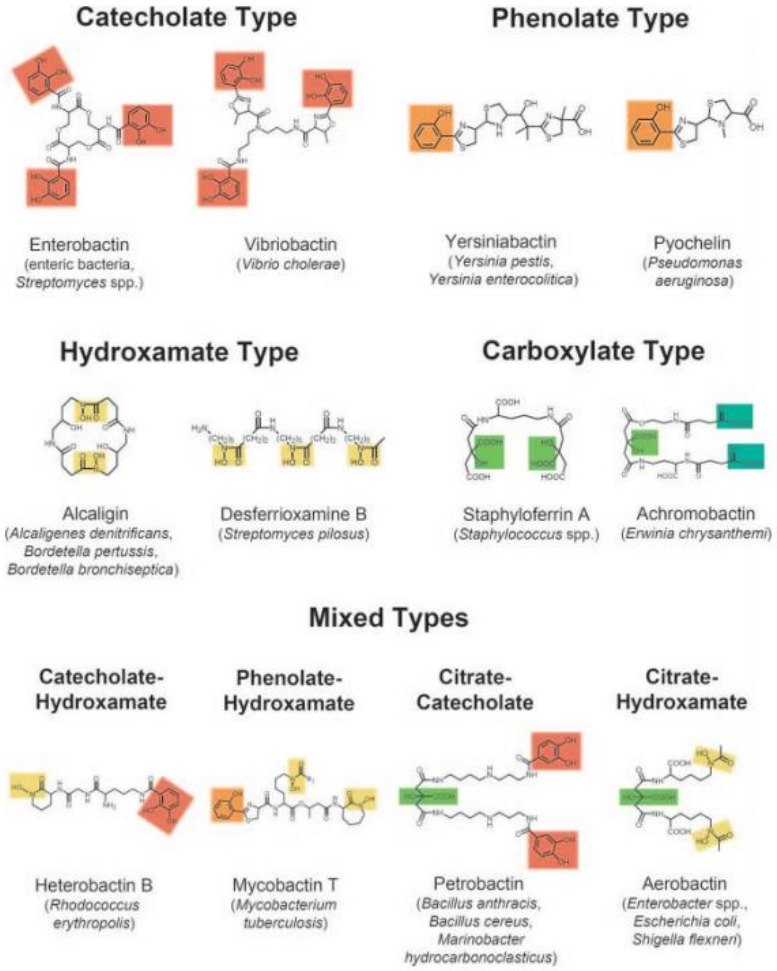


Figure 1.7 Types of siderophore and their binding groups. Moieties involved in iron coordination are as follow: catecholates (red), phenolates (orange), hydroxamates (yellow), α-hydroxy-carboxylates (light green) and α-keto-carboxylates (cyan) From Miethke et al, 2007.

Siderophores are low molecular weight iron chelating compounds synthesised by many Gram-negative and Gram-positive bacteria to scavenge Fe(III) ions from the environment. They are often assembled by nonribosomal, cytoplasmic peptide synthases using dihydroxybenzoic acid, hydroxamate groups containing N^5-acyl-N^5- hydroxyornithine or N^6-acyl-N^6-hydroxylysine and hydroxycarboxylates consisting of citric acid or β-hydroxyaspartic acid as building blocks (Winkelmann, 2002). They are then excreted upon formation (Wandersman and Delepelaire, 2004). There are more than 500 different siderophores identified with various nonprotein amino acid analogues (Wandersman and Delepelaire, 2004). Optimal selection of metal binding units and

stereochemical arrangement affect the selectivity of siderophores for Fe(III). Siderophores have three major types of iron binding functional groups: α-hydroxycarboxylate (as in pyochelin), catecholate (as in enterobactin) and hydroxamate (as in ferrichrome and aerobactin) which can be arranged into different structures (Boukhalfa and Crumbliss, 2002).

The most important factor in siderophore architecture is denticity (the number of donor groups). They can be categorised into hexadentate or tetradentate siderophores with the former having the optimal denticity to satisfy the 6 coordination sites on Fe(III) (Boukhalfa and Crumbliss, 2002). Most siderophores with hydroxamate and/or catecholate binding functional groups convey hexadentate denticity. For example, desferrioxamine B which contains three hydroxamate units is able to achieve full iron coordination (Boukhalfa and Crumbliss, 2002). In terms of tetradentate siderophores, due to the inability to form a simple 1:1 complex (such as those found in hexadentate trihydroxamate ligands), they are unable to satisfy the preferred octahedral coordination geometry of Fe(III) (Spasojevic et al, 2001). Dihydroxamate siderophore form bimetallic complexes with a minimal stoichiometry of Fe_2L_3 to coordinate metal ions (Spasojevic et al, 2001). Thus, two or three molecules are needed to form the six iron coordination sites, giving a range of stoichiometries (Fe_2L_3, FeL(LH) etc.) (Boukhalfa and Crumbliss, 2002). Alcaligin and rhodotorulic acid are the best characterised tetradentate siderophores. In general, both hexadentate and tetradentate siderophores are selective for Fe(III) over Fe(II) but hexadentate siderophores have higher affinity for Fe(III) than tetradentate siderophores (Boukhalfa and Crumbliss, 2002).

1.4.2.1.1 Siderophore receptors

Ferric siderophores translocate into the periplasmic space of gram-negative bacteria with the aid of highly specific OM receptors as the complex exceeds the molecular cut off weight of the porins. *E.coli* strain K-12 possesses eight known TonB-dependent transporters, 7 for ferric chelators and 1 for vitamin B12 (Yue et al, 2003). Of the 7 transporters, 6 of them recognise catecholates and hydroxamate siderophores while the other is ferric citrate transport (Yue et al, 2003). All the OM transporters require energy for the transport of ferric-siderophores. This is provided by the TonB

complex found in the cytoplasmic membrane (CM). The complex transduces proton motive force (pmf) to enable internalisation of substrate to the periplasmic space (Wandersman and Delepelaire, 2004). Crystallographic analysis of 3 ferric-siderophore receptors from *E.coli* has been performed: FhuA, FepA and FecA which transport ferrichrome, enterobactin and ferric citrate respectively. Despite low sequence similarity between these 3 receptors (FecA/FepA: 15.8%; FecA/FhuA: 16%; FepA/FhuA: 12.9%), they share a common 'plug and β-barrel' structure (Yue et al, 2003). The plug is a globular domain derived from the first 160 residues at the N-terminus and sits inside a barrel composed of 22 transmembrane β-strands (Andrews et al, 2003).

1.4.2.1.1.1 β-barrel

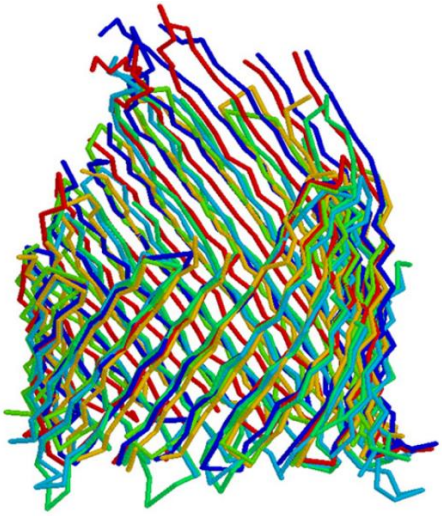


Figure 1.8 Superposition of the Cα backbones of BtuB, FpvA, FepA, FecA, and FhuA. From Krewulak and Vogel, 2008.

The β-barrel has three main features: ten 2 to 10 residue short, periplasmic loops; a barrel made up of 22 antiparallel β-strands which is tilted at a 45° angle relative to the main axis and 11 extracellular loops. β-barrel varies in height, ranging from 55 Å (BtuB) to 70 Å (FepA) (Krewulak and Vogel, 2008). Inter-strand hydrogen bonds and salt bridges between the plug and inner surface of the β-barrel stabilises the structure (Wandersman and Delepelaire, 2004). The presence of highly conserved Phe or Trp in the C-terminal is important for correct folding and insertion to the outer membrane (Krewulak and Vogel, 2008). The 11 extracellular loops of the β-barrel range from 2 to 37 residues and are able to extend 30 to 40 Å above the OM (Krewulak and Vogel, 2008). They interact with ferric-siderophores and close the opening of the barrel to prevent entry of unnecessary solutes. An

example of the importance of extracellular loops is shown in FecA where surface loops 7(L7) and 8(L8) are involved in the binding of ferric citrate and the transport of ferric citrate into the periplasm by closing the entrance to prevent the escape of the substrate (Sauter and Braun, 2004). Deletion of L7 and L8 abolishes the induction and transport of ferric citrate (Sauter and Braun, 2004). Similarly, deletion of L7 and L8 in FepA also eliminated the binding and uptake of enterobactin (and colicins), indicating the participation of loops in recognition and transport of substrate (Newton et al, 1999). However, deletion of L7 and L8 only reduced but did not abolish the function in FhuA. The absence of L7 or L8 causes the escape of ferrichrome into the medium and reduces transport rates. The deletion of L3 and L11 abolished FhuA transport activity (Endriss and Braun, 2004). This clearly shows that although loops are important in transport of substrate, they do not convey the same function in all transporters

1.4.2.1.1.2 Plug domain

Figure 1.9 C^{α} superposition of the plug domains in FecA, Fep A and FhuA. The structures of FecA(gold), Fep(magenta) and unliganded FhuA(light blue) were used for the superposition. From Yue et al, 2003.

The N-terminal or plug domain of OM receptors are similar in structures. They consists of a central mixed four-stranded β-sheet. The plug domain is held in position in the barrel by 40-70 hydrogen bonds and two salt bridges created from four highly conserved residues: 2 Glu residues in the β-barrel domain and 2 Arg residues in the globular domain. They are involved in siderophore uptake

through the unwinding of the 'switch helix' which has a role in the 'TonB box' within the plug domain. The TonB box interacts with the TonB protein which powers the translocation of substrate through the OM receptor by coupling to the pmf (Krewulak and Vogel, 2008).

The plug domains of FecA of *E.coli* and FpvA of *P.aeruginosa* have an extra 80 residues which are involved in regulation of iron uptake and are called N-terminal signalling domain. The *E.coli* *fecABCDE* and *fecIR* system is the most elaborate. Upon binding of ferric-citrate to FecA, conformational change of the N-terminal signalling domain of FecA is transferred to FecR which causes the release of FecI into the bacterial cytoplasm. FecI binds RNA polymerase, acting as an alternative sigma factor, which then binds to the promoter of the *fecABCDE* operon, initiating ferric-citrate uptake gene transcription. After acquiring enough iron, Fur-Fe^{2+} (the global iron regulator of most Gram negative bacteria) represses *fecIR* and *fecABCDE* by binding to the promoter (Krewulak and Vogel, 2008).

1.4.2.1.2 TonB Complex

Translocation of ferric-siderophores and other substrates across the OM receptor into the periplasmic space is an active process, requiring a functional TonB system to provide the pmf for the transport. The TonB complex transduces energy from pmf into conformational changes in the OM receptors, allowing passage of substrates (Perkins-Balding et al, 2004). TonB complex is made up of three proteins: TonB which interacts with OM and is anchored in the CM; and two CM embedded proteins, ExbB and ExbD (Postle and Kadner, 2003). Quantification of the subunits suggests a ratio of 1:7:2 for TonB: ExbB: ExbD (Andrews et al, 2003).

ExbB consists of the transmembrane domains with bulk protein in the cytoplasm. It functions to stabilise both TonB and ExbD. Using formaldehyde, TonB can be cross linked to ExbB via transmembrane domains and to ExbD though periplasmic domain (Postle and Kadner, 2003). ExbD is 17kDa and, like TonB, contains a single transmembrane domain with bulk sequence in the periplasmic space (Postle and Kadner, 2003). TonB can be divided into three functional domains with numerous conserved sequence motifs. The N-terminal domain is made up of a 32-residue

transmembrane helix and a short cytoplasmic region (Krewulak and Vogel, 2008). Ser-16 and His-20 are essential for the conversion to the energised form as well as active transport. Replacement of these Ser and His residues or deletion of amino acids from the region between them causes activity defect (Postle and Kadner, 2003). It also contains a striking and characteristic Pro-rich central domain (amino acids 66-120) with a series of Pro-Glu and Pro-Lys repeats. It has an unusual highly structured conformation, appearing as a rigid extended rod of about 10nm (Postle and Kadner, 2003), allowing it to span the periplasm and contact with the C-terminal domain of OM receptors (Andrews et al, 2003). Following the Pro-rich domain is a 'genetic suppressor' site of the TonB box mutation, residue 160, which causes a TonB-uncoupled phenotype. Frame deletion around this site inactivates TonB. The C-terminal domain of TonB is a highly ordered strand-exchange dimer composed of antiparallel β-sheets which functions in OM association (Postle and Kadner, 2003). ExbB and ExbD utilise the pmf of the CM to energise TonB; this energy is then transferred to the OM transporter via TonB (Postle and Kadner, 2003). The necessity for TonB to transduce energy was shown in experiments by separating the periplasmic and cytoplasmic domain using a leader peptidase cleavage site in TonB; this caused loss of function due to disconnection from the pmf energy source (Postle, 1993). Conformation change after depolarisation of TonB requires ExbB and ExbD. Although TonB is able to change occasionally to the energised conformation, ExbB and ExbD are required for this to occur efficiently (Postle and Kadner, 2003).

1.4.2.1.3 Periplasmic binding protein and transport into cytoplasm

Upon entering into the periplasm, siderophores are escorted to the CM for transport into the cytoplasm by periplasmic binding proteins (PBP). Each binding protein is able to bind one molecule and deliver it to the cytoplasmic ABC (ATP binding cassette) permease. Gram-positive bacteria lack an OM and a periplasmic space, so the binding proteins are anchored to the membrane via an N-terminal glyceride-cysteine domain (Tam and Saier Jr., 1993). The binding proteins are categorised into 8 groups according to their sequence similarity which correlates to the molecular sizes and solute binding specifications (Tam and Saier Jr., 1993). Cluster 1 and cluster 8 consist of iron related

binding proteins. Cluster 1 is specifically for oligosaccharides, α-glycerol phosphate and iron. It is made up of the larger CHO-binding protein and smaller iron-binding proteins. Iron binding proteins in this cluster include SfuA of *Serratia marcescens* and FbpNme of *Neisseria gonorrhoeae* and *N. meningitidis* (Tam and Saier Jr., 1993). Cluster 8 is specifically for iron complexes binding protein. It includes FepB, FecB and FhuD from *E.coli* and FatB from *Vibrio anguillarum* (Tam and Saier Jr., 1993). In *E.coli,* the presence of various PBPs is due to the chemical distinctness of each siderophores requiring a specific PBP for their transport. FepB, FecB and FhuD each carry catecholate, citrate and hydroxyamate-type siderophores respectively. However, they share similar structures. Domains of PBPs are connected by either a long α-helix or 2 or 3 β-strands. Each domain is composed of a mixture of α/β strands. The two domains move in a 'venus fly trap' fashion for binding and release siderophores (Krewulak and Vogel, 2008). FhuD from *E.coli* is the best characterised PBP. Binding of siderophore is mediated by hydrophilic and hydrophobic interactions. A mutation in Trp68 causes a decreased ferrichrome binding. Its N-terminal domain consists of a twisted five-stranded parallel β-sheet while the C-terminal domain contains a five-stranded mixed β-sheet. Both sheets are sandwiched between layers of α-helices. A kinked α-helix introduced by Pro167 which crosses the domains once links them together. FhuD adopts a novel fold which lacks the flexible hinge as seen in classical PBPs (Koster, 2001). There are two highly conserved residues in FhuD which are involved in hydrogen bonding with the hydroxamic acid moieties in siderophores: Tyr106 and Arg84. The former form bonds with a carbonyl oxygen while the latter bonds with a carbonyl oxygen and/or a nitroxyl oxygen (Krewulak and Vogel, 2008).

The entry of siderophore into the cytoplasm is provided by ABC permease complex which couples ATP hydrolysis to transfer of the siderophore from the periplasm into the cytoplasm. The ABC permease complex consists of two transmembrane domains forming a channel allowing the ferric-siderophore to pass through and two nucleotide binding domains which hydrolyse ATP (Krewulak and Vogel, 2008). The two transmembrane modules can be either two independent subunits (FepD and FepG), two copies of the same subunits (haem uptake) or one large two-module subunit (FhuB)

(Andrews et al, 2003). FhuB is an extremely hydrophobic polytopic integral membrane protein while FhuC functions as an ATP-binding component (Koster, 2001). FhuB has a central role in the system, interacting with FhuD and FhuC as well as various ferric hydroxamates. FhuD binds to FhuB to deliver ferric hydroxamates to the CM. The interaction of FhuD and FhuB prevents the degradation of FhuB by proteolysis and they can be cross-linked (Koster, 2001). FhuC acts as an ATP hydrolase, energising the transport process which is likely to be conformational changes of the permease complex (Koster et al, 2001).

Once being transported to the cytoplasm, iron must be liberated from the ferri-siderophore complex to be used. The process is thought to involve ferric reducing enzymes (YgjH, FhuF, flavin reductase, sulphite reductase and flavohaemoglobin) which reduce the bound iron to the ferrous form, causing it to dissociate due to the low affinity of siderophore for ferrous iron (Andrews et al, 2003).

1.4.2.2 Transferrin/Lactoferrin Uptake

Neisseria species do not synthesise and secrete siderophores for iron acquisition but instead have the ability to obtain iron from transferrin (Tf) and lactoferrin (Lf). They have two specific bipartite receptors (TfR and LfR) on the cell surface which binds to Tf and Lf respectively (Gray-Owen and Schryvers, 1996). *Neisseria gonorrhoeae* is extremely efficient in utilising Tf and Lf as iron sources for growth. A 5% iron saturation of either transferrin or lactoferrin is sufficient to support growth provided enough protein is present (McKenna et al, 1988). Transferrin and lactoferrin are too large to be internalised, thus iron is removed from these proteins at the external surface prior to transport into the cell (Krewulak and Vogel, 2008). The receptors are TbpA and TbpB for TfR and LbpA and LbpB for LfR. The molecular masses of TbpAB varies between different species, ranging from 93 to 98kDa for TbpA and 68 to 85kDa for TbpB. LbpA and LbpB on the other hand are 98kDa and 84kDa respectively (Perkins-Balding et al, 2004). TbpA and LbpA are capable of binding to their respective substrate with high affinity while TbpB and LbpB acts as facilitators that help to distinguish between the apo- and holo- substrate (Perkins-Balding et al, 2004). Iron utilisation from Tf and Lf is TonB dependent. Upon entering the periplasm, the Fbp (ferric-binding protein) system is involved in the

import of the ferric iron acquired. The Fbp periplasmic binding protein consist of 2 similar domains resembling a Venus flytrap that binds to the ferric iron. It then transports it to the ABC permease to be translocated into the cytoplasm (Andrews et al, 2003).

1.4.2.3 Ferrous Iron Uptake

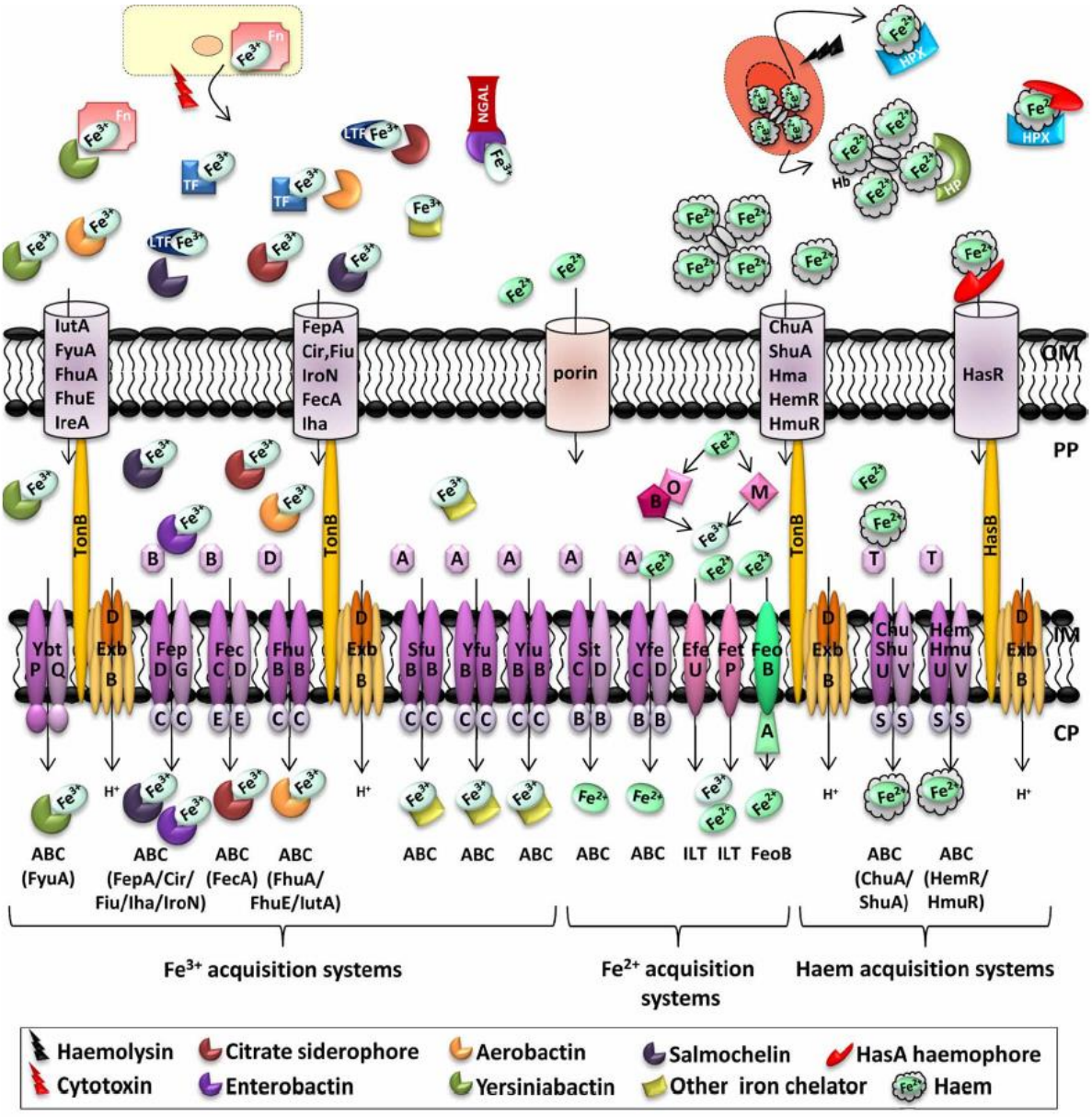


Figure 1.10 Iron transporters and iron acquisition systems. Bacteria utilises a variety of transport proteins to cater for the different forms of iron in the process of acquiring iron from its environment. From Porcheron et al, 2013.

As well as ferric iron, bacteria are also able to utilise ferrous iron and it is taken up via Feo (*Ferr*ous iron transport) system (Cartron et al, 2006). Ferrous iron is only present in anaerobic or low pH environments and is the preferred iron form due to its high solubility which allows direct transport

(Cartron et al, 2006). Feo acts independently from ferric iron uptake pathway as mutation in ferric iron uptake genes does not affect its activity (Cartron et al, 2006). The Feo system is encoded by the *feoABC* operon, producing three predicted proteins: FeoA, a small 75-residue hydrophilic protein with 'flexible' C-terminal domain as in DtxR (Andrews et al, 2003); FeoB, a large 773-residue transmembrane protein acting as a ferrous permease and FeoC, a small 78-residue hydrophilic protein (Cartron et al 2005). A *feoB* mutant showed a reduced ability to colonise mouse intestine, suggesting an inability to utilise ferrous iron (Tsolis et al, 1996). Mutants containing *feoB* and *tonB* defects showed a higher clearance rate than the wild-type, showing the inability to utilise both ferric and ferrous iron (Tsolis et al, 1996).

1.4.2.4 Haem and Haem-containing proteins uptake

Due to the high abundance of haem and haem-containing proteins in the host, they serve as a valuable bacterial iron source. Through evolution, bacteria developed novel strategies enabling acquisition of iron from host iron-sequestering proteins. Bacteria first secrete exotoxins which include cytolysins, haemolysins and proteases which lyse cells and release the enclosed haem compounds. Subsequently bacteria have specific OM receptors that can bind directly to the released haem or haem-containing proteins as well as haemophores (siderophore-like molecules) which bind haem, haemoglobin, haem-haemopexin and haemoglobin-haptoglobin.

1.4.2.4.1 Haemophore mediated uptake

Some bacteria are able to synthesise haemophore which extract haem from haemoglobin and haemopexin. Once removed, the complex is then delivered to the OM receptor where the haem is transported into the cell. HasA (haem acquisition system) is a haemophore secreted by *Serratia marcescens, Pseudomonas aeruginosa, Pseudomonas fluorescens, Yersinia pestis and Erwinia carotovora* to acquire haem (Krewulak and Vogel, 2008). Upon the capturing of haem, HasA shuttles it to specific cell receptor, HasR.

HasA is a monomer capable of binding one haem molecule with very high affinity. Although its receptor, HasR is sufficient for uptake of haem, HasA-HasR is a more efficient system for haem delivery to cells (Letoffe et al, 1999). The structure of HasA is described as 'fish biting the haem'. This

globular protein has 2 faces: a curved, 7-stranded antiparallel β-sheet on one face with 4 α-helices on the other face (Krewulak and Vogel, 2008). The binding site is solvent exposed, residing between 2 loops at the α/β interface of HasA. Upon binding, the loop containing His 32 closes on the haem molecule. His 32 is highly conserved between species (Krewulak and Vogel, 2008). HasA may directly capture haem from haemoglobin or bind to haem during spontaneous dissociation as it has higher affinity than globin for haem (Letoffe et al, 1999).

Uptake of haem like the uptake of ferric iron is a TonB dependent process. The OM receptor HasR, is a TonB-dependent receptor, which binds to HasA and allows the entry of haem. However, HasA is not internalised during haem uptake unlike siderophores, indicating an unknown mechanism is present at the cell surface which extracts the bound haem. A direct protein-protein interaction is formed between HasA and HasR in which HasR is able to bind both apo- and holo-HasA with high affinity. Since HasR binds to both loaded and unloaded HasA at high affinity, during haem uptake, apo-HasA may rapidly dissociate to allow a loaded HasA to bind or transfer haem from holo-HasA to the bound apo-HasA for uptake (Letoffe et al, 1993).

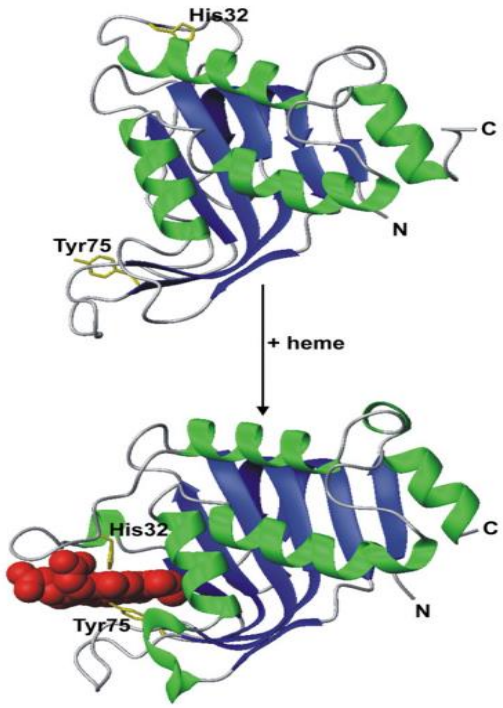


Figure 1.11 Structure of apo- and holo-HasA bound to haem. The loop containing His32 will close upon binding a haem molecule. From Krewulak and Vogel, 2008.

1.4.2.4.2 Haemoglobin and Haptoglobin-haemoglobin uptake

Two independent haem transport systems have been identified in *Neisseria meningitidis* for the uptake haemoglobin and haptoglobin-haemoglobin respectively. HmbR receptor is involved in haemoglobin uptake while HpuAB is required for haptoglobin-haemoglobin. These receptors are TonB-dependent and differ from each other whereby HpuAB contains an accessory lipoprotein and is able to facilitate iron acquisition from haptoglobin-haemoglobin complexes compared to HmbR (Rohde et al, 2002).

1.4.2.4.2.1 HmbR

HmbR is an 89-kDa protein located at the OM with a signal peptidase I recognition sequence in the amino-terminal region. The putative Loop 7 contains an invariable histidine residue and highly conserved amino acid sequence motifs FRAP and NPNL present in all haem-haemoglobin receptors. The putative extracellular Loop7 of HmbR is essential for haemoglobin utilisation but not required for haemoglobin binding. An amino-terminal deletion affects haemoglobin usage but not binding of haemoglobin, suggesting a role of the cork domain in the utilisation of haem (Perkins-Baling, 2004). The amino-terminal end also contains a putative six-amino-acid TonB box which interacts with TonB to supply energy to HmbR. HmbR binds haemoglobin with high affinity, capturing haem from haemoglobin and transport it into the periplasm (Perkins-Balding et al, 2004).

1.4.2.4.2.2 HpuAB

HpuAB is a two-component, TonB-dependent receptor which binds to haemoglobin, haptoglobin-haemoglobin and apo-haemoglobin (Rohde et al, 2002). HpuA is a 37kDa outer-surface anchored lipoprotein while HpuB is a 85kDa transmembrane protein that forms a gated porin in the OM, predicted to adopt the 'plug and barrel' structure (Rohde et al, 2002). HpuB is required for haem utilisation from haemoglobin or haptoglobin-haemoglobin . HpuAB is closely related to the transferrin (TbpAB) and lactoferrin (LbpAB) OM receptor systems. HpuB shares 25 and 40% similarity to TbpA and LbpA but Hpu A shows no homology with TbpB and LbpB. Both Hpu A and Hpu B are indispensable for receptor function; mutants lacking either component display the inability to utilise iron from haemoglobin or haptoglobin-haemoglobin (Rohde et al, 2002). Consistent with this

observation, HpuAB has only one high-affinity binding site and has a preference of binding oxidised methemoglobin over reduced haemoglobin (Rohde et al, 2002). Haem binds only weakly to HpuAB, indicating that haem is not the primary contact site of HpuAB (Rohde et al, 2002).This might be due to a minor recognition site for haem. There is no protein/protein complex with a recognition site less than 1000 $Å^2$ (Genco and Dixon, 2001). Haem is bound to haemoglobin, giving it limited exposure thus forming a minor portion of the recognition site upon binding to the receptors. The rest of the recognition specificity might largely be due to haemoglobin itself (Genco and Dixon, 2001). The initial recognition and binding of haemoglobin involves interactions of the receptor with the globin chains, inducing a conformational change in the ligand that reduces its affinity for haem (Rohde et al, 2002). Once extracted from haemoglobin and haptoglobin, the entire haem moiety is internalised into the cell (Rohde et al, 2002)

1.5 Gut Microbiota

The surface area of the human gut is estimated to be $200m^2$, approximately the size of a tennis court and it is major site for microbial colonisation. The nutrient rich environment of the gut also makes it a preferred colonisation site (Sekirov et al, 2010). It is packed with 100 trillion (10^{14}) microbes which is 10 times greater than the number of human cells present in the body (Sekirov et al, 2010) and encodes 100-fold more unique genes than the human genome (Qin et al, 2010). The collective microbes found in the gut are termed 'the microbiota', 'microflora' or 'normal flora' (Sekirov et al, 2010). The stomach and small intestine contain a low number and few species of bacteria due to the presence of acid, bile and pancreatic secretion, killing most ingested microorganisms. The large intestine however harbours a complex and dynamic microbial community with concentrations up to 10^{11} or 10^{12} cells/g of luminal contents (Guarner and Malagelada, 2003). The relationship between the host and microbiota is often referred as commensal rather than mutualistic whereby it involved various functions that benefit the host including protective function, structural function and various metabolic functions (O'Hara and Shanahan, 2006). With the advances in molecular techniques, it is now possible to determine and classify the microbial community present in the human gut. The use

of 16S rRNA together with various sequencing methods enables the detection of cultivatable and uncultivatable species present in the gut. The intestinal habit contains 300-500 different species of bacteria (Guarner and Malagelada, 2003) whereby strict anaerobes dominate over facultative anaerobes and aerobes by two to three orders of magnitude (Sekirov et al, 2010). Although there are many different species, they only belong to two major phyla that dominate the gut, namely Firmicutes and Bacteroidetes (Arumugam et al, 2011). Other phyla includes Actinobacteria, Proteobacteria, Verrucomicrobia and Fusobacteria (Arumugam et al, 2011). Bacterial composition also varies along the gut. The small intestine is enriched in the Bacilli class of the Firmicutes and Actinobacteria while Bacteroidetes and Lachnospiraceae families of Firmicutes are more abundant in the colon (Sekirov et al, 2010). There are four distinct bacterial habitats in the intestinal tract: the surface of epithelium, the crypts, the mucous gel layer and the intestinal lumen (Fanaro et al, 2003). Microbial activities is influenced by substrate availability, pH, O_2 tension, redox potential and distribution in the colon (Fanaro et al, 2003).

1.5.1 Acquisition of early gut microbiota

Various factors are involved in determining the composition of the infant gut: the mode of delivery, hygiene levels, infant diet and medications (O'Hara and Shanahan, 2008). The first encounter with the microbial world for the baby is during delivery. The mode of delivery, natural (vaginal delivery) or Caesarean (C-section), has a dynamic role in determining their microflora composition. In vaginal delivery, babies encounters the large number of bacteria present in the vaginal canal and perineal area, thus natural birth allows babies to acquire the same microflora as the mother (Orrhage and Nord, 1999).

The intestinal microbiota of infants showed similarities with the vaginal microbiota, indicating a transfer of microbiota (Sekirov et al, 2010). The gastric content of 5- to 10-minute old babies showed similar composition to their mothers' cervix, further suggesting that vaginal microflora are the first colonisers during vaginal delivery (Morelli, 2009).

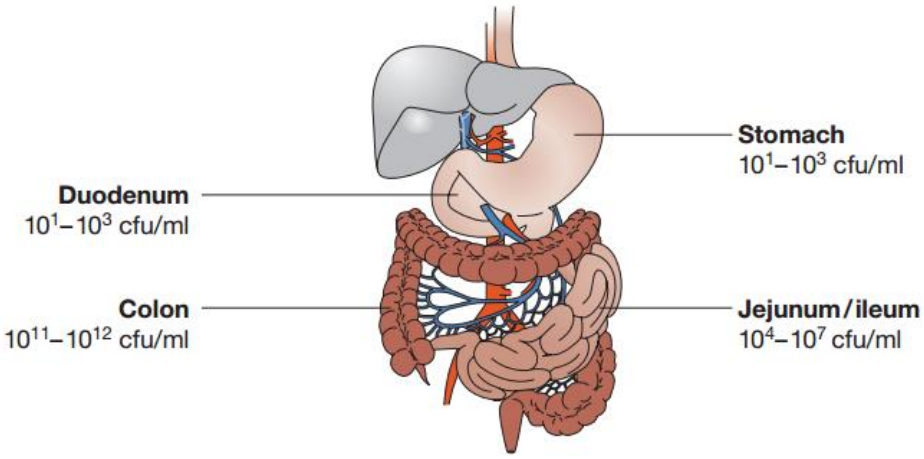


Figure 1.12 The distribution of bacteria along the human gut. The numbers of bacteria increases as it moves down the gastrointestinal tract. From O'Hara and Shanahan, 2006.

In C-section, the bacteria are introduced from the environment or hospital staff (Bezirtzoglou, 1997). They acquire a different microbiota from babies delivered vaginally. Newborns delivered via C-section have a reduced bacterial number compared to natural delivery and the appearance of bifidobacteria is delayed by up to 6 months (Morelli, 2008). Penders and colleagues showed that the colonization rate and counts of *B.fragilis* differed significantly between C-section and natural delivery. C-section newborns have a lower count of *B.fragilis* colonising their gut at 1 month of age and a higher frequency of *C.difficile* (Penders et al, 2006). C-section newborns also have a delayed acquisition of other *Bacteroides* species and *E.coli* (Adlerberth and Wold, 2009).

Besides delivery method, the environment in which babies are delivered can play a role in affecting gut composition. Babies born in a hospital (vaginally or C-section) acquire higher levels of *C.difficile* compared to babies born vaginally at home (Penders et al, 2006). This is considered to be due to the presence of *C.difficile* in hospital personnel and from the neonatal intensive care unit (NICU). This is also the case for preterm babies who spend most of the time in the NICU, where the effect is more significant with the combined use of antibiotics. The use of antibiotics suppresses the growth of most anaerobic bacteria, leaving clostridia as the only bacteria at detectable levels (Fanaro et al, 2003). Infants born before 37 weeks of gestation have the highest carriage rate of *C.difficile* compared to term infants (Penders et al, 2006). Sanitary condition of the delivery environment also

affect the initial gut microbiota of infants. C-section delivered infants in developing countries acquired *Bacteroides, E.coli* and *Bifidobacterium* very early. The gut is also colonised by *Enterobacteriaceae,* enterococci and lactobacilli earlier upon delivery than infants in the Western societies (Adlerberth and Wold, 2008).

The first bacteria to colonise the gut of newborns are usually facultative anaerobes which lower the positive redox potential of the gut at birth; this then facilitates the growth of strict anaerobes (Bezirtzoglou, 1999). These includes facultative anaerobes bacteria such as *Staphylococcus, Streptococcus* and *Enterobacteriaceae* species. The strict anaerobic bacteria that follow on include *Bifidobacterium, Clostridia, Eubacterium, Lactobacillus, Peptostreptococcus* and *Fusobacterium* (Bezirtzoglou, 1999). Orrhage and Nord compiled a table showing the shift of aerobic to anaerobic bacteria following birth to three months of age (Orrhage and Nord, 1999). Anaerobes are present at a low number during birth but there is an increase over time, highlighting the temporal changes of bacterial composition in the gut (Orrhage and Nord, 1999). The microbiota during the first year of life is simple and varies between individuals as well as time. After 1 year of age, the infant gut microbiota starts to stabilise and resembles that of a young adult (Sekirov et al, 2010).

1.5.2 Factors Affecting the Composition of Gut Microbiota

1.5.2.1 Diet

Diet is one of the main factors affecting the composition of gut microbiota of both infants and adults. In infants, besides the mode of delivery and delivery environment, the mode of feeding is also strongly influences the bacterial community (Matamoros et al, 2013). Breast and bottle-fed infants have different microbial composition. Infant formulas are made up of cow's milk with extensive modification. The composition of breast milk and infant formulas differs significantly which can contribute to the difference in the gut microbiota between the two groups.

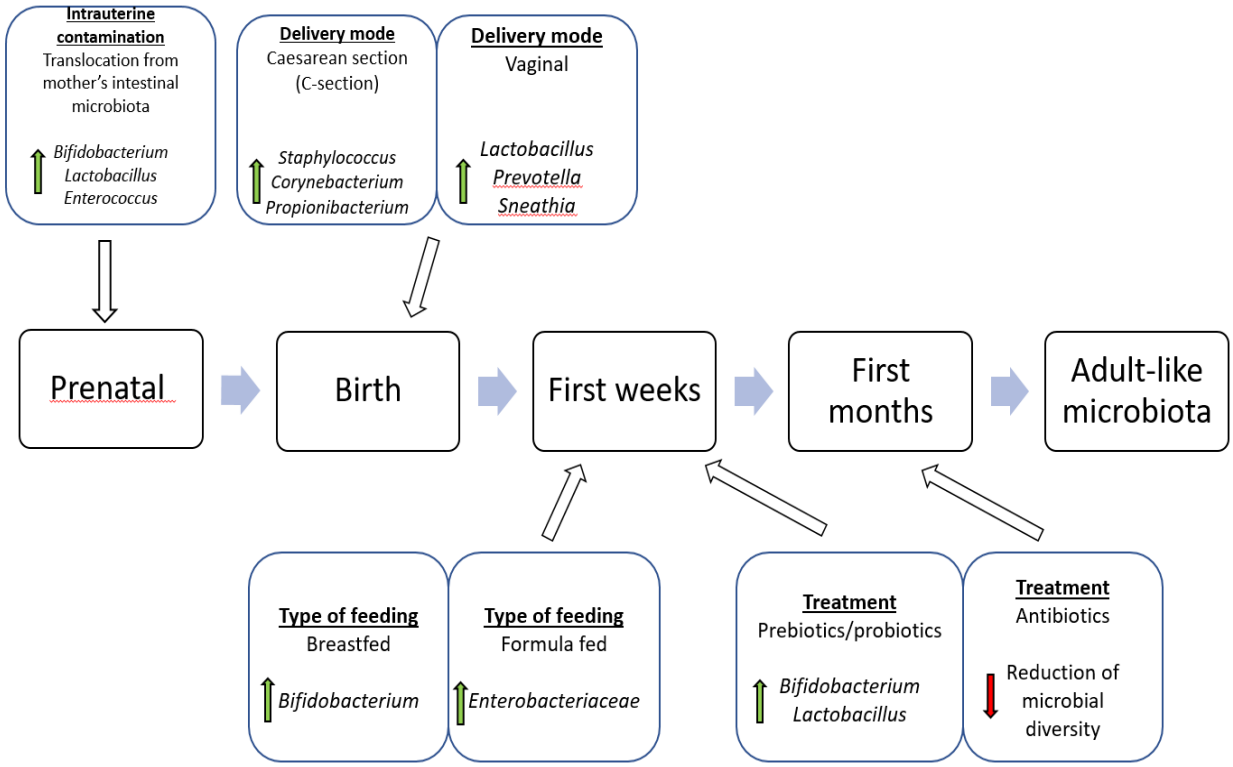


Figure 1.13 Factors affecting the bacterial composition of infant gut. Various factors are involved in the modulation of an infant gut microbiota which can be varied through delivery mode, feeding and use of medication.

The gut of a breastfed infant is predominantly colonised by *Bifidobacterium* (Bezirtzoglou et al, 2011; Balmer and Wharton, 1989; Bullen et al, 1976; Benno et al, 1984) and *Staphylococcus* (Balmer and Wharton, 1989). *Bacteroides, E.coli* and *Atopobium* are also present in the gut of a breastfed infant. (Bezirtzoglou et al, 2011). Bottle-fed infants carry a more diverse bacterial community. Although *Bifidobacterium* also dominates their guts, it occurs at a lower number (Bezirtzoglou et al, 2011; Benno et al, 1984). *E.coli, Clostridium* (*parputrificum, perfringens, difficile and tertium* species), *Bacteroides* species (*fragilis, thetaiotaomicron, uniformis* and *eggerthii*) and lactobacilli colonise the gut of exclusively formula-fed infants more so compared to breastfed infants (Penders et al, 2006; Benno et al, 1984; Balmer and Wharton, 1989). Clostridia were frequently found in formula-fed infant and can only be isolated from the guts of breastfed infants upon weaning (Benno et al, 1984). Enterobacteria and enterococci were also significantly higher in formula-fed infants compared to breastfed infants (Fanaro et al, 2003).

Breast milk is a good vehicle for bacterial introduction to the infant gut since babies consume roughly 800ml of milk per day which amounts to between $1x10^5$ to $1x10^7$ bacteria daily (Fernandez et al, 2013). Human breast milk is abundant in milk oligosaccharides which can be utilised by bifidobacterial and lactobacilli phylotypes (Fernandez et al, 2013). *Streptococcus, Staphylococcus, Bifidobacterium* and *Lactobacillus* are present in breast milk which could explain the early dominance of these bacteria in the infant gut as well as their role as probiotic bacteria (Matamoros et al, 2013). Bifidobacteria can appear as early as 4 days after birth for full-term breastfed infants and a diet of breast milk creates an environment favouring the growth of bifidobacteria after a week (Fanaro et al, 2003). The lower diversity of microbiota seen in breastfed infants could be due to the low buffering capacity of breast milk. Acid production from bacterial fermentation lowers the pH of the gut, making it a favourable growth environment for bifidobacteria to predominate (Bullen et al, 1976). The high buffering capacity of formula milk prevents the lowering of pH, creating a favourable growth environment for bacteria such as *E.coli* (Bullen et al, 1976).

Introduction of solid food has a significant effect on the gut microbiota. Although *Bifidobacteria* species still dominate the gut microbiota, there is a significant decrease in number and the bacterial composition is more diverse. The proportion of clostridia increases and the facultative anaerobes show a significant reduction in number. There is no change in *Bacteroides* species and these still represent one of the most predominant groups in the gut (Fallani et al, 2011). The pre-weaning method, either breastfed or formula-fed, has an impact on the gut microbiota after weaning started. Breastfed infants show a slower increase in *C.leptum* and a faster reduction of *C.difficile* and *C.perfringens* compared to formula or mixed fed (breastfed and formula-fed) infants (Fallani et al, 2011). The mode of delivery also has an impact on the bacterial community after weaning. In the C-section group, the delayed and low number of *Bacteroides* species colonisation persists and infants in this group show a higher proportion of *Bacteriodes* and *Atopobium* species. *Bacteroides* species were higher in vaginal delivery post-weaning (Fallani et al, 2011). As the amount of solids increase, infants acquire a gut microbiota similar to an adult (Stark and Lee, 1982).

Microbiota response to diet has been shown in an *in vitro* gut model. A diet high in protein is less favourable to bacteria belonging to the phyla of Firmicutes, Actinobacteria, Fusobacteria and Verrucomicrobia while it favours the growth of members in the Bacteroidetes phyla such as *Bacteroides ovatus* and *Bacteroides fragilis*. Propionate production is higher in the high-protein diet and this showed a correlation with the *Bacteroides* and *Prevotella* species (Aguirre et al, 2015). This clearly shows the different bacterial distribution among different diet regimes.

In a mouse model, mice fed with high fat diet showed reduced bacteria levels of the Cytophaga-Flavobacter-Bacteroides phylum. There was also a reduced number of bifidobacteria and members of the *Eubacterium rectale-Clostridium coccoides* group (Cani et al, 2007). While the mice used by Cani and colleagues are conventionally raised mice, Turnbaugh and colleagues studied the effect of diet and gut microbiota using humanised gnotobiotic mice which is more representative of the human gut. They inoculated mice with fresh human faecal samples between feeding them with a high-fat, high-sugar Western diet or a low-fat, polysaccharide-rich diet. Mice on the Western diet had an increase in the Erysipelotrichi class of bacteria within the Firmicutes phylum. The increase was most closely related to *Clostridium innocuum, Eubacterium dolichum* and *Catenibacterium mitsuokai*. There was also an increase in Bacilli and a significant decrease in members of the Bacteroidetes phylum. Mice on the low-fat, polysaccharide-rich diet were dominated by bacteria of the Bacteroidetes phylum and had a reduced number of bacteria in the Firmicutes phylum as compared to the mice on the Western diet (Turnbaugh et al, 2009).

A protein-rich, low carbohydrate diet that is usually used in weight management has caused a shift in the gut microbiota of humans. *Roseburia* spp. and *E. rectale,* a subgroup of the clostridial cluster XIVa showed a significant decrease as a fraction of bacteria as the carbohydrate intake was reduced. Numbers of bifidobacteria also were reduced with a decreasing carbohydrate intake. The reduction in population of *Roseburia* spp., *Bifidobacterium* spp. and *E. rectale* group may be due to the limited carbohydrate supply which was insufficient to support good growth. The reduced production of short chain fatty acids (SCFA) as an end production increases the luminal pH which is unfavourable

for the growth of these bacteria. Total SCFA production was lowered in the reduced carbohydrate group compared to the normal diet group (Duncan et al, 2007). Similar results were reported by Russell and colleagues using similar composition of the diet (Russell et al, 2011). Another report by Walker and colleagues clearly showed the relationship between diet and gut microbiota. Previous reports separated individuals into groups and compared their composition while Walker used the same individual but a different diet regime over a 10-week period. Subjects were provided successively with a control diet, a high resistant starch or non-starch polysaccharide diet and a high protein weight loss diet. The *Bacteroides/Prevotella* group of bacteria and *Faecalibacterium prausnitzii* where similar among all the diet groups and showed little change with the *Bacteroides/Prevotella* group being the most abundant as determined by qPCR. This might suggest that they are a dominant group in the gut and that diet has little effect on their composition. The *E. rectale, Roseburia* spp. and Ruminococcaceae group of bacteria increased significantly in response to resistant starch but showed a drop in the non-starch polysaccharide diet. *Bifidobacterium* spp. has similar abundance in diets containing carbohydrates but show a huge drop in the low carbohydrate weight loss diet. *E. rectale, Roseburia* spp. and *Bifidobacterium* spp. all showed a drop in numbers for the high protein, low carbohydrate weight loss diet. The reduction in numbers is in line with previous observations by Duncan et al (2007) and Russell et al (2011). The results from this experiment show that the gut microbiota is able to change over time depending on the diet regime of the host (Walker et al 2011).

Another study comparing children in Europe and rural Africa showed an impact of the diet on the gut microbiota. African children living in Boulpon in Burkina Faso had a diet mainly of cereals, legumes and vegetables, giving an extremely high carbohydrate and fibre diet, with very low animal protein. Italian children had a typical western diet high in sugar, fat, starch, animal proteins and low in fibre. The gut bacteria of the children belonged to 4 phyla: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. However, there was a difference in the proportion of the 4 phyla between the 2 groups of children. Actinobacteria and Bacteroidetes were more represented in

African children while Italian children have a higher abundance of Firmicutes and Proteobacteria. *Prevotella, Xylanibacter* and *Treponema* were present exclusively in African children which could be the consequence of high fibre intake such that their gut microbiota was adapted to allow the maximium metabolic energy extraction from the indigestible dietary components. It is not surprising that African children also had a higher total SCFA compare to Italian children as their diet is mainly carbohydrate which is fermentable to SCFA by bacteria (De Filippo et al, 2010).

1.5.2.2 Age

Gut microbiota changes with age and we acquire a different microbiota in different stages in life. The infant microbiota is very different from that of an adult and an adult microbiota is very different from that of an elderly person. Aging causes a decrease in intestine motility, leading to a longer transit time, affecting the dynamics of nutrient turn-over. Elderly individuals may have reduced dentition, reducing their chewing strength which can limit the food choice and affect microbial growth (O'Toole and Claesson, 2010). Biagi and colleagues analysed the gut composition of young adults (20-40 years old), elderly (60-80 years old) and centenarians (over a 100 years old). Centenarian gut microbiota was mostly enriched with Proteobacteria (*E. coli., Haemophilus, Klebsiella pneumoniae, Leminorella, Proteus, Pseudomonas, Serratia, Vibrio* and *Yersina*) and Bacilli (*Bacillus, Staphylococcus*). *Clostridium* cluster XIVa was lower in centenarians than in the elderly and young adult groups. *Papillibacter cinnamovorans* and *F. prausnitzii* showed a significant decrease in centenarians, while there was a significant increase in *C. leptum, Sporobacter termiditis, Anaerotruncus colihominis* and *Clostridium orbiscindens*. *Bacteroidetes* and *Firmicutes* still dominated the gut of centenarians but there were specific changes in relative proportion of *Firmicutes,* with a decrease in *Clostridium* cluster XIVa, an increase in Bacilli and rearrangement of *Clostridium* cluster IV composition. This rearrangement is seen with a change in the population of butyrate producers where there is a decrease in *Roseburia, Ruminococcus, E. rectale, E. hallii* and *Papillibacter cinnamovorans* coupled with an increase in *Anaerotruncus colihominis* and *Eubacterium limosun.* Bifidobacteria were also significantly lower in this extreme age group compared to young

adult (Biagi et al, 2010). In another study comparing elderly Irish adults (>65 years old) and younger adults (28-46 years old), there were huge differences in their gut compositions. The elderly Irish subjects had a lower proportion of *Firmicutes* compared to the younger population which is in line with the observation by Biagi mentioned above. Gut microbiota of the elderly subjects were made up mainly of *Bacteroidetes* (53%), including *Bacteroides, Alistipes* and *Parabacteroides* compared to only 8-27% in younger population. There was also a clear distinction between the *Clostridium* clusters. *Clostridium* cluster IV was predominant in older subjects whereas cluster XIVa is more prevalent in the younger group (Claesson et al, 2011), as observed by Biagi et al (2010). The shift in human GI microbiota was also shown in institutionalised elderly subjects. The elderly subjects had a lower bacterial count and diversity compared to younger subjects. In addition, they had a reduced abundance of bifidobacteria and *Clostridium* cluster IV (Zwielehner et al, 2009), again reflecting the results of Biagi et al (2010). All the above observations showed that gut microbiota is affected by age.

1.5.2.3 Antibiotics

Antibiotics are prescribed to treat infections. Different antibiotics are used to target specific pathogens, Gram-positive or Gram-negative bacteria. Antibiotics cause short term and long-term alteration to the gut microbiota. Administration has been shown to reduce the abundance and diversity in the digestive tract (Panda et al, 2014). β-Lactam antibiotics binds to penicillin-binding proteins located in bacterial cell walls and interfere with cell wall synthesis. They have a broad-spectrum activity against Gram-negative and Gram-positive bacteria. Fluoroquinolone antibiotics are broad-spectrum antibacterial agents but they have limited effect on anaerobic bacteria. They are often used to treat intracellular pathogens such as *Legionella pneumophila* and *Mycoplasma pneumoniae* (Panda et al, 2014). Seven days antibiotic treatment causes a global change in microbial community structure. Antibiotic treatments affect microbial abundance and composition (Panda et al, 2014). Administration of amoxiclav® and levofloxacin® causes a significant decrease in microbial diversity by around 20% while increasing the ratio of Bacteroidetes/Firmicutes (Panda et al, 2014).

Subjects on ciprofloxacin® (Cp) treatment showed a significant decreased taxonomic richness. The *Clostridiales* were completely eliminated after Cp treatment. *Bilophila wadsworthia* was reduced post-Cp treatment but had a tendency to rebound to pre-Cp treatment levels after treatment. However, during Cp treatment, butyrate-producing organisms are maintained or increased, including *Butyrivibrio fibrisolvens* and *Roseburia intestinalis* (Dethlefsen et al, 2008). Antibiotic therapy involving ampicillin/sulbactam and cefazolin shifted the dominance of Firmicutes towards Bacteroidetes. Prior to administration of antibiotics, the majority of the gut bacterial belonged to the Firmicutes phylum. However, there was a marked shift towards Bacteroidetes and an increase in β-proteobacteria. Antibiotic treatment also causes a loss of taxa post treatment. This includes *Slackia* and *Bifidobacterium* genera (Actinobacteria), *Gemmiger* genus (Betaproteobacteria), *Streptococcus* genus (Streptococcaceae), *Barnesilla* genus (Porphyromonadaceae) and *Eubacterium* and *Subdoligranulum* genera (Clostridiales). Administration of antibiotics reduced the bacterial diversity during the course of treatment but its diversity was slowly restored once the treatment ended. *Firmicutes* also regained their dominance after the antibiotic therapy (Perez-Cobas et al, 2012).

1.5.3 Functions of Gut Microbiota

1.5.3.1 Vitamin Synthesis

Intestinal microbiota are involved in the production of a series of vitamins that can be absorbed and utilised by the host. Vitamins are essential micronutrients that participate in various vital biochemical reactions within the cells. The intestinal microbiota is capable of synthesising vitamin K as well as B vitamins, including cobalamin, folates and riboflavin (LeBlanc et al, 2013). In contrast to the uptake of dietary vitamin which occurs in the small intestine, bacterial synthesis of vitamins occurs in the colon (LeBlanc et al, 2013).

Folate is a cofactor involved in many metabolic reactions such as biosynthesis of building blocks of DNA and RNA (Sybesma et al, 2003). Bacterially synthesised folate and cobalamin can affect the host DNA methylation patterns whereas the acetate produced from microbial fermentation is able to modify chromatin structure and gene transcription by histone acetylation (Kau et al, 2011). Folate is usually synthesised by lactic acid bacteria using GTP, *p*-aminobenzoate (PABA) and glutamate as

building blocks (Sybesma et al, 2003). *Lactobacillus* strains with the exception of *L. plantarum* are unable to produce folate. *L. plantarum, L. delbrueckii, L. sakei, L. helveticus, L. reuteri* and *L. fermentum* contains folate biosynthesis clusters which have genes encoding the dihydropteroate synthase and all genes for DHPPP biosynthesis which the exception of alkaline phosphatase (Rossi et al, 2011). These bacteria are thought to be potential folate producers in the presence PABA. Analysis of nucleotide sequence data revealed that members of *Lactobacillus* are unable to synthesise PABA *de novo* except for *L. plantarum* which has a complete shikimate pathway for chorismate production, making it the only member of the *Lactobacillus* capable of producing folate (Rossi et al, 2011).

Bifidobacteria are expected to produce chorismate as they contain the entire set of genes for the shikimate pathway. However, only strains of *B. adolescentis* and *B. dentium* possess the 4-amino-4-deoxychorismate lyase which is able to complete the de novo biosynthesis of PABA. The cluster of *fol* genes encoding dihydropteroate synthase and some enzymes for DHPPP biosynthesis is found in all bifidobacteria, enabling them to perform the condensation reaction between PABA and DHPPP (Rossi et al, 2011). Although bifidobacteria contains most of the genes needed for folate production, only *B. adolescentis* and *B. dentium* possess the complete set of genes, making them the only bifidobacterial species capable of producing folate *de novo*. *B.longum* is also capable of *de novo* folate production but it requires the presence of PABA. The ability of bifidobacteria to produce folate has been shown in animal models. Pompei and colleagues showed that administration of bifidobacteria as a probiotic in Wistar rats gave a significant increase in their serum folate concentration compared to rats in the control and prebiotic groups. The effects were enhanced by administrating a symbiotic (prebiotic plus probiotic). The marked increase in folate concentration is due to the abundance of folate producing bifidobacteria (Pompei et al, 2007b). In another study by Krause and colleagues, different kinds of milk (human, cow, goat) were administered to Sprague-Dawley rats. Human milk gave significantly higher numbers of bifidobacteria than the other kinds of milk in the experiment. The mean concentration of bifidobacteria in the cecal and colonic samples of

rats consuming human milk solids was 7-fold and 2-fold greater respectively than the other treatment group. At the end of the folate-repletion period, the mean plasma folate concentration of rats in the human milk group was significantly higher than the other group. The plasma folate concentration was significantly correlated with the abundance of bifidobacteria presence in the ceca and the colons. The increase in colonisation of bifidobacteria in rats consuming human milk solids enhanced the net microbial folate production, improving the folate status of the rats (Krause et al, 1996).

Riboflavin has an important role in many cellular metabolism pathways. It is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) where both molecules are hydrogen carriers participating in many biological redox reactions (LeBlanc et al, 2011). Milk and dairy products, meat, cereals and green leafy vegetables are a good source of dietary riboflavin. Riboflavin deficiency is more prevalent in developing countries but it has also been observed in adolescent girls in the UK and among the Irish population (Burgess et al, 2009). *Bacillus subtilis* and *E.coli* have been studied extensively for their ability to produce riboflavin. The biosynthesis of riboflavin requires precursors GTP and ribulose-5-phosphate (Burgess et al, 2009). Riboflavin is synthesised via a series of dephosphorylation, condensation and dismutation reactions using GTP and ribulose-5-phosphate as building blocks. Riboflavin formation requires 1 equivalent of GTP and 2 equivalents of ribulose-5-phosphate (Bacher et al, 2001). Riboflavin is then converted by the bifunctional flavokinase/FAD synthetase to its coenzyme forms, FMN and FAD (Burgess et al, 2009). Bacterial production of riboflavin has been shown in animal studies. LeBlanc and colleagues fed riboflavin depleted Wistar rats with yoghurt prepared with riboflavin producing bacteria as a starter culture and showed improved riboflavin status in erythrocytes compared to conventional yoghurt and yogurt prepared with non-riboflavin producing bacteria as starter culture (LeBlanc et al, 2006). The rats showed a similar vitamin B2 status as the rats receiving 0.5mg/kg vitamin B2 supplement, indicating the riboflavin produced in the yogurt showed similar bioavailability as commercially available pure riboflavin. Riboflavin deficiency has been linked to stunted growth due to its failure to participate in

various biochemical reactions. However, rats fed with bacterial produced yoghurt had significant increase in growth compared to the other groups, indicating the presence of riboflavin in supporting growth (LeBlanc et al, 2006). This shows the ability of bacteria to produce riboflavin needed to sustain growth.

1.5.3.2 Degradation of Complex Carbohydrates

The mammalian genome does not encode enzymes needed for polysaccharide degradation. To overcome this, mammalian hosts developed a complex mutualistic dependence with symbiotic gut microbes that have the ability to access this abundant source of energy (Flint et al, 2012). During the degradation process, bacteria demonstrated a cross-feeding pattern that generates various end products that are utilised by the host. This relationship is shown in coculture of *B.adolescentis* L2-32 and *E.hallii. B.adolescentis* L2-32 grown on potato starch as substrate produces lactate as an end product. Butyrate-producing *E.hallii* L2-7 is unable to grow in a pure starch environment but is able to utilise the lactate produced by other strains. A drop in lactate concentrations and an increase in butyrate formation is observed in the presence of *E.hallii* L2-7, suggesting the cross-feeding relationship (Belenguer et al, 2006). In the gut environment, cross-feeding of lactate produced by bifidobacteria is able to stimulate the growth of butyrate-producing bacteria, increasing the concentration of butyrate (Belenguer et al, 2006).

There are a total of 130 families of glycoside hydrolases, 22 of polysaccharide lyases and 16 of carbohydrate esterases identified in all life forms and most of them are encoded in the microbial genome (Flint et al, 2012). Around 20-60g of dietary carbohydrates are estimated to reach the colon each day. They are usually resistant starch, plant cell wall polysaccharides and non-digestible oligosaccharides (Flint et al, 2012).

Bacteroides spp. are able to break down a range of indigestible dietary plant polysaccharides. *B. thetaiotaomicron* in particular can breakdown a wide range of polysaccharide. This ability is due to its possession of the largest repertoire of genes involved in the polysaccharide acquisition and

metabolism. It can encode 226 predicted glycoside hydrolases and 15 polysaccharide lyases as well as 163 paralogues of 2 outer membrane proteins involved in binding and importing of starch. More than half of the enzymes produced to degrade carbohydrates by *B.thetaiotaomicron* are predicted to be secreted into the periplasmic or extracellular space, thus liberating mono- and oligosaccharide from dietary polysaccharide (Sonnenburg et al, 2005). *Bacteroides* and *Roseburia* are xylanolytic bacteria, which are able to digest hemicelluloses extensively in the human gut (Chassad et al, 2006). End products of the microbial fermentation of the substrate are acetate, succinate, propionate and lactate by *Bacteroides* and butyrate, formate and lactate for *Roseburia* (Chassad et al, 2006). Bifidobacterial strains have been shown to have the ability to degrade starch, amylopectin and pullulan. Ryan and colleagues identified 11 strains of *Bifidobacterium* that are able to digest all 3 polysaccharides, 5 strains from *B. breve,* 3 from *B. pseudolongum* while one strain each from *B. dentium, B. infantis* and *B. thermophilum* (Ryan et al, 2006). The ability of bifidobacteria to digest pullulan suggests that it can be used as a novel prebiotic to stimulate the growth of bifidobacteria. The ability of bifidobacteria to utilise complex carbohydrate enables the development of prebiotics which consist mainly of oligosaccharides that can stimulate growth of these beneficial bacteria. These includes xylo-oligosaccharides (XOS), galacto-oligosaccharides (GOS), inulin and fructo-oligosaccharides (FOS). The use of prebiotics to enhance the growth of bifidobacteria can be beneficial as these bacteria demonstrated antibacterial activity such as competitive exclusion of *E.coli*, anticarcinogenic effect by metabolising nitrosamine and stabilising the gut mucosal barrier (Gomes and Malcata, 1999).

Intestinal microbial fermentation of dietary carbohydrates results in the formation of short chain fatty acids (SCFA). They are 2-carbon to 5-carbon weak organic acids which includes acetate, butyrate, propionate and valerate. The production of these acids lowers luminal pH in the proximal colon which boosts the formation of butyrate as the acidic environment favours the growth of butyrate-producing bacteria (Canani et al, 2011). Butyrate has the ability to exert a powerful proabsorption stimulus on NaCl transport and an anti-secretory effect on Cl^- secretion. Butyrate has

been shown to have an anticancer effect. Butyrate is able to inhibit the proliferation of and induce apoptosis of tumour cells. In the human colon cancer cell lines, butyrate acts as a HDAC (histone deacetylase) inhibitor which hyperacetylates histones. This increases p21 gene expression through selectively regulating the degree of acetylation of gene-associated histones and induces G1 cell cycle arrest. In contrast to tumor cells, butyrate stimulates the proliferation of cells in the basal crypts of the colon (Canani et al, 2011). Butyrate stimulates the *MUC2* gene and induces mucin synthesis which affects the mucus layer, giving an enhanced protection against luminal agents. The HDAC inhibitory effects of butyrate increase the *LL-37* gene which can strengthen the innate immune system (Canani et al, 2011). Administration of butyrate to mice prevents the development of dietary obesity and insulin resistance. It also reduces obesity and increases the insulin sensitivity in obese mice. Butyrate may increase energy expenditure and fatty acid oxidation, exhibiting an antiobesity effect. There is also an increase in PGC-1α, PPAR-γ and CPT1b. As mentioned earlier, the HDAC inhibitory properties of butyrate may contribute to the increased expression of these genes (Gao et al, 2009). An increase in SCFA is able to increase the solubility of calcium, promoting proliferation of enterocytes which increases the absorption as well as the expression of calcium binding proteins (Scholz-Ahrens et al, 2007).

1.5.4 Effects of iron on the gut microbiota

Various factors affect the composition of human gut microbiota with diet as the predominant factor as mentioned above. However, the composition can be manipulated through supplementation, notably prebiotics which selectively stimulate the growth of certain bacterial groups (Tuohy et al, 2003). A lot of research has been focusing on the effects of macronutrients on gut microbiota but little and limited research has been conducted to investigate the relationship between micronutrients and their effect on gut bacteria. Although micronutrients are needed in small quantities as compared to the macronutrients, they exert significant effects on the gut microbiota and can affect the overall wellbeing of the host. Few early studies have looked at the effect of iron and the gut microbiota but due to the lack of a sophisticated analysis method at that time,

researchers were only able to identify the bacteria based on culturing techniques rather than using the more advanced techniques such as NGS (454 pyrosequencing, Illumina sequencing) that are present today. Culturing bacteria on plates may underestimate the presence of certain bacteria species due nutrient limitation and growth environment. The culturing technique was also unable to differentiate the bacteria into more specific taxonomic rank and tend to group the bacteria at a higher rank. Nevertheless, it provides some useful information and shows the link between iron intake and its effect on the gut microbiota.

1.5.4.1 Human studies

Balmer and colleagues showed the difference between the microbiota acquired by infants receiving different feeding methods, breast- or bottle-fed. Although the composition of breast milk and formula milk differs slightly, one major difference is the amount of iron between them. Formula milk contains about 10.5 times more iron than breast milk, thus it can be assumed that iron contributes to this difference in the gut bacteria makeup but the different types of proteins may also be a potential factor. Human breast milk contains more whey than casein while commercially available formula milk may either be whey- or casein-rich (Balmer et al, 1989c). In order to show the relationship between dietary intake and the gut microbiota, Balmer and colleagues conducted a series of experiments, addressing both the effect of protein types as well as the iron content, which eliminates bias in the results (Balmer et al, 1989b; Balmer et al, 1989c). Staphylococci and bifidobacteria are the predominant species found in breast-fed infants compared to formula-fed infants which enterococci dominate the gut. An increase in coliforms, staphylococci and bifidobacterium and a decrease in enterococci numbers is seen in breast milk infants while the reverse trend occurs in formula-fed infant. Formula-fed infants has a reduced number of staphylococci and an increase in clostridia, enterococci, *E.coli* and lactobacilli (Balmer and Wharton, 1989a). Results obtained by Balmer are in agreement with an earlier study by Benno and colleagues which also looked at the same area (Benno et al, 1984). A similar result is seen when comparing the types of proteins fed to the infants (Balmer et al, 1989b). Breastfed infants have higher lactobacilli

and bifidobacterium counts and a reduced number of *E.coli* compared to the formula-fed infants (whey and casein). In addition, differences can be seen comparing whey and casein formulae as the former have higher counts of bifidobacterium and showed an increase in coliforms, resembling closely to the breastfed infants (Balmer et al, 1989b). This is in agreement as breast milk contains more whey than casein as mentioned before. Although proteins in milk affect the gut microbe composition, Balmer also showed the effect of iron by supplementing formula milk with either lactoferrin or lactoferrin and iron (Balmer et al, 1989c). Lactoferrin is found in high abundance in human milk where concentrations can reach 7mg/ml and at least 1mg/ml in colostrum and mature milk respectively (Brock, 1980). Lactoferrin binds two ferric ions and two bicarbonate ions synergistically and this has been linked to limit the availability of iron to bacteria as a way of preventing the colonisation of harmful pathogens in the infant's gut (Brock, 1980). Babies that were given formula milk fortified with iron and lactoferrin had an increase in *E.coli* and lower staphylococci counts. In addition, bifidobacteria were eliminated after 2 weeks of feeding compared to the control or the group that contains only lactoferrin. The researchers concluded that addition of iron shifted the gut microbiota away from those seen in breastfed babies (Balmer et al, 1989c). However, surprisingly the addition of lactoferrin does not show any affect to the gut microbiota but the effect is only apparent with the addition of iron. Babies taking milk containing lactoferrin harvest a microbiota similar to the control rather than to that of the breastfed babies. The researchers explained that the use of bovine lactoferrin rather than human lactoferrin may induce a foreign protein response, making it ineffective. Another possible explanation is the lack of bovine antibodies (IgG), lysozyme and bicarbonate which inactivate bovine lactoferrin, thus making it inactive (Balmer et al, 1989). In a follow up study 2 years later, Balmer and colleagues fortified whey and casein formula milk used in previous study with iron and noticed a significant change in the gut microbiota. Regardless of the types of milk, iron encourages the growth of enterococci and clostridia while it limits the growth of staphylococci and bacteroides. In addition, the presence of iron unfavourably promotes the growth of *E.coli* and lowers the beneficial bifidobacteria and lactobacilli which can

affect the health and wellbeing of the infant. Together, this can clearly prove that iron affects the bacteria residing in the gut (Balmer et al, 1991).

1.5.4.2 Animal models

In animal models, mice kept on an iron-deficient diet has significant higher counts of lactic acid bacteria (enterococcus and lactobacillus) compared to iron replete and iron excess diet (Tompkins et al, 2001). In addition, a lower overall bacterial number is observed in iron-supplemented mice, possibly due to the increased redox potential caused by iron (Tompkins et al, 2001). Similar results are obtained in an iron depletion and repletion study. *Lactobacillus/Leuconostoc/Pediococcus* spp. and *Enterobacteriaceae* significantly decreased in iron-depleted rats while *Bacteroides* spp. and *Roseburia* spp./*E.rectale* increased in number (Dostal et al, 2012). The opposite trend is seen in iron-sufficient rats and also during iron repletion with ferrous sulphate and electrolytic iron, indicating iron has an effect on the gut microbiota (Dostal et al, 2012). Another study conducted using weaning pigs showed that feeding piglets with increasing concentrations of iron has no effect on *Bifidobacterium* spp., *Lactobacillus* spp., *Clostridium* spp. as well as on the total anaerobic bacterial counts but there is an increase in coliform counts in the iron-supplemented groups. Analysis of diarrheal faeces (possibly caused by gastrointestinal infection or irritant effect of iron) showed a lower total bacterial number, *Bifidobacterium* spp., *Lactobacillus* spp., *Clostridium* spp. and a higher coliform count compared to normal faeces indicating that extremely high levels of iron may cause changes to the microbiota (Lee et al, 2008). The growth-stimulating effect of iron on coliform causes an increase in *E.coli,* the major coliform bacterium which can competitively exclude *Bifidobacterium* spp. and *Lactobacillus* spp., thus reducing their population as seen in diarrheal faeces (Lee et al, 2008). Pigs are more tolerant to dietary iron which can explain the same microbiota isolated in normal faeces in both supplemented and non-supplemented groups. The limitation of both studies is that the investigators only isolate lactic acid bacteria and not other bacteria species which limits our understanding of the effect of iron on the microbiota using an animal model.

1.6 Effects of iron on health and wellbeing

Iron is primarily absorbed in the small intestine, mainly the duodenum and upper jejunum via DMT-1 found on the surface of the enterocytes (Abbaspour et al, 2014). However, a large amount of iron failed to be absorbed and is passed to the colon which is subsequently discarded from the body. Faecal iron concentrations of healthy individuals can reach on average 100µg/g wet weight faecal samples or equivalent to 1.8mM and this value can increase nearly 3.5 times with supplementation (Lund et al, 1999). The high levels of iron reaching the colon may be explained by its low bioavailability and reduced rate of absorption by the host. As iron passes through the gastrointestinal tract (GI), it faces fluctuations in pH, moving from a very acidic environment in the stomach to a near neutral pH in the colon (Evans et al, 1988). The increase in pH further down the GI reduces the solubility of ferric iron as well as promotes the oxidation of ferrous iron in the presence of oxygen, which together lowers its bioavailability. Another possible explanation for this event is the formation of iron complexes with food components, notably polyphenols and phytates. Polyphenols are found in large quantities in daily favoured drinks such as tea and coffee while the latter is found in legumes, grains and nuts. Many studies have shown the antinutritional effects of these compounds as they bind tightly to various di- and trivalent cations (Fe, Zn, Ca, Mg) which prevents the host from absorbing them. Hurrel and colleagues as well as Morck and colleagues have both demonstrated the effects of tea and coffee on iron absorption respectively (Hurrel et al, 1999; Morck et al, 1983). Hurell tested a range of beverages but black tea had the greatest inhibitory effect on iron absorption. It showed a 70% smaller absorption rate even when it is diluted to 5% of the initial concentration, indicating the strong binding capacity of polyphenols (Hurrell et al 1999). In terms of coffee, a greater inhibitory effect was seen. Depending on the types of coffee, drip or instant coffee showed a reduced absorption rate of 72% and 83% respectively when ingested with a meal (Morck et al, 1983). The inhibitory effect of food compounds on iron absorption will lead to an accumulation of iron in the gut.

Although iron is an essential metal to the human body, an excess of iron can have negative effects on the host. It can form harmful radicals that damage biological component, trigger bacterial infection and cause diseases by stimulating bacterial growth as well as complicating intestinal diseases namely Inflammatory Bowel Disease (IBD). Various factors can contribute to the excessive accumulation of iron in the large intestine which includes complex formation with food compounds, admission of iron supplementation or inflammatory conditions such as celiac and Crohn's disease.

1.6.1 Iron and free radicals

Iron is involved in many cellular activities such as oxygen utilisation, cell growth, enzymatic activities and immune responses (Jomova and Valko, 2011). However, iron is also able to participate in Fenton and Haber-Weiss reactions which produce highly reactive hydroxyl radical. Although the half-life of the hydroxyl radical in aqueous solution is less than 1ns, the production of free radicals near DNA can react with both its purine and pyrimidine bases as well as the deoxyribose backbone resulting in damaged bases or strand breaks (Jomova and Valko, 2011; Valko et al, 2006). Figure 1.14 shows an example of the reaction between a free radical and purine base (guanine) and the formation of 8-hydroxyguanine (8-OH-G) which is carcinogenic and mutagenic.

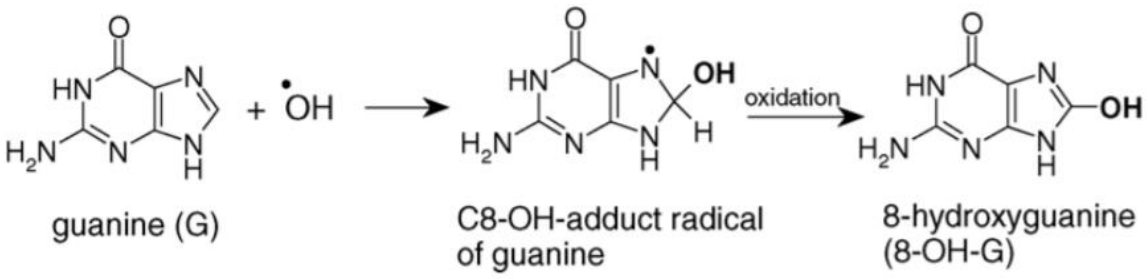


Figure 1.14 Reaction of guanine with the hydroxyl radical. Guanine is attacked by a free hydroxyl radical which forms an intermediate before being oxidised to of 8-hydroxyguanine. From Valko et al, 2006

In addition to damaging DNA, hydroxyl radicals can trigger a chain reaction that generates more free radicals by reacting with compound such as lipids and proteins. Metal-induced oxygen radicals are able to oxidise polyunsaturated fatty acids, causing lipid peroxidation. Lipid peroxidation occurs in three stages: initiation, propagation and termination with malondialdehyde (MDA) and 4-hydroxy-2-

nonenal (HNE) formed as end products. During the propagation stage, free iron can target the lipid hydroperoxides produced, initiating additional lipid peroxidation which affects the integrity of membrane structure as well as function (Emerit et al, 2001). Iron supplementation has been shown to increase oxidative stress in anaemic women as displayed by an increase in lipid peroxidation products accompanied by a decrease in vitamin C and vitamin E levels. Vitamin C participates in the scavenging of free radicals in the gut as well as the reduction of ferric iron to ferrous iron for absorption which can explain the lower levels of radicals upon iron supplementation. Lipid-soluble vitamin E on the other hand is a chain-breaking antioxidant which terminates the propagation of free radicals and the decrease in concentration of radicals post-supplementation (Tiwari et al, 2011). However, iron supplementation improves the blood profile of anaemic women as well as restores and reactivates antioxidant enzymes such as catalase and superoxide dismutase, which helps in scavenging free radicals (Tiwari et al, 2011).

As for protein, hydroxyl radicals have the tendency to abstract a hydrogen atom from the protein backbone, forming carbon centred radicals which react with dioxygen to form peroxyl radicals which subsequently convert to alkyl peroxides upon reacting with protonated superoxide. Side chains of amino acid are most susceptible to attack by various radical species, forming a range of end products (table 6.1) (Valko et al, 2006).

Table 1.2 End products of reaction between various amino acids and radical species

Amino acid residues	End product (s)
Arginine	Glutamic semialdehyde
Glutamate	4-hydroxy-glutamate
Histidine	2-oxo-histidine
Tyrosine	3,4-dihydroxy phenylalanine
	Tyr-tyr cross-linked proteins
	3-nitro-tyrosine
Valine	3,4-hydroxy-valine
Cysteine	cys-S-S-cys
	cys-S-S-R
Methionine	Methionine sulphone
	sulphoxide

Inter- and intra-protein cross linkages can also be formed as a result of protein oxidation by radicals. These linkages include addition of amino groups to carbonyl groups of oxidised proteins, interaction between two carbon-centred radicals formed through the abstraction of hydrogens from the protein backbone by hydroxyl radicals, oxidation of tyrosine to form –tyr-tyr– crosslinks and oxidation of sulphydryl groups of cysteine to form –S-S– crosslinks (Valko et al, 2006).

1.6.2 Iron and bacterial infection

In recent years, Zimmermann and colleagues also demonstrated the effects of iron on gut microbiota in African children. Iron-fortified biscuits were given to anaemic schoolchildren over a 6 month trial period. Iron not only did not improve the iron status or cure anaemia, but caused an increased growth of harmful pathogens which induce gut inflammation (Zimmermann et al, 2010). Although there was no significant change in total bacterial numbers between the start and end of the trial, an increase in enterobacteria and a decrease in lactobacilli occurs in the iron supplemented group. *Salmonella* spp. was present at low numbers in the children initally but it was more prevelant in children receiving iron-fortified biscuits than the control after 6 months, indicating an increased risk of infection (Zimmermann et al, 2010). The low bioavailability of elemental iron powder and poor iron absorption capacity caused by systemic inflammation of the children reduced the uptake of iron, leading to the failure to improve iron status as well as accumulation of a large amount of iron in the gut, potentially feeding the microbiota (Zimmermann et al, 2010). Thus, this clearly showed a negative effect of over-supply of iron on the gut microbiota.

Iron supplementation has been linked to increased susceptibility of the host to infection. Although the host secretes various proteins to bind freely available iron such as the secretion of lactoferrin at the mucosal surfaces as part of the mechanism to prevent bacteria from accquring iron, pathogenic bacteria are able to overcome this through the secretion of siderophores that strip iron from lactoferrin or expression of lactoferrin receptor on their surface (Cassat and Skaar, 2013; Kortman et al, 2014). Studies conducted on guinea pigs showed that intraperitoneal injection of *E.coli* 0111/B4/H2 combined with iron compounds had an increased virulence and is able to kill the host

within 2 days with just a few hundred bacteria. Without any iron supplementation, it took at least 1000-fold more bacteria to kill the host. The bacterial virulence increases with the concentration of iron supplemented, showing the enhancing effect of iron in aiding the colonisation and infection of pathogens (Bullen et al, 1968). Iron also plays an important role in bacterial colonisation and survival in the host. FeoB mutants of *Campylobacter jejuni* which have an impaired iron uptake system fails to transport iron into the cytosol, leading to an accumulation in the periplasmic space (Naikare et al, 2006). These mutant strains also accumulated 1.5-fold less iron and showed poorer growth compared to the wild-type. A lack of ferrous iron uptake affects the survival of *C.jejuni* within the intestinal cells as well as their ability to colonise the gut. Survivability test of both wild-type and mutant strain using human INT-407 embryonic intestinal cells and porcine IPEC-1 small intestine epithelial cells indicates a 5- and 8-fold inhibition in the cell lines repectively at 72h, although they showed the same persistence for 48h. This effect might be due to the depletion of its iron stores that is sufficient for the first 48h and the failure to import iron quickly diminishes their survival capability (Naikare et al, 2006).

The colonisation capacity is highly reduced in FeoB mutants when they are introduced into animal models. Pathogenic bacteria such as *C.jejuni*, *H.pylori* and *S.typhimurium* that carry a *feoB* mutation all fail to colonise their host compared to the wild-type (Naikare et al, 2006; Velayudhan et al, 2000; Tsolis et al, 1996). When chicks and piglets are orally innoculated with *C.jejuni,* the mutants are significantly affected in their colonising ability and are outcompeted by the wild-type strain. Interestingly, *C.jejuni* which harbours a ferric-siderophore uptake system mutation also have an altered colonisation ability and fail to colonise the host, indicating the importance of iron in ensuring successful colonisation and infection establishment in the host (Naikare et al, 2006).

Iron increases the tendency of pathogens to adhere to epithelial cells which explains the failure to colonise the gut of the host in mutant strains. *S.typhimurium, C.ferundii and E.coli* all have increased adhesion ability after pre-incubation with ferric citrate. In addition, *S.typhimurium* adheres better to mucus-producing E12 cells than Caco-2 cells which better reflect the condition of the human

intestine (Kortman et al, 2012). In order for *S.typhimurium* to cause systemic infection, it needs to translocate across the epithelial barrier and this was enhanced by pre-incubation with iron. The efficiency increases with an increasing concentration of iron but a high concentration has the reverse effect which is probably due to the disturbance in invasion factors under excessive iron (Kortman et al, 2012). Together, excessive iron accumulation in the gut can have a negative effect on the host's overall health and wellbeing.

1.6.3 Iron and inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) is a collective medical term that consists of ulcerative colitis (UC) and Crohn's disease (CD) which are relapsing, chronic and immunologically mediated disorders (Sartor, 2005). Pathogenesis of IBD is hypothesised to be caused by immune response towards a subset of commensal enteric bacteria in genetically susceptible hosts while the reactivation of the disease can be triggered by environmental factors (Sartor, 2005). Recurrent anaemia occurs in a third of IBD patients and this has significant effect on their quality of life (Gasche et al, 2004). Factors that contribute to anaemia in IBD are usually both iron deficiency and anaemia of chronic disease but it can also happen as the consequence of some drugs (salazopyrine, azathioprine) used in treating IBD which have myelosuppressive effects (Gomollon and Gisbert, 2009).

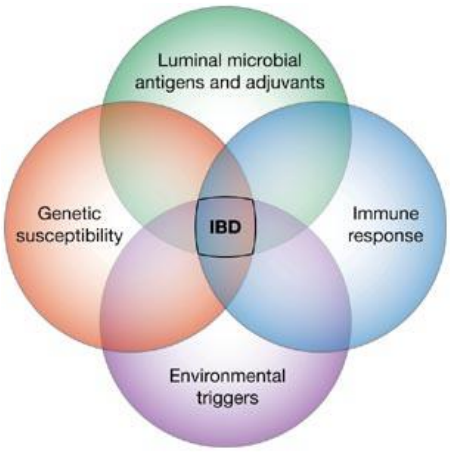


Figure 1.15 The interactions between different factors in the pathogenesis of IBD. These factors are interlinked and no single factor is sufficient to cause IBD. From Sartor (2005).

Iron deficiency is often caused by excessive blood loss in the intestine, impaired iron absorption in the small intestine due to inflammatory activity, resection or even inadequate dietary intake. Together, these factors contribute to the development of anaemia in IBD patients (Oldenburg et al, 2001). Anaemia of chronic disease on the other hand occurs as a result of changes in iron metabolism as an attempt to lower the concentration of iron through the deployment of a 'iron withholding mechanism' (Oldenburg et al, 2001). Proinflammatory cytokines such as TNF-α, γ-IFN, IL-1 and IL-6 can participate in the development of anaemia of chronic disease by interferring with erythropoietin synthesis and blunting the response of the erythron to erythropoietin, directly reducing the proliferation of the erythron. In addition, TNF-α is not only able to aggravate the effect of hepcidin, which blocks the transport of absorbed iron into circulation, it also prevents the release of iron from the macrophages and monocytes (Weiss and Gasche, 2010). Besides this, the biological half-life of erythrocytes is reduced during an inflammatory condition, thus stimulating erythrophagocytosis, further lowering the concentration of iron (Weiss and Gasche, 2010). In short, the reduced rate of proliferation of erythron together with less recirculation of iron results in anaemia of chronic disease. However, the low iron environment is seen as having a protective role in inhibiting the growth of infiltrating microorganisms as well as the generation of reactive oxygen species that causes secondary damage to tissues, thus preventing any disease complications (Oldenburg et al, 2001).

Anaemia is usually corrected by supplementing 200mg (or 400mg in some cases) of iron (Gomollon and Gisbert, 2009). A maximum of 10-20mg of the supplemented iron can be absorbed per day and given the inflammatory condition of IBD patients, absorption could be less than that amount, leading to the accumulation of a large quantity of unabsorbed iron in the colon, which can cause undesired side effects (Gomollon and Gisbert, 2009; Semrin et al, 2006). However, a reduced rate of iron absorption only occurs in disease-active patients but not in disease-inactive patients, showing that supplementing iron in the former group of patients may have limited benefits (Semrin et al, 2006). Indeed, many researchers have shown the undesirable effect of oral iron supplementation using

mouse model. Oxidative stress and intestinal inflammation are increased in iron supplemented mice due to the activation of Fenton reaction in which superoxide and hydrogen peroxide produced by neutrophil at the inflammed site reacts with iron, resulting in the production of hydroxyl radicals (Carrier et al, 2001). Oxidative stress caused by the overproduction of free radicals subsequently leads to tissue damage and has the capability to amplify inflammation, causing more severe complications in IBD (Carrier et al, 2001). Dextran sulphate sodium (DSS)-induced colitis rats have significantly shorter colonic length compared to control rats and experiences further shortening when iron is supplemented. A higher rate of lipid peroxidation accompanied by lower levels of antioxidants (vitamin C and vitamin E), indicating high levels of free radicals are produced in iron-supplemented DSS rats compared to DSS alone, thus showing the detrimental effect of iron in colitis patients (Carrier et al, 2001). Similar results have been obtained when iron is injected intraperitoneally (Aghdassi et al, 2001), suggesting high levels of iron accumulating in the gut can have a deleterious effect in IBD patients. Besides this, iron administration to DSS mice exhibited a more severe inflammation and extended to a larger area. A high dosage of iron can cause severe mucosal injury and bleeding, leading to the death of DSS mice (Seril et al, 2002). In addition, DSS mice supplemented with iron have a higher chance of developing colorectal cancer (Seril et al, 2002). In conclusion, care should be taken when supplementing iron to IBD patients as an effort to correct anaemia.

The only advantage of using an oral iron supplement in treating anaemia in IBD patients is convenience. These supplements can be either in liquid or tablet form and are fairly cheap and easy to obtain. However, their efficacy is hindered by the reduced absorption in the inflamed gut and the gastrointestinal side-effects such as stomach upset, nausea and vomiting. An alternative approach such as parenteral iron therapy or intravenous iron therapy (IV) has been used in treating anaemic IBD patients. Due to its excellent safety record and wide availability, iron sucrose has been the standard care in IBD. In recent years, more and more IV preparations have appeared on the market which include low molecular weight iron dextran (Cosmofer®), ferric carboxymaltose (Ferinject®),

Ferumoxytol (Feraheme®) and Iron isomaltoside 1000 (Monofer®) (Stein and Dignass, 2013). Although new products have higher efficacy and become more convenient as fewer infusions were needed, the downside of these products is their cost which can be 2-3 times higher. IV iron therapy has an advantage over oral iron as haemoglobin and ferritin levels showed better response. Besides this, IV iron therapy also has a reduced incidence of gastrointestinal adverse events such as abdominal pain, diarrhea and vomiting which are prevelant in oral iron treatment (Avni et al, 2013). Thus, IV iron therapy should be considered a routine in treating anaemia in IBD patients if given the chance.

1.7 The impact of phytic acid on human health

1.7.1 Phytic acid

Phytic acid or phytate (salt of phytic acid) or *myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate (IP_6) was discovered around 1855 to 1856 by Hartig when small particles resembling the size of potato starch grains were reported in plant seeds (Schlemmer et al, 2009). However, the particles did not react with iodine, indicating the particles were not starch but another nutrient reserve involved in the germination of seeds. The particles were later shown to liberate phosphoric acid and inositol upon hydrolysis by hydrochloric acid (Schlemmer et al, 2009). Phytic acid serves as a phosphate store in plants which accumulates during seed development (representing up to 3% of seed mass) and is broken down during the germination stage. In addition to phosphate stores, phytic acid can also act as a counter-ion for di- and trivalent mineral cations such as zinc, manganese, magnesium and calcium which can be mobilised during germination (Raboy, 2003). Due to its function as a phosphate store in plants, it can contain up to 75% of a seed's total phosphate (Raboy, 2003).

1.7.2 Phytate as an antinutrient

Although phytic acid has an important role in plants, it can have a negative impact on humans, particularly as an antinutrient which binds tightly to numerous minerals such as zinc, iron, copper, calcium, manganese and magnesium, lowering their bioavailability (Kumar et al, 2010). Phytic acid is strongly negatively charged over a wide range of pH and can easily interact with the cations (Persson et al, 1998). Phytic acid forms complexes with cations by either binding to one of the phosphate

groups or by bridging two different phosphate groups from different molecules (Persson et al, 1998). The tendency to bind minerals and form complexes depends on the degree of phosphorylation whereby the more phosphate groups present, the more cation it is able to bind. In addition, pH can also affect the binding of cations by phytic acid, in which highest binding occurs between pH 4-6 (Persson et al, 1998). Various studies have shown that binding capability of phytic acid differs from one mineral to another. Earlier studies showed that phytic acid tends to bind to metals in the following order: Zn^{2+}>Cu^{2+}>Co^{2+}>Mn^{2+}>Ca^{2+} (Maddaiah et al, 1964). A similar experiment conducted later showed that the order of phytic acid to bind metals tends to be Cu^{2+}>Zn^{2+}>Ni^{2+}>Co^{2+}>Mn^{2+}>Fe^{3+}>Ca^{2+} (Vohra et al, 1965). Although there is a difference between the order of the first two metals, it can be seen that Cu^{2+} and Zn^{2+} bind strongly to phytic acid. A more recent study also showed that phytic acid binds more strongly to Cu^{2+} than Zn^{2+} but there are very little differences between the capability of phytic acid to bind these metals which could explain the discrepancy between studies (Persson et al, 1998). The complexes formed between phytic acid and minerals can precipitate at physiological pH, thus lowering their bioavailability and reducing mineral absorption in humans (Schlemmer et al, 2009). However, the solubility of these inositol phosphates increases with decreasing phosphorylation or phosphates groups, the less phosphate groups in the molecule, the more soluble the complex is at physiological pH. Besides this, lower inositol phosphates also show lower affinity to minerals, thereby increasing mineral availability for absorption (Schlemmer et al, 2009).

Various studies on the inhibitory effect of phytic acid conducted using Caco-2 cells indicate that the presence of phytic acid reduces the uptake of metals into cells and uptake increases with a lower number of phosphates groups. A study carried out by Han and colleagues showed that the addition of phytic acid significantly lowers the uptake of both zinc and iron (Han et al, 1994). The effect of reduction in uptake of metals is more prominent for zinc than iron, which is in agreement with the results obtained by Vohra whereby phytic acid binds more strongly to zinc than to iron (Han et al, 1994). The presence of phytic acid reduces the uptake of iron by Caco-2 cells by roughly 50% and

that of zinc by 70%, but inositol and phosphate individually or inositol and phosphate together have no effect on the uptake of iron and zinc. The inhibitory effect is only seen when phytic acid is present, indicating its antinutrient properties (Han et al, 1994). A later study carried out by Skoglund and colleagues also showed similar results in which phytic acid reduces the uptake of iron in Caco-2 cells but they obtained up to 77% reduced uptake when phytic acid was present (Skoglund et al, 1999). A more recent study showed that the presence of phytic acid also reduces the uptake of ferritin, an iron storage protein, in Caco-2 cells but the effect was not significantly different to the control (Kalgaonkar and Lonnerdal, 2008). In human studies, the administration of phytic acid reduces the absorption of nonhaem-iron in a dose-dependent manner. In the study conducted by Hallberg and colleagues, the presence of just 2 mg phytic acid is sufficient to reduce iron absorption and 250 mg of phytic acid shows an ~7-fold reduction compared to the control (Hallberg et al, 1989). The authors also indicate that the intake of 250 mg of phytic acid is not uncommon especially in a vegetarian diet or diets in developing countries, which can significantly reduce the nutrient absorbed from the diet (0.3-3 g phytic acid are typically consumed per day; Schlemmer, 1995). Another study carried out by Siegenberg and colleagues also showed the inhibitory effect of phytic acid on the absorption of iron in a dose-dependent manner but the level used was much lower compared to the observation of Hallberg (Siegenberg et al, 1991). Both studies showed that the addition of ascorbic acid is able to reverse the inhibitory effect of phytic acid, thus improving its absorption. Supplementation with as little as 30 mg of ascorbic acid is able to offset the effect of phytic acid (Siegenberg et al, 1991) and the presence of 50 mg of ascorbic acid can overcome the effect of 250 mg phytic acid (Hallberg et al, 1989). The number of phosphate groups present has also been shown to affect the ability of inositol phosphates in binding nonhaem-iron. Iron absorption increases as the number of phosphate reduces and IP_3 and IP_4 showed no significant effect on iron absorption (Sandberg et al, 1990). Although the presence of either IP_3 or IP_4 does not cause any inhibitory effect, when both IP_3 and IP_4 are present, iron absorption can be reduced by up to 54% (Sandberg et al, 1990). This effect may be due to the presence of certain isomers that can bind to iron, thus

reducing its absorption or the interaction between lower and higher inositol phosphates which can increase mineral binding capacity (Sandberg et al, 1990).

1.7.3 Phytate depletion

Since phytic acid is able to bind various minerals, lowering their bioavailability, it is important that phytic acid is removed from the food sources prior to consumption, thus various methods are available for the removal of phytic acid. These methods include enzymatic breakdown, thermal processing or germination and soaking, which significantly reduce the amount of phytic acid present.

Thermal processing involves the application of heat to the food product. The simplest form of thermal processing is boiling in which the product is boiled in water for a period of time. Few studies showed that the length of cooking as well as temperature has an effect on phytic acid. The higher the temperature and the longer the time of cooking reduces the amount of phytic acid in an increasing manner. Schlemmer and colleagues showed that cooking raw brown beans at 110 °C for 30 and 90 min reduces the phytic acid content from 86% initially to 85% and 66%, respectively. By increasing the temperature to 120 °C, the phytic acid reduces to 68% at 30 min and 47% at 90 min. The temperature is achievable using a pressure cooker in a household setting but when temperature is increased to 140°C on an industrial scale, 90 min of cooking reduces the phytic acid to 21% from an initial 86% (Schlemmer et al, 2009). Tabekhia and Luh also showed that cooking is able to reduce the phytic acid content, however different types of beans showed different responses. Pink beans showed a reduction of 26.4% while red kidney beans only reduced to 7.7% after cooking for 3 h, however the authors did not specify the temperature (Tabekhia and Luh, 1980). Canning which involved adding the beans to a brine and subsequent heat processing for cooking and sterilisation would reduce the phytic acid content. Canned black-eyed beans showed more than 90% reduction in phytic acid, with only 8.5% was left in the final product. Canning processes can reduce on average two-thirds of the phytic acid content in beans (Tabekhia and Luh, 1980). Rehman and Shah also showed that autoclaving legumes as a means of cooking is able to reduce their phytic acid content in both a time and temperature dependent manner. Besides lowering the phytic acid content,

autoclaving also affects the protein and starch digestibility of legumes. Protein digestibility reduces with time and temperature while starch digestibility increases with time (Rehman and Shah, 2005). However, it is also noted that the researchers soak the seeds in water prior to autoclaving which will remove some of the phytic acid content.

Germination, malting or soaking is able to reduce the amount of phytic acid present in legumes and cereals. Numerous studies have investigated different conditions utilised during this process, with successful results (Nkhata et al, 2018; Bartnik and Szafranska, 1987; Archana et al, 1998; Badau et al, 2005; Yasmin et al, 2008; Rumiyati et al, 2012; Onyango et al, 2013; Ogbonna et al, 2012; Gustafsson and Sandberg, 1995). During soaking, phytases can be activated or the phytic acid leaches out into the liquid which causes the content inside the legumes or cereals to decrease. The temperature, pH and length of soaking affects the phytic acid in the final product (Gustafsson and Sandberg, 1995). In the latter study, there was a gradual reduction in pH of the water with time, indicating the leaching of inositol phosphates. Besides this, the drop was more significant when the water temperature was at 55 °C compared to room temperature (Gustafsson and Sandberg, 1995). The studies also found that soaking the beans in Tris-buffer at pH 7 resulted in the greatest reduction in phytic acid, coupled by an increase in lower inositol phosphates, showing the activity of phytases in degrading phytic acid (Gustafsson and Sandberg, 1995). The authors also indicate that the optimum condition for brown bean phytases is pH 7 and 55 °C, which complements other work (Gustafsson and Sandberg, 1995). This may explain the results obtained by Yasmin and colleagues in which soaking did not show a reduction in phytic acid content as the beans were soaked at room temperature (Yasmin et al, 2008). Germination of cereals (Bartnik and Szafranska, 1987; Larsson and Sandberg, 1992; Egli et al, 2002) and legumes (Egli et al, 2002; Yasmin et al, 2008) gives a gradual reduction in phytic acid throughout the process. During germination, phytases are activated to release the phosphate from phytic acid to support growth of the seeds. Bartnik and Szafranska showed that phytase activity increases with the time of germination, coupled with a reduction in phytic acid as the seeds germinate. However, the activity varies between different cereals, with highest activity in rye and lowest in oats (Bartnik and

Szafranska, 1987; Egli et al, 2002). Temperature also plays a role in reducing phytate during germination with the optimum temperature of oats being between 37-40 °C while wheat but 55 °C for rye (Larsson and Sandberg, 1992). Thus, is it important to have the optimum condition for the maximum reduction of phytic acid.

1.7.4. Phytases

Phytases are more specifically called '*myo*-inositol (1,2,3,4,5,6) hexa*kis*phosphate phosphohydrolases' which are involved in the sequential release of phosphate from phytic acid, resulting in free inorganic phosphorous and eventually inositol when all phosphate groups have been liberated (Kumar et al, 2010). Phytases can be divided into 3-phytases or 6-phytases depending on the position of the phosphate released, with the former releasing from the C3 position and the latter at C6 position (Kumar et al, 2010). Phytases can be isolated from either plant or microbial sources. A few plant based phytases has been isolated from rice (Hayakawa et al, 1989), canola seed (Houde et al, 1990), soy (Hamada, 1996), mung bean (Mandal et al, 1972) and wheat (Nakano et al, 1999). As for microbial sources, they can originate from either fungi or bacteria, e.g.: yeasts such as *Schwanniomyces castellii* (Segueilha et al, 1992) and *Pichia anomala* (Vohra and Satyanarayna, 2002); fungi such as *Aspergillus niger* (van Hartingsveldt, 1993), *Aspergillus carneus* (Ghareib, 1990), *Aspergillus fumigatus* (Pasamontes et al, 1997) and *Thermomyces lanuginosus* (Berka et al, 1998); bacteria such as *Klebsiella terrigena* (Greiner et al, 1997), *Lactobacillus sanfranciscensis* (De Angelis et al, 2003), *Bacillus* (Kim et al, 1998) and *E. coli* (Greiner et al, 1993). Some thermostable phytases from *Aspergillus fumigatus* or *Bacillus* which show enzymatic activity above 80 °C can be used in the production of animal feed whereby high temperature is utilised during the process, thus improving the nutritional quality of the feed. Recombinant microbial phytases from *Aspergillus niger* and *Peniophora lycii* are commercially available under the trade name Natuphos and Ronozyme respectively (Augspurger et al, 2003). An *E. coli* derived phytase has also been produced which showed better performance than the commercial phytases in releasing phosphate from phytic acid (Augspurger et al, 2003). The addition of phytases into animal feeds, which is usually comprised of

cereal and legumes, resulted in higher weight gain compared to the control, which can translate to better economic outcome for the farmer (Augspurger et al, 2003; Augspurger and Baker, 2004; Selle et al, 2003a; Selle et al, 2003b; Kornegay et al, 1996; Simons et al, 1990). In addition, phytases from microbial sources have better efficiency over plant phytases as plant phytases are more heat labile and so cannot withstand the processing temperatures and are also inactivated at low pH in the animals (Selle et al, 2003b).

1.7.4.1 Phytases of E. coli

A few earlier studies have shown and isolated an acid phosphatase from *E. coli* able to hydrolyse *p*-nitrophenol phosphate to liberate free phosphate (von Hofsten and Porath, 1962; Dvorak et al, 1967; Hafckenscheid, 1968; Dassa et al, 1980). It differed from alkaline phosphatase as it worked at a lower pH as compared to alkaline phosphatases. A later study found that the *appA* gene is responsible for the production of this acid phosphatase in *E. coli* (Dassa and Boquet, 1985; see Figure 1.16). Activity of hexose phosphatase and alkaline phosphatase was not modified in the mutant but phosphatase activity at pH 2.5 showed a 90% reduction compared to the wild-type, suggesting the acid phosphatase is encoded by *appA.* Greiner and colleagues managed to isolate phytases from *E. coli* which showed the ability to hydrolyse phytate to lower inositol phosphate forms. A search in the database revealed similar amino-terminal sequence with the pH 2.5 acid phosphatase (Greiner et al, 1993). A later study by Golovan and colleagues showed that the acid phosphatase of *E. coli* exhibits both phytase and phosphatase activity which could explain the results obtained by both Dassa and Greiner (Golovan et al, 2000).

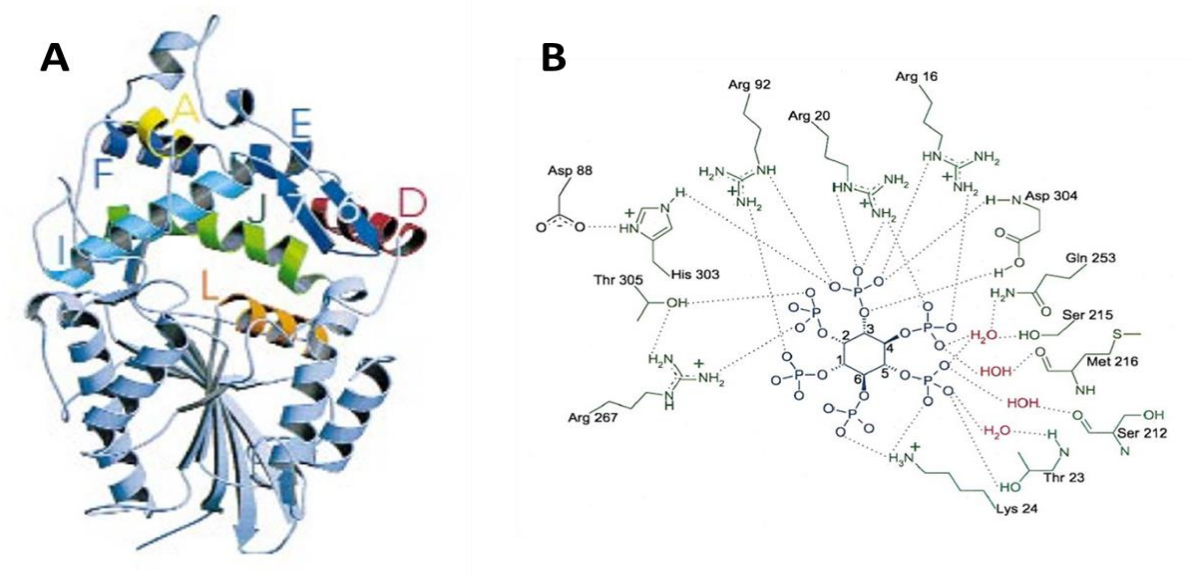


Figure 1.16 Structure of *E. coli* phytase. A. Three-dimensional structure of *E. coli* periplasmic phytase (*appA*). **B**. Schematic diagram of phytate binding to AppA (adapted from Lim et al, 2000).

Glucose-1-phosphatase (G1Pase), with a primary function of scavenging glucose from glucose-1-phosphate, is encoded by the *agp* gene in *E. coli.* Acid phosphatase and G1Pase are homologous histidine acid phosphates sharing a common RHGxRxP active site motif (Dassa et al, 1990). G1Pase (Figure. 1.17) has been shown to be involved in the utilisation of glucose-1-phosphate as both carbon and phosphate source to support the growth of *E. coli* (Pradel and Boquet, 1991), which suggests its ability to hydrolyse phosphorylated compounds. A later study by Cottrill and colleague confirmed the ability of G1Pase to hydrolyse phytic acid as seen by the appearance of lower inositol phosphate forms when phytic acid was incubated with the enzyme (Cottrill et al, 2002). However, G1Pase can rapidly be inactivated by pepsin due to the presence of 10 additional pepsin cleavage sites in the amino acid sequence (Cottrill et al, 2002).

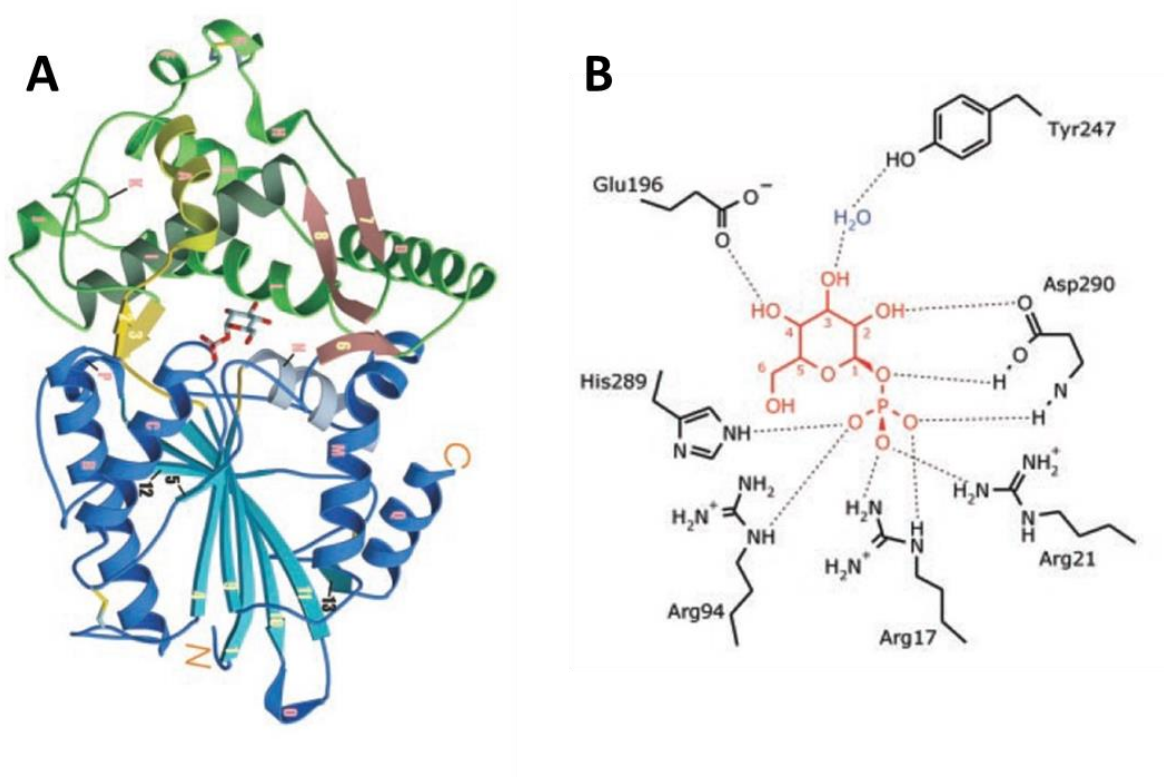


Figure 1.17 Structure of *E. coli* Glucose-1-phosphatase. A. The three dimensional structure *E. coli* glucose-1-phosphotase. **B**. Schematic diagram of glucose-1-phosphate bound to the enzyme (adapted from Lee et al, 2003).

Alkaline phosphatase of *E. coli* is encoded by the *phoA* gene and is a zinc and magnesium-containing metalloprotein, consisting of two identical subunits (Kamitani et al, 1992). Alkaline phosphatase catalyses the transphosphorylation and hydrolysis of phosphate monoesters (Stec et al, 2000). Although studies on alkaline phosphatases and their ability to hydrolyse phytic acid are scarce, Wilson and colleagues have shown the ability of alkaline phosphatase to hydrolyse *p*-nitrophenol phosphate and liberate free phosphate (Wilson et al, 1964; Trentham and Gutfreund, 1968), suggesting its potential as a phytase. Although *E. coli* possess these enzymes which can hydrolyse phytic acid to liberate free phosphate, there have been limited studies in investigating the ability of *E. coli* in utilising phytic acid as a phosphate/carbon source to support its growth; this will be investigated as part of this thesis.

1.8 Impact of zinc on the gut microbiota

1.8.1 Chemistry of zinc

Zinc makes up roughly 0.02% of the Earth's crust and is the 23rd most abundant element on Earth with an atomic weight of 65.37 and atomic number of 30 (Kaur et al, 2014; Brown et al, 2001). Zinc normally exhibits only one oxidation state (+2) and is an essential micronutrient with an important role in biological systems (Brown et al, 2001). In addition, the absence of redox properties in zinc enables it to be transported in biological systems without causing any oxidant damage (Brown et al, 2001). Because of its unique chemical properties, zinc acts as a component (co-factor, catalytic factor or structural component) in almost 1000 enzymes that carry out various biological functions in the body such as cell division, metabolism of macronutrients and the synthesis of DNA and proteins (Tubek et al, 2008; Deshpande et al, 2013). Besides this, zinc is also involved in stabilising cell membranes and regulating its function, as well as a role in blood clot formation and cell signalling (Tubek et al, 2008; Maret, 2017).

1.8.2 Zinc homeostasis in human

As zinc play an important role in numerous biological functions in the body, it is of no surprise that zinc is the second most abundant trace mineral in the body, just behind iron (Livingstone, 2015). The adult human has a total zinc content of between 1.5-2.5 g in which the majority is found intracellularly, predominantly located in liver, muscle, bone and various organs (Brown et al, 2001). Unlike iron which has a dedicated storage protein, zinc does not have any storage protein despite its abundance in the body. In addition, a large proportion of the zinc reserves turn over slowly, thus making it not readily available for metabolism. However, the presence of a small functional pool of zinc (10% of intracellular zinc) is vital in maintaining all zinc-dependent biological functions. This zinc pool is able to move in and out of the plasma with a relatively short turnover period and the size of the pool is sensitive to the zinc absorbed from diet, thus a constant supply of zinc is necessary to meet the requirement of zinc for growth and maintenance. The zinc in the plasma is mostly bound to albumin and alpha-2-macroglobulin, and its concentration is maintained by redistribution and conservation (Livingstone, 2015; Brown et al, 2001).

As mentioned above, dietary zinc is important in meeting the requirement for growth and maintenance. The current zinc daily intake recommendation for adult is 9.5 mg/d for males and 7 mg/d for females, and up to a maximum of 25 mg/d is zinc recommended in the form of supplements. Food derived from animal sources contains high zinc levels as do seeds and nuts (Brown et al, 2001). The zinc content of liver or kidney ranges from 4.2 to 6.1 mg/100 g with absorbable zinc at up to 3.1 mg/100 g. Although seeds and nuts contain high zinc levels (up to 7.8 mg/100 g), the presence of phytate greatly reduces its bioavailability to 0.3-0.8 mg/100 g. Animal sources are typically phytate free and thus the bioavailability of zinc is high as compared to cereals or legumes. Phytate irreversibly binds zinc in the intestinal lumen, making it unavailable for absorption. Food with a phytate-zinc molar ratio greater than 15 is considered to have relatively poor zinc bioavailability and a molar ratio of less than 5 is considered to provide good bioavailability. Ratios between 5 and 10 are considered to afford medium bioavailabaibility (Brown et al, 2001).

The gastrointestinal tract (GI) plays a major role in the regulation of zinc homeostasis which is achieved by adjusting both zinc absorption and endogenous excretion. Zinc is absorbed throughout the small intestine with the jejunum being the site of maximal absorption (Lee et al, 1989). The fraction of absorption of dietary zinc is inversely proportional to oral zinc intake and is typically between 16-50% (Maares and Haase, 2020). Thus, humans may benefit from a higher fractional absorption by having a low dietary zinc intake (Hunt, Beiseigel and Johnson, 2008). However, the net absorption of zinc is affected by body zinc status with a higher absorption occurring in zinc-deficient individuals (Maares and Haase, 2020). Excess zinc on the other hand can be secreted and excreted from the body in the faeces. There are several potential sources of endogenous zinc which includes pancreatic and biliary secretions, transepithelial flux from intestinal cells, gastroduodenal secretions and sloughing of mucosal cells (Krebs, 2000). Through these mechanisms, the body is able to maintain zinc homeostasis so that all biological systems are able to function normally.

1.8.3 Zinc uptake in human

The movement and transport of zinc into and out cells require zinc transporters that fall into two families: the ZnT and ZIP families. The ZnT family consists of 10 members and is responsible for lowering the intracellular zinc levels by either transporting zinc into organelles or the extracellular space. The ZIP family on the other hand consists of 14 members and has a role in increasing the intracellular zinc level by moving zinc from organelles or extracellular space into the cytoplasm (Litchen and Cousins, 2009; Cousins, 2010). The absorption of zinc from the small intestine involves a saturable carrier-mediated mechanism which shows a linear increase in uptake with increasing concentration of zinc up to a concentration of 1.8mM; higher zinc concentration does not allow an increase in zinc absorption, thus showing it to be a rate limiting step (Lee et al, 1989; Gopalsamy et al, 2015; Maares and Haase, 2020). Absorption of zinc occurs at the intestinal brush border membrane where it is transported from the lumen into the enterocytes and subsequently secreted at the basolateral side into the portal circulation. The absorption of zinc into the enterocyte is mainly mediated by ZIP4 which is responsible for the import of zinc from the lumen into cell (Gopalsamy et al, 2015; Maares and Haase, 2020; Cousins, 2010). ZIP4 is predicted to have eight transmembrane domains with both N- and C-terminal ends located outside the plasma membrane. It contains a His-rich region between transmembrane domains III and IV and zinc is assumed to be transported into the cell through binding at this region (Kambe et al, 2004). Although ZIP4 is highly expressed in the small intestine, as the site of zinc absorption, it can also be found in the stomach, colon, kidney and pancreatic β cells, but at a lower abundance (Dufner-Beattie et al, 2003; Bafaro et al, 2017). ZIP4 expression is upregulated during zinc deficiency and the proteins accumulates on the surface of enterocytes as a method of ensuring maximum zinc absorption takes place (Dufner-Beattie et al, 2003; Cragg et al, 2005). Conversely, its expression is downregulated when zinc is supplemented as a response to maintain zinc homeostasis in the body (Cragg et al, 2005). Another zinc transporter that is involves in uptake of zinc into the enterocyte is ZnT-5 variant B. Although it belongs to the ZnT family, it is a unique protein which functions in a bidirectional manner, capable of transporting zinc

into the cell and cellular ions back to the lumen (Cragg et al, 2002; Valentine et al 2007; Cousins 2010; Maares and Haase, 2020). Upon entering the cell, zinc is can be mobilised into four intracellular pools: 1) zinc bound tightly to metalloproteins as cofactor or structural component; 2) zinc bound to metallothioneins as a reservoir and buffer of cytosolic zinc; 3) zinc compartmentalised into intracellular organelles; and 4) cytosolic free zinc (Kambe et al, 2004). Zinc is exported from the enterocytes into the portal circulation with the help of ZnT transporters. Members of the ZnT family are predicted to have six transmembrane domains with intracellular N- and C-termini. They also contain a His-rich loop between transmembrane domains IV and V (Kambe et al, 2004). ZnT-1 is found on the basolateral side of the enterocyte and has a role in exporting zinc from the cell (Lichten and Cousins, 2009; Maares and Haase, 2020). In animal models, the expression of ZnT-1 is regulated by zinc levels whereby a high zinc intake (particularly zinc supplementation) increased its expression (McMahon and Cousins, 1998; Liuzzi, Blanchard and Cousins, 2001). However, zinc supplementation leads to a downregulation of both Znt-1 mRNA and its protein in humans, suggesting a different regulatory response between animals and humans (Cragg et al, 2005). Under zinc deficient conditions, ZnT-1 is endocytosed and degraded through lysosomal and proteasomal pathways as a mean to conserve zinc (Nishito and Kambe, 2019).

1.8.4 Zinc uptake in bacteria

Zinc is an essential trace mineral in bacteria whereby zinc-binding proteins constitute around 5%-6% of the proteome in bacteria (Andreini, Bertini and Rosato, 2009). Although the zinc-requiring proteins in prokaryotes are lower than eukaryotes (~9%), zinc likewise plays important roles in various biological systems such as protein synthesis and DNA replication and so is needed for the growth and development of prokaryotes. In addition, although not desirable for humans/animals, zinc is involved in the pathogenicity of certain bacteria such as *Salmonella enterica* (Ammendola et al, 2007), uropathogenic *E. coli* (Velasco et al, 2018) and *Listeria monocytogenes* (Corbett et al, 2012). Due to the importance of zinc, bacteria have developed high affinity ABC transporters to effectively transport zinc into the cell. ZnuABC is a high affinity zinc transporter found in Gram-

negative bacteria which consists of a periplasmic protein (ZnuA), a transmembrane permease (ZnuB) and an ATPase (ZnuC) (Blindauer, 2015; Hantke, 2005). An additional auxiliary periplasmic component (ZinT) has been identified which is able to collaborate with ZnuA in the transportation of zinc (Petrarca et al, 2010; Ilari et al, 2013). The presence of ZinT enhances the growth of bacteria under zinc-deficient conditions (Petrarca et al, 2010) and it is thought to act as a Zn^{2+}-buffering protein that delivers zinc to ZnuA (Ilari et al, 2013). Another component, ZnuD, has been identified in *Neisseria meningitidis* which is located in the outer membrane. ZnuD is a TonB-dependent receptor that requires mechanical energy derived from the inner-membrane TonB/ExbB/ExbD complex to transport zinc across the outer membrane (Calmettes et al, 2015). The expression of ZnuABC is regulated by Zur whereby its expression is repressed under zinc sufficient condition. Zur is a dimeric protein which can bind 2 zinc ions due to having a structural Zn^{2+} site and a second Zn^{2+} sensing site (Chandrangsu, Rensing and Helmann, 2017). Bacteria possess another low affinity transporter, ZupT, which mediates the uptake of zinc (Grass et al, 2002; Grass et al, 2005; Cerasi et al, 2014). The role of ZupT in zinc uptake has been shown in *E. coli* (Grass et al, 2002) and *Salmonella enterica* (Cerasi et al, 2014) whereby the mutants showed a reduced growth compared to the wild-type and the effect is more pronounced in strains lacking both ZnuABC and ZupT, thus indicating their roles in zinc uptake. Although ZupT mediates zinc uptake, the metals that it can transport is not limited to only zinc as it is a broad-range metal transporter which is also able to transport Fe^{2+}, Co^{2+} and possibly Mn^{2+} (Grass et al, 2005). The presence of a high affinity ZnuABC and a low affinity ZupT transport system ensures that the corresponding bacteria are able to obtain zinc needed for growth and development efficiently at under a range of environment zinc concentrations. When the zinc concentration in the cell is elevated or reaches a toxic level, it needs to be exported out of the cell to prevent any unwanted damage from happening. Zinc export or detoxification is primarily achieved via P-type ATPase and cation diffusion facilitators (CDF). ZntA is a P-type ATPase found in *E. coli* which has been shown to exhibit zinc export capabilities (Rensing, Mitra and Rosen, 1997) and it is regulated by ZntR whereby ZntA is upregulated under high zinc stress condition to reduce its

intracellular zinc concentration (Porcheron et al, 2013; Wang, Hosteen and Fierke, 2012). ZntA couples hydrolysis of ATP to transport zinc out of the cytoplasm and has several unique features which includes an intracellular, negatively charged ion-catching funnel, a high-affinity Zn^{2+}-binding site and an extracellular Zn^{2+} release pathway (Wang et al, 2014). In addition to being a zinc exporter, ZntA is capable of exporting cadmium (Cd^{2+}) and lead (Pb^{2+}) and both of these metals can also be considered as inducer of the system (Binet and Poole, 2000). ZitB and YiiP are cation diffusion facilitators which are also involved in the removal of excess intracellular zinc. Both transporters are obligatory Zn^{2+}/H^{+} antiporters which utilise free energy from H^{+} influx to couple export of Zn^{2+} from the cytoplasm (Porcheron et al, 2013; Chao and Fu, 2004; Wei and Fu, 2006).

1.8.5 Zinc and the gut microbiota

Although the amount of research involving zinc and the gut microbiota is lower compared to iron, numerous studies have been conducted over the years, mostly using animal models. Using pigs as a study model, zinc supplementation in pig feed has been shown to reduce diarrhoea, improve growth performance and act as an antimicrobial agent in pigs (Pieper et al, 2020; Villagomez-Estrada et al, 2020). 2000-3000 mg/kg of zinc is usually supplemented in the feed during weaning as zinc oxide and causes changes in the gut microbiota compared to the control. However, the changes to the composition of the gut microbiota vary from study to study which may be due to different DNA extraction methods, methods of data analysis as well as differences in the microbial community of the pigs due to housing, type of diet or environmental conditions. Supplementation of high levels of zinc oxide showed a significant reduction in certain species belonging to the *Lactobacillus* (Starke et al, 2013), *Clostridium* (Hu et al, 2012), *Alistipes*, *Megasphaera, Dialister*, *Acidaminococcus* and *Ruminoccocus* (Pieper et al, 2020) genera. The presence of lower levels of zinc in gut on the other hand increases the abundance of *Methanobrevibacter* (Yu et al, 2017), *Bacteroides, Parabacteroides, Faecalibacterium, Collinsella, Blautia* and *Eubacterium* (Pieper et al, 2012) genera. Although the supplementation of zinc oxide can be used as an alternative to antibiotics in pigs, high levels of zinc can have adverse effect. This was illustrated in two different studies (Bednorz et al, 2013; Ciesinski et

al, 2018) whereby they supplemented the feed with zinc oxide at concentration which are used commercially to determine its impact on antimicrobial resistance. The abundance of multi-resistant *E. coli* was increased in the supplemented group compared to the control, thus showing a possible role in producing multi-resistant *E. coli.* The results suggest that although high concentration of zinc oxide may be economically beneficial as it improved the growth of pigs, it can also be damaging to the environment due to pollution as well as causing multidrug resistance in bacteria.

In mice, zinc supplementation at 250 mg/kg as $ZnCl_2$ over a 2-week period did not significantly affect the diversity of the gut microbiota as indicated by the alpha diversity indices (Foligne et al, 2020). The addition of zinc showed little impact on the gut microbiota at higher taxonomic level (phylum and family) as the microbiota showed similar abundance pre- and post-treatment. At lower taxonomic level, the supplementation of zinc significantly reduced the abundance of *Candidatus arthromitus* and *Lactobacillus* spp. In addition, the abundance of *Coprococcus*, *Ruminococcus* and *Akkermansia* spp. also showed a reduction in the presence of zinc. Conversely, the abundance of *Sutterella*, *Bacteroides* and *Allobaculum* spp. showed an increase with the supplementation of zinc (Foligne et al, 2020). Besides mice and pigs, there are also some studies conducted in chickens. The addition of zinc in the form of $ZnSO_4$ decreased the abundance of *Lactobacillus, Enterococcus* and Enterobacteriaceae spp. while *Streptococcus* showed an increase in the ileal section of the chicken. In the cecal section, the presence of zinc reduced the relative abundance of Clostridales and *Coprobacillus* but *Lactobacillus,* Erysipelotrichaceae and Enterobacteriaceae showed an increase in this section (Bortoluzzi et al, 2019). In humans however, there are numerous studies on zinc supplementation and health but research on the effect of zinc on the gut microbiota is limited, thus this aspect will be considered in this study in the later sections.

1.9 Aim and Objectives

1.9.1 Aim

To determine the effect of different iron/zinc regimes on the composition of human gut microbiota

1.9.2 Objectives

1. To determine the growth and composition of gut microbiota with the presence/absence of iron

2. To determine the growth and composition of gut microbiota using different forms of iron

3. To determine the growth and composition of gut microbiota with the presence/absence of zinc

4. To determine the growth and composition of gut microbiota with different concentration of zinc

5. *To determine the link between iron/zinc nutrition, gut microbiota and human health*

6. To determine the ability of E. coli *to utilise phytic acid as a phosphate/carbon source to support growth*

7. To determine the ability of gut microbiota to utilise phytic acid as a phosphate/carbon source in a phosphate/carbon restricted environment

8. To determine the impact of phytic acid on the gut microbiota

1.9.3 Methodology

Anaerobic mixed-human faecal cultures will be used to inoculate a single stage batch culture which allow us to investigate the influence of different iron regimens on gut microbiota using a well-controlled *in vitro* approach. Cultures will be 'fed' with defined gut-model medium and inoculated with microbiota (faecal slurries) from human volunteers. An initial aim will be to test the effect of iron and haem depleted gut model medium on the growth and activity of microbiota from different donors. Iron-limited gut model growth conditions are established to allow the application of various forms of iron in an attempt to reverse iron limitation.

In order to test the impact of zinc on the gut microbiota, a similar approach will be used but with varying concentrations of zinc sulphate to mimic the daily supplementation doses. The effect of zinc restriction and zinc supplementation will also be investigated using a three-stage gut model that mimics the human colon which allows studies on the impact on the gut microbiota in more detail.

The composition of the microbiota will be determined by NGS-dependent 16S rRNA community profiling. Total bacterial numbers will be determined by flow-FISH using universal oligo probes and 14 taxon-specific probes, allowing an estimation of numbers for each bacterial genus/species when combined with the NGS data. Iron in the medium and faecal slurries will be assayed by ICP-OES/MS, indicating iron availability during growth thus allowing accurate correlation of metal content with

microbiota profiles. Faecal inocula will be at a low volume ratio (1:100) so will contribute minimally to culture Fe content.

The ability of bacteria to utilise phytic acid as a phosphate/carbon source to support growth will be studies using M9 minimal medium with a defined phosphate/glucose concentration to produce a phosphate or carbon restrictive environment. Growth will be monitored over 24 h using a 200-well Bioscreen C microbial-growth monitoring system under aerobic, shaking conditions. The effect of pH on phytic acid utilisation will also be determined as the phytase enzymes have different pH optimum. A series of mutants will also be generated to understand the mechanisms and enzymes involved in phytic acid utilisation. In addition, the impact of phytic acid on the gut microbiota will also be investigated using single-stage batch cultures with the focus on utilisation of phytic acid as a phosphate/carbon source by the gut microbes.

Chapter 2 Materials and methods

2.1 Reagents

All reagents were of analytical grade or higher quality and were generally purchased from Fisher Scientific and Sigma-Aldrich unless otherwise stated. qH_2O (18 mΩ-cm) was used throughout the experiment (NANOpure Diamond, UK; Suez, UK)

2.2 Faecal donors

All donors were between 21-37 of age and on normal mixed diets without any special dietary requirement or supplementation. They were generally healthy and had not taken any antibiotics within 1 year prior to the study.

2.3 Media and solutions

2.3.1 Preparation of gut model media (GMM)
The gut model medium was prepared according to Macfarlane et al (1998) with slight modification. The culture medium contained the following components (g/L) in qH_2O: starch, 5.0; peptone water, 5.0; tryptone, 5.0; yeast extract, 4.5; NaCl, 4.5; KCl, 4.5; mucin (porcine stomach type III), 4.0; casein, 3.0; pectin (citrus), 2.0; xylan (beech wood) (Serva Electrophoresis GmbH), 2.0; arabinogalactan (larch wood), 2.0; $NaHCO_3$, 1.5; $MgSO_4$, 1.25; guar gum, 1.0; inulin, 1.0; L-cysteine hydrochloride, 0.8; KH_2PO_4, 0.5; K_2HPO_4, 0.5; bile salts No.3, 0.4; $CaCl_2.6H_2O$, 0.15; $FeSO_4.7H_2O$, 0.005; haemin, 0.05; Tween 80, 1 ml; vitamin K (100µl/ml), 10 µl; resazurin (250mg/L), 4 ml.

2.3.1.1 Preparation of modified gut model media (mGMM)
The gut model medium was prepared as in section 2.3.1 with slight modification to reduce the metal content. The medium contained the same components as the GMM with the exception of tryptone, yeast extract and mucin and 10g of peptone water is used instead of 5g. Vitamin and mineral solution (section 2.3.1.2) were also added to the medium.

2.3.1.2 Preparation vitamin and mineral solutions
A 1000X stock solution of vitamin and mineral solution was prepared to be added to the modified gut model media at a volume of 1µl/L media. The mineral solution contained the following

ingredients (g/L): $Na_3C_6H_5O_7$, 2.1; $MnSO_4 \cdot H_2O$, 0.5; $CoCl_2$.6H2O, 0.1; $CuSO_4 \cdot 5H_2O$, 0.1; $KAl(SO_4)_2$, 0.01; BH_3O_3, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.1; $NiCl_2 \cdot 6H_2O$, 0.025; Na_2SeO_3, 0.2; VCl_3, 0.1; $Na_2WO_4 \cdot 2H_2O$, 0.01.

The vitamin solution contained the following ingredients (g/L): biotin, 0.002, folic acid; 0.002; pyridoxine, 0.01; thiamine, 0.005; riboflavin, 0.005; nicotinic acid, 0.005; calcium pantothenate, 0.01; vitamin B12, 0.0005; *p*-aminobenzoic acid, 0.005; lipoic acid, 0.005; menadione, 0.001.

Both vitamin and mineral solution were filter sterilised and kept at 4 °C until needed.

2.3.2 L-broth and L-agar

L-broth is used for bacterial growth and contains the following ingredients (g/L): tryptone, 10; yeast extract, 5; NaCl, 5. L-agar contains the same ingredients with the addition of 15 g/L agar. Antibiotics were added to the broth and agar when necessary: ampicillin (100 µg/ml) and chloramphenicol (50 µg/ml).

2.3.3 Preparation of M9 minimal media and M9-agar

M9 minimal medium was prepared by dissolving 10 g of 5X M9 minimal salts (Sigma-Aldrich) in 990 ml of qH_2O. After autoclaving, 1 ml of 1 M $MgSO_4$, 10 ml of 20% glucose, 0.5 ml of 1% thiamine and 1 ml of 0.1 M $CaCl_2$ were added into the medium. Ferric citrate (10 mM) was added to the required medium to give a 10 µM final concentration. M9-agar was made by preparing the medium and agar separately. A 10 g quantity of 5X M9 minimal salts was dissolved in qH_2O to give a final volume of 500 ml and 15 g of agar were also dissolved in qH_2O to give a final volume of 500 ml. After autoclaving and the addition of all the necessary supplements, the medium and agar were combined, mixed and poured into plates.

2.3.3.1 Preparation of M9 minimal media with low phosphate content

A 4X salt and phosphate solution was made separately according to the content of commercially available M9 minimal salts (Sigma-Aldrich). The salt solution contained (g/L): NH_4Cl, 4; NaCl, 2. The phosphate solution contained (g/L): Na_2HPO_4, 27.12; KH_2PO_4, 12. A 2X solution was made by combining 25 ml of the 4X salt solution, 1.25 ml phosphate solution and 23.75 ml of sterile qH_2O. The solution was then supplemented with 0.1 ml of 1 M $MgSO_4$, 1 ml of 20% (w/v) glucose, 50 µl of 1% (w/v) thiamine and 0.1 ml of 0.1 M $CaCl_2$.

2.3.4 SOC medium

SOC medium was used to recover bacteria after electroporation. The medium contained (g/100 ml): tryptone, 2; yeast extract, 0.5; NaCl, 0.058; $MgSO_4$, 0.12. After autoclaving, 1 ml of sterile 1 M $MgCl_2$ and 2 ml of sterile 1 M glucose were added.

2.3.5 Basal medium

The culture medium contained the following components (g/L) in qH_2O: peptone water, 2; NaCl, 0.1; $NaHCO_3$, 2; $MgSO_4$, 0.01; L-cysteine hydrochloride, 0.5; KH_2PO_4, 0.04; K_2HPO_4, 0.04; bile salts No.3, 0.5; hemin, 0.05; Tween 80, 2 ml; vitamin K(100µl/ml), 10 µl; resazurin (250mg/L), 4 ml; yeast extract, 2.

2.3.5.1 Basal medium with low phosphate content

The culture medium is similar to basal medium with slight modification to lower the phosphate content. It contained the following components (g/L) in qH_2O: NaCl, 0.1; $NaHCO_3$, 2; $MgSO_4$, 0.01; L-cysteine hydrochloride, 0.5; bile salts No.3, 0.5; hemin, 0.05; Tween 80, 2 ml; vitamin K(100µl/ml), 10 µl; resazurin, 4 ml; starch, 5.

2.3.6 Preparation of 4% paraformaldehyde

A 2 g quantity of paraformaldehyde, 30 ml of qH_2O and 100 µl of 1 M NaOH were added to a Duran® bottle and incubated at 50 °C in a water bath with constant stirring for 15 min until all powder was dissolved. Then, 100 µl 1 M HCl and 16.6 ml 3 x PBS (adding 3 PBS tablet to 100ml of water) were added to the bottle and the final volume was made up to 50 ml, followed by filter sterilising through a 0.22 µm filter into a sterile container and storage at 4 °C.

2.3.7 Preparation of hybridisation buffer and washing buffer

The hybridisation buffer contained (µl/ml): 5 M NaCl, 180; 1 M Tris-HCl pH 8.0, 20; formamide, 300; qH_2O, 499 µl; 10% sodium dodecyl sulphate (SDS), 1.

The washing buffer contained (µl/ml): 5 M NaCl, 12.8; 1 M Tris-HCl pH 8.0, 20; 0.5 M EDTA pH 8.0, 10; qH_2O, 956.2 µl; 10% SDS, 1.

The hybridisation buffer and washing buffer were sterilised, using a 0.22 µm filter, into a a sterile Falcon tube.

2.4 Fluorescence *in situ* hybridisation coupled with flow cytometry (Flow-FISH)

2.4.1 Fixation of batch culture samples for Flow-FISH

A 750 µl volume of culture was taken at different time points from each fermentation vessel and transferred to a sterile 1.5 ml microcentrifuge tube. The samples were then spun at 12,045 x g (Eppendorf Minispin, Eppendorf, Germany) for 5 min. The supernatant was transferred to a new 1.5 ml microcentrifuge tube and stored immediately at -80 °C for further analysis. The pellet was resuspended in 375 µl of ice cold 1x PBS and 1.125ml of 4% paraformaldehyde solution. Samples were stored at 4 °C for 4 h and subsequently spun at 12,045 x g for 5 min. The supernatant was discarded while the pellet was resuspended in 1 ml of 1x PBS and re-spun at 12,045 x g for 5 min. This step was repeated twice to wash off any residual paraformaldehyde. The supernatant was removed using a pipette and the pellet was resuspended thoroughly in 300 µl of 1x PBS, followed by 300 µl of ethanol (96%). Samples were kept at -20 °C until further analysis.

2.4.2 Addition of probes and analysis

Fixed batch culture samples were dispersed and mixed by vortexing for 10 s. Sample volumes of 50-100 µl (depending on the density) were added to a 1.5 ml microcentrifuge tube containing 500 µl of 1 X PBS and the suspensions were mixed by pipetting. The tubes were then vortexed gently and spun at 12,045 x g (Eppendorf Minispin) for 5 min. The supernatant was discarded and the pellet was resuspended in TE-FISH buffer (0.1 M Tris-HCl, pH 8, 0.05 M EDTA, pH 8) containing lysozyme (1 mg/ml) and subsequently incubated in a dark at room temperature (20°C) for 10 min, followed by vortexing and centrifugation as before. The supernatant was discarded and the pellet was resuspended in 1x PBS to wash off any residual lysozyme. Tubes were then gently vortexed and spun again at 12,045 x g for 5 min. Subsequent steps were carried out in the fume cabinet as formamide used in the hybridisation buffer is hazardous to health. The supernatant was discarded and the pellet was resuspended in 150 µl hybridisation buffer as prepared in section 2.3.3. After vortexing and spun at 12,045 x g for 5 min, the pellet was resuspended in 1 ml of hybridisation buffer and was subsequently aliquoted into 14 individual 1.5 ml microcentrifuge tubes, each containing 50 µl of the sample. A 4 µl volume of a specific probe (Bif, Lab, Bac, Erec, Rrec, Ato, Prop, Fprau, DSV and Chis;

Table 2.1), at 50 ng/ml, was added individually to each microcentrifuge tube together with 4 µl of Alexa-488 labelled probe Eub338-I, -II and –III (Table 2.1) (prepared by combining equal volumes of each of the three Eub388 probes). Four control tubes were prepared by adding only 4 µl of Alexa-488 linked Non-Eub and Eub I/II/III as well as Alexa-647 linked Non-Eub and Eub I/II/III. All 14 tubes were incubated overnight at 35 °C using a heating block.

The next day, 150 µl of hybridisation buffer were added to each tube, tubes were vortexed gently and then spun at 12,045 x g for 5 min. The supernatant was then discarded and the pellet was washed with 200 µl of washing buffer (as prepared in section 2.3.3), vortexed gently and incubated at 37 °C for 20 min using a heating block. The tubes were spun and supernatants were discarded. Then, 400 µl of 1x PBS were added to each tube and the samples were analysed using a flow cytometer (Accuri C6, BD Biosciences, USA) with a medium flow rate and a fixed count of 100,000 counts.

Table 2.1 Oligonucleotides fluorescence probes used during analysis. Working concentration 50 ng/ml.

Probe Name	Sequence (5' to 3')	Fluorescence
Non Eub	ACTCCTACGGGAGGCAGC	Alexa-488
Eub338I	GCTGCCTCCCGTAGGAGT	Alexa-488
Eub338II	GCAGCCACCCGTAGGTGT	Alexa-488
Eub338III	GCTGCCACCCGTAGGTGT	Alexa-488
Non Eub	ACTCCTACGGGAGGCAGC	Alexa-647
Eub338I	GCTGCCTCCCGTAGGAGT	Alexa-647
Eub338II	GCAGCCACCCGTAGGTGT	Alexa-647
Eub338III	GCTGCCACCCGTAGGTGT	Alexa-647
Bif164	CATCCGGCATTACCACCC	Alexa-647
Lab158	GGTATTAGCAYCTGTTTCCA	Alexa-647
Bac303	CCAATGTGGGGGACCTT	Alexa-647
Erec482	GCTTCTTAGTCARGTACCG	Alexa-647
Rrec584	TCAGACTTGCCGYACCGC	Alexa-647
Ato291	GGTCGGTCTCTCAACCC	Alexa-647
Prop853	ATTGCGTTAACTCCGGCAC	Alexa-647
Fprau655	CGCCTACCTCTGCACTAC	Alexa-647
DSV687	TACGGATTTCACTCCT	Alexa-647
Chis150	TTATGCGGTATTAATCTYCCTTT	Alexa-647

2.5 DNA extraction

2.5.1 DNA isolation by bead beating and column based (RBB+C) purification

The DNA extraction method was adapted from Yu and Morrison (2004). Acid-washed glass beads (BioSpec Products; 0.1 g, 0.1 mm) were added to 2 ml screw cap microcentrifuge tubes. Cells stored in 50% glycerol/PBS were harvested by centrifugation at 12,045 x g (Eppendorf Minispin) for 5 min, resuspended in 500 µl lysis buffer (500 mM NaCl, 500 mM Tris-HCl, pH 8.0, 50 mM EDTA, 4% SDS) and then transferred to microcentrifuge tubes containing the beads. The tubes were homogenised using a FastPrep®-24 Instrument (MP Biomedicals, USA) at speed setting 6 for 1 min, and were then placed on ice for 1 min. The cycle was repeated twice and then all tubes were incubated at 95 °C using a heating block for 15 min. The samples were then spun at 4 °C at 12,045 x g for 5 min and the supernatants were transferred to new 1.5 ml microcentrifuge tubes. A 130 µl volume of ice cold 10M ammonium acetate was added to each tube and the tubes were left to incubate on ice for 5 min before being spun at 4 °C at 12,045 x g for 10 min. The resulting supernatant were transferred to new 1.5 ml microcentrifuge tubes. Ice-cold iso-propanol (750 µl) was added to each tube and tubes were incubated on ice for 30 min. Samples were spun at 4 °C at 12,045 x g for 15 min and supernatants were discarded. The nucleic acid pellet was washed with 500 µl 70% ethanol by pipetting up and down a few times until the pellet was resuspended completely. After resuspension, samples were spun again at 4 °C at 12,045 x g for 15 min and left to air dry for 30 min before adding 200µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). The DNA samples were then purified using a QIAamp DNA Mini Kit (Qiagen, Germany) to remove RNA and proteins. This involved adding 2 µl of PureLink® RNAse A (10 mg/ml) to each tube and incubation at 37 °C for 15 min using a heating block. Proteinase K (15 µl) and 200 µl of buffer AL (provided in the kit) were added to the tubes which were then incubated at 70 °C for 10 min. Ethanol (200 µl) was subsequently added and the samples were transferred to a QIAamp column and spun for 1 min. The flow-through was discarded and 500 µl of Buffer AW1 (provided in the kit) were added and centrifuged again for another minute. This step was repeated with Buffer AW2 (provided in the kit) and the tubes were

spun for 1 min to dry the columns. To elute the DNA, 50 µl of Buffer AE (provided in the kit) were added to each column followed by incubation at room temperature (20°C) for 1 min before being spun for 1 min at 12,045 x g. The eluted DNA was kept at -20 °C until further processing.

2.5.2 DNA isolation using commercial kit

2.5.2.1 DNA isolation using a FastDNA™ SPIN Kit for Soil

DNA was isolated using a FastDNA™ SPIN Kit for Soil (MP Biomedicals, USA). Cells stored in 50% glycerol/PBS were harvested by centrifugation at 12,045 x g (Eppendorf Minispin) for 5 min. The supernatant was discarded and the pellet was washed with 500 µl 1 X PBS by pipetting up and down until the pellet was suspended completely being spun at 12,045 x g for 5 min. The supernatants were discarded and the pellets were resuspended in 978 µl of sodium phosphate buffer (provided in the kit) which was then transferred to lysing matrix E (provided in the kit). The subsequent steps were carried out according to the manufacturer's instruction. Eluted DNA was stored at -20 °C until further processing.

2.6 Polymerase chain reaction and gel electrophoresis

2.6.1 Polymerase chain reaction (PCR)

The V4-V5 region of bacterial 16S rRNA was amplified using primers U515F (GTGYCAGCMGCCGCGGTA) and U927R (CCCGYCAATTCMTTTRAGT). Each 50 µl of PCR mixture contained the following components in a 0.5 ml thin wall PCR tube: 36.75 µl of qH_2O, 5 µl of DreamTaq™ buffer, 5 µl dNTPs mixture (10 mM), 1 µl of each primer, 1.25U DreamTaq™ polymerase and 1 µl DNA template. All PCR amplifications were performed using a T100™ Thermal Cycler (Bio-Rad, USA) with the lid heated to 105 °C. After initial denaturation at 95 °C for 2 min, samples were subjected to 36 thermal cycles (denaturation at 95 °C for 30 s, annealing at 49.5 °C for 30 s and extension at 72 °C for 30 s), followed by a final extension at 72 °C for 10 min and hold at 4 °C upon completion. PCR products were confirmed by agarose-gel electrophoresis.

2.6.2 Agarose gel electrophoresis

Gel electrophoresis was used to determine the size and quality of DNA as well as the presence of PCR products. Gels were generally 0.6% (w/v) agarose in 0.5X TBE buffer (40 mM Tris, 40 mM

borate, 1 mM EDTA (pH 8)). Gel staining was performed by adding 1 µl of GelRed™ fluorescence DNA stain (Biotium, USA) in 50 ml of gel solution. DNA samples (2 µl) were loaded with 2 µl DNA loading dye (10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% v/v glycerol, 60 mM EDTA) and 6 µl qH_2O. GeneRuler™ 1kb DNA ladder was used as DNA size marker (0.5 µl of GeneRuler™, 2 µl DNA loading dye, 20 µl qH_2O). Samples were electrophoresed for 60 min at a constant voltage of 60 V in a Mini-Sub® Cell GT cell (Bio-Rad, USA) and were visualised using G:Box (Syngene, UK).

2.8 Analysis of microbial community

Isolated microbiota DNA was subject to 16S rRNA microbial community analysis by deep sequencing (APHA, UK) using a MiSeq Sequencing System (Illumina, USA). The results were analysed using the Microbial Genomics module of the CLC Genomics Workbench program (Qiagen, Germany). The Greengenes data base (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) was used as the reference database to identify the OTUs obtained.

2.9 Analysis whole-cell and medium metal content by ICP-OES

The cell pellet was resuspended in 735 µl of metal-free nitric acid (TraceMetal™ Grade, Fisher Scientific) and transferred to a 15 ml Falcon tube. The supernatant (2 ml) was transferred to a 15ml Falcon tube and 735µl of 67-69%metal-free nitric acid was added. Both tubes are then incubated at 80 °C for 24 h. 9.265 ml of qH_2O and 7.265 ml of qH_2O were then added to the pellet and supernatant containing solutions, respectively. The tubes were then kept at 4°C until analysis.

2.10 Short chain fatty acid analysis by GC

Standards were prepared as listed in the table below:

Table 2.3 Standards used in short chain fatty acid analysis

Acid	Acid volume (µl)	Water volume (µl)
Acetic	573	9427
Butyric	914	9086

Formic	397.3	9603
Propionic	746	9254
Lactic	877	9123
Isobutyric	918	9082
Isovaleric	1104	8896
Valeric	1088	8912
Caproic	1253	8747
Sodium succinate	2.7g	10000

All the acids were reagent grade (>95%) and in liquid form with the exception of sodium succinate which is in powder form. A supermix was then prepared by mixing 3 ml of each acid to a 15 ml Falcon tube and vortexing briefly. A serial dilution were made to obtain give 0, 20, 40, 60, 80 and 100% supermix concentrations in order to provide a standard curve. An internal standard was prepared by adding 1.258ml of 2-ethyl butyric to 100ml of HPLC grade water (Fisher Scientific). Volumes of 500 µl of each of the acids (standards), supermix and samples were added to separate 10 ml glass vials and 25 µl of internal standard was added to each vial. A 250 µl volume of concentrated hydrochloric acid was subsequently added to each vial, followed by 1.5 ml of diethyl ether. The vials were then vortexed for 1 min and spun at 268 x g for 10 min. The liquid separated into two phases and 200 µl of the upper layer were taken and added to a new 4 ml glass vial. Then, 25 µl of *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) were added to each vial and the lid was closed tightly. All vials were left at room temperature (20°C) for 3 days and subsequently analysed by HPLC

2.11 Effects of iron on gut microbiota

A 1 ml volume of fresh 10% (w/v) faecal slurry was inoculated into four separate Hungate tubes (experiment carried out in duplicate) containing 9 ml of gut model medium, with and without iron

supplementation. Gut model medium with iron was prepared as described in section 2.3.1 while the medium without iron was prepared in similar manner except with omission of $FeSO_4$ and haemin. Sampling time points were 0 and 24 h. DNA and Flow-FISH samples were collected and processed at both time points. All work was conducted in an anaerobic environment and all tubes were incubated at 37 °C in the anaerobic cabinet (Whitley A95 Workstation, Don Whitley Scientific, UK).

2.11.1 Sample collection and processing

2.11.1.1 DNA

A 1 ml volume of the culture was taken at 0 h and 24 h from every tube and transferred to sterile 1.5 ml microcentrifuge tubes. The samples were then spun at 12,045 x g (Eppendorf Minispin) for 5 min. Supernatant was transferred to a fresh 1.5 ml microcentrifuge tube and stored at -20 °C for further analysis. The pellet was resuspended in 1 ml 50% glycerol/PBS and stored at -20 °C.

2.11.1.2 Flow-FISH

A 750 µl volume of culture was taken at 0 h and 24 h from every tube and transferred to a sterile 1.5 ml microcentrifuge tube. The cells were fixed immediately according to the method described in section 2.4.1.

2.12 The use of buffer as an alternative to pH regulation by titration with acid and alkali

Gut model medium was prepared as described in section 2.3.1 and the pH was adjusted using 10 M HCl and 5 M NaOH to the desired value. The medium was autoclaved immediately after pH adjustment and kept in the anaerobic cabinet to equilibrate at least 24 h before use. Fresh 10% (w/v) faecal slurry was added to the gut model medium to create a master mix before distribution into individual 50 ml Falcon tubes. Filter-sterilised qH_2O and 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH range 5.5-6.7, pKa 5.97, 37°C) were used as negative and positive controls, respectively. A 5 ml volume of qH_2O, 1, 2 or 3 M MES buffer was added to the Falcon tubes individually. Each tube had a final volume of 50 ml and a final concentration of 1% faecal slurry with 0, 0.1, 0.2 or 0.3 M MES buffer. Sampling time points were 0, 4, 8, 12, 24, 30, 36 and 48 h. All work was conducted in an anaerobic environment and all tubes were incubated at 37 °C in the anaerobic cabinet.

2.12.1 Sample collection and processing

2.12.1.1 DNA

A 1 ml volume of the culture was taken at 0 h and 24 h from every tube and transferred to sterile 1.5 ml microcentrifuge tubes. The samples were then centrifuged at 12,045 x g (Eppendorf Minispin) for 5 min. The supernatant was transferred to a fresh 1.5 ml microcentrifuge tube and stored at -20 °C for further analysis. The pellet was resuspended in 1 ml 50% glycerol/PBS and stored at -20 °C.

2.12.1.2 Flow-FISH

A 750 µl volume of culture was taken at 0, 4, 8, 12 and 24 h from every tube and transferred to a sterile 1.5 ml microcentrifuge tube. The cells were fixed immediately according to the method describe in section 2.4.1

2.12.1.3 pH

A 1 ml volume of culture was taken at 0, 4, 8, 12, 24, 30, 36 and 48 h from every tube and transferred to a sterile 1.5 ml microcentrifuge tube. The pH of the samples was then measured using a pre-calibrated pH meter (calibrated with pH 4 and pH 7 buffers).

2.13 The effect of iron and haem on the gut microbiota using batch culture fermentations

Sterile batch culture vessels (280ml) were filled with 198 ml of gut model medium as described in section 2.3.1 and allowed to equilibrate under anaerobic gas (O_2-free N_2) for 24 h prior to inoculation. Four vessels were set up, each representing a different condition: no iron (negative control), 18 µM $FeSO_4$, 77 µM haem or both 18 µM $FeSO_4$ and 77 µM haem. A 2 ml volume of fresh 1% (w/v) faecal slurry was added to each vessel and the pH was maintained at 5.5 (to stimulate the proximal region of the colon) throughout the experiment by the automatic addition of 1 M HCl or 1 M NaOH using a pH meter. The vessels were incubated at 37 °C and samples were taken at t0, t12, t24, t36 and t48. Samples collected were stored directly on ice and processed accordingly for community profiling (NGS), bacterial counts (Flow-FISH), metal analysis (ICP-OES) and short chain fatty acid analysis (GC). The processed samples were stored at -80 °C (NGS and GC) or -20 °C (Flow-FISH and ICP-OES). The experiment was repeated with four different healthy donors.

2.13.1 Sample collection and processing

2.13.1.1 DNA

A 1 ml volume of the culture was taken at 0, 12, 24, 36 and 48 h from every vessel and transferred to a sterile 1.5 ml microcentrifuge tube. The samples were then spun at 12,045 x g (Eppendorf Minispin) for 5 min. Supernatant was transferred to a fresh 1.5 ml microcentrifuge tube. Both pellet and supernatant were immediately placed at -80 °C until further processing.

2.13.1.2 Flow-FISH

A 750 µl volume of culture was taken at 0, 12, 24, 36 and 48 h from every vessel and transferred to a sterile 1.5 ml microcentrifuge tube. The cells were fixed immediately according to the method describe in section 2.4.1.

2.13.1.3 Metal analysis by ICP-OES

A 2 ml volume of the culture was taken at 0, 12, 24, 36 and 48 h from every vessel and transferred to a sterile 2 ml microcentrifuge tube. The samples were then spun at 12,045 x g (Eppendorf Minispin) for 5 min. Supernatant was transferred to a fresh 2 ml microcentrifuge tube. Both pellet and supernatant were immediately placed at -20°C until further processing (section 2.9).

2.13.1.4 Short chain fatty acid analysis by GC

A 1 ml volume of supernatant was taken at each time point and kept at -80 °C until further processing (section 2.10).

2.14 The effect of zinc on the gut microbiota using batch culture fermentations

Condition were as above except for use of modified gut model medium (2.3.1.1) and the use of zinc in place of iron or haem, as follows: four vessels were containing either no zinc (negative control), 77 µM, 192 µM or 770 µM $ZnSO_4$. A 200 µl volume of the vitamin and mineral stock solutions were added to each vessel before the addition of faecal slurry. Same analysis were carried out as described in section 2.13.1

2.15 The effect of zinc on the gut microbiota using a continuous three-stage gut model

A continuous three-stage gut model was used to examine the effect of zinc on different parts of the colon. The system consists of three vessels connected in series representing the proximal, transverse and distal colon. The fresh culture medium feeds into the first vessel (V1), the output of V1

subsequently feeds into the second vessel (V2), and the output of V2 then feeds into the third vessel (V3), with output of V3 collected in the waste bottle. Each vessel was maintained at a specific working volume (V1, 80 ml; V2, 100 ml; and V3, 120 ml and pH (V1, 5.5; V2, 6.2; and V3, 6.8).

A 4-litre culture medium was prepared using the modified gut model medium as described in section 2.3.1.1 and 4 ml of the vitamin and mineral stock solutions were added to the medium. The culture medium was autoclaved and equilibrated under anaerobic gas (O_2-free N_2) for 2 days. Just like the batch culture fermentation, sterile vessels were filled with culture medium and left to equilibrate under anaerobic gas for 24 h prior to the experiment. V1 was filled with 51.43 ml of culture medium, V2 with 66.67ml and V3 with 82.50ml. On the day of the experiment, fresh 20% (w/v) faecal slurry was prepared and inoculated into each vessel at different volume: V1 (28.57 ml), V2 (33.33 ml) and V3 (37.50 ml). After inoculation, the bacteria were left to propagate in the vessels for 24 h before starting the medium pump. At 24 h, fresh culture medium was introduced to V1 through an automated pump and the gut model is allowed to continue for two weeks in order to reach a steady state (SS) for sampling. Fresh medium was pumped at a rate of 300 ml/48 h to represent colonic movement in humans. Once the steady state was reached, samples were taken and a new bottle of fresh 4-litre culture medium was provided. The gut model was left to continue for another two weeks until the next steady state was achieved.

Samples were taken at t0, t24, SS1, SS2, SS3 and SS4. Samples collected were stored directly on ice and processed accordingly for community profiling (NGS), bacterial counts (Flow-FISH), metal analysis (ICP-OES) and short chain fatty acid analysis (GC). The processed sample is stored at -80 °C (NGS and GC) or -20 °C (Flow-FISH and ICP-OES). Sample processing were as described above (section 2.13.1).

Table 2.3 Conditions tested at each steady state of the gut model.

	2 weeks	4 weeks	6 weeks
	SS1	SS2	SS3
Gut model 1	mGMM (section 2.3.1.1) with 16 µM Zn	mGMM	mGMM with 93 µM Zn
Gut model 2	mGMM (section 2.3.1.1) with 16 µM Zn	mGMM (section 2.3.1.1) with 93 µM Zn	mGMM (section 2.3.1.1) with 786 µM Zn
Gut model 3	GMM (section 2.3.1)	mGMM (section 2.3.1.1) with 16 µM Zn	

2.16 Phytic acid utilisation in bacteria

2.16.1 Bacterial strains, plasmids and primers

Table 2.4 Sources and sequence of bacterial strains and plasmid

Type	Source
Bacterial strain	
***E.coli* BW25113**	Keio collection (Baba et al, 2006)
E.coli* BW25113Δ*appA	
E.coli* BW25113Δ*agp	
E.coli* BW25113Δ*phoA	
E.coli* BW25113Δ*appA*Δ*agp	This work
E.coli* BW25113Δ*appA*Δ*phoA	
E.coli* BW25113Δ*agp*Δ*phoA	
E.coli* BW25113Δ*appA*Δ*agp*Δ*phoA	
Plasmid	
pKD46	Lab Stock
pKD3	
pCP20	

Table 2.5 Sequence of primers used

Primers used	Sequence
pKD3-appA-F	GCATCAGGCAATCAATAATGTCAGATATGAAAAGCGGAAACATATCGATGAAAGCGATCTTGTGTAGGCTGGAGCTGCTTC
pKD3-appA-R	CGTTTGTCATCAGCCTCAGAGCATTCAGGTAACTGAATGCTCTTTTTTATGCATTACAAACCATATGAATATCCTCCTTAGT
pKD3-agp-F	ATTTCTGTCACACTCTTTAGTGATTGATAACAAAAGAGGTGCCAGGAATGAACAAAACGTGTGTAGGCTGGAGCTGCTTC
pKD3-agp-R	AAAACGTTTAACCAGCGACTCCCCCGCTTCTCGCGGGGGAGTTTTCTGTTATTTCACCATATGAATATCCTCCTTAGT
appA-F	GGCGCATTAGCATCGCATCAG

appA-R	CAGCCTCAGAGCATTCAGGTAAC
agp-F	TGCGAACCTGTTCCCGGAA
agp-R	GGCGAACAGACCATCGAACGT
phoA-F	CCGATGCCAGCATTCCTGACG
phoA-R	GTTCTCTCGGCAGCGCCGAC
Kan-F	CATCAGAGCAGCCGATTGTCTGTTG
Kan-R	ACGTACTCGGATGGAAGCCGGTCT
Cat-F	GGCCGGATAAAACTTGTGCTT
Cat-R	GTTACGGTGAAAACCTGGCC
appA100-F	GCTCTCCACCCTTGTGTTGGTATG
appA100-R	CTCCGAGAAAGAGTAAAACTGAAAGTGGC
appA200-F	GACAACAGAAACTCTCCGCCGTAACGA
appA200-R	CAGGCAGGCGTCAATATTAAAGGCGC
agp100-F	GCATAGTTTGCGTCAAACCTTGCCTG
agp100-R	GAAGAAGGTAGTGATGAAGTGTTAGTCAAAGCC
agp200-F	GTCTCTGTATTGGTAACGCCGCAG
agp200-R	GGCATCTCTGCGCAATACGTCGCC
phoA100-F	GCTGCTGCGCGATTACGTAAAGAAGTT
phoA100-R	GTAATGTTATTTTCATAGCACCATCCCTCTTC
phoA200-F	GACCAACAGCGGTTGATTGATCAGG
phoA200-R	CTGCTGATTACAGGAGGTCATACGC

2.16.2 Generation of double and triple mutants

2.16.2.1 Generation of PCR products

Gene-specific mutagenic PCR products carrying a Kn^R cassette were generated using pKD3 DNA as template with primers pkD3-appA or pkD3-agp (Table 2.4) using conditions mentioned in section 2.6.1. Once the PCR product was confirmed using agarose-gel electrophoresis, the desired band was excised from an agarose gel and the product was recovered using a gel extraction kit.

2.16.2.2 Generation of electrocompetent cells

In order to generate electrocompetent cells, 0.5 ml of an overnight culture of the desired bacterial strain was inoculated into 50 ml of L-broth and incubated in a shaking incubator at 37 °C. Once the OD_{600} reached 0.5, the cells were harvested by centrifugation at 12045 x g and washed twice with 25 ml ice cold 10% glycerol. Cells were resuspended in the residual liquid and 50 µl of cells were aliquoted into ice cold 1.5 ml microcentrifuge tubes and used directly or kept at -80 °C until required.

2.16.2.3 Introduction of pKD46 by electroporation

A 1 µl volume of plasmid DNA was added to electrocompetent cells before transfer to ice-cold electroporation cuvettes and application of a single electrical pulse at 1.8kV. A 1 ml volume of SOC

(section 2.3.4) medium was directly added to the mixture which was incubated at 30 °C for 1-2 h. The cells were then plated onto the appropriate antibiotic plates and incubated at 30 °C for 24 h.

2.16.2.4 Generation of mutants

A 0.5 ml volume of transformants containing the *red gam*-encoding plasmid, pKD46, was inoculated into 50 ml LB containing 100 μg/ml ampicillin and 100 mM arabinose. The mixture was incubated in a shaking incubator at 30 °C. The transformants were harvested and subjected to electrotransformation as described above (2.16.2.3) except that 1 μg of PCR product (2.16.2.3) was used in place of pKD46. The cells were then plated onto ampicillin L-agar and incubated at 30 °C. Colonies were confirmed by colony PCR using antibiotic cassette specific primers (Table 2.5).

2.16.3 Phytic acid as a phosphate and carbon source for bacterial growth

Once the medium had been prepared as in sections 2.3.3 and 2.3.3.1, they were utilised to determine the effect of phytate on bacterial growth. Normal M9 minimal medium with/without glucose) was used for tested phytate as a carbon source, while a low-phosphate M9 minimal medium was used to test phytate as a phosphate source. Phytate was added to tubes at varying concentrations and the OD_{600} for overnight bacterial cultures was adjusted to 0.01. The growth of the bacteria was measured using the Bioscreen C (automated growth curve reader) with continuous shaking and measurement at 30 min interval. Each condition was performed in triplicate.

2.16.4 The effect of pH on the activity of phosphatases

A combination of three different buffers were mixed to create a master mix. Three solutions, one for each buffer at 300 mM concentration, were mixed together in a 1:1:1 ratio to create a final concentration of 100 mM each. The buffers used were: 2-(N-morpholino)ethanesulfonic acid (MES) (pH range 5.5-6.7, pKa 5.97, 37°C); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH range 6.8-8.2, pKa 7.31, 37°C); [tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS) (pH range 7.7-9.1, pKa 8.18, 37°C). The pH of the master mix was then adjusted to 5.5, 7 and 8.5 to simulate acidic, neutral and alkaline environment. A 1 ml volume of the master mix was added to 9 ml of medium to achieve a final concentration of 10 mM. An overnight culture was added and the starting OD_{600} was adjusted to 0.01. The growth of the bacteria was measured using the Bioscreen C

with continuous shaking and measurement at 30 min interval. Each condition was performed in triplicate.

2.16.5 Utilisation of phytate by gut microbiota

2.16.5.1 Phytic acid as a phosphate source

Sterile batch culture vessels (280ml) were filled with 198 ml of basal medium, as described in section 2.3.5.1, and allowed to equilibrate under anaerobic gas (O_2-free N_2) for 24 h prior to the inoculation. Four vessels were set up, each representing a different condition: no phosphate (negative control), phosphate (0.04g of KH_2PO_4 and 0.04g of K_2HPO_4), 10 mM phytate or both phosphate and phytate. A 200 µl volume of the vitamin and mineral stock solutions (section 2.3.1.2) was added to each vessel before the addition of faecal slurry. A 2 ml volume of fresh 1% (w/v) faecal slurry was added to each vessel and the pH was maintained at 5.5 throughout the experiment by the automatic addition of 1 M HCl or 1 M NaOH using a pH meter to stimulate the proximal region of the colon. The vessels are incubated at 37 °C and samples were taken at t0, t12, t24 and t48. Samples collected were stored directly on ice and processed accordingly for community profiling (NGS), bacterial counts (Flow-FISH), metal analysis (ICP-OES) and short chain fatty acid analysis (GC) (sections 2.13.1). The processed sample were stored at -80 °C (NGS and GC) or -20 °C (Flow-FISH and ICP-OES). The experiment was repeated with four different healthy donors.

2.16.5.2 Phytic acid as a carbon source

Details were as above except that the four vessels were set up as follows: no starch (negative control); 5g of starch; 10 mM phytic acid or both 5 g starch and 10 mM phytic acid.

Chapter 3: Effect of iron on the gut microbiota using Hungate tubes

3.1 Introduction

Iron is a micronutrient that is indispensable for most living organisms, with the exception of *Lactobacillus*, as it is involved in numerous biological functions required for growth and development (Andrews et al, 2003). Although iron is important in maintaining the wellbeing of humans, iron deficiency remains one of the most prevalent public health problems, affecting an estimated 2 billion people worldwide both in developing and industrialised countries with women and children most at risk (Zimmermann and Hurrell, 2007). Efforts have been made to overcome iron deficiency which includes oral supplementation of ferrous iron salts, and fortification of food and biofortification of staple foods (Zimmermann and Hurrell, 2007). Although the use of iron supplementation and fortification is beneficial to humans from the nutrition perspective, from a gut microbiota standpoint, it can also have negative impacts as it has been shown to reduce the level of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* coupled with an increase of Enterobacteriaceae which shifts the gut microbiota towards a pathogenic profile (Zimmermann et al, 2010; Jaeggi et al, 2014). The experiment described in this chapter was designed to determine the effect of iron on the gut microbiota. Gut model medium (GMM) was utilised in the experiment with the normal iron ingredients, $FeSO_4$ and haem, either included or excluded. Thus, two conditions were employed: normal iron (GMM with 18 µM iron sulphate and 77 µM haem); and iron-reduced medium (GMM without inclusion of ferrous sulphate or haem; background iron at 28 µM). This experiment was performed to test whether simplistic anaerobic Hungate tube cultures could be used as an effective experimental tool to understand how the gut microbiota react towards iron/haem regime change in the gut of a healthy human, and as a preliminary test to determine whether an impact of iron regime could be discerned that would justify further experimentation. Since the experimental condition needed to be anaerobic, all preparation and experimental procedure were carried out in the anaerobic cabinet. Hungate tubes were filled with 9 ml of either the control medium (GMM without haem and $FeSO_4$) or normal GMM (with haem and $FeSO_4$). 1ml of

faecal innocula (10% w/v) from 3 different healthy subjects (1 male, 2 female; no antibiotic usage 3 months prior) were separately inoculated into the medium and growth was allowed to proceed at 37 ˚C for 24 h (without shaking). Samples were taken via a needle and a syringe to ensure sterility and to prevent contamination of the medium, at t0 and t24, to be processed as in section 2.11.

3.2 Impact of iron on the bacterial numbers

Following anaerobic growth in triplicate at 37 °C for 24 h, total bacteria were determined by Flow-FISH using Eub I-II-III probes (Figure 3.1). Since both regimes showed similar bacterial counts at t0, the t0 values were averaged to ensure a more uniform comparison. At t0, the average total bacterial number was $2.17x10^9$ cells/ml medium. A slight overall 10% increase in cell number was seen in the control (-Fe/haem) at 24 h, whereas the +Fe/haem regime showed a slight decrease of 7%. Thus, compared with the control, the presence of iron and haem causes a 1.18-fold diminished growth of the bacteria. However, this effect was not significant and differences are slight indicating little overall growth. The reason for the weak overall growth might be related to the high starting bacterial cell numbers (resembling those seen at the end of batch gut cultures; Khalil et al, 2014, Costabile et al, 2014; Beards, Tuohy and Gibson, 2010) combined with the lack of pH control.

Table 3.1 Total bacterial numbers at each time point and the fold difference against t0. Data are derived from Figure. 3.1.

Probe	t0	t24		Fold Changes		
				t24/t0		Fe/Control
		Control	Fe	Control	Fe	
Total Bacteria	2.17E+09	2.39E+09	2.02E+09	1.10	0.93	0.85

Table 3.2 Total bacterial number and fold changes of different bacterial groups at phylum level determined by Flow-FISH. The total bacterial number of different bacterial groups were calculated at t0 and t24 for the control and the iron regime. Bacterial counts are expressed as cells/ml medium The results are the average of 3 subjects. One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. Values in bold indicates significant difference ($p<0.05$) with respect to t0 time point.

Phylum	t0	t24		Fold Changes		
				t24/t0		Fe/Control
		Control	Fe	Control	Fe	
Actinobacteria	2.07E+08	1.41E+09	1.23E+09	6.83	5.94	0.87
Bacteroidetes	7.24E+07	5.50E+08	**9.97E+08**	7.59	**13.8**	1.81
Firmicutes	6.41E+08	6.74E+08	8.78E+08	1.05	1.37	1.30

Proteobacteria	1.22E+08	4.02E+07	6.51E+07	0.33	0.53	1.62

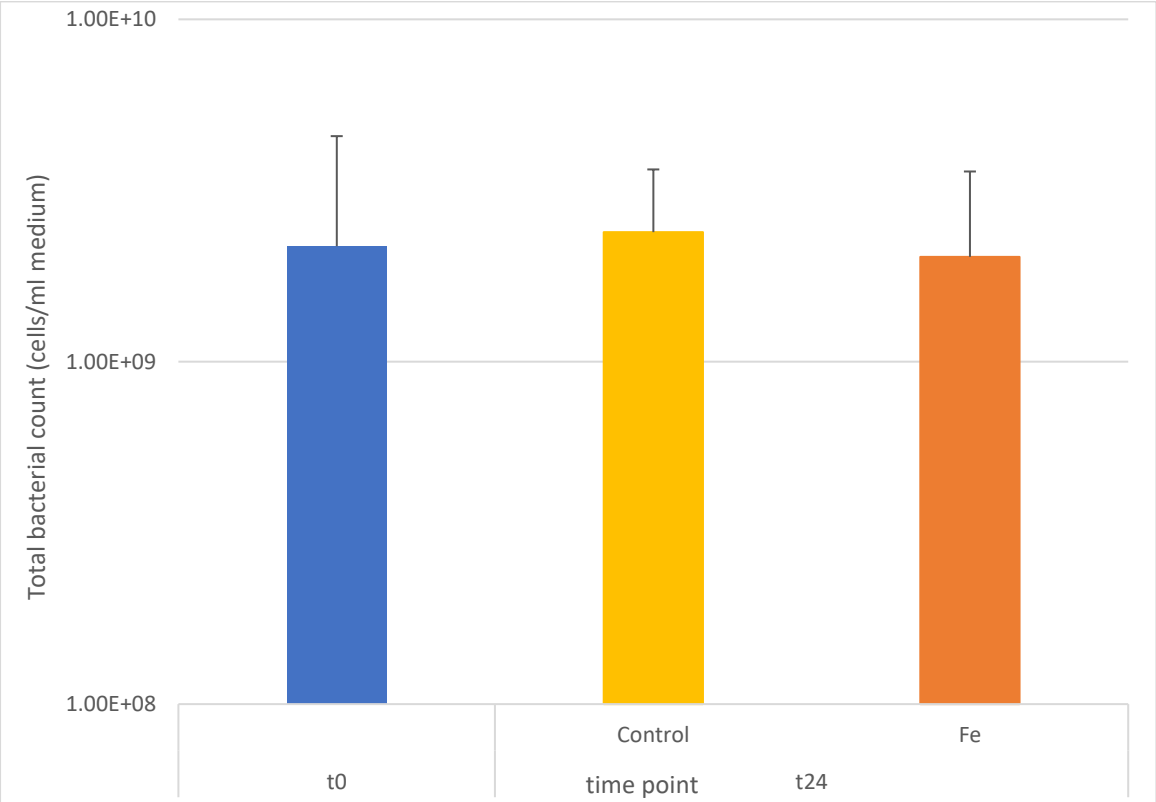


Figure 3.1 Effect of Iron regime on total bacterial counts. Total bacterial counts for each regime were determined by Flow-FISH using Eub I-II-III probes. Each bar indicates a different regime/time point: blue (t0) for both conditions (data combined); yellow (GMM -Fe/haem) and orange (GMM +Fe/haem). Bacterial counts are expressed as cells/ml medium. Results are average of 3 subjects and error bars represent SD. One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. No significant difference is seen ($p>0.05$).

3.3 Impact of iron and haem on the gut microbiota determined by Flow-FISH

3.3.1 Impact of iron and haem at phylum level

In order to determine the impact of iron/haem on key taxa, Flow-FISH analysis was performed using the following probes: Actinobacteria (Bif164, Ato291), Bacteroidetes (Bac303), Firmicutes (Lab158, Erec482, Rrec584, Prop853, Fprau655, Chis150) and Proteobacteria (DSV687).

As seen from table 3.2, Bacteroidetes showed the biggest change among all the bacteria with 7.59-fold increase in the control and a significant increase of 13.8-fold ($p<0.05$) for the +Fe/haem condition as compared to t0. The former increased from 7.24×10^7 cells/ml medium to 5.50×10^8 while the latter increased to 9.97×10^8 cells at t24. The presence of iron and haem increased the total Bacteroidetes by 1.81-fold compared to the control.

The other 3 phyla showed no significant change for either regime ($p>0.05$) but both Actinobacteria and Firmicutes were increased in number at t24 while Proteobacteria showed a decrease. Both the control and the +Fe/haem regime showed an increase in Actinobacteria of 6.83- and 5.94-fold, respectively, increasing total Actinobacteria bacterial counts from $2.07x10^8$ cells/ml medium to $1.41x10^9$ and $1.23x10^9$ at t24, respectively. Firmicutes also showed an increase in both regimes with numbers in the control increasing 1.05-fold) and the +Fe/haem regime increased by 1.37-fold at t24, as compared to t0. The presence of haem/iron benefitted the Firmicutes phylum as the iron regime contained 1.30-fold more Firmicutes bacteria than the control, but it negatively affected the Actinobacteria bacteria as there were 1.15-fold less bacterial counts for the +Fe/haem regime than compared to the control. Proteobacteria, on the other hand, showed a decrease of 3.03- and 1.88-fold for the control and the +Fe/haem regime respectively at t24, reducing its total counts from $1.22x10^8$ cells/ml medium (t0) to $4.02x10^7$ and $6.51x10^7$, accordingly. Even though both regimes showed a decrease in Actinobacteria numbers, the presence of iron/haem caused a smaller decrease such that the +Fe/haem regime had 1.62-fold more bacteria than the control at t24.

3.3.2 Impact of iron at genus level

At lower taxonomic level (table 3.3), Bac and DSV showed the same result as the Bacteroidetes and Proteobacteria phylum as they are the only bacteria in that particular phylum, thus they will not be mentioned further in this section.

Table 3.3 Total bacterial number and fold changes of different bacterial groups at genus level determined by Flow-FISH. The total bacterial number of different bacterial groups were calculated at t0 and t24 for the control and the iron regime. Bacterial counts are expressed as cells/ml medium The results are the average of 3 subjects. One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. Values in bold indicates significant difference ($p<0.05$) with respect to t0 time point.

Probe	t0	t24		Fold Changes		
				t24/t0		Fe/Control
		Control	Fe	Control	Fe	
Bif164	2.00E+08	1.10E+09	1.13E+09	5.48	5.65	1.03
Bac303	7.24E+07	5.50E+08	**9.97E+08**	7.59	**13.77**	1.81
Lab158	3.48E+06	2.97E+07	3.49E+07	8.54	10.05	1.18

Erec482	2.23E+08	2.11E+08	3.33E+08	0.94	1.49	1.58
Rrec584	6.76E+07	7.62E+07	1.64E+08	1.13	2.42	2.15
Ato291	6.56E+06	**3.16E+08**	9.73E+07	**48.17**	14.84	0.31
Prop853	4.65E+07	4.65E+07	3.34E+07	1.00	0.72	0.72
Fprau655	2.84E+08	2.98E+08	3.06E+08	1.05	1.08	1.03
Chis150	1.57E+07	1.31E+07	6.11E+06	0.84	0.39	0.47
DSV687	1.22E+08	4.02E+07	6.51E+07	0.33	0.53	1.62

The *Atopobium* cluster (Ato291) showed the biggest increase among all the bacterial groups with a significant increase of 48.2-fold in the control ($p<0.05$), increasing total counts to $3.16x10^8$ cells/ml medium (t24) from $6.56x10^6$ at t0. The +Fe/hae regime showed a non-significant increase of 14.8-fold to $9.73x10^7$ cells/ml. The presence of iron/haem caused a 3.25-fold decrease in *Atopobium* cell number as compared to the control. Among all the other bacterial groups, none of the groups showed any significant changes when tested statistically ($p>0.05$). *Bifidobacterium* (Bif164), another member of the Actinobacteria phylum, showed similar growth between the control and the +Fe/haem regime indicating little impact of iron/haem. As for the Firmicutes phylum, *Lactobacillus* (Lab158) showed the greatest increase followed by *Roseburia* (Rrec584). *Lactobacillus* displayed a 8.54- and 10.1-fold increase for the control and the +Fe/haem regime, respectively. *Roseburia* on the other hand increased to $7.62x10^7$ cells/ml (control; 1.13-fold) and $1.64x10^8$ cells/ml (iron regime; 2.42-fold) from $6.76x10^7$ cells/ml medium at t0. As for the *Clostridium* clusters, the presence of iron/haem caused a 1.49 increase in *Clostridium* cluster XIVa and XIVb (Erec482), but a slight decrease in the control, indicating an enhancement by Fe/haem. In contrast, there was a decrease of 0.70 fold in +Fe/haem regime, but no effect in the control, for the *Clostridium* cluster IX (Prop853). *Faecalibacterium prausnitzii* (Fprau655) showed little difference between the control and iron regime at t24 with the former increasing 1.05-fold to $2.98x10^8$ cells and the latter increasing 1.08-fold to $3.06x10^8$ cells from $2.84x10^8$ cells/ml medium (t0). *Clostridium* cluster I and II (Chis15) was the only group that showed a decrease in both the control and the +Fe/haem regime whereby the former showed a decrease of 1.20-fold to $4.02x10^7$ cells/ml and the latter experienced a decrease of 2.56-fold to $6.51x10^7$ cells/ml. Thus, among the members in the Firmicutes phylum, the

+iron/haem regime caused enhanced growth for 3 of its members (Lab158, Erec482 and Rrec584), with Rrec584 benefitted the most. Prop853 and Chis150 were inhibited by the +Fe/haem regime whereby they had a reduced growth of 1.39- and 2.14-fold, respectively, as compared to the control. Fprau655 was minimally affected by the +Fe/haem regime (Table 3.3).

3.4 Impact of iron on the gut microbiota determine by NGS

3.4.1 Impact of iron at phylum level

Since the presence of iron and haem resulted in differences in composition as determined by Flow-FISH, the effect of +Fe/haem regime was further explored by 16S rRNA gene amplicon NGS analysis, which provides better detail regarding the bacteria present at various taxonomic levels.

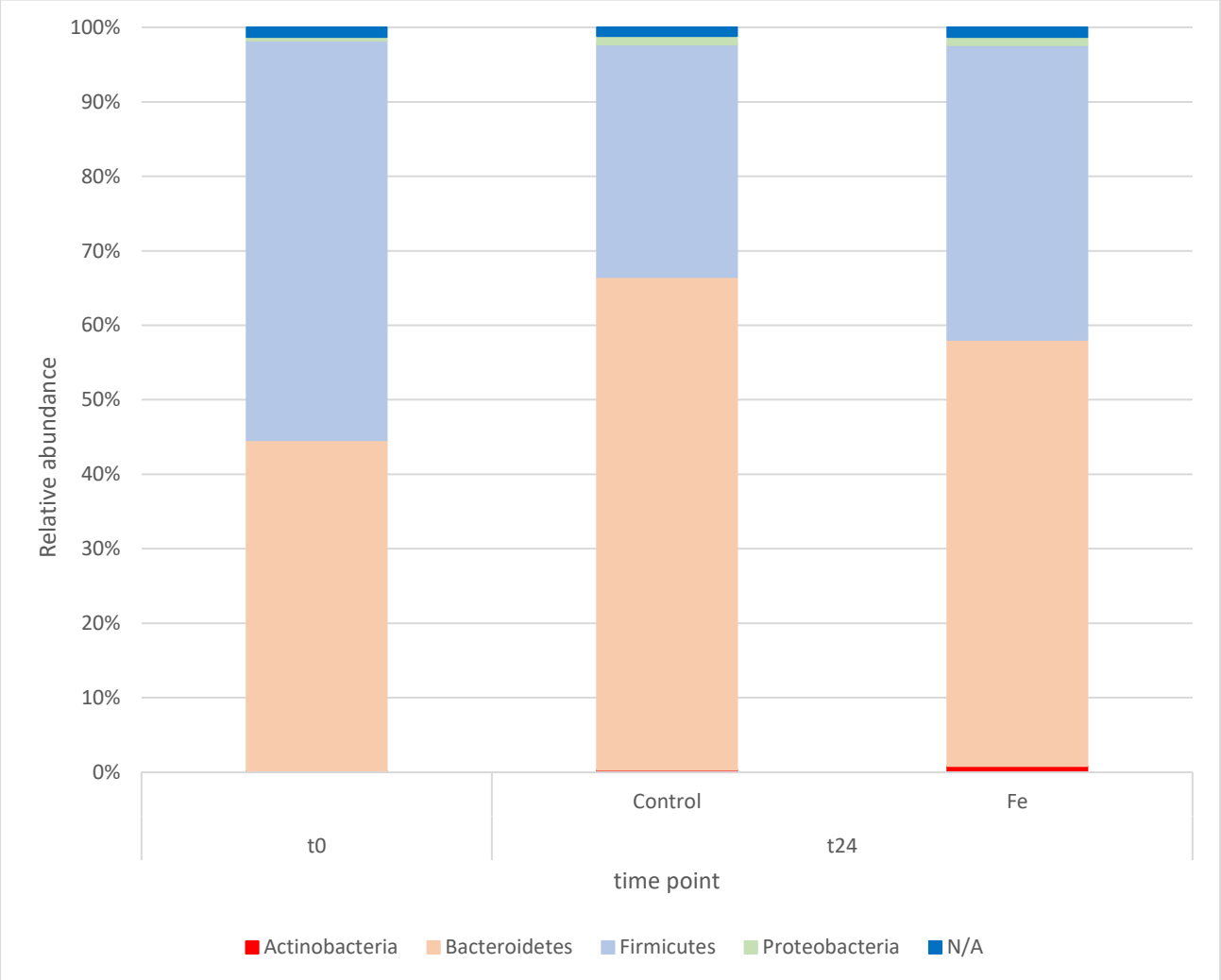


Figure 3.2 Composition of gut microbiota at phylum level determine by NGS. The gut microbiota is classified at phylum level for the control and +Fe/haem regime at t0 and t24.

Table 3.4 Relative abundance and fold changes of different bacterial groups at phylum level determined by NGS. The relative abundance of different bacterial groups at t0 and t24 for the control and the iron regime which they are expressed as a percentage of the total microbial community profile. The results are the average of 3 subjects. One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. No significant difference was detected ($p>0.05$)

Phylum	t0	t24		Fold Changes		
				t24/t0		Fe/Control
		Control	Fe	Control	Fe	
Actinobacteria	0.16	0.30	0.86	1.87	5.31	2.84
Bacteroidetes	44.39	66.19	57.18	1.49	1.29	0.86
Firmicutes	53.63	31.20	39.54	0.58	0.74	1.27
Proteobacteria	0.48	1.14	1.12	2.36	2.33	0.99
N/A	1.31	1.17	1.28	0.89	0.98	1.09

As seen from Figure 3.2, Bacteroidetes and Firmicutes constituted over 90% of the bacteria present in both the control and the +Fe/haem regime while Actinobacteria, Proteobacteria and other unidentified species made up the remaining portion. At t0, Firmicutes was the most abundant phylum with a relative abundance of 53.6%, followed by Bacteroidetes with 44.4%. Actinobacteria and Proteobacteria had a combined abundance of roughly 0.5% at t0. Comparing the change between t0 and t24, the trends observed for each phylum were not affected by the regime; thus the relative abundance of Actinobacteria, Bacteroidetes and Proteobacteria increased while Firmicutes showed a decreased. Bacteroidetes showed a 1.49- and 1.29-fold increase in the control and the +Fe/haem regime, respectively, increasing in abundance to 66.2% and 57.2%, making it the most abundant phylum at t24. However, the presence of iron/haem resulted in a slight reduction in Bacteroidetes (1.16-fold). Actinobacteria increased 1.87- (control) and 5.31-fold (+Fe/haem regime), to 0.30 and 0.86% abundance, respectively, at t24, thus indicating a growth enhancing effect of iron/haem on the Actinobacteria. Proteobacteria on the other hand showed similar increases in the control and +Fe/haem regime whereby the former increased 2.36-fold to 1.14% and the latter increased 2.33-fold to 1.12% at t24. The +Fe/haem regime thus had little effect on the growth of the Proteobacteria. Firmicutes being the only phylum which had a decrease over time showed a 1.72- and 1.36-fold reduction for the control and +Fe/haem regime, reducing in abundance to 31.2 and

39.5%, respectively. Although the Firmicutes showed a decrease at t24, the presence of iron/haem enhanced the growth by 1.27-fold as compared to the control.

3.4.2 Impact of iron at species level

The microbiota composition was also examined at lower taxonomic level to provide a more detailed indication of the effect of the +Fe/haem regime on the gut microbiota (table 3.5).

Table 3.5 Effect of iron regime on relative abundance and fold changes at the species level determined by NGS. The relative abundance of bacterial groups within the gut microbiota at species levels is indicated at t0 and t24 for both control and the +Fe/haem regime. Data are expressed as a percentage of the total microbial community profile. The results are the average of 3 subjects. Only bacterial species present at >0.5% for at least one time-point are shown. Values in bold indicate significant difference ($p<0.05$) with respect to t0. One-way Anova with Bonferroni Post Hoc was employed for statistical analysis. Values in green indicate an increase while values in red indicate a decreaseThe t=0 time point data have been averaged for both regimes (sp, species; N/A, not identifiable).

Phylum	Order	Species	t0	t24		Fold Changes		
						t24/t0		Fe/Control
				Control	Fe	Control	Fe	
Actionobacteria	Bifidobacteriales	*Bifidobacterium* sp	0.02	0.08	0.56	3.30	22.90	6.94
		Bifidobacterium adolescentis	0.01	0.19	**0.21**	17.92	**19.97**	1.11
Bacteroidetes	Bacteroidales	*Bacteroides* sp	19.35	16.33	18.43	0.84	0.95	1.13
		Bacteroides fragilis	0.19	0.11	0.14	0.57	0.74	1.30
		Bacteroides ovatus	0.27	0.15	0.19	0.55	0.72	1.32
		Bacteroides uniformis	5.05	1.63	1.51	0.32	0.30	0.93
		Prevotella sp	0.18	0.20	0.13	1.06	0.69	0.65
		Prevotella copri	17.89	46.17	35.68	2.58	1.99	0.77
		Prevotella stercorea	0.29	1.03	0.78	3.57	2.68	0.75
	Lactobacillales	*Lactobacillus* sp	0.15	7.24	5.68	46.73	36.70	0.79
Firmicutes		*Streptococcus* sp	1.55	0.40	2.23	0.26	1.44	5.57
	Clostridiales	*Blautia* sp	3.39	2.72	2.88	0.80	0.85	1.06
		Clostridiales sp	2.50	0.24	1.44	0.10	0.57	5.95
		Clostridium sp	0.69	0.40	0.63	0.58	0.91	1.58
		Dialister sp	1.79	0.82	0.79	0.46	0.44	0.97
		Faecalibacterium prausnitzii	17.88	**2.75**	7.50	**0.15**	0.42	2.73
		Lachnospiraceae sp	4.99	**0.78**	2.03	**0.16**	0.41	2.61
		Lachnospira sp	0.45	**0.08**	0.26	**0.17**	0.58	3.44
		Roseburia faecis	0.82	0.01	0.34	0.01	0.41	40.67
		Ruminococcaceae sp	9.61	5.36	5.31	0.56	0.55	0.99
		Ruminococcus sp	0.88	0.08	0.66	0.09	0.75	8.82

		Ruminococcus bromii	1.16	0.27	0.26	0.24	0.23	0.97
	Erysipelotrichales	*Catenibacterium* sp	2.14	4.55	3.08	2.12	1.44	0.68
		Erysipelotrichaceae sp	0.52	0.92	1.97	1.76	3.76	2.14
		Eubacterium biforme	2.22	3.90	3.34	1.76	1.50	0.86
Proteobacteria	Burkholderiales	*Sutterella* sp	0.25	0.53	0.64	2.09	2.53	1.21
	Enterobacteriales	*Enterobacteriaceae* sp	0.17	0.60	0.46	3.59	2.75	0.77
N/A			1.31	1.17	1.28	0.89	0.98	1.09

In the Actinobacteria phylum, both *Bifidobacterium* sp and *Bifidobacterium adolescentis* which had a relative abundance of 0.02 and 0.01%, respectively, at t0 showed an increase in their relative abundance over time regardless of the regime. *Bifidobacterium* sp increased 3.30-fold in the control to 0.08% but in the +Fe/haem regime there was a more substantial increase of 22.9-fold to 0.56%. Thus, the addition of haem/iron caused an enhanced growth of 6.94-fold compared to the control. *Bifidobacterium adolescentis* on the other hand showed a 17.9-fold increase in the control, increasing its abundance to 0.19% at t24. When iron/haem was applied to the medium, this species showed a significant increase of 20.0-fold to 0.21% ($p<0.05$). However, the provision of Fe/haem had little effect as shown by the similar relative abundances (Table 3.5).

As for the Bacteroidetes phylum, the detected species were either *Bacteroides* or *Prevotella*. *Bacteroides* sp and *Prevotella copri* were the most abundant Bacteroidetes at t0 with relative abundance of 19.4 and 17.9%, respectively, followed by *Bacteroides uniformis* at 5.05%, while the rest of the members of this phylum had a relative abundance of less than 0.5%. At t24, although there were changes to the abundance of these bacteria, none of the changes were significant ($p>0.05$). *Prevotella* were the only group that showed an increase while the *Bacteroides* species experienced reductions in relative abundance. *Bacteroides uniformis* showed the biggest decrease with a reduction of 3.10- and 3.35-fold in control and +Fe/haem regime, respectively, reducing in abundance to 1.63 and 1.51%, respectively. The rest of the *Bacteroides* showed a decrease between 1.19- to 1.82-fold in the control and between 1.05- to 1.38-fold in the iron regime. Although the

Bacteroides showed a decrease in their relative abundance over time, the presence of iron/haem enhanced their growth by up to 1.32-fold as compared to the control. However, this does not apply to *Bacteroides uniformis* which showed a reduced growth in the presence of iron/haem, by 1.08-fold, with respect to the control. As for *Prevotella* species, *Prevotella stercorea* had the greatest increase at 3.57- and 2.68-fold for the control and Fe/haem regime, respectively, which increased in abundance to 1.03 and 0.78% at t24. *Prevotella copri* increased 2.58-fold in the control to 46.2% and 1.99-fold in the iron regime to 35.7%, making it the most abundant species in both regimes. However, the addition of iron/haem reduces the growth of bacteria by 1.33- and 1.29-fold for *Prevotella stercorea* and *Prevotella copri*.

As for the Firmicutes phylum, all bacteria belonging to the Clostridales order showed a decrease in abundance over time while those in the Lactobacillales and Erysipelotrichales orders displayed an increase over time. *Lactobacillus* sp showed the biggest increase among all these bacteria with 46.7- and 36.7-fold increases in the control and +Fe/haem regime, respectively, increasing in abundance from 0.15 (t0) to 7.24 and 5.68%, respectively, at t24. *Streptococcus* sp (another member in the Lactobacillales order) decreased of.87-fold in the control, reducing in abundance from 1.55 to 0.40%, but the presence of iron/haem causes an increase of 1.44-fold, giving an abundance of 2.23% at t24. The addition of iron/haem caused a reduced growth in the *Lactobacillus* sp of 1.27-fold but increased the abundance of *Streptococcus* sp by 5.57-fold. As for the Clostridales order, although all of the bacteria in this group showed a decrease in abundance over time, the majority benefitted from the presence of iron/haem as shown by the higher abundance in the +Fe/haem regime compared to the control, with an enhanced growth of up to 40.7-fold. *Faecalibacterium prausnitzii*, which was the most abundant bacterium in the Firmicutes phylum at 17.9%, showed a significant decrease of 6.50-fold in the control ($p<0.05$), reducing its abundance to 2.75% at t24. However, in the +Fe/haem regime this species showed a non-significant decrease of 2.38-fold, decreasing in abundance to 7.50%. *Lachnospiraceae* sp and *Lachnospira* sp also showed a significant decrease in the control (6.41- and 5.93-fold decreases, respectively; $p<0.05$). Similar to *Faecalibacterium*

prausnitzii, these bacteria showed a non-significant reduction of 2.46- and 1.73-fold, accordingly, tin response to Fe/haem. Besides these three species, the remaining bacteria in the Firmicutes phylum showed no significant changes in the abundance when tested ($p>0.05$). *Roseburia faecis* showed the greatest reduction with a 98.2- and 2.41-fold decrease in the control and +Fe/haem regime, respectively, reducing in abundance from 0.82 to 0.01 and 0.34%, respectively, at t24. Clostridales sp and *Ruminococcus* sp also showed substantial decreases, particularly in the control, whereby the relative abundance of the former species was reduced by 10.4-fold while the latter decreased by 11.8-fold, reducing in abundance from 2.50 to 0.24% and from 0.88 to 0.08%, respectively. The iron regime showed a more modest reduction of 1.18-fold for Clostridales sp and 1.33-fold for *Ruminococcus* sp. The rest of the species in the Clostridales order dosplayed more modest decreases (in both regimes) of 1.25- to 6.50-fold in the control and between 1.10- to 2.46-fold in the +Fe/haem regime. Of the 11 species in the Clostridales order, 3 (*Dialister* sp, *Ruminococcaceae* sp and *Ruminococcus bromii*) showed a negligible reduction growth in the presence of iron/haem while the rest of the other 8 species benefitted from the additional iron as shown by their higher abundance in the iron regime as compared to the control. *Roseburia faecis* showed the greatest increase (40.7-fold) while the remaining 7 species showed an increase of up to 8.82-fold induced by iron/haem. As for the bacteria in the Erysipelotrichales order, all species showed an increase in their abundance over time. *Erysipelotrichaceae* sp displayed the greatest increase with 1.76- and 3.76-fold increases for the control and +Fe/haem regime, respectively, increasing its abundance from 0.52 to 0.92 and 1.97%, respectively, at t24. *Catenibacterium* sp, on the other hand, showed a 2.12- and 1.44-fold increase for the control and +Fe/haem regime, respectively, while *Eubacterium biforme* showed a 1.76- and 1.50-fold increase for the same regimes. The presence of iron/haem benefitted *Erysipelotrichaceae* sp only whereby a 2.14-fold enhanced abundance was achieved compared to the control. *Catenibacterium* sp and *Eubacterium biforme* showed a 1.48- and 1.17-fold reduced growth when iron/haem was provided.

For the Proteobacteria phylum, both species showed an increase in both regimes over time. *Enterobacteriaceae* sp showed a greater increase compared to *Sutterella* sp with a 3.59- and 2.75-fold increase in the control and +Fe/haem regime, increasing in abundance from 0.17 to 0.60 and 0.46%, respectively. *Sutterella* sp increased 2.09- and 2.53-fold, respectively, reaching an abundance of 0.53 and 0.64% (from 0.25%), respectively. The presence of iron/haem enhanced the abundance of *Sutterella* sp by 1.21-fold but reduced that of *Enterobacteriaceae* sp by 1.30-fold.

3.5 Comparison between Flow-FISH and NGS

Both Flow-FISH and NGS have their advantages and disadvantages in enumerating the bacteria present. Flow-FISH gives the exact number of bacterial cells present but it is highly dependent on the specificity of the probes used. NGS on the other hand gives a more detailed information regarding the bacteria at various taxonomic levels, but the PCR step involved in amplifying the DNA can introduce bias, causing overestimation or underestimation of the bacteria, and depends on equivalent extraction of DNA from all species present. A comparison between results obtained by the two methods is presented in tables 3.6 and 3.7. The absolute numbers in NGS were obtained by multiplying the relative abundance with the total bacterial counts at t0 or t24. The relative abundance for Flow-FISH was obtained by dividing the bacterial numbers of a particular bacterial group over the total bacterial number to obtain the percentage value.

Table 3.6 Comparison between the results obtained from NGS and Flow-FISH at phylum level. Results were derived from table 3.2 and 3.4. The values were expressed in relative abundance and absolute numbers for comparison.

	Phylum	NGS			Flow-FISH		
		t0	t24		t0	t24	
			Control	Fe		Control	Fe
Relative abundance (%)	Actinobacteria	0.16	0.30	0.86	19.86	52.80	38.81
	Bacteroidetes	44.39	66.19	57.18	6.95	20.52	31.44
	Firmicutes	53.63	31.20	39.54	61.49	25.17	27.70
	Proteobacteria	0.48	1.14	1.12	11.71	1.50	2.05
Absolute number (cells/ml medium)	Actinobacteria	3.54E+06	7.27E+06	1.75E+07	2.07E+08	1.41E+09	1.23E+09
	Bacteroidetes	9.65E+08	1.58E+09	1.16E+09	7.24E+07	5.50E+08	9.97E+08
	Firmicutes	1.17E+09	7.46E+08	8.00E+08	6.41E+08	6.74E+08	8.78E+08
	Proteobacteria	1.05E+07	2.71E+07	2.28E+07	1.22E+08	4.02E+07	6.51E+07

The Flow-FISH data were converted to relative abundance for comparison with the NGS results. The bacteria were compared at phylum level to enable their comparison. As seen from table 3.6, Actinobacteria were substantially higher in the Flow-FISH at all time points as compared to NGS data, while Bacteroidetes were higher in NGS data as compared to the Flow-FISH data.

Table 3.7 Comparison between the results obtained from NGS and Flow-FISH at genus/species level. Results were derived from table 3.2 and 3.4. The values were expressed in relative abundance and absolute numbers for comparison.

	Probe	Corresponding bacteria	NGS			Flow-FISH		
			t0	t24		t0	t24	
				Control	Fe		Control	Fe
Relative abundance (%)	Bif164	*Bifidobacterium*	0.04	0.27	0.77	19.23	41.00	35.74
	Bac303	*Bacteroides*	24.86	18.21	20.27	6.95	20.52	31.44
	Lab158	*Lactobacillus*	0.15	7.24	5.68	0.33	1.11	1.10
	Rrec584	*Roseburia*	0.82	0.01	0.34	6.48	2.85	5.17
	Fprau655	*Faecalibacterium prausnitzii*	17.88	2.75	7.50	27.27	11.13	9.67
Absolute number (cells/ml medium)	Bif164	*Bifidobacterium*	7.64E+05	6.43E+06	1.57E+07	2.00E+08	1.10E+09	1.13E+09
	Bac303	*Bacteroides*	5.41E+08	4.35E+08	4.10E+08	7.24E+07	5.50E+08	9.97E+08
	Lab158	*Lactobacillus*	3.37E+06	1.73E+08	1.15E+08	3.48E+06	2.97E+07	3.49E+07
	Rrec584	*Roseburia*	1.78E+07	1.99E+05	6.86E+06	6.76E+07	7.62E+07	1.64E+08
	Fprau655	*Faecalibacterium prausnitzii*	3.89E+08	6.58E+07	1.52E+08	2.84E+08	2.98E+08	3.06E+08

Firmicutes levels were comparable between the two methods, while Proteobacteria levels were higher at t0 in the Flow-FISH data but the abundance at t24 was similar for both methods. As for comparison with the specied specific probes (table 3.7), only Bif, Bac, Lab, Rrec and Fprau were selected as the corresponding bacteria can be found in NGS while the bacteria for the other probes were either too general (*Clostridium*) or unavailable (Ato, DSV). *Bifidobacterium* (Bif164) were substantially higher in the Flow-FISH at all time points with the biggest difference occurring at t0. As for *Bacteroides* (Bac303) and *Faecalibacterium prausnitzii* (Fprau655), both groups share a similar trend whereby the NGS data showed a higher abundance at t0 but the results for t24 (both control and iron regime) were modestly higher in the Flow-FISH data. *Lactobacillus* (Lab158) showed the

opposite trend as that seen for *Bacteroides* whereby the NGS data showed a lower abundance at t0, but a substantially higher value at t24 for both regimes as compared to Flow-FISH. *Roseburia* at t0 showed a bigger difference between the two methods when calculated using relative abundance as compared to absolute numbers. At t24, the bacteria showed a substantially lower abundance (and bacterial numbers) in the NGS as compared to Flow-FISH, particularly in the control.

3.6 Discussion

As seen from tables 3.3 and 3.5, the presence of iron sulphate and haem affected the gut microbiota composition, as shown by the differences obtained between the control and iron regime, although none of these differences were significant. Gut model medium contains a background iron concentration of 28 μM, mainly due to the presence of mucin and yeast extract. As for the +Fe/haem regime, 18 μM $FeSO_4$ and 77 μM haemin were added to the medium, resulting in a total of 123 μM iron. Since bacteria only require 0.3 -1.8 μM of iron for optimal growth (Kim et al, 2009), the background iron concentration of the control is in excess and more than sufficient to support the growth of bacteria. This may explain why no significant differences were seen between the two regimes. Besides this, the pH in Hungate tubes was not regulated, unlike the controlled batch cultures and gut models whereby the pH is maintained within a specific range through the addition of acid and base. Bacteria display optimal pH ranges for growth and the lower pH achieved through the production of SCFAs favours the lactic acid bacteria such as *Bifidobacterium* and *Lactobacillus* species. These 2 bacterial groups showed the greatest increase over time, possibly due to their ability to tolerate low pH a reduced competition by other, acid-sensitive elements of the gut microflora. The changes in pH in Hungate tube will be discussed further in Chapter 4. Although use of Hungate tubes has limitations, mainly due to its inability to regulate pH, it can still be a quick and easy method for preliminary experiment.

Interestingly, *Roseburia faecis* showed the greatest increase in both NGS and Flow-FISH when iron/haem was added to the medium, indicating that this species benefitted from the iron/haem added. Iron availability has been shown to affect the abundance of *Roseburia* whereby a very low

iron concentration resulted in a decrease in *Roseburia* (Dostal et al, 2013) and iron supplementation with Vitamin E significantly increased the abundance of *Roseburia* (Tang et al, 2017). The results obtained from this experiment are in agreement with the 2 publications whereby the presence of iron favoured the growth of *Roseburia*. *Bacteroides*, which has an absolute requirement for haem (or PPIX and iron) (Rocha et al, 2019), also showed an increase in the +Fe/haem regime as compared to the control. Results from both Flow-FISH and NGS showed an increase in total numbers and relative abundance of *Bacteroides* when iron/haem is present in the medium. *Lactobacillus*, which has no requirement for iron (Archibald, 1983), showed a decrease in the iron regime possibly due to the increase in *Bacteroides*, causing a lower abundance in the +Fe/haem regime.

Numerous techniques are available for the enumeration and identification of the gut microbiota (Fraher, O'Toole and Quigley, 2012), each with their own advantages and disadvantages. Culture-based techniques were used for the identification of gut microbiota in 1990s (Fraher, O'Toole and Quigley, 2012), which is a relatively cheap approach but labour intensive and many of the members of the gut microbiota are unculturable (<30% of gut microbiota has been cultured). However, the culture-based technique is still useful for isolation and identification of any organisms of interest. qPCR (quantitative polymerase chain reaction) and FISH (Fluorescence in situ hybridization) are able to quantify the bacteria present in the given sample but are highly dependent on the specificity of the probe sequences utilised and are unable to detect unknown species (Fraher, O'Toole and Quigley, 2012). NGS (next generation sequencing) allow massively-parallel nucleotide sequencing 16S rRNA gene amplicons at a lower cost and a faster rate as compared to traditional sequencing (Sanger sequencing) but suffers from drawbacks such as PCR bias which could underestimate or overestimate certain taxa and the identity of a given taxon depends on the availability of a corresponding reference genome. Although numerous studies on the gut microbiota use the abovementioned techniques (Collado et al, 2008; Kalliomäki et al, 2008; Zimmermann et al, 2010, Tang et al, 2017; Dostal et al, 2013; Jaeggi et al, 2014; Dostal et al, 2014), none of these studies had compared the results obtained between 2 different techniques, thus comparison between results

obtained from NGS and Flow-FISH was performed here (table 3.7). For the comparison between NGS and Flow-FISH data, as the two methods utilise different approaches for enumerating the bacteria of the gut microbiota, it is not surprising to find that the same result is not obtained for the two methods. NGS is able to detect all of the bacteria present and categorise them according to different taxonomic levels but it heavily relies on the information available on the database, proportion PCR amplification, and equivalent yield of DNA from each species. Flow-FISH on the other hand relies on the specificity of the probe used to detect the bacteria and the recovery of bacteria in single form suitable for detection by flowcytometry. Using the Bacteroidetes phylum as an example, *Bacteroides* is the only bacteria indicated in that phylum by Flow-FISH, whereas *Prevotella* is also detected in NGS. This may help to explain the discrepancies in data presented by NGS and Flow-FISH at phylum level (table 3.6). Similarly, for the Firmicutes phylum, only probes for the bacterial groups of interest were used (while NGS provides data for all the bacteria present within that phylum) causing an underestimation of bacteria in the Flow-FISH as compared to NGS.

In summary, the addition of ferrous sulphate and haem had no significant effect on the gut microbiota, although there were changes in abundance that may become significant with sufficient replicates. Indeed, this is the major advantage of the Hungate method – the capacity to deploy multiple replicates to enhance the potential to detect significant changes. In addition, the iron concentration in the control was sufficient to support growth of the microbiota, which would also be expected to contribute to the lack of significant difference obtained between the control and the +Fe/haem regime. In addition, because pH of the medium was not regulated, the anticipated decrease in pH over time may affect the composition of the gut microbiota, which would further complicate the results obtained but would not be expected to result in failure of iron regime to cause a change in microbiota composition. The use of a 1% final inoculum may also be too high such that the growth of the microbiota is hindered, leading to small changes in the total bacterial counts (table 3.1). Similar results have been reported in batch cultures using the same protocol (Khalil et al, 2014, Costabile et al, 2014; Beards, Tuohy and Gibson, 2010). In order to address these issues, future

experiment in the later chapters (chapter 5, 7 and 8) will be conducted in a pH regulated batch culture using a 0.1% final inoculum.

Chapter 4: The use of buffer to regulate the pH in Hungates tube

4.1 Introduction

The gut microbiota produces SCFAs as metabolic end products which can lower the pH of the surrounding medium, thus creating an acidic environment over time which can be unfavourable for some bacterial species. Unlike the single vessel batch cultures or the three-stage gut model which use HCl and NaOH to regulate the pH, keeping the pH of the medium within the desired range of pH 5.5. 2-(*N*-morpholino)ethanesulfonic acid (MES) is one of the Good's buffer with a useful pH range of 5.5 to 6.7 and a pKa of 5.97 at 37°C. Here, an experiment is conducted using Hungate tubes over 48 h with constant sampling in between. Faecal slurry (1%), 5ml and 5 ml of either 1, 2, 3 M MES (pH 5.5) or 1 ml of sterile water (control) were added to 40 ml of gut model medium to achieve a final concentration of 0.1, 0.2 or 0.3M buffer. The impact of autoclaving and the pH of medium at t0 on the overall pH changes was also investigated to determine whether an initial pH change would affect the efficiency of the buffer. The aim was to determine whether buffered Hungate tubes could be used to perform high throughput studies on the gut microbiota under a stable pH regime.

4.2 Effects of buffer on pH changes

A change in pH of the environment will affect the composition and growth of the bacteria. Thus, it is important to keep the pH within strict limits. MES was used to restrict pH changes throughout the experiments with an increasing concentration. Regardless of the presence of buffer, all samples showed a similar trend, indicating similar growth responses (Figure 4.1). Addition of MES buffer lowered the pH of the medium to 5.98-6.06 from an initial pH of 7.48. There was a constant drop in pH for the first 8 h of the experiment followed by a steady, but more slight increase towards 24 h for all MES samples. The samples subsequently showed a decrease, on average, to t36 followed by an increase towards the end. The negative control on the other hand showed a sharp decrease at t4 (from pH 7.35 to 4.68) and a further decrease towards t8 (pH 4.59). It then showed an increase to t24 and levelled off to roughly pH 4.9. All samples have the lowest pH at t8 with control being the lowest, followed by 1, 2 and 3 M MES (pH 4.82, 5.04 and 5.53 respectively). At the end of the

experiment (t48), only 2 and 3 M MES were able to maintain the pH within the range desired with pH 5.43 and 5.59 values achieved, accordingly. The control and 1 M conditions, which had pH values of 4.96 and 5.02, were well below the lower limit of the desired range. The presence of MES was able to maintain pH within the range wanted and there is a noticeable effect with an increase in concentration of the buffer (Figure 4.1). The higher the concentration, the nearer the pH towards the range wanted. Although MES buffer showed better pH control, only 3M was able to maintain the pH within the 5.4-5.6 range throughout the experiment. As compared to MES buffer, tubes without buffer showed lowest pH among all samples, with pH lower than pH 5 throughout the experiment, clearly showing the beneficial effect of MES buffer on restricting pH changes.

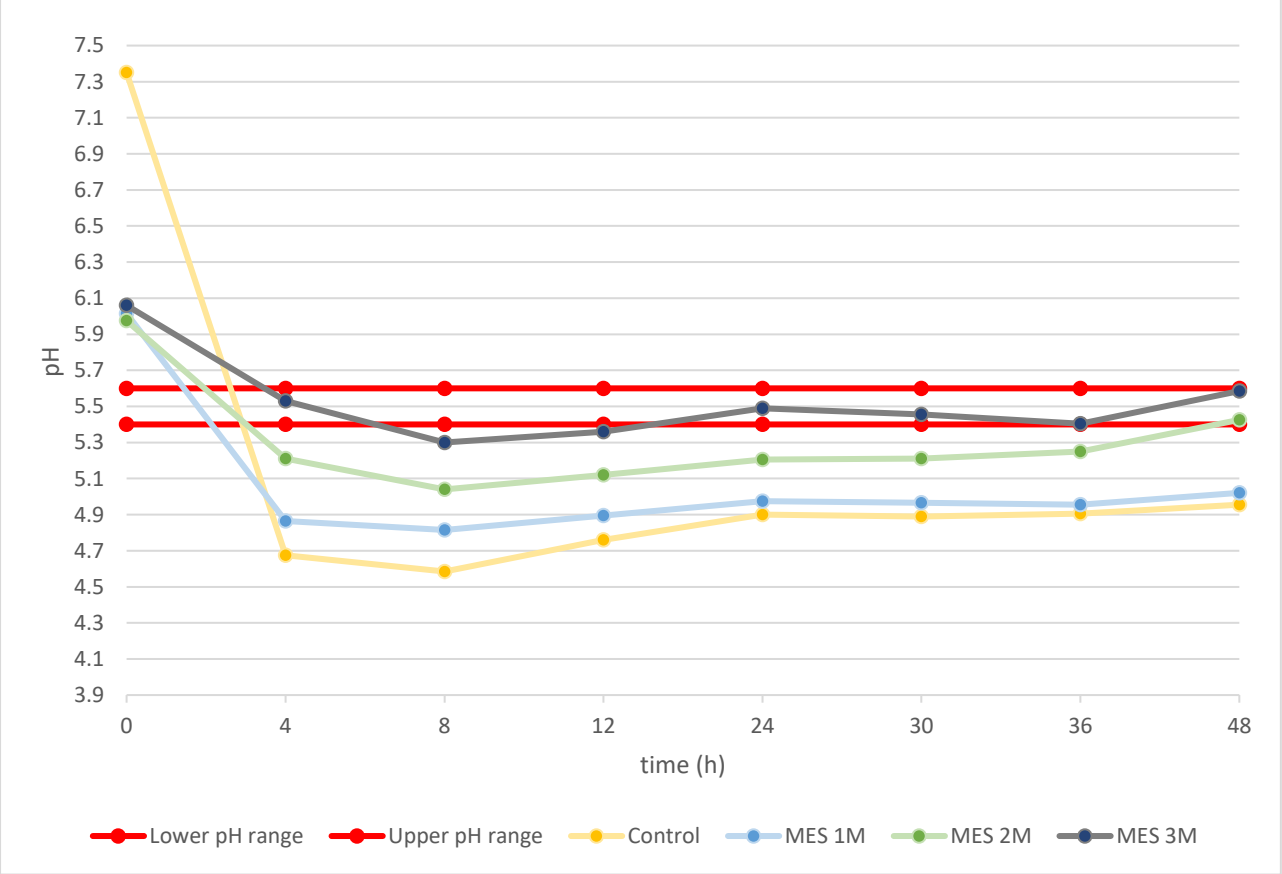


Figure 4.1 Changes in pH over 48 h with 1-3 M MES buffer. pH of the medium was not adjusted prior to autoclaving. The initial pH indicates the pH of the medium before the addition of MES or water (control) with a pH of 7.48. t0 shows the pH after the addition of the buffer or water. The red lines indicate the desired pH range (5.4-5.6) corresponding to that of the ascending colon. Results shown are the mean of 3 subjects.

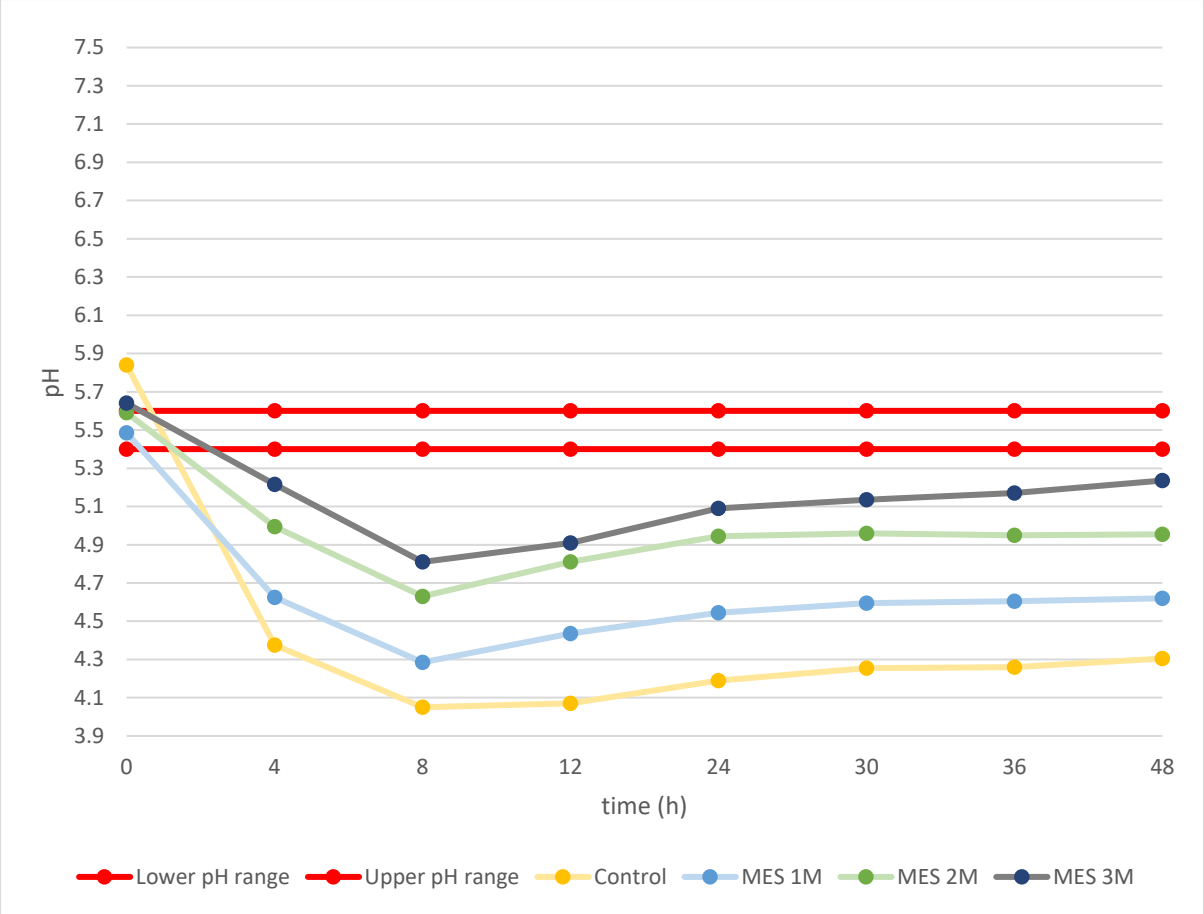


Figure 4.2 Changes in pH over 48 h with 1-3 M MES buffer, with pH adjustment before autoclaving. The pH of the medium was adjusted to 5.5 prior to autoclaving. The initial pH indicates the pH of the medium before the addition of MES or water (control) with a pH of 5.9. t0 shows the pH after the addition of the buffer or water. The red lines indicate the desired pH range (5.4-5.6). Result shown was the mean of 3 subjects.

The above experiment was repeated, but this time the starting pH of the medium was adjusted prior to autoclaving in an attempt to ensure that the starting pH is within the desired range. Indeed, the pH of the medium was lowered to 5.5 at t=0, which is within the range wanted (Figure. 4.2). However, the results indicated that a starting (t=0) pH within the desired range results in an excessively low subsequent (t≥4) pH. Although lowering the starting pH did not affect the overall trend of the pH as shown by the similarity in trend compared to Figure 4.1, the overall pH was lowered and moved towards acidity, with no samples maintaining the pH within the desired range (Figure 4.2). All samples shared the same trend throughout the experiment but there is a clear distinction between the buffered medium compared to the control. The starting pH of all samples

were close to one another, with pH ranging from 5.49 to 5.84. There was a decrease in pH from t0-t8 and a steady increase till t24 and finally a levelling off. Unbuffered samples had the lowest pH among all, with lowest pH of 4.05 at t8 and an average pH of 4.3 which is about 1.2 units lower than the intended range. There is an increase in MES efficiency with concentration, an observation similar to the one in Figure 4.1. Compared to the negative control, the lowest pH achieved by the buffered samples was at t8:4.29, 4.63 and 4.81, for 1, 2 and 3 M MES, respectively. Although no samples were able to maintain the pH of the medium within the range wanted, 3M MES showed the closest with a final pH of 5.24, followed by 2 and 1 M with 4.96 and 4.62 respectively. Unbuffered medium had the lowest pH of all samples with a pH of only 4.31. This clearly shows MES is able to prevent large pH changes in the medium compared to unbuffered media.

4.3 Effects of initial pH on overall pH changes

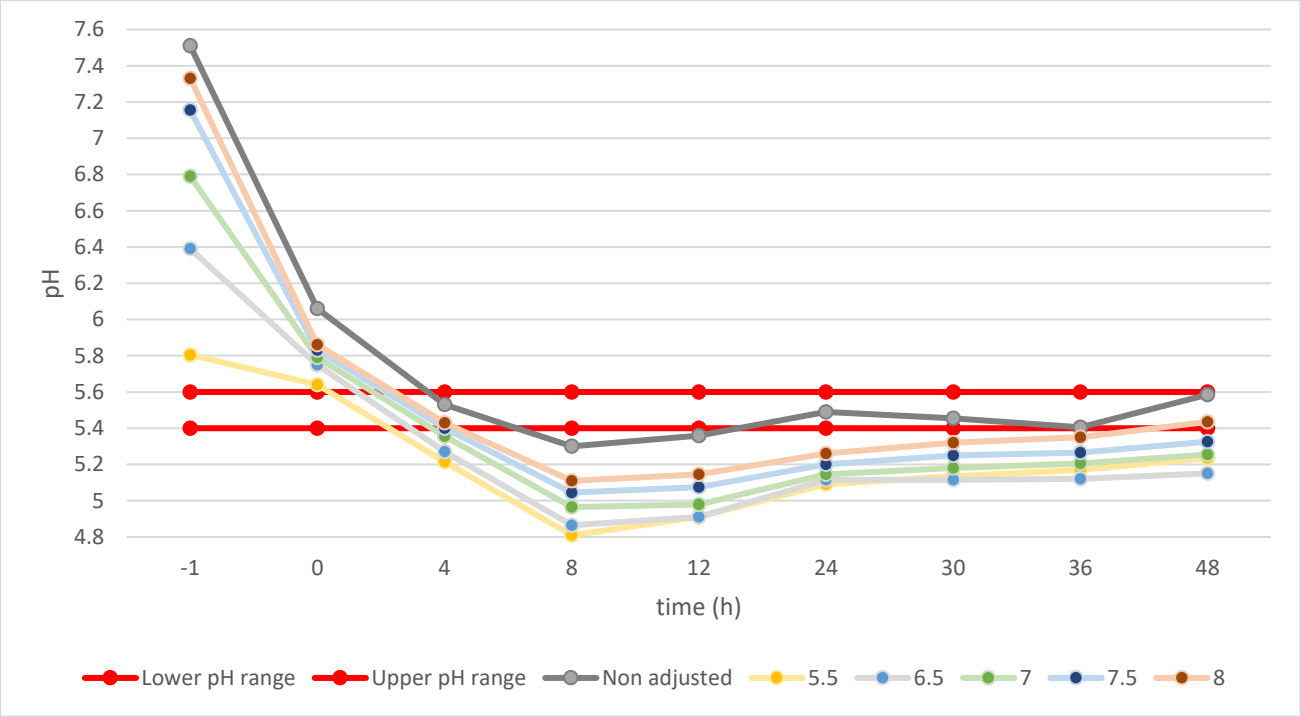


Figure 4.3 Changes in pH over 48 h with 3 M MES buffer when initial pH of medium was adjusted to 5.5-8 prior to buffer addition. All conditions contain 300mM final concentration MES buffer (pH 5.5) from t=0 onwards. At t-1, the pH of the medium after autoclaving is shown (either adjusted to pH 5.5-8, or non-adjusted); t0 indicates the pH after the addition of the 0.3 M MES buffer. The red lines indicate the desired pH range (5.4-5.6). Results shown are the mean of 3 subjects.

As shown from previous results, 3 M MES is the most effective concentration in preventing pH fluctuations throughout the experiment and the starting pH has a major impact on pH drift during

growth. Thus, one of the interesting observation is that the initial pH of the medium has an important effect on the final pH obtained. Thus, a range of different initial pH values was examined to determine the effect on subsequent pH changes using 3 M MES. Similar trends in pH change occurred across all samples regardless of their initial pH, indicating similar bacterial growth effects to the medium pH. All conditions had a similar starting pH (t0) between 5.86-6.06 upon addition of buffer. There was an initial drop from t0 to t8 and a steady increase towards t48. pH was lowest at t8 across all conditions with the 'pH 5.5 condition' achieving the lowest pH of 4.81 while conditions starting at pH 6.5, 7 and 7.5, and the non-pH-adjusted sample had values of 4.87, 4.96, 5.05, 5.11 and 5.3 accordingly. At the end of the experiment (t48), only the non-adjusted sample and the pH 8 condition are within the desired range with pH 5.59 and 5.44 respectively. For pH conditions 5.5, 6.5, 7 and 7.5, at the end of the experiment the pHs were 5.24, 5.15, 5.26 and 5.33 which were slightly below the lower limit of the range. As mentioned before, the initial pH has an effect on the overall pH change over 48 h as shown in Figure 4.2. From the Figure, the higher the initial pH, the closer the pH is towards the desired range. Although 3 M MES caused the starting pH to be similar at the start of the experiment (t0), a higher initial pH is better at restricting pH fluctuations to within the desired range during growth. An initial pH of 7.48 (non-adjusted sample) was able to maintain the pH within the range throughout the experiment with the lowest pH occurring at t8 while sample 5.5 had the lowest value among all other samples, further suggesting an effect of the initial medium pH.

4.4 Effects of buffer on bacterial number

To determine whether control of medium pH with MES had an impact on growth, Flow-FISH was used to estimate total bacterial numbers at each time point for Figure 4.1 (Figure 4.4). The results showed that there is a difference in growth between the presence and absence of buffer. It is evident that although there is an increase in bacteria number, MES is able to prevent pH changes as compared to the negative control which showed a larger drop in pH as bacteria number increases at t12.

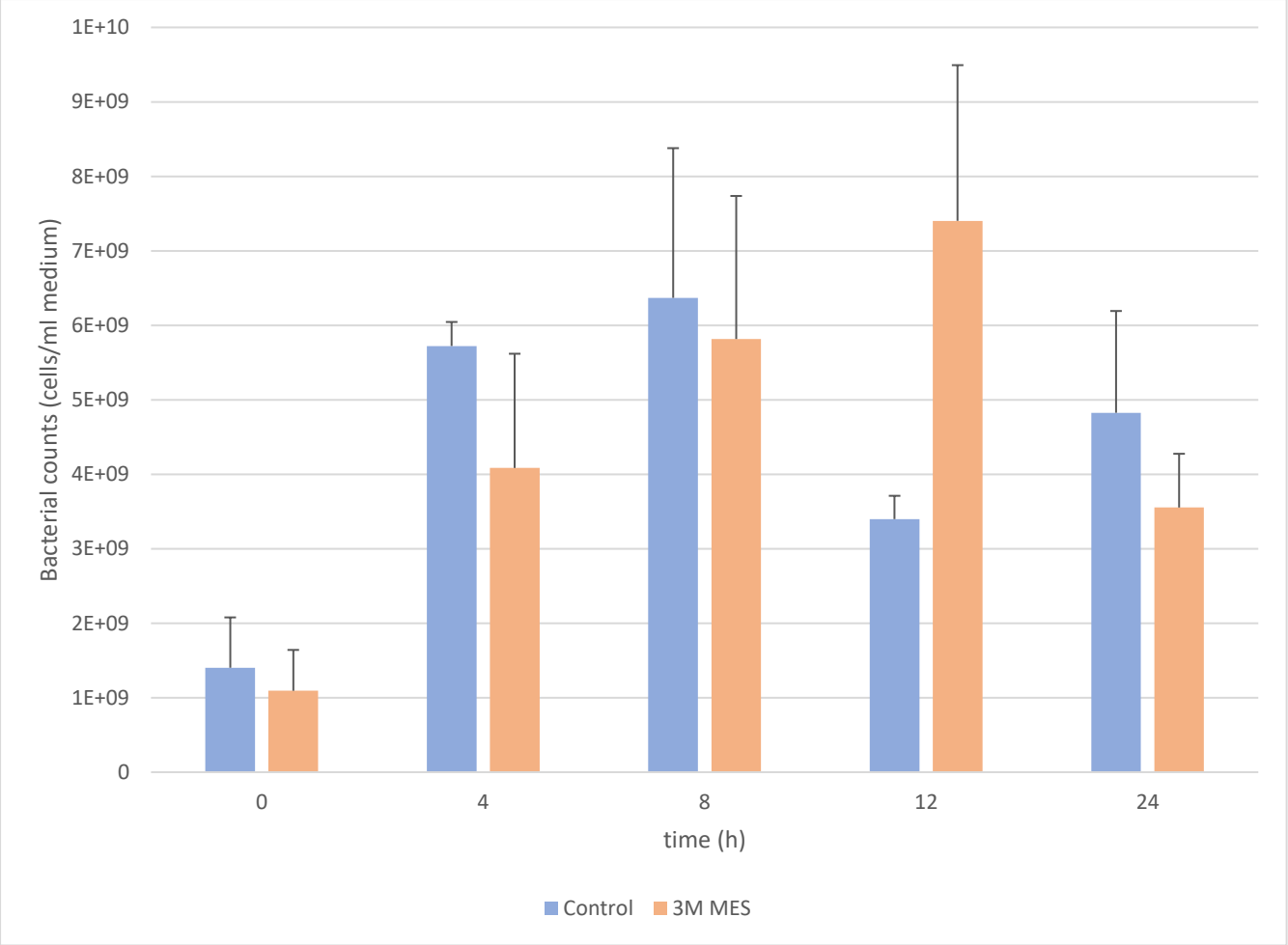


Figure 4.4 Bacterial growth over 24 hours with and without the presence of 3 M MES buffer (pH 5.5). pH of the medium was not adjusted prior to autoclaving and had a mean initial pH of 7.48. Bacterial counts are the mean of 3 subjects and error bars represents mean ± SD. No significant difference is seen between the regimes. Data corresponds to that in Figure 4.1.

A lower starting bacterial number is observed in the buffered medium compared to the negative control with $1.4x10^9$ vs $1.1x10^9$ cell/ml medium. The presence of buffer affected the growth response. Bacteria in the buffered medium grew steadily and peaked at t12, followed by a decrease towards the end. The negative control on the other hand showed an increase and peaked at t8 which then dropped in number and increased again towards t24. At its peak, the buffered medium has a total of $7.4x10^9$ cells/ml medium compared to the negative control which only has $6.3x10^9$ cells/ml medium, roughly 1.2-fold higher. At the end of the experiment (t24), the control had a higher bacterial count compared to the buffered medium with $4.83x10^9$ and $3.55x10^9$ cells/ml medium, respectively.

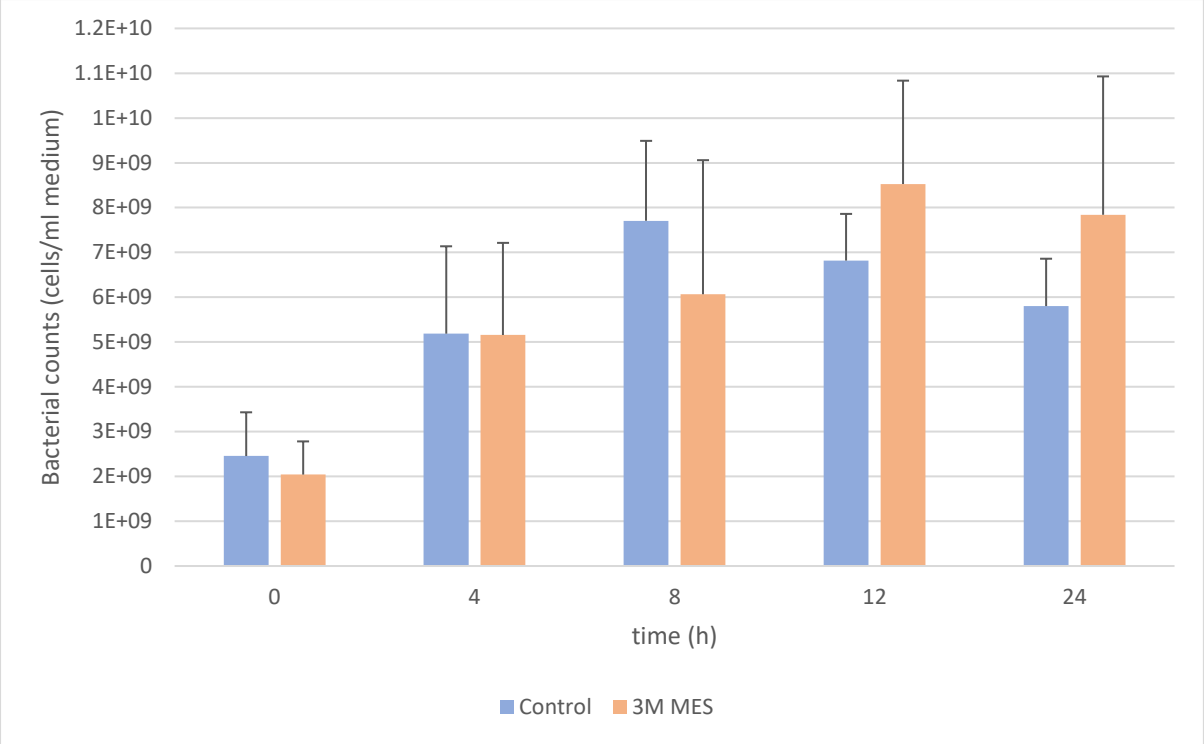


Figure 4.5 Bacterial growth over 24 hs with and without 3 M MES buffer (pH 5.5). pH of the medium was adjusted to 5.5 prior to autoclaving with a mean initial pH of 5.9. Bacterial counts are the means of 3 subjects and error bars represents mean ± SD. No significant difference is seen between the regimes. Data corresponds to that in Figure 4.2.

When the initial pH was lowered, there was an increase in bacterial counts compared to the results above where there was a higher starting pH as shown in Figure 4.4 and 4.6. In addition, the two conditions showed a different growth response, as seen with the higher initial pH. By lowering the pH, both conditions had higher initial bacterial counts compared to the non-pH-adjusted conditions (Figure 4.3) but the MES samples still had lower counts than the control at t0. Negative control started with 2.45×10^9 while the positive control had an initial count of 2.04×10^9 cells/ml medium. At lower initial pH (5.5), the buffered condition gave a similar growth response the unadjusted pH condition (Figure 4.4). Both showed a constant increase towards t12, followed by a decrease in bacterial counts. The negative control showed a steady rise from the start towards t8 and subsequently declining towards t24. The unbuffered condition had a higher bacteria count at early stages but was overtaken by the buffered condition after t8 when the bacterial numbers started to drop.

At its peak, the buffered medium had roughly 1.1-fold more bacterial counts with respect to the control with $8.53x10^9$ against $7.71x10^9$ cells/ml medium, showing an effect of the buffer on the bacterial growth. At t24, the buffered condition had a higher bacterial count compared to the negative control with final bacterial counts of $7.84x10^9$ and $5.8x10^9$ cells/ml medium, respectively (~1.4-fold higher).

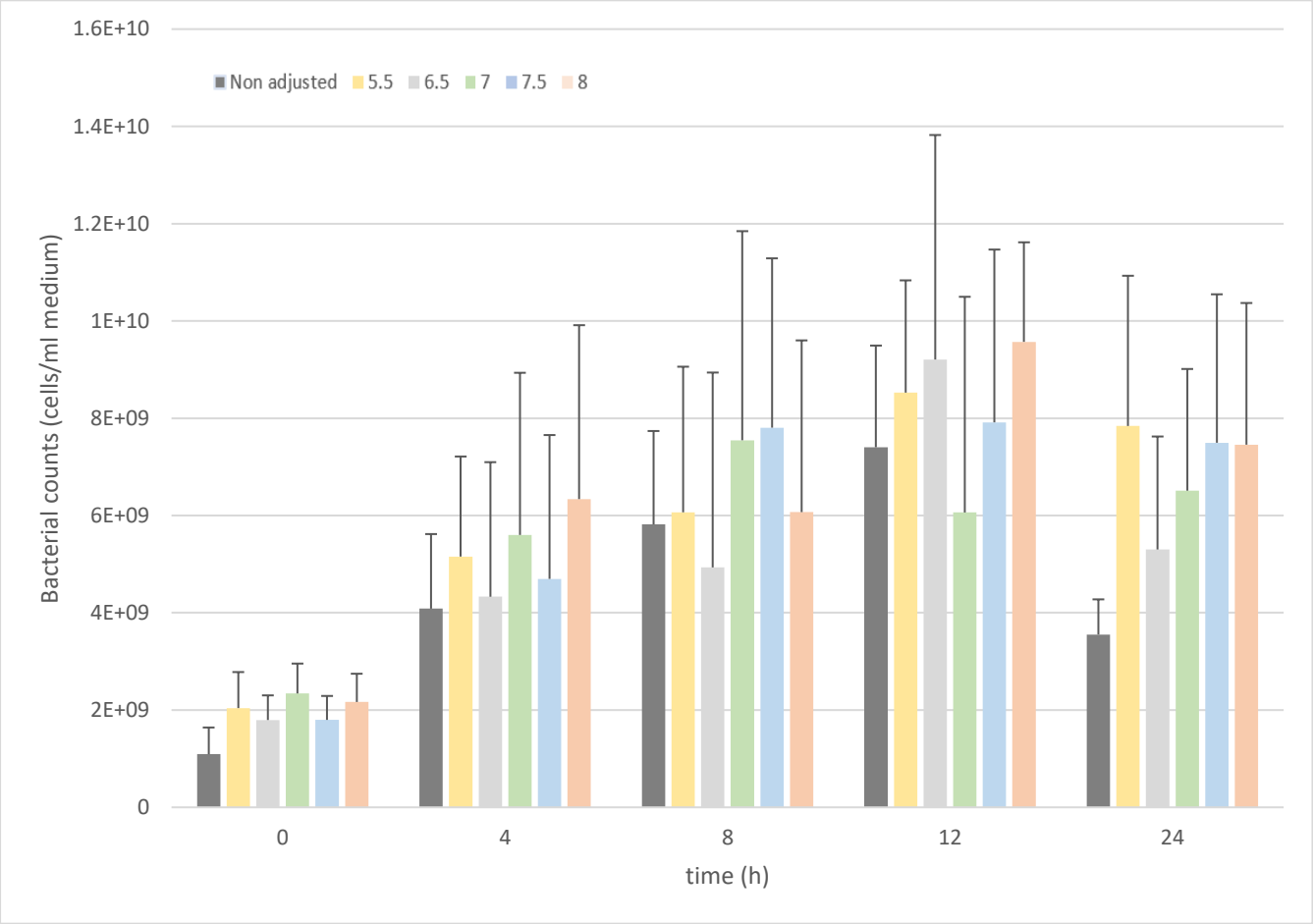


Figure 4.6 Bacterial growth over 24 hours with the presence of 3 M MES and different initial pH. pH of the media was either non adjusted or adjusted to 5.5, 6.5, 7, 7.5 and 8 prior to autoclaving. Bacterial counts are means of 3 subjects and error bars represents mean ± SD. No significant difference is seen between the regimes. Data corresponds to that in Figure 4.3.

As seen from previous results (Figure 4.4; Figure 4.5), the initial pH of the medium has an effect on the growth of the microbiota in Hungate tubes. Also, the starting pH has a major impact on the pH observed during subsequent growth of the microbiota (Figure. 4.3). To determine if this had an effect on bacterial numbers, total counts were again measured by Flow-FISH (Figure. 4.6). The microbiota showed different growth responses when they were inoculated in medium at different

initial pH values. From Figure 4.6, it is clear that initial pH has an influence on the overall growth of the bacteria.

At the start of the experiment (t0), the non-pH-adjusted conditions (Figure 4.4) had the lowest bacterial counts with only $1.09x10^9$ cells/ml medium compared to buffered conditions which had an average of $2.03x10^9$ cells/ml medium (Figure 4.5), nearly twice as much with respect to the non pH adjusted conditions. The pH 7 condition has the highest initial bacterial counts with $2.34x10^9$ cells/ml medium, followed by the pH 8 and 5.5 conditions, at $2.17x10^9$ and $2.04x10^9$ cells/ml medium, respectively.

The non pH adjusted condition and the pH 5.5, 6.5 and 7.5 conditions had similar growth patterns with an increase from t0 to t12 and a decrease towards the end. The pH 7 and 8 conditions both showed drops in number partway through the growth experiment, followed by a subsequent increase. Bacterial counts peaked at t12 for all samples with the exception of the pH 7 condition which peak at t8. The pH 8 condition had the highest bacterial number among all conditions at its peak with $9.57x10^9$cells/ml medium, followed closely by the pH 6.5 condition with $9.21x10^9$ cells/ml medium while the pH 7 condition had the lowest bacterial counts with only $6.06x10^9$ cells/ml. The pH 5.5, 7.5 and non pH adjusted conditions have a peak bacterial counts of $8.53x10^9$, $7.92x10^9$ and $7.4x10^9$ cells/ml medium, accordingly.

At the end of the experiment, the pH 5.5, 7.5 and 8 conditions had relatively high bacterial counts at $7.84x10^9$, $7.5x10^9$ and $7.46x10^9$ cells/ml medium, respectively. The unadjusted samples had the lowest bacterial counts with $3.55x10^9$ cells/ml medium.

4.5 Discussion

In vitro studies of the human gut are often conducted using flow fermenters or bioreactors. These vessels can be used individually to simulate specific regions of the colon or several vessels can be set up in parallel to simulate different region of the entire colon, thus mimicking the whole human colon. The use of flow fermenters or bioreactors has an advantage over *in vivo* studies in that it gives the investigators better control of the environment and the media as well as the ability to test a

wide range of substrates. However, setting up the vessels is time consuming and it requires constant monitoring to ensure the conditions are suitable for bacterial growth.

The use of Hungate or Falcon tubes provides an easier alternative to the vessels in that they are easier to set up and multiple tubes can be set up in one setting, giving the ability to test multiple samples in one go. The volume required in these tubes is far less than that in the vessels, typically 10-50 ml compared to 150 ml typical for gut culture vessels. This allow the investigators to use smaller amount of the test substrates rather than a huge amount needed in the vessels. The disadvantage of using Hungate or Falcon tubes is that they are not pH regulated compared to the vessels which use acid and alkali as pH regulators. In addition, the tubes are static as compared to the vessels which have individual stirrers in them but this can be easily overcome by placing the tubes on a shaker or a roller.

It is evident that SCFAs produced as a metabolic end product of bacteria lowers the pH of the medium and it can have an effect on the bacteria composition (Walker et al, 2005; Duncan et al 2009). Thus, using the tubes as an alternative may be subject to the effect of pH, subsequently affecting the bacterial composition and masking the effect of test substrate. The use of MES buffer can restrict pH fluctuation, thus eliminating the effect of pH on the gut microbiota. Another benefit of using buffer as a pH regulator is that it produces a closed system which limits the entry of unwanted compounds into the medium through base/acid titration, and this is particularly important in experiment involving micronutrients. The use of acid and alkali can introduce excess micronutrients to the medium over time, thus affecting growth of bacteria in response to micronutrients.

MES is one of the Good's buffer developed by Norman Good and colleagues with the aim of providing buffers that work well between pH 6 and 8 to be used in biological research (Good et al, 1966). It is a zwitterionic amino acid with *N*-substituted taurines and has a pKa of 6.1 (25°C) or 5.97 (37°C). The addition of buffer is able to maintain the pH of the samples within the range of the

proximal colon (pH 5.4-5.6), indicating the possible use as a pH regulator in Hungate tubes. However, the initial pH of the medium has an effect on the ability of the buffer to maintain the pH within the desired range. A higher initial pH is better at maintaining the pH within the range compared to a lower initial pH. One possible explanation is that at high initial pH, more conjugated base is produced and thus are better at restricting pH changes caused by the production of SCFAs, hence have the ability to maintain the pH within the range wanted throughout the experiment (Figure 4.3). When the initial pH of the medium was adjusted to 5.5, the buffer would remain undissociated. Thus, when the pH of the medium is lowered as a result of increased SCFA concentration, the buffer is not as effective in maintaining the pH, resulting in a drop in pH as seen in Figure 4.2.

Faecal slurry was added to the medium to create a 'master mix' prior to aliquoting into individual tubes to ensure even distribution of bacteria in all samples, followed by the addition of positive (MES buffer) and negative (water) controls. The initial bacterial counts of the buffered medium were lower compared to the control possibly due to pH shock experienced by the gut microbiota when they are moved from the faecal slurry to the medium. Faecal slurry has a more alkaline pH compared to the gut model medium. Thus, some bacteria may lyse as a result of the shock. In addition, MES buffer was added after inoculating the medium with the gut bacteria, which further lowers the pH of the medium to around pH 5.5, causing the bacteria to experience a second pH shock, potentially leading to more bacterial lysis. Together, this results in a decrease in initial bacterial number.

Based on the results obtained, adjusting the pH of the medium to around pH 8 and providing MES at 3M gives the best overall result. Although non-pH-adjusted medium showed better growth over time, it had very low bacterial number possibly due to a bigger shock experienced by the bacteria moving from a very high pH to low pH. Adjustment to pH 8 resulted in a reasonable bacterial number and bacterial growth as well as a pH closer to the range wanted.

However, the use of a single vessel batch culture or a three stage gut model would give a more desirable (controlled) condition for future experiments. They would also avoid the addition of any

strong buffer that might influence the microbiota and additionally deliver trace metals. Thus, although the preliminary experiments above with buffered medium in anaerobic Hungate tubes gave promising results in terms of pH control and overall growth. Further work is required to determine to what degree the microbiota composition is affected with respect to that obtained using traditional, pH regulated batch cultures.

Chapter 5: Effect of iron on the gut microbiota using single-stage batch cultures

5.1 Introduction

In the experiment reported in this chapter, the aim was to determine the effect of different forms of iron on the gut microbiota rather than the effect of different levels of iron. Thus, a gut model medium depleted of iron was used for the selective addition of desired forms of iron. The preliminary analysis (chapter 3) on the effect of haem/iron supplementation on the gut microbiota was performed using Hungate tubes without pH control. Although haem/iron dependent changes in composition were observed, these were not significant. In order to more fully determine how the different forms of iron (haem and $FeSO_4$) impact on the composition of gut microflora, a pH controlled single vessel batch culture was employed over 48 h, using faecal inocula (0.1% w/v final concentration) from four healthy subjects (2 male and 2 females, age 27-40) representing four biological replicates. The gut model medium used contained either no added iron (control; 28 µM residual iron), haem (77 µM), $FeSO_4$ (18 µM) or both 77 µM haem and 18 µM $FeSO_4$.

5.2 Impact of iron on the bacterial numbers

Following the completion of the batch cultures, samples collected were analysed for total bacterial numbers by Flow-Fish (section 2.4); the results are presented in Figure 5.1 below.

As seen from Figure 5.1, the microbiota showed a different response to the forms of iron provided (haem or $FeSO_4$). Total bacteria number increased over time in all cases, however, bacteria in the haem-containing medium showed a slower growth rate compared to the non-haem containing condition, which was particularly apparent at 12 h.

Table 5.1 Total bacterial numbers at each time point and the fold difference against t0. Data are derived from Figure. 5.1 (see Figure 5.1 for details).

Regime	Bacterial counts (cells/ml medium)					Fold Difference			
	t0	t12	t24	t36	t48	t12/t0	t24/t0	t36/t0	t48/t0
Control	1.49E+07	2.91E+08	3.99E+08	4.7E+08	4.21E+08	19.55	26.86	31.61	28.30
Haem	9.57E+06	1.17E+08	2.78E+08	3.13E+08	4.19E+08	12.24	29.10	32.70	43.75
$FeSO_4$	1.11E+07	2.88E+08	3.91E+08	4.46E+08	4.2E+08	25.92	35.17	40.15	37.75
Haem & $FeSO_4$	1.24E+07	1.02E+08	3.43E+08	3.38E+08	4.11E+08	8.28	27.73	27.30	33.19

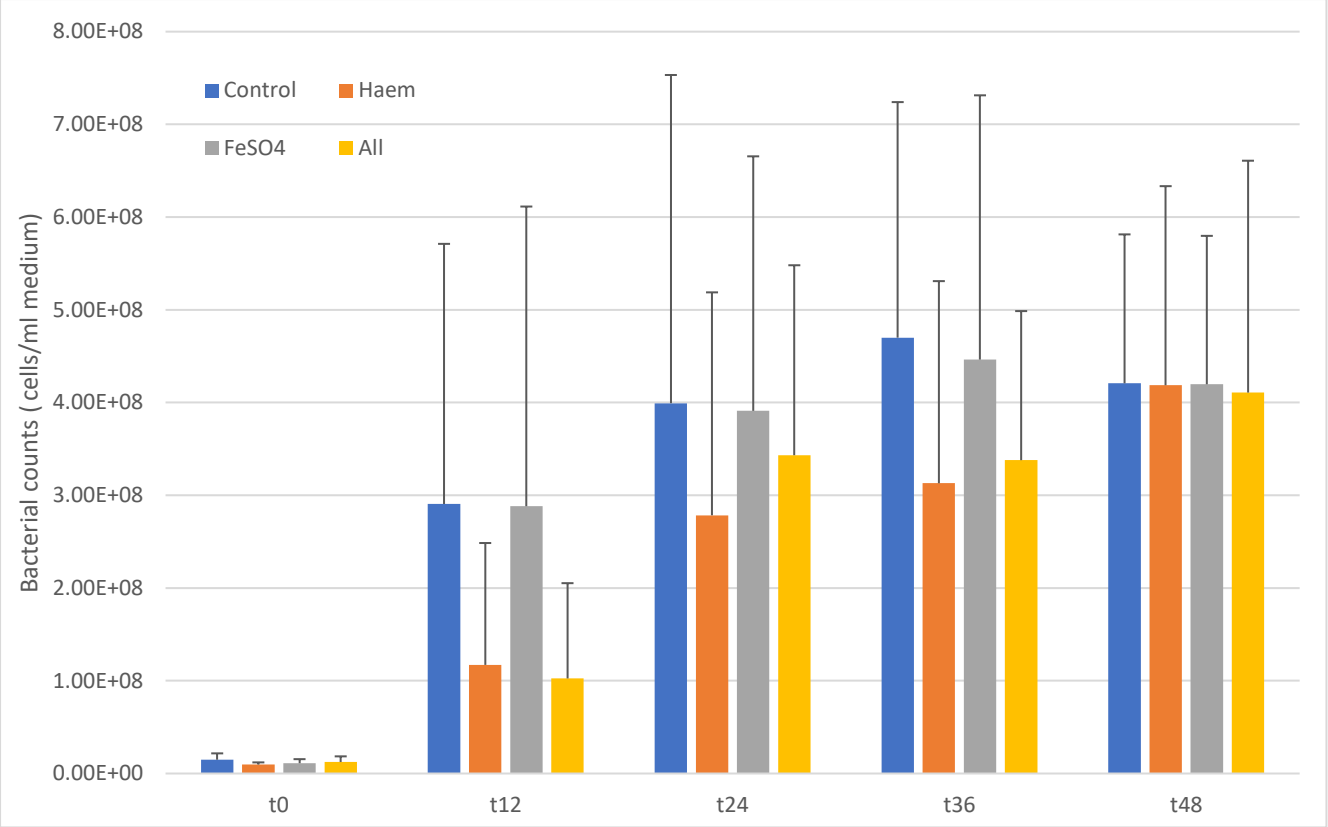


Figure 5.1 Effect of iron regime on total bacterial counts. Total bacterial counts for each iron regime were determined by Flow-FISH using Eub I-II-III probes. Each bar indicates a different regime: blue (control), orange (haem), grey ($FeSO_4$) and yellow (haem & $FeSO_4$). Bacterial counts are expressed as cells/ml medium. Results are average of 4 subjects and error bars represent SD. One-way Anova with Tukey Post Hoc was employed for statistical analysis. No significant difference was seen ($p>0.05$).

In the absence of haem, total bacterial growth was faster with higher numbers observed at 12-36 h with respect to the haem-containing condition, and a peak growth at t36 followed but a decline at 48 h. In the presence of haem, total bacteria gradually, but more slowly, increased to reach a maximum at t48. Importantly, the haem-containing condition showed lower bacterial counts at t12, t24 and t36 compared with the non-haem containing growth (control and $FeSO_4$ only). At t48, all four conditions showed similar cell counts with no significant difference between them ($p>0.05$). Although the control and $FeSO_4$-containing conditions showed higher bacterial counts than the haem-containing conditions (haem and both haem & $FeSO_4$) at t12, t24 and t36, total bacteria numbers were not significantly different from one another when tested statistically.

When no iron was present, the control showed a 19.6-, 26.7-, 31.6- and 28.3-fold increase for t12, t24, t36 and t48 respectively. The bacterial counts increased from an initial $1.49x10^7$ cells/ml medium to $2.91x10^8$ at t12, $3.99x10^8$ at t24, followed by $4.7x10^8$ at t36 and finally $4.21x10^8$ cells. When both forms of iron were included (haem & $FeSO_4$), there was an 8.28-, 27.7-, 27.3- and 33.2-fold increase for the same period of time. The vessel contained $1.24x10^7$ cells/ml medium initially, which then increased to $1.02x10^8$ cells 12 hours later and subsequently $3.43x10^8$ at t24. The bacterial counts dropped slightly to $3.38x10^8$ at t36 but continued to increase to $4.11x10^8$ at t48. When haem-only was added to the medium, the bacterial numbers increased 12.2-, 29.1-, 32.7-and 43.8-fold from t12 to t48. The number of cells increased from $9.57x10^6$ at t0 to $1.17x10^8$ at t12 and $2.78x10^8$ at t24. The bacterial counts continued to rise to $3.13x10^8$ at t36 and finally peaked at $4.19x10^8$ cells/ml medium at t48. When $FeSO_4$-only was included, relatively strong growth was observed. The bacterial counts increased 25.9-, 35.2-, 40.2- and 37.8-fold from t12 to t48. The bacteria in the $FeSO_4$ vessel gradually increased from $1.11x10^7$ cells/ml medium at t0 to $2.88x10^8$ at t12, followed by $3.91x10^8$ cells at t24. The bacteria number peaked at t36 with $4.46x10^8$ cells and subsequently showed a slight drop to $4.2x10^8$ cells/ml medium at t48.

Table 5.2 Fold difference between different iron regimes against the control. Data are derived from Figure. 5.1.

Regime	Fold Difference			
	t12	t24	t36	t48
Haem/Control	0.40	0.70	0.67	1.00
$FeSO_4$/Control	0.99	0.98	0.95	1.00
Haem & $FeSO_4$/Control	0.35	0.86	0.72	0.98

In order to further determine the effect of iron on the total number of bacterial cells, the cell numbers in the iron regimes were compared with those obtained in the control (table 5.2). As seen from table 5.2, the presence of iron caused a slight decrease (non significant) in the total bacterial counts of up t 1.05 fold, but at t48 all the regimes showed comparable counts with the control. However, the presence of haem caused a larger decrease compared to $FeSO_4$. The bacterial number showed the biggest decrease at t12 with the addition of haem whereby the total number in the

haem only and haem & $FeSO_4$ regime was 2.48- and 2.84-fold lower than the control, respectively. At t24 and t36, both haem-containing regimes showed a smaller decrease with the haem-only regime having a 1.43- and 1.50-fold reduction compared to the control while the haem & $FeSO_4$ regime which was reduced by 1.16- and 1.39-fold with respect to the control. The results thus suggest that the inclusion of ferrous sulphate partly reversed the negative impact of haem on growth, although it should be pointed out that the effects observed are not significant.

5.3 Impact of iron on the composition of the gut microbiota

5.3.1 Impact of iron on the composition of gut microbiota at phylum level

To determine whether the iron regimes employed had any impact on the relative abundance of the gut microbiota population composition, particularly under conditions where iron causes a lower growth than the control, 16S rRNA gene amplicon NGS analysis was performed. From the 48 samples (at 0, 24 and 48 h only) thus analysed, a total 740,392 reads were assigned to OTUs with an average of 20,566 reads per sample.

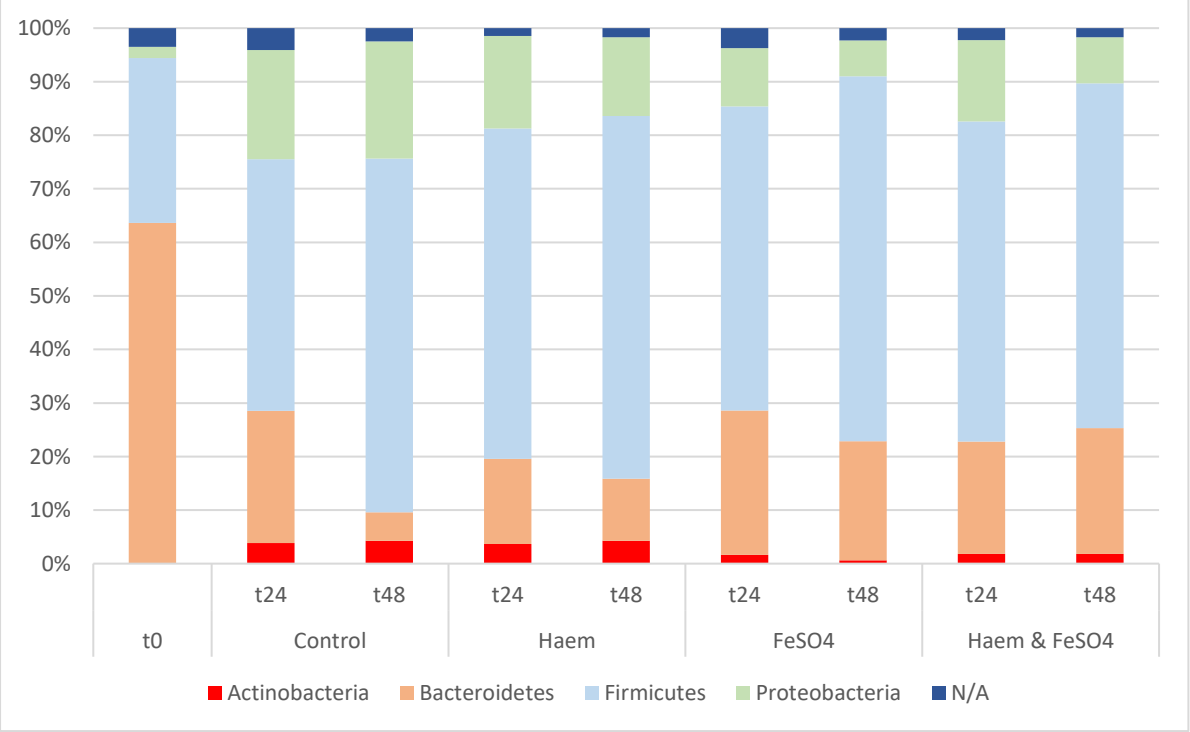


Figure 5.2 Composition of gut microbiota at phylum level. The gut microbiota is classified at phylum level for all the iron regime (control, haem only, $FeSO_4$ only and haem & $FeSO_4$) at both t24 and t48.

Table 5.3 Relative abundance of different bacterial groups at phylum level. The relative abundance of different bacterial groups at t0, t24 and t48 and they are expressed as a percentage of the total microbial community profile. The results are the average of 4 subjects. One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. Values in bold indicates significant difference ($p<0.05$) with respect to t0 time point.

Phylum	t0	Control		Haem		$FeSO_4$		Haem & $FeSO_4$	
		t24	t48	t24	t48	t24	t48	t24	t48
Actinobacteria	0.04	3.85	4.28	3.68	4.25	1.61	0.61	1.80	1.78
Bacteroidetes	63.56	24.67	**5.28**	15.87	**11.63**	26.96	22.25	20.99	23.52
Firmicutes	30.77	46.98	66.09	61.70	67.69	56.80	68.15	59.81	64.42
Proteobacteria	2.12	20.41	21.86	17.30	14.71	10.90	6.70	15.14	8.61
N/A	3.47	4.08	2.49	1.45	1.72	3.73	2.29	2.25	1.67

Table 5.4 Fold changes in abundance at phylum level with respect to t0. Fold changes of the bacteria at phylum level at t24 and t48 compared to t0. Data derived from table 5.3. One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. Values in bold indicate significant difference ($p<0.05$) with respect to t0 time point.

Phylum	Control		Haem		$FeSO_4$		Haem & $FeSO_4$	
	t24/t0	t48/t0	t24/t0	t48/t0	t24/t0	t48/t0	t24/t0	t48/t0
Actinobacteria	103.23	114.75	98.66	114.13	43.26	16.26	48.28	47.87
Bacteroidetes	0.39	**0.08**	0.25	**0.18**	0.42	0.35	0.33	0.37
Firmicutes	1.53	2.15	2.01	2.20	1.85	2.21	1.94	2.09
Proteobacteria	9.64	10.32	8.17	6.95	5.14	3.16	7.15	4.07

As seen from table 5.3, Bacteroidetes and Firmicutes made up the majority of the gut microbiota at t0 with a relative abundance of 63.6 and 30.8%, respectively, while Proteobacteria and Actinobacteria only contributed 2.12% and 0.04%, respectively. Bacteroidetes is the only phylum that showed a decrease throughout all the conditions tested while the other phyla increased in their relative abundance at the expense of Bacteroidetes. Bacteroidetes showed the biggest decrease when iron was absent (control) with a drop of 2.58- and 12.0-fold at t24 and t48, respectively, reducing in abundance to 24.7% and 5.28%. When iron was added to the medium, Bacteroidetes continued to show a decrease in relative abundance but to a lesser extent, with an average decrease of 3.15-fold across all iron-containing conditions. The haem-only condition showed a lower relative Bacteroidetes abundance than the $FeSO_4$-only and haem & $FeSO_4$ conditions indicating that although haem caused a small increase in Bacteroidetes relative abundance, $FeSO_4$-only and haem & $FeSO_4$

gave a greater increase. For the Bacteroidetes phylum, only t48 of the control and the haem-only regime showed a significant difference ($p<0.05$) between regimes while the differences in the other time points in all regimes were not significant when tested statistically ($p>0.05$). Firmicutes overtook Bacteroidetes to become the dominant phylum in all the regimes tested. The addition of iron and/or haem had little impact on Firmicute levels at 48 h with an average increase of ~2-fold seen under all conditions. However, at t24 the addition of $FeSO_4$ increased Firmicute levels slightly with respect to the control (1.53 versus 1.85 fold increase); the haem conditions also showed higher Firmutes increases than the control at t24, but it should be noted that there was also a lower overall growth level at this time point in the presence of haem that could account for such differences.

Although Actinobacteria showed a substantial increase in all the regimes, particularly in the control and haem-only media (average increases of 109- and 106-fold over time, respectively), the overall relative abundance of Actinobacteria was smaller (<4.3%) than seen for the other phyla, thus the Actinobacteria made up only a small proportion of the entire population. Actinobacteria did not show any significant difference between regimes when tested statistically ($p>0.05$). However, the addition of $FeSO_4$ (with or without haem) appeared to reduce the expansion of the Actinobacteria population by up to 7-fold. The Proteobacteria showed an increase in all the regimes tested with the highest increase occurring in the control whereby it showed a 9.64- (t24) and 10.3-fold (t48) increase, increasing in relative abundance to 20.4% and 21.9%, respectively. However, the presence of iron resulted in a small increase in the Proteobacteria compared to the control, particularly in the $FeSO_4$-only medium where the lowest increase among all the regimes was observed, with an average increase of just 4.15-fold over time. Among the iron-supplemented conditions, inclusion of haem-only resulted in the biggest increase, although the increase observed was still less than that seen for the control.

Table 5.5 Changes in relative abundance between the iron-supplemented media compared to the iron deficient medium at phylum level. The abundance data for the iron regime (control, haem only, $FeSO_4$ only and haem & $FeSO_4$) were compared with the control (0 μM) at both t24 and t48. One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. No significant difference is seen when tested statistically ($p>0.05$).

Phylum	Haem/Control		$FeSO_4$/Control		Haem & $FeSO_4$/Control	
	t24	t48	t24	t48	t24	t48
Actinobacteria	0.96	0.99	0.42	0.14	0.47	0.42
Bacteroidetes	0.64	2.20	1.09	4.21	0.85	4.45
Firmicutes	1.31	1.02	1.21	1.03	1.27	0.97
Proteobacteria	0.85	0.67	0.53	0.31	0.74	0.39

In order to determine the effect of iron on the growth of the bacteria, the relative abundance of the bacteria in the iron containing medium were compared to the control where no iron is present at t24 and t48 (Table 5.5). Both Actinobacteria and Proteobacteria showed a decrease in their relative abundance in the presence of iron, particularly when only $FeSO_4$ was added to the medium, with Actinobacteria having a 2.39- (t24) and 7.06-fold (t48) lower growth than the control and Proteobacteria a 1.87- (t24) and 3.26-fold (t48) lower growth. Both phyla also share a similar trend whereby the reduction in abundance was bigger at t48 compared to t24. However, in the same regime ($FeSO_4$ only), Bacteroidetes had the highest increase among all the iron regimes, increasing 1.09- and 4.21-fold for t24 and t48, respectively, indicating that the presence of $FeSO_4$ gave Bacteroidetes a growth advantage over the other phyla. When haem was present in the medium, Bacteroidetes displayed a slight decrease in relative abundance at t24 before increasing at t48. Bacteroidetes showed a 1.55- (haem only) and 1.18-fold (haem & $FeSO_4$) lower relative abundance compared to the control at t24 but showed an increase of 2.20- and 4.45-fold, respectively, at t48. Although the presence of haem caused Bacteroidetes to decrease at t24, the addition of $FeSO_4$ reduced the degree of reduction at t24 and stimulated a bigger increase at t48. Firmicutes showed a modest increase in relative abundance in the presence of iron compared to the control with an average increase between 1.12- to 1.17-fold. In addition, Firmicutes showed a bigger increase in growth at t24 compared to t48 across all the iron regimes tested. Bacteroidetes and Firmicutes

showed an increase in their relative abundance in all the regimes tested compared to the control, indicating that the presence of iron enhances their growth.

5.3.2 Impact of iron on the composition of gut microbiota at species level over time

In order to determine the effect of the iron on the gut microbiota at a lower taxonomic level, the composition of the microbiota was analysed at species level to provide a clearer view of the impact of iron. A total of 30 major species were identified: 2 from Actinobacteria, 8 from Bacteroidetes, 18 from Firmicutes and 2 from Proteobacteria.

Table 5.6 Effect of iron regime on relative abundance at the species level. The relative abundance of bacterial groups within the gut microbiota at species levels is indicated at t0, t24 and t48. The colour indicates the different regime: Control (orange), Haem only (yellow), $FeSO_4$ only (green) and Haem & $FeSO_4$ (grey). Data are expressed as a percentage of the total microbial community profile. The results are the average of 4 subjects. Only bacterial species present at >0.5% for at least one condition/time-point are shown. Values in bold indicates significant difference ($p<0.05$) with respect to t0. One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis (values in bold show significant difference with respect to t=0). The t=0 time point data have been averaged for all 4 regimes (sp, species; N/A, not identifiable).

Phylum	Order	Species	t0	Control		Haem		$FeSO_4$		Haem & $FeSO_4$	
				t24	t48	t24	t48	t24	t48	t24	t48
Actinobacteria	Bifidobacteriales	*Bifidobacterium* sp	0.01	2.16	2.44	1.71	1.90	1.24	0.29	1.00	1.39
	Coriobacteriales	*Collinsella aerofaciens*	0.00	0.99	0.97	0.94	1.41	0.09	0.14	0.08	0.02
Bacteroidetes	Bacteroidales	*Bacteroides* sp1	1.87	0.50	0.51	1.40	1.15	1.49	1.73	2.26	2.57
		Bacteroides sp2	22.11	4.84	3.12	11.25	7.24	11.66	15.47	13.58	13.29
		Bacteroides fragilis	0.23	0.10	0.09	0.12	0.14	0.15	0.06	0.20	0.55
		Bacteroides ovatus	2.67	0.44	0.56	1.68	1.44	1.60	1.60	2.43	2.66
		Bacteroides uniformis	1.88	0.49	0.36	0.79	0.87	0.98	0.77	1.34	1.72
		Parabacteroides sp	0.67	0.44	0.21	0.23	0.41	0.16	1.52	0.55	1.46
		Prevotella copri	29.58	17.13	0.15	0.01	0.01	10.25	0.40	0.16	0.34
		Rikenellaceae sp	3.16	0.34	0.12	0.25	0.18	0.35	0.11	0.17	0.21
Firmicutes	Lactobacillales	*Lactobacillus* sp1	0.01	0.12	5.98	6.76	7.01	6.72	4.87	7.72	6.28
		Lactobacillus sp2	0.02	0.63	3.66	2.23	4.66	5.31	2.22	3.99	2.52
		Lactobacillus delbrueckii	0.01	0.12	10.21	11.93	10.62	9.02	9.28	11.64	10.87
	Clostridiales	*Acidaminococcus* sp	0.38	15.56	20.91	7.54	17.92	10.45	13.66	18.31	22.03
		Clostridiales sp	0.72	2.32	2.48	1.46	2.06	1.43	2.31	1.39	2.50
		Clostridium sp	0.22	3.74	3.48	6.78	4.15	1.81	4.84	2.76	4.48
		Clostridium butyricum	0.00	0.07	0.00	9.88	4.05	0.30	1.39	0.00	0.00
		Dialister sp	0.03	1.97	3.36	1.56	3.65	0.76	1.05	1.48	3.17
		Dorea formicigenerans	0.05	0.11	1.13	0.04	0.88	1.50	3.09	0.03	1.05
		Faecalibacterium prausnitzii	15.24	**3.11**	**0.74**	**0.32**	**0.20**	**0.53**	**0.57**	**0.29**	**0.26**

		Lachnospiraceae sp	1.88	1.27	0.81	0.58	4.85	2.45	6.14	0.50	1.00
		Megamonas sp	0.01	0.27	0.07	0.50	0.10	5.48	3.07	0.18	0.04
		Megasphaera sp	0.01	7.83	3.56	8.86	2.79	1.58	3.84	4.85	3.33
		Mitsuokella multacida	0.01	1.71	2.46	0.04	0.08	0.99	1.66	0.72	0.98
		Ruminococcaceae sp1	3.90	**0.80**	**0.23**	**0.08**	**0.08**	**0.28**	**0.18**	**0.11**	**0.11**
		Ruminococcaceae sp2	2.60	**0.89**	**0.29**	**0.18**	**0.14**	**0.43**	**0.39**	**0.21**	**0.10**
		Ruminococcus sp	1.16	0.33	0.19	0.09	0.06	0.18	0.03	0.07	0.03
		Veillonella dispar	0.19	0.59	1.84	0.49	1.40	2.49	2.95	0.29	0.31
Proteobacteria	Burkholderiales	*Sutterella* sp	1.85	10.13	9.06	7.00	5.55	8.26	5.09	6.22	4.63
	Enterobacteriales	Enterobacteriaceae sp	0.02	10.23	12.75	10.15	9.11	2.62	1.60	8.88	3.96
N/A			3.47	4.08	2.49	1.45	1.72	3.73	2.29	2.25	1.67

Table 5.7 Fold changes in abundance at species level with respect to t0. Fold changes of the bacteria at species level at t24 and t48 compared to t0. Data derived from table 5.6. The colours indicate the different growth pattern (see Figure 5.3) that the bacteria displayed (yellow: pattern A; green: pattern B; blue: pattern C; red: pattern D). One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. Values in bold indicates significant difference with respect to t0 ($p<0.05$)

Phylum	Order	Species	Control		Haem		$FeSO_4$		Haem & $FeSO_4$	
			t24	t48	t24	t48	t24	t48	t24	t48
Actinobacteria	Bifidobacteriales	*Bifidobacterium* sp	189.28	214.55	149.78	166.62	108.80	25.82	88.18	121.75
	Coriobacteriales	*Collinsella aerofaciens*	300.80	296.09	287.36	430.46	25.87	41.39	24.56	4.88
Bacteroidetes	Bacteroidales	*Bacteroides* sp1	0.27	0.27	0.75	0.61	0.80	0.93	1.21	1.38
		Bacteroides sp2	0.22	0.14	0.51	0.33	0.53	0.70	0.61	0.60
		Bacteroides fragilis	0.41	0.41	0.53	0.59	0.64	0.26	0.87	2.37
		Bacteroides ovatus	0.16	0.21	0.63	0.54	0.60	0.60	0.91	0.99
		Bacteroides uniformis	0.26	0.19	0.42	0.46	0.52	0.41	0.71	0.92
		Parabacteroides sp	0.66	0.31	0.35	0.61	0.24	2.27	0.83	2.18
		Prevotella copri	0.58	0.01	0.00	0.00	0.35	0.01	0.01	0.01
		Rikenellaceae sp	0.11	0.04	0.08	0.06	0.11	0.03	0.05	0.07
Firmicutes	Lactobacillales	*Lactobacillus* sp1	24.48	1179.06	1332.40	1381.64	1324.82	959.30	1520.99	1238.40
		Lactobacillus sp2	36.35	212.14	128.82	270.05	307.39	128.67	231.03	146.16
		Lactobacillus delbrueckii	19.57	1669.98	1951.90	1737.45	1476.49	1518.74	1903.66	1779.18
	Clostridiales	*Acidaminococcus* sp	40.42	54.32	19.58	46.56	27.14	35.49	47.58	57.23
		Clostridiales sp	3.23	3.45	2.03	2.86	1.99	3.21	1.93	3.47
		Clostridium sp	16.70	15.53	30.28	18.55	8.10	21.59	12.32	19.98
		Clostridium butyricum	50.05	1.70	6969.32	2861.02	213.92	980.44	1.85	1.48
		Dialister sp	63.38	108.30	50.32	117.51	24.39	33.91	47.70	102.05
		Dorea formicigenerans	2.30	23.18	0.77	17.92	30.78	63.30	0.66	21.48
		Faecalibacterium prausnitzii	**0.20**	**0.05**	**0.02**	**0.01**	**0.03**	**0.04**	**0.02**	**0.02**

		Lachnospiraceae sp	0.67	0.43	0.31	2.58	1.30	3.26	0.27	0.53
		Megamonas sp	30.47	7.59	56.00	11.06	612.29	342.33	19.60	4.93
		Megasphaera sp	813.17	370.14	919.64	289.18	164.34	398.65	503.56	346.28
		Mitsuokella multacida	233.05	334.49	4.78	10.43	134.17	225.24	97.74	132.73
		Ruminococcaceae sp1	**0.20**	**0.06**	**0.02**	**0.02**	**0.07**	**0.05**	**0.03**	**0.03**
		Ruminococcaceae sp2	**0.34**	**0.11**	**0.07**	**0.05**	**0.16**	**0.15**	**0.08**	**0.04**
		Ruminococcus sp	0.29	0.16	0.07	0.05	0.15	0.02	0.06	0.03
		Veillonella dispar	3.02	9.49	2.52	7.20	12.80	15.18	1.49	1.62
Proteobacteria	Burkholderiales	*Sutterella* sp	5.46	4.89	3.78	2.99	4.45	2.74	3.36	2.50
	Enterobacteriales	Enterobacteriaceae sp	434.49	541.12	430.89	386.79	111.28	67.81	376.89	168.16
N/A			1.18	0.72	0.42	0.49	1.07	0.66	0.65	0.48

In order to better understand the impact of iron has on the gut microbiota, the bacteria were grouped according to their growth pattern over time which can be categorised into 4 different types: growth pattern A (modest increase over time, less than 50-fold), growth pattern B (substantial increase over time, less than 1000-fold increase), growth pattern C (increase over 1000-fold), growth pattern D (decrease over time). In the control vessel, where no iron was added, members of the Actinobacteria phylum displayed growth pattern B whereby *Bifidobacterium* sp increased 189- (t24) and 215-fold (t48) to 2.16 and 2.44% while the relative abundance of *Collinsella aerofaciens* increased to 0.99% and 0.97%, showing a 301- and 296-fold increase at t24 and t48, respectively. However, neither species showed significant changes when tested statistically ($p>0.05$).

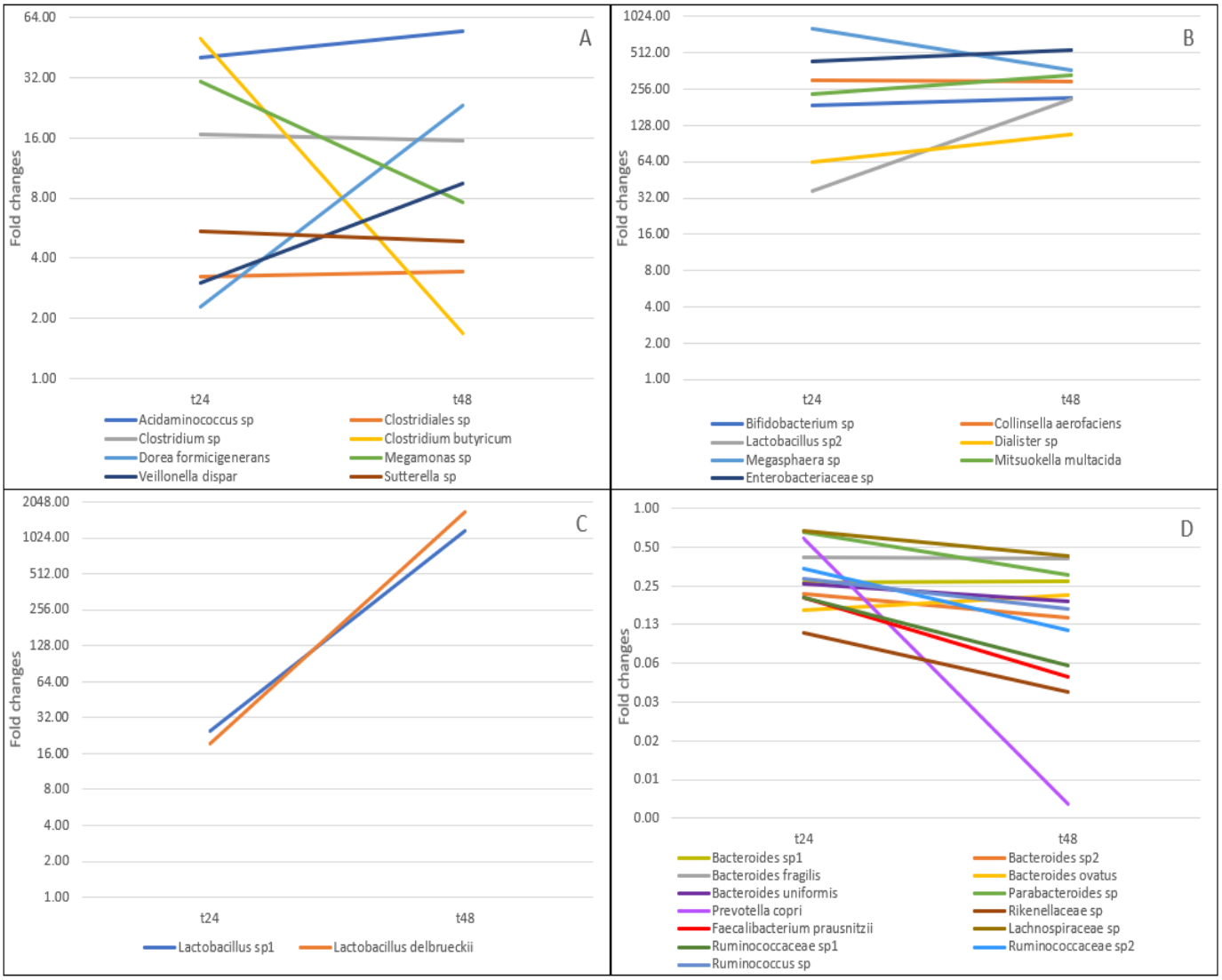


Figure 5.3 Change in abundance patterns of the most common bacterial species over time (0-48 h) during growth in the control regime (no iron). The data from Table 5.7 were plotted to determine similar patterns of change. In this way, all 30 species could be grouped into four distinct growth pattern types: **A**, modest increase over time, less than 50-fold. **B**, substantial increase over time, less than 1000-fold increase. **C**, increase over 1000-fold. **D** Decrease over time. The fold differences are expressed as $\log_2$.

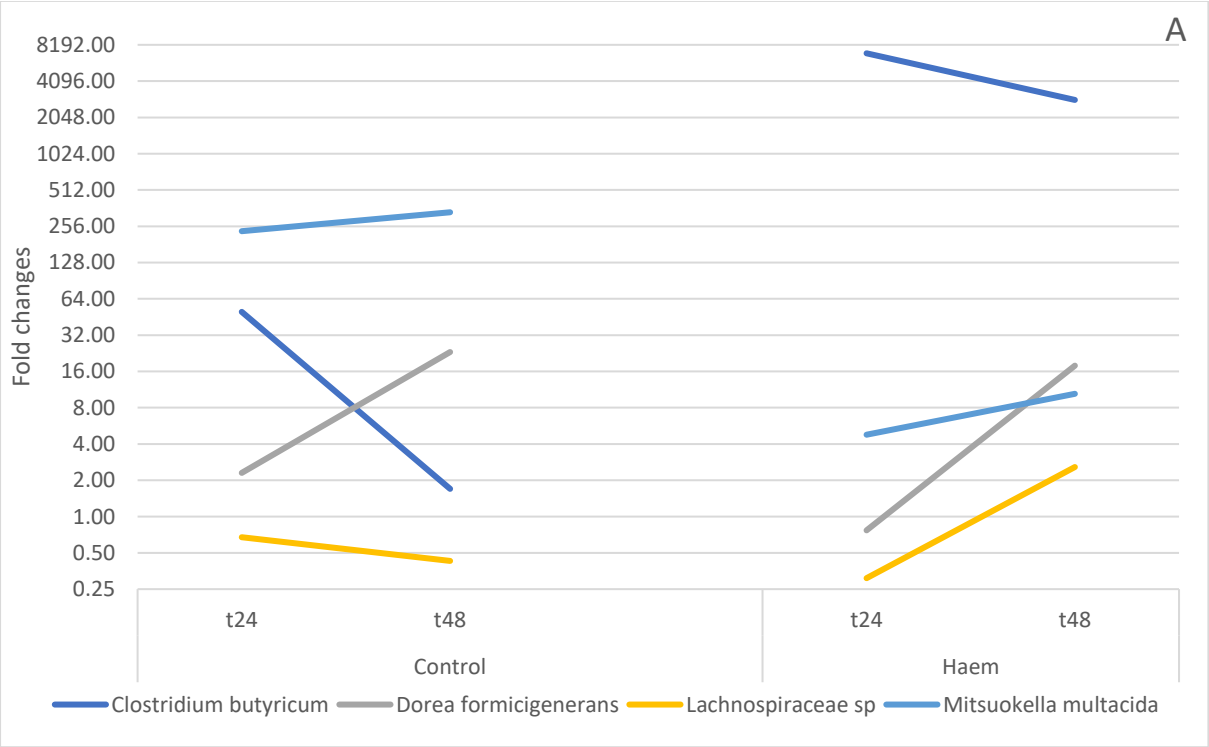
In the absence of iron, all members of the Bacteroidetes phylum followed growth pattern D where they all showed a decrease in relative abundance over time but none of the bacteria had a significant difference when tested ($p>0.05$). *Prevotella copri*, which was the most abundant species at t0, had the biggest decrease among all the bacterial groups with a reduction of 1.73- (t24) and 198-fold (t48) followed by Rikenellaceae sp which decreased 9.22- (t24) and 26.9-fold (t48). The *Bacteroides* and *Parabacteroides* spp. on the other hand showed a more modest decrease from t0 with a reduction in relative abundance of 1.52- to 6.12-fold at t24 and 3.25- to 7.10-fold at t48.

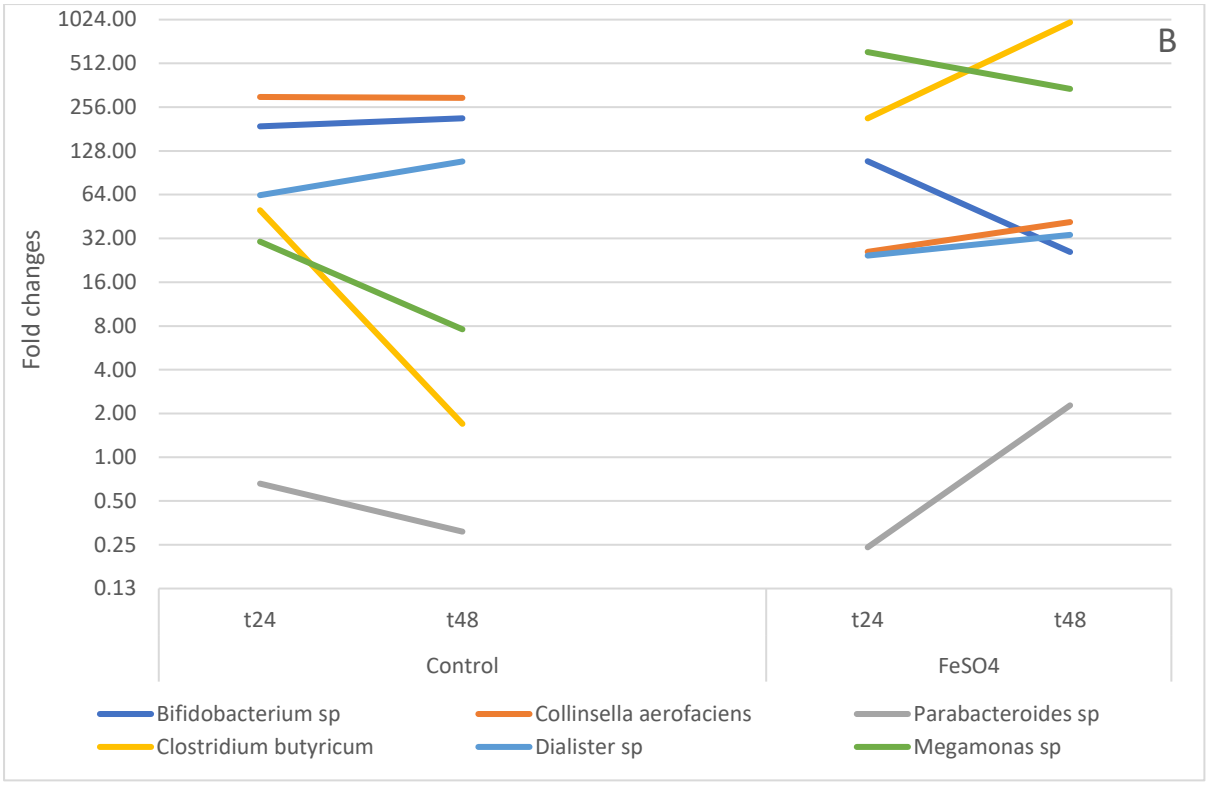
Among these species, *Bacteroides* sp2 which was the second most abundant at t0, showing the biggest decrease of 4.57- (t24) and 7.10-fold (t48), reducing its abundance from 22.1 to 4.84 and 3.12% respectively. As for the Firmicutes, a mixture of different growth patterns was seen. Although not significant ($p>0.05$), members of the Lactobacillales order generally showed an increase over time with a smaller increase at t24 of 19.6- to 36.4-fold and larger increase at t48: *Lactobacillus* sp2 increased 212-fold while *Lactobacillus* sp1 and *Lactobacillus delbrueckii* increased 1180- and 1670-fold, respectively. Clostridales sp, *Clostridium* sp, *Clostridium butyricum*, *Dorea formicigenerans, Acidaminococcus* sp, *Megamonas* sp and *Veillonella dispar* displayed growth pattern A where they showed a more modest increase over time with *Acidaminococcus* sp having the highest increase among these bacteria with an increase of 40.4- (t24) and 54.3-fold (t48), increasing its abundance to 15.6 and 20.9% from 0.38%. The rest of the species in this group showed an increase of 3.02- to 50.1-fold at t24 and between 1.70- to 23.2-fold at t48. However, none of the increased were significant when tested statistically with Bonferroni correction ($p>0.05$). *Dialister* sp, *Megasphaera* sp and *Mitsuokella multacida* displayed growth pattern B whereby they showed a more substantial increase in their relative abundance than the previous group. *Megasphaera* sp showed the highest increase among the 3 species with an increase of 813- and 370-fold for t24 and t48, reaching a relative abundance of 7.83 and 3.56%, respectively, while the other 2 species showed an increase of 63.4- to 334-fold over time, but none of these differences were significant ($p>0.05$). Lachnospiraceae sp, Ruminococcaceae sp1, Ruminococcaceae sp2, *Faecalibacterium prausnitzii* and *Ruminococcus* sp were the only Firmicutes species that displayed growth pattern D whereby they showed a decrease in their relative abundance over time. *F. prausnitzii* were the third most abundant bacteria at t24 but displayed the biggest decrease among this group, with a significant decrease of 4.91- and 20.5-fold at t24 and t48, reducing in abundance from 15.2 to 3.11 and 0.74%, respectively ($p<0.05$). Ruminococcaceae sp1 and sp2 also showed a significant decrease at both t24 and t48 with a reduction of 4.89- and 16.7-fold for the former and 2.92- and 8.92-fold for the latter, respectively ($p<0.05$). Lachnospiraceae sp and *Ruminococcus* sp had a modest non-significant decrease of 1.48-

and 6.07-fold over time. In the Proteobacteria phylum, *Sutterella* sp and Enterobacteriaceae sp displayed growth pattern A and B, respectively, with *Sutterella* sp increasing 5.46- and 4.89-fold at t24 and t48, while Enterobacteriaceae sp showed an increase of 434- and 541-fold for the same time point.

5.3.2.1 Impact of haem-only on the change-in-abundance patterns of the gut microbiota over time

The addition of iron caused some species to exhibit a change in growth pattern; these are presented in Figure 5.4 below (only species that showed a change in pattern compared to the control are included in Figure 5.4).





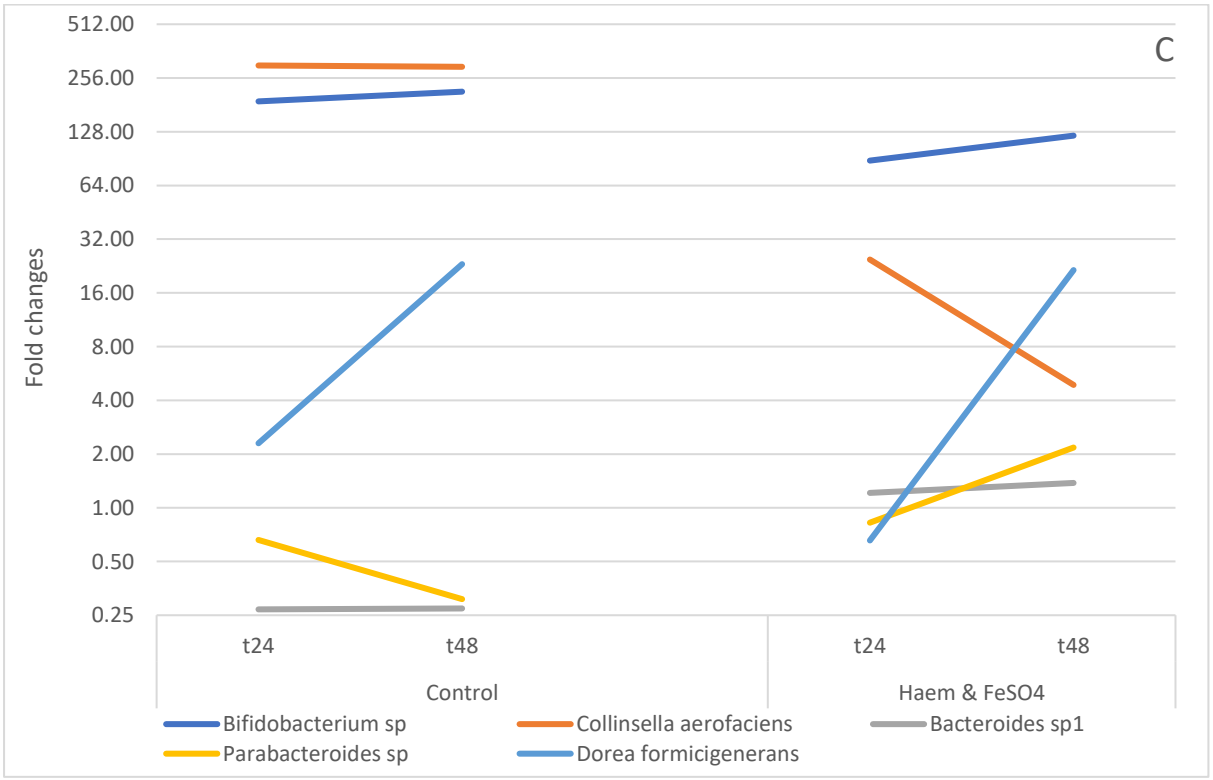


Figure 5.4 Change in growth pattern over time (0-48h) in the presence of iron. The data from Table 5.7 were plotted to determine the effect of zinc on the growth pattern of the bacterial groups. Only bacterial groups that showed a change in growth are shown when different concentrations of zinc were added to the medium: **A,** Haem only **B,** $FeSO_4$ only **C,** Haem & $FeSO_4$. The original growth pattern is shown on the left of each graph while the changed pattern is shown on the right. The fold differences are expressed as $\log_2$

When haem was added to the medium, most of the species showed similar growth patterns but with some moving towards a different pattern. Although both *Bifidobacterium* sp and *C. aerofaciens* maintain the same growth pattern (growth pattern B) in the presence of haem, *Bifidobacterium* sp showed a lesser increase (158-fold vs 202-fold, on average) while *C. aerofaciens* showed a bigger increase (359-fold vs 298-fold, on average) over time compared to the control. Members of the Bacteroidetes phylum also show a decrease in their relative abundance over time (growth pattern D) but to a much lesser extend as compared to the control with the exception of *P. copri* which showed a greater reduction in the presence of haem. *P. copri* displayed a 2080- and 3240-fold decrease at t24 and t48, reducing in abundance from 29.6 to 0.01% at both time point. The other members in the Bacteroidetes phylum showed a decrease of 1.34- to 12.8-fold at t24 and between 1.63- to 17.5-fold at t48. As for the Firmicutes phylum, the *Lactobacillus* spp. displayed the same growth pattern (growth pattern B & C) as the control but the presence of haem caused these species to present a greater increase in relative abundance with *L. delbrueckii* showing the biggest increase among them. These species showed an increase of 129- to 1950-fold for t24 and 270- to 1740-fold for t48. *C. butyricum,* Lachnospiraceae sp and *D. formicigenerans* were the only species that showed a change in their growth pattern in the presence of haem compared to the control. *C. butyricum* moved from growth pattern A (modest increase with less than 50-fold) to growth pattern C (increase over 1000-fold) whereby it increased 6970- and 2860-fold at t24 and t48, increasing in abundance to 9.88 and 4.05%, respectively. On the other hand, Lachnospiraceae sp and *D. formicigenerans* moved from growth pattern D and growth pattern A accordingly to a new pattern whereby they showed a decrease at t24 followed by a subsequently increase at t48. The former bacteria decreased 3.24-fold at t24 before then increasing 2.58-fold at t48, while the latter decreased 1.30-fold and increased 17.9-fold for the same time points. The presence of haem changed the growth pattern of *M. multacida* from growth pattern B (substantial increase with less than 1000-fold) to growth pattern A (modest increase with less than 50-fold) where it showed a 4.78- and 10.4-fold increase for t24 and t48, respectively. Ruminococcaceae sp1, Ruminococcaceae sp2, *F. prausnitzii* and

Ruminococcus sp remained the only 4 bacterial species that showed a decrease in relative abundance over time (growth pattern D) but the presence of haem causes the bacteria to have a greater decrease compared to the control. *F. prausnitzii* had the highest reduction among them, with a significant decrease of 47.0- and 75.1-fold at t24 and t48, respectively, reducing in abundance to 0.32 and 0.20%, respectively ($p<0.05$). Ruminococcaceae sp1 and sp2 also showed a significant decrease at both time points with the former bacteria decreasing 46.9- (t24) and 51.7-fold (t48) while the latter decrease 14. 7- (t24) and 18.3-fold (t48) ($p<0.05$). *Ruminococcus* sp1 showed a non-significant decrease of 13.7- and 21.1-fold at t24 and t48, reducing in abundance to 0.09 and 0.06%, respectively. *Sutterella* sp and Enterobacteriaceae sp showed the same trend as the control but the presence of haem caused a smaller increase in their relative abundance over time.

5.3.2.2 Impact of $FeSO_4$-only on the change-in-abundance patterns of the gut microbiota over time

When $FeSO_4$ was added to the medium, both members of the Actinobacteria phylum changed from growth pattern B (substantial increase with less than 1000-fold) to growth pattern A (modest increase with less than 50-fold) whereby *Bifidobacterium* sp showed an average increase of 67.3-fold over time while *C. aerofaciens* increased on average 33.6-fold in the presence of $FeSO_4$. Similar to the previous 2 regimes (control and haem only), bacterial in the at Bacteroidetes phylum still showed a decrease in their relative abundance over time (growth pattern D) in the $FeSO_4$-only regime. However, the presence of $FeSO_4$ as an iron source caused the majority of the species to display a smaller reduction in their relative abundance compared to the previous regimes. The bacterial species showed a more modest reduction of 1.26- to 8.99-fold at t24 and of 1.08- to 73.4-fold at t48 with *P. copri* having the biggest decrease followed by Rikenellaceae sp. In addition, instead of showing a continuous decrease over time, *Parabacteroides* sp showed a 4.16-fold decrease at t24 but a 2.27-fold increase in its relative abundance at t48, showing a similar growth pattern as Lachnospiraceae sp and *D. formicigenerans* in the haem-only regime. As for the Firmicutes phylum, the majority of the bacterial groups maintained the same growth pattern from the previous regimes with only 4 species showing a change in growth pattern in the presence of

$FeSO_4$. Both *C. butyricum* and *Megamonas* sp moved from growth pattern A (modest increase with less than 50-fold) in the control to growth pattern B (substantial increase with less than 1000-fold) where the former showed a 214- and 980-fold increase at t24 and t48 and the latter increased 612- and 342-fold for the same time point compared to the control. Lachnospiraceae sp and *Dialister* sp changed to growth pattern A from growth pattern D and growth pattern B, respectively. Lachnospiracaeae sp had a 1.30- and 3.26-fold increase at t24 and t48, while *Dialister* sp showed a 24.4- and 33.9-fold increase. Although all members of the Lactobacillales order showed a higher increase and the same pattern as the control, the relative abundance of *Lactobacillus* sp1 (1140- vs 1360-fold, on average) and *L. delbrueckii* (1500- vs 1840-fold, on average) showed a lower increase in the presence of $FeSO_4$ than the haem-only regime while *Lactobacillus* sp2 had a greater increase for the same regime (218- vs 199-fold, on average). The addition of $FeSO_4$ to the medium causes 10 bacterial species to display a smaller increase over time compared to the control and 5 to show a greater increase despite having the same growth pattern as the control. *Acidaminococcus* sp, Clostridales sp, *Clostridium* sp, *Dialister* sp, *Megasphaera* sp and *M. multacida* were among the bacterial groups that had a smaller increase in their relative abundance as compared to the control with an average increase between 2.60- to 281-fold. *F. prausnitzii*, Ruminococcaceae sp1 and sp2, and *Ruminococcus* sp had a bigger reduction in their relative abundance compared to the control. *F. prausnitzii*, and Ruminococcaceae sp1 and sp2 all showed a significant decrease with an average reduction of 27.8-, 17.0- and 6.34-fold, respectively ($p<0.05$) while *Ruminococcus* sp showed a non-significant reduction of 11.3-fold on average over time. Both members of the Proteobacteria phylum also showed a smaller increase compared to the control with *Sutterella* sp having a modest increase of 3.60-fold on average over time while Enterobacteriaceae sp increased on average 89.5-fold.

5.3.2.3 Impact of haem & $FeSO_4$ on the change-in-abundance patterns of the gut microbiota over time

When both haem and $FeSO_4$ were added to the medium, *Bifidobacterium* sp retained the same growth pattern as in the control but with a smaller increase while *C. aerofaciens* shifted from growth pattern B (substantial increase with less than 1000-fold) to growth pattern A (modest increase with

less than 50-fold) where it had an increase of 24.6- and 4.88-fold for t24 and t48, respectively. For the Bacteroidetes phylum, 3 species displayed a change in their growth pattern where they showed an increase in their relative abundance while the rest of the members still showed a decrease over time (growth pattern D). *Bacteroides* sp1 moved from growth pattern D to growth pattern A where it had a 1.21- and 1.38-fold increase at t24 and t48, increasing its abundance to 2.26 and 2.57%, respectively. *B. fragilis* and *Parabacteroides* sp changed from a continuous decrease pattern to a modest decrease at t24 followed by an increase at 48. The former species decreased 1.15-fold at t24 and increased 2.37-fold at t48 while the latter had a 1.21-fold reduction at t24 followed by a 2.18-fold increase. With the exception of *P. copri* and Rikenellaceae sp, the rest of the *Bacteroides* species showed the lowest reduction in their relative abundance among all the regimes tested with decreases of 1.01- to 2.66-fold over time. The relative abundance of *P. copri* decreased 182- and 86.2-fold at t24 and t48, reducing in abundance to 0.16 and 0.34%, respectively. Interestingly, *P. copri* showed a higher reduction when haem was provided with the highest decrease occurring in the haem-only medium and the second highest in the haem & $FeSO_4$ medium, indicating haem has an inhibitory effect on the growth of *P. copri* over time. Although Rikenellaceae sp showed the highest reduction in the haem & $FeSO_4$ regime with a decrease of 18.9- (t24) and 14.7-fold (t48), it showed a similar degree of reduction throughout all the regimes tested. Only 1 bacterial species showed a change in growth pattern in the Firmicutes phylum, while the rest of the showed similar patterns to those in the control. When both haem and $FeSO_4$ were added to the medium, *D. formicigenerans* changed from growth pattern A to the pattern seen in the haem-only regime (decrease at t24 followed by an increase at t48), indicating that the presence of haem caused this observed pattern. *F. prausnitzii*, and Ruminococcaceae sp1 and sp2 are the 3 species that showed a significant decrease in their relative abundance over time ($p<0.05$). Similar to *P. copri,* these 3 species showed a higher decrease when haem was present in the medium as compared to the control and the $FeSO_4$-only medium, indicating an inhibitory effect caused by haem. *F. prausnitzii* showed the highest decrease among them with a reduction of 52.7- and 59.4-fold for t24 and t48

while the other 2 species gave a decrease of 12.1- to 37.2-fold over time, respectively. *Sutterella* sp and Enterobacteriaceae sp also maintained the same growth pattern as the control but the presence of both haem and $FeSO_4$ caused them to have a smaller increase in their relative abundance as compared to the control.

5.3.3 Impact of iron regime on the composition of the gut microbiota at species level

In order to determine the effect of iron on the abundance of the bacteria species, the relative abundance at t24 and t48 for each iron regime was compared with the control at the same time point. The presence of iron caused an increase in majority of the member in the Bacteroidetes phylum while members in the Actinobacteria, Firmicutes and Proteobacteria phyla showed a decrease with iron.

Table 5.8 Changes in relative abundance between the iron containing medium compared to the iron deficient medium. The abundance data for the iron regime (control, haem only, $FeSO_4$ only and haem & $FeSO_4$) were compared with the control (no iron/haem added) at both t24 and t48. Numbers with green background indicate an increase and numbers with red background indicate a decrease compared to the control. Bacterial groups with green background showed an overall increase in the presence of iron while bacterial groups with red background an overall decrease with the addition of iron. Bacterial groups with white background showed an increase at certain iron regime. One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. No significant difference was obtained when tested ($p>0.05$).

Phylum	Order	Species	Haem/Control		$FeSO_4$/Control		Haem & $FeSO_4$/Control	
			t24	t48	t24	t48	t24	t48
Actinobacteria	Bifidobacteriales	*Bifidobacterium* sp	0.79	0.78	0.57	0.12	0.47	0.57
	Coriobacteriales	*Collinsella aerofaciens*	0.96	1.45	0.09	0.14	0.08	0.02
Bacteroidetes	Bacteroidales	*Bacteroides* sp1	2.78	2.25	2.95	3.40	4.49	5.04
		Bacteroides sp2	2.32	2.32	2.41	4.97	2.80	4.27
		Bacteroides fragilis	1.29	1.46	1.54	0.65	2.10	5.83
		Bacteroides ovatus	3.84	2.56	3.67	2.86	5.56	4.73
		Bacteroides uniformis	1.61	2.43	2.01	2.15	2.74	4.80
		Parabacteroides sp	0.53	2.00	0.36	7.39	1.25	7.07
		Prevotella copri	0.00	0.06	0.60	2.70	0.01	2.30
		Rikenellaceae sp	0.72	1.54	1.03	0.93	0.49	1.83
Firmicutes	Lactobacillales	*Lactobacillus* sp1	54.43	1.17	54.12	0.81	62.13	1.05
		Lactobacillus sp2	3.54	1.27	8.46	0.61	6.36	0.69
		Lactobacillus delbrueckii	99.74	1.04	75.45	0.91	97.28	1.07
	Clostridiales	*Acidaminococcus* sp	0.48	0.86	0.67	0.65	1.18	1.05

		Clostridiales sp	0.63	0.83	0.62	0.93	0.60	1.01
		Clostridium sp	1.81	1.19	0.48	1.39	0.74	1.29
		Clostridium butyricum	139.25	1685.43	4.27	577.58	0.04	0.87
		Dialister sp	0.79	1.09	0.38	0.31	0.75	0.94
		Dorea formicigenerans	0.33	0.77	13.39	2.73	0.29	0.93
		Faecalibacterium prausnitzii	0.10	0.27	0.17	0.77	0.09	0.35
		Lachnospiraceae sp	0.46	6.01	1.93	7.60	0.39	1.24
		Megamonas sp	1.84	1.46	20.10	45.13	0.64	0.65
		Megasphaera sp	1.13	0.78	0.20	1.08	0.62	0.94
		Mitsuokella multacida	0.02	0.03	0.58	0.67	0.42	0.40
		Ruminococcaceae sp1	0.10	0.32	0.35	0.77	0.13	0.47
		Ruminococcaceae sp2	0.20	0.49	0.48	1.35	0.24	0.33
		Ruminococcus sp	0.26	0.29	0.54	0.13	0.21	0.18
		Veillonella dispar	0.83	0.76	4.24	1.60	0.49	0.17
Proteobacteria	Burkholderiales	*Sutterella* sp	0.69	0.61	0.81	0.56	0.61	0.51
	Enterobacteriales	Enterobacteriaceae sp	0.99	0.71	0.26	0.13	0.87	0.31
		N/A	0.36	0.69	0.91	0.92	0.55	0.67

Members of the Actinobacteria phylum generally showed a lower relative abundance compared to the control in the presence of iron particularly in the $FeSO_4$-only regime and the haem & $FeSO_4$ regime whereby *Bifidobacterium* sp was on average 2.88- and 1.94-fold lower than the control while *Collinsella aerofaciens* was 8.86- and 20.4-fold lower compared to the control, respectively. In the haem-only regime, *Bifidobacterium* sp showed an average 1.28-fold lower abundance than the control but *C. aerofaciens* was 1.20-fold higher than the control. Although the relative abundance of members belonging to the Bacteroidetes phylum decreased over time, the presence of iron enhanced their abundance compared to the control. Most of the species in this phylum shared a similar trend whereby they showed the greatest increase when both haem and $FeSO_4$ were added to the medium compared to when they were individually added. These species showed a 1.16- to 5.15-fold higher abundance compared to the control with *Bacteroides ovatus* showing the biggest increase followed by *Bacteroides* sp1 and *Parabacteroides* sp. With the exception of *Bacteroides fragilis* and Rikenellaceae sp, the other species showed the second highest increase in the $FeSO_4$-only

regime followed by the haem-only regime. In the $FeSO_4$-only regime, *Parabacteroides* sp had the biggest increase with an average increase of 3.88-fold, followed by *Bacteroides* sp2 and *B. ovatus* with 3.69- and 3.26-fold increases, respectively. The relative abundance of the rest of the species was on average between 1.09- to 3.17-fold higher than in the control. In the haem-only regime, *B. ovatus*, and *Bacteroides* sp1 and sp2 were among the species that showed the biggest increase in relative abundance, being on average 3.20-, 2.51- and 2.32-fold higher than the control; the remaining bacterial species where 1.13- and 2.02-fold greater in abundance than the control. Interestingly, *Prevotella copri* showed on average 32.3-fold lower abundance than the control in the haem-only medium but not in the other 2 media, indicating that *P. copri* prefers $FeSO_4$ as its iron source as it shows an increase in the presence of $FeSO_4$. On the other hand, Rikenellaceae sp abundance was unaffected by $FeSO_4$-only (displaying an average 1.02-fold lower growth than in the control) but was enhanced in the conditions containing haem, indicating that haem enhances the growth of Rikenellaceae sp.

As for the Firmicutes phylum, most of its members showed a lower relative abundance than the control when iron was provided. In the condition with both haem and $FeSO_4$, the highest number (13 out of 18) of Firmicutes species showed a reduced growth compared to the control, followed by the haem-only regime with 11 and the $FeSO_4$-only regime with 10. The presence of haem tended to have an inhibitory effect on the species in this phylum as the majority showed a reduced abundance when haem was added to the medium. The Firmicutes species reacted differently towards the iron regimes but there was some similarity in the patterns between them such they can be categorised accordingly. Clostridales sp, Ruminococcaceae sp1 and sp2, *Faecalibacterium prausnitzii, Ruminococcus* sp1, *Dialister* sp, *Megasphaera* sp and *Mitsuokella multacida* all showed a lower relative abundance than the control in the presence of iron (regardless of its form). Clostridales sp, Ruminococcaceae sp1, *Faecalibacterium prausnitzii* and *Mitsuokella multacida* all showed the biggest reduction in the haem-only regime with *M. multacida* being the most affected displaying an average 38.7-fold lower abundance than the control while the other species showed a decreased

growth of 1.37- to 5.30-fold (on average). These 4 species showed the second highest reduction in growth in the haem & $FeSO_4$ regime followed by the $FeSO_4$-only regime, thus indicating that haem has an inhibitory effect on their growth while $FeSO_4$ is able to reduce its effect. Ruminococcaceae sp2 and *Ruminococcus* sp had the biggest reduction when both haem & $FeSO_4$ were provided to the medium with the former and latter having an average 3.52-and 5.23-fold lower abundance, respectively, than the control. Similar to the bacterial species indicated above, haem had an inhibitory effect on Ruminococcaceae sp2 and *Ruminococcus* sp as the haem-only regime had the second highest growth reduction followed by the $FeSO_4$-only regime. $FeSO_4$ did not appear to reduce the effect of haem in these cases, but instead the combination of haem and $FeSO_4$ negatively affected their abundance. *Dialister* sp and *Megasphaera* sp displayed the biggest decrease in the $FeSO_4$-only regime followed by the haem & $FeSO_4$ regime and the haem-only regime. These bacteria were more affected by $FeSO_4$ than haem - *Dialister* sp and *Megasphaera* sp were on average 2.87- and 1.56-fold lower in relative abundance than the control. However, in the haem-only regime, there was a negligible difference (1.06- and 1.05-fold reduction respectively) implying that haem had little impact on their growth. *Clostridium butyricum*, Lachnospiraceae sp and *Megamonas* sp only showed a reduced growth when both haem & $FeSO_4$ were present in the medium with a modest reduction on average of between 1.22- to 2.20-fold. However, when haem and $FeSO_4$ were added separately, these species tended to show an increase in abundance with *C. butyricum* showing the biggest increase (912-fold for haem-only and 291-fold for $FeSO_4$-only) while the other 2 species showed an increase between 1.65- to 32.6-fold (on average). *Clostridium* sp and *Veillonella dispar* showed opposing reactions in the $FeSO_4$-only regime with *Clostridium* sp showing little change (decrease of 1.07-fold) and *V. dispar* (2.92-fold) showing an increase in relative abundance compared to the control. This indicates that the growth of *Clostridium* sp is enhanced by the presence of haem as it shows an increase in the 2 haem-containing conditions while haem had an inhibitory effect on *V. dispar* as shown by the reduced abundance in the presence of haem. The *Lactobacillus* spp. all showed an increase in the presence of haem with *L. delbrueckii* having the

biggest increase among them. The relative abundance of *L. delbrueckii* showed on average a 50.4-, 38.2- and 49.2-fold higher abundance than the control in the haem-only, $FeSO_4$-only and haem & $FeSO_4$ regimes, respectively; this indicates a dependence on the added iron sources for raised abundance. *Lactobacillus* sp1 increased 27.8-, 27.5- and 31.6-fold while *Lactobacillus* sp2 increased 2.41-, 4.53- and 3.52-fold for the same regimes. Both members of the Proteobacteria phylum showed a decrease in all of the regimes tested. Thus, these *Lactobacillus* species show a surprising growth enhancement in response to the iron/haem supplements. *Sutterella* sp had the biggest reduction when both haem & $FeSO_4$ were added to the medium, showing a 1.78-fold lower growth than the control while the haem-only and $FeSO_4$-only regimes showed a 1.53- and 1.45-fold reduction, respectively. The relative abundance of Enterobacteriaceae sp was 5.24-fold lower than the control in the $FeSO_4$-only regime while the haem-only and haem & $FeSO_4$ regime showed a decrease of 1.17- and 1.70-fold, indicating that Enterobacteriaceae sp is able to utilise haem to support its growth.

In summary, the growth of members belonging to the Bacteroidetes phylum was enhanced by the presence of iron especially when both haem and $FeSO_4$ were present, but this combination negatively affected the growth of members in the Firmicutes phylum. The presence of iron, irrespective of its form, was generally deleterious to bacteria belonging to the Actinobacteria, Firmicutes and Proteobacteria phyla as shown by their reduced abundance when iron was added to the medium.

5.4 Discussion

The presence of iron, particularly haem, caused a slower growth rate for the gut microbiota as shown in Figure 5.1, while addition of $FeSO_4$, on the other hand, gave a comparable growth rate with the control. The presence of iron (haem and $FeSO_4$) caused a lower total bacterial count as compared to the control. Although the presence of iron has an effect on the growth rate, the bacteria showed no significant difference in their numbers between regimes. At phylum level, despite showing a reduced growth over time, the presence of iron greatly enhanced the growth of

Bacteroidetes. The growth of Firmicutes was also slightly enhanced by the presence of iron while both Actinobacteria and Proteobacteria showed a lower growth when iron was added to the medium. At species level, most of the Bacteroidetes showed an increase in growth with all of the *Bacteroides* showing an enhanced growth in the presence of iron particularly when both haem and $FeSO_4$ were present. As for the Firmicutes, most of its members showed a decrease in the iron supplemented medium but *Lactobacillus* showed a substantial increase in growth in all of the iron regimes. *Faecalibacterium prausnitzii* and *Ruminococcus* showed a decrease in all the iron regimes with a bigger decrease when haem was present in the medium. *Sutterella* and Enterobacteriaceae from the Proteobacteria phylum, as well as *Bifidobacterium* and *Collinsella aerofaciens* from the Actinobacteria phylum, showed a reduced growth compared to the control when iron was added to the medium. Although the growth of the Enterobacteriaceae and *Bifidobacterium* species was reduced in the iron regimes, both bacteria showed a better growth in the presence of haem than its absence (i.e. the $FeSO_4$-only regime).

Iron is essential for most living organisms with the exception of a few, such as *Lactobacillus* species and *Borrelia burgdorferi*, whereby the lack of iron does not affect their growth nor biological function (Andrews et al, 2003). Since iron is indispensable in most bacteria, they have developed numerous mechanisms for acquiring iron which includes the production of siderophores, possession of multiple iron transporters and receptors, as well as has the ability to utilise various forms of iron (Andrews et al, 2003) to ensure that they are able to compete and uptake iron from the environment. The iron regime batch culture experiments described in this chapter were designed to determine the effect of two different forms of iron on the gut microbiota rather than the concentration of iron. The medium was prepared according to Macfarlane and colleagues (Macfarlane, Macfarlane and Gibson, 1998). No extra iron was added to the gut model medium used in this experiment and the concentration of iron present was the same as the original recipe used. However, the iron was selectively added, haem only, $FeSO_4$ only or both haem & $FeSO_4$. The concentration of haem and $FeSO_4$ usually employed in the gut model medium is 77 and 18 μM,

respectively (Macfarlane, Macfarlane and Gibson, 1998), thus both the haem-only and $FeSO_4$-only regimes had lower total iron contents than the standard gut model medium (which matches that of the haem & $FeSO_4$ regime). Neither extra haem nor $FeSO_4$ was added to medium to compensate for the difference as the study was designed to study the effect of the forms of iron that are normally present in the gut model medium on the gut microbiota and adding additional iron would increase the concentration of iron beyond that normally present in the gut model medium. However, the gut model medium has a background iron concentration of 28 µM, mainly contributed by mucin and yeast extract (data not shown), thus the background levels of iron provided by the medium are relatively high.

One of the disadvantage of using the batch culture is the development of the Enterobacteriaceae in high abundance over time. Due to the experiment being run for only 48 h, the batch culture approach provides the fast-growing, bacteria such as Enterobacteriaceae, an advantage over the slow-growing bacteria, resulting in an increase in their final abundance which diminishes the abundance of other bacteria. Members of the Enterobacteriaceae such as *E. coli* have a doubling time of 20 min under laboratory conditions (Gibson et al, 2018), thus it is of no surprise that this bacterial group is able to outgrow many of the other bacteria. As seen from table 5.6, the abundance of Enterobacteriaceae was only 0.02% at t0 but showed an average increase of 315-fold across all the regimes tested to an abundance between 1.60 and 12.75%. An increase in Enterobacteriaceae in batch culture has been reported in other studies regardless of the experimental design or substrate tested (Takagi et al, 2016; Ahmadi et al, 2019; Kristek et al, 2019; Wiese et al, 2018; Ding et al, 2019) and this may cause an underestimation of the impact of the corresponding growth regime on other bacterial groups.

At t0, when faecal samples were directly inoculated into the vessels, the composition of the microbiota was dominated by Bacteroidetes and Firmicutes which are the two main phyla of the human gut microbiota (Arumugam et al, 2011; Rinninella et al, 2019). However, over time, the

Firmicutes overtook the Bacteroidetes to become the dominant phylum. Short term diet changes have led to an altered composition of the gut microbiota (David et al, 2014), thus propagating human gut microbiota in the gut model medium would be expected to introduce alterations to the gut microbiota whereby some bacteria have an advantage and whilst others a disadvantage when shifting to the new environment, which would explain the decrease or increase in the relative abundance of different species over time.

As seen from tables 5.6 and 5.7, the relative abundance of the gut microbiota undergoes some changes over the 48 h period, with some species having a greater growth and some with diminished growth. Since bacteria species compete for the limited nutrients present in the medium, bacterial species which have distinct requirements or the ability to utilise a alternative nutrients will have an advantage over other bacterial groups. This effect is shown by the increase in the abundance of Firmicutes, which was mainly due to increases in *Lactobacillus* and *Acidaminococcus* species. *Lactobacillus plantarum* is known to be independent of iron and can contain less than 2 iron atoms per cell which is considered to have no conceivable biological function (Archibald, 1983). *Lactobacillus* spp. appear to use other metals such as cobalt and manganese instead of iron (Archibald, 1983) which can give them a competitive advantage over the other bacteria which are iron-dependent when iron levels are limited. *Acidaminococcus*, on the other hand, is a Gram-negative diplococci which utilises amino acids (mainly glutamic acid) as energy source (Jumas-Bilak et al, 2007), which gives it an advantage over the other bacterial species that compete for other energy sources. *Faecalibacterium prausnitzii*, which was one of the most abundant species at t0, showed the greatest reduction among all members of the Firmicutes phylum, possibly due to its slow growth rate of only 0.13 h^{-1}, putting it at a disadvantage compared to the other bacteria (Heinken et al, 2014). Comparing with other members of the Firmicutes phylum, *Lactobacillus* has a growth rate between 0.12 to 0.21 h^{-1} (grown on MRS broth) (de Mesquita, 2017) but its unique properties of not requiring iron may give it an advantage in competing for other nutrients. *Ruminococcus gnavus* which is a symbiont in the human gut showed a growth rate between 0.87 to

1 h^{-1} (grown on basal medium with glucose as carbon source) depending on the strain (Crost et al, 2013) while growth studies done on *Clostridium* species from a clinical microbiology lab showed that it has an average doubling time of 30.8 min (Sottile II and Zabransky, 1977). Members of the Actinobacteria phylum, such as *Bifidobacterium*, have a growth rate between 0.17 and 0.76 h^{-1} depending on its species (Rios-Covian et al, 2015). Most of the members of the Bacteroidetes showed a decrease in their abundance over time irrespective of the iron regime indicating that they are being outcompeted by the bacteria in the other phyla which have a faster growth rate as mentioned above. Indeed, the growth rate of *Bacteroides fragilis* in a batch culture has been shown to be between 0.12 to 0.52 h^{-1} depending on the availability of glucose (Dalland and Hofstad, 1974) while *Prevotella copri* has a growth rate of 0.52 h^{-1} (Huang et al, 2021).

Although the *Bacteroides* showed a decrease in the relative abundance over time, the presence of iron caused an increase in abundance compared to the control particularly when both haem and $FeSO_4$ were added to the medium. *Bacteroides* are known to have an essential requirement for haem or inorganic iron plus protoporphyrin to support their growth (Rocha et al, 2019). *Bacteroides* lack the genes required for the synthesis of the macrocycle tetrapyrrole ring protoporphyrin IX (PPIX), thus they require an exogenous source of haem for growth. However, these bacteria can synthesis haem *in vitro* if PPIX and inorganic iron are made available (Rocha et al, 2019). This is in agreement with the results obtained from the iron regime batch cultures whereby the haem & $FeSO_4$ regime had the greatest increase among all the regimes tested. In an *in vitro* study conducted by Dostal and colleagues, the presence of iron (through supplementation of $FeSO_4$) had minimal impact on *Bacteroides* but did reduce the abundance of the Ruminococcaceae family by 2.5-fold which is similar to the observed results in this chapter (Dostal et al, 2013). However, under severe iron restriction (chelation by 2,2'-dipyridyl and Chelex®), there was a decrease in the abundance of the *Bacteroides*, thus indicating that these organisms have a need for iron (Dostal et al, 2013). In addition, Enterobacteriaceae showed an increase in the presence of iron (Dostal et al, 2013) but results from table 5.8 showed that Enterobacteriaceae had a decrease in their abundance when iron

is added to the medium. Besides the opposing results obtained in *Bacteroides* and Enterobacteriaceae, the remaining bacterial groups showed similar results between the *in vitro* study and those presented in this chapter whereby the presence of iron increased the abundance of *Lactobacillus* spp. but decreased abundance for *Bifidobacterium* sp and *Faecalibacterium prausnitzii* (Dostal et al, 2013). It is also worth noting that the sequencing results presented by the authors contained a large number of unidentified genera (~ 75% unclassified) which may have impacted the findings. In another *in vitro* study using the TIM-2 model, iron was supplemented as either ferrous sulphate (50 µM or 250 µM), ferric citrate (50 µM or 250 µM) or haemin (50 µM). The results showed that the microbiota reacted in a similar manner towards ferrous sulphate and ferric citrate, indicating no difference between the 2 forms of iron supplemented but formed a separate cluster when haemin was added. Although the forms of iron were not mentioned, the presence of iron caused a reduction in the abundance of *Lactobacillus* and *Bifidobacterium* spp. while the abundance of *Prevotella, Acidaminococcus* and *Ruminococcus* spp. showed an increase (Kortman et al, 2016). Interestingly, results from table 5.8 showed some opposing observations to those of Kortman and colleagues, particularly with respect to *Lactobacillus* and *Ruminococcus*. *Lactobacillus* showed a substantial increase while *Ruminococcus* showed a decrease in all the iron regime tested. *Prevotella* showed a decrease in the haem only regime but showed an increase when $FeSO_4$ was present which agrees with the results from Kortman and colleagues. *Acidminococcus* on the other hand showed a decrease in the haem-only and $FeSO_4$-only regimes but the presence of both haem and $FeSO_4$ increased the abundance of this species. The discrepancies in results obtained between this experiment and those of Kortman et al. (2016) may be due to the experimental design. The experiment conducted by Dostal and colleagues was done in a continuous batch culture which was ran for a total of 70 days (10 days for each different condition used) and a child gut microbiota immobilised on a gellan-xanthan beads were used (Dostal et al, 2013). This experiment utilised fresh faecal inoculum from healthy adult subjects and the batch culture was only ran for 2 days. Kortman and colleagues on the other hand used the TIM-2 (TNO Intestinal model-2) and was run for 72 h with

sampling at certain time points. The TIM-2 model is a computer-controlled model with multiple sensors that mimics the condition of the human proximal colon. In addition, TIM-2 enables the removal of metabolites via a dialysis system which would be expected to affect growth of the gut microbiota. The experiment also utilised a cryopreserved pooled human microbiota instead of fresh separate human faecal samples. Perhaps the most significant difference between the two experiments is the medium used. The medium used by Kortman and colleagues had higher amounts of carbohydrate and protein compared to the medium used in this experiment and was thus was much richer in nutrients: pectin (9 vs 2 g), xylan (9 vs 2 g), arabinogalactan (9 vs 2 g), starch (74.6 vs 5 g) and casein (43.7 vs 3 g) (Kortman et al, 2016).

Based on human studies performed in Africa, iron has been shown to increase the number of Enterobacteriaceae coupled with a decrease in *Lactobacillus* spp. numbers (Zimmermann et al, 2010) and *Bifidobacterium* spp. levels (Jaeggi et al, 2014). However, the results from the iron regime batch culture reported in table 5.8 gave a different result. Although the abundance of Enterobacteriaceae increased over time, comparing the iron regimes with the control showed a decrease in their number (table 5.8). Furthermore, *Lactobacillus* levels increased in the presence of iron compared to the control. However, *Bifidobacterium* did show a decrease in their abundance in the presence of iron which agrees with the observation in the human studies. The difference in results obtained is likely to be mainly due to the very different experimental approach whereby the two human studies were performed in school children (Zimmermann et al, 2010) and infants (Jaeggi et al, 2014) while the batch culture is an *in vitro* model which uses faecal sample from healthy adults. In addition, the socioeconomic backgrounds and diets would be considerably different to those of the adult faecal donors used here. Further, the school children recruited into the study were mostly anaemic, indicating a long-term iron deficient diet. Fortification was done with electrolytic iron, which is poorly available to the human host (<5% absorption rate; Jaeggi et al, 2014) and so passes into the gut where it is considered to be available to the gut microbiota. Here, highly soluble ferrous iron was used rather than poorly bioavailable electrolytic iron, and it should be

noted that levels of iron in the unsupplemented medium (control) were relatively high such that iron restriction is unlikely.

Enterobacteriaceae possess various iron-uptake mechanisms which makes them effective at scavenging environmental iron. This may explain why these bacteria appeared to display an enhanced gut colonisation capacity with respect to other elements of the microbiota during the iron fortification trial (Zimmermann et al, 2010). In the study involving infants, the infants recruited were just 5.5 months, thus it can be assumed that they were being breastfed prior to the study as Actinobacteria made up 64.3% of the total reads at baseline (Jaeggi et al, 2014). The study involved iron fortification in maize porridge, meaning that the infants were transitioned to a solid food diet. The transition to solid foods usually causes a decrease in *Bifidobacterium* and *Lactobacillus* but an increase in other bacteria depending on the food given (Milani et al, 2017). The decrease in *Bifidobacterium* may not be entirely due to iron fortification but the iron fortified group did show a lower number compared to the non-fortified group. The combination of weaning and iron may cause an increase in Enterobacteriaceae as observed by the authors while the decrease in Enterobacteriaceae in the batch culture is possibly due to an increase in other bacterial groups which reduces the relative abundance of Enterobacteriaceae. In addition, fortifying with high iron (12.5 mg ferrous fumarate) showed an increase in *Bacteroides* and *Clostridium* which is in agreement with the results obtained from the batch culture. Besides the difference in subjects involved between the human studies and the batch culture, the length of the experiment differed hugely with the batch cultures being operated for only for 48 h while the school children trial was conducted for 6 months and the infants trial was ran for 4 months, thus allowing more time for the gut microbiota to adapt to dietary iron regimes deployed.

Chapter 6. Impact of phytate on the gut microbiota

6.1 Introduction

Phytic acid (PA) is the primary source of phosphate and inositol storage in plants and it is degraded during germination of seeds (Raboy, 2003). Phytic acid is an important component in plants but it can have antinutrient properties on human nutrition. Due to the presence of negatively charged phosphate groups of the phytic acid, it is able to form a stable complex with positively charged minerals such as iron, zinc, calcium and magnesium, thus lowering their bioavailability for absorption (Lopez et al, 2002). On average, vegetarian diets can contribute to a daily intake of 2-2.6 g (3-4 mM) of phytic acid while a mixed diet usually contains 0.15-1.4 g (0.23-2 mM) of phytic acid (Greiner and Konietzny, 2006). Legume- and cereal-based foods are the major contributor of phytic acid in the diet with certain food such as peanuts (9.2-19.7 mg/g PA) and wild rice (12.7-21.6 mg/g PA) containing high levels of phytic acid (Greiner and Konietzny, 2006). Humans lack phytases to degrade phytic acid but certain bacteria have the ability to degrade phytic acid.

Like many other bacteria, *E. coli* contains genes encoding phosphatases which are able to break down phosphate-containing molecules, thus providing a source of phosphate for utilisation (Greiner et al, 1993). The *appA* gene product, acid phosphatase, has been shown to have the ability to degrade phytic acid (Greiner et al, 1993). Recombinant phytases from *E. coli* showed better performance than commercially available phytases from *Aspergillus niger* (Natuphos, BASF) and *Peniophora lycii* (Ronozyme, Roche) (Augspurger et al, 2003). Phytases have been used commercially to breakdown phytic acid in food or animal feed in order to increase the nutritional value of the product (Augspurger et al, 2003; Kornegay et al, 1996; Greiner and Konietzny, 2006). However, the ability of *E. coli* and other elements of the gut microbiota, to break down phytic acid and utilise it as a phosphate and/or a carbon source is rarely tested. Most of the studies on phytase from *E. coli* mainly focuses on its ability to degrade phytic acid and the optimum condition (Greiner et al, 1993; Dassa and Boquet, 1985) but the utilisation of phytic acid as a phosphate/carbon source has yet to be studied. Besides this, glucose-1-phosphatase (G1Pase) which is encoded by the *agp* gene, has the

ability to hydrolyse phytic acid (Cottrill et al, 2002) and is related to the *appA* gene product (Dassa et al, 1990), thus this gene is included in this study. Alkaline phosphatase which is encoded by the *phoA* gene has the ability to hydrolyse *p*-nitrophenol phosphate (Wilson et al, 1964; Trentham and Gutfreund, 1968), suggesting its ability to degrade phosphorylated compounds but the ability to degrade phytic acid has yet to be shown. In addition, the ability of phytic acid to act as a chelator that restricts iron availability to *E. coli* (as a representative and easy-to-study member of the gut microbiota) is also tested to determine whether phytate prevents access of metals to the gut microbiota in the same way as it is reported to do so for the human host (Kumar et al, 2010).

The aim of the experiment described below was to determine the ability of bacteria to utilise phytic acid as a phosphate and carbon source. The objectives of this experiment were as follow:

1) Determine the ability of *E. coli* to degrade phytic acid and utilise the phosphate liberated to support its growth in a phosphate-restricted medium
2) Determine the ability of *E. coli* to utilise phytic acid as a carbon source to support growth
3) Identify the phosphatases involved (and not involved) in hydrolysis of phytic acid
4) Determine the ability of the gut microbiota to utilise phytic acid as a phosphate source in a low-phosphate environment
5) Determine the ability of gut microbiota to utilise phytic acid as a carbon source in a carbon-free medium
6) Determine the impact of phytic acid on the composition of the gut microbiota and its metabolites

6.2 Can phytate act as a phosphate source for *E. coli*?

6.2.1 Phosphate-restricted growth

In order to determine whether phytic acid can act as a phosphate source for *E. coli* BW25113, it was necessary to explore the impact of the phosphate content of the growth medium on the growth of this bacterium to show that growth-limitation could be achieved on the basis of phosphate availability. This would allow the impact of phytic acid as a sole or major phosphate source to be

determined. Thus, the phosphate content of glucose M9 medium was lowered to 5-50% (section 2.3.3.1) of that normally present and the impact of growth assessed (Figure 6.1). There was a clear concentration-dependent growth reduction in response to phosphate level, with a culture OD_{600} of 0.17 after 24 h with 5% (3.5 mM) phosphate as compared to 1.33 when using normal M9 minimal medium (70 mM phosphate). Thus, a modified M9 minimal medium with 5% phosphate content (3.5 mM PO_4^{3-}) can used to generate phosphate-growth restricted conditions to test the effect of phytic acid as a phosphate source.

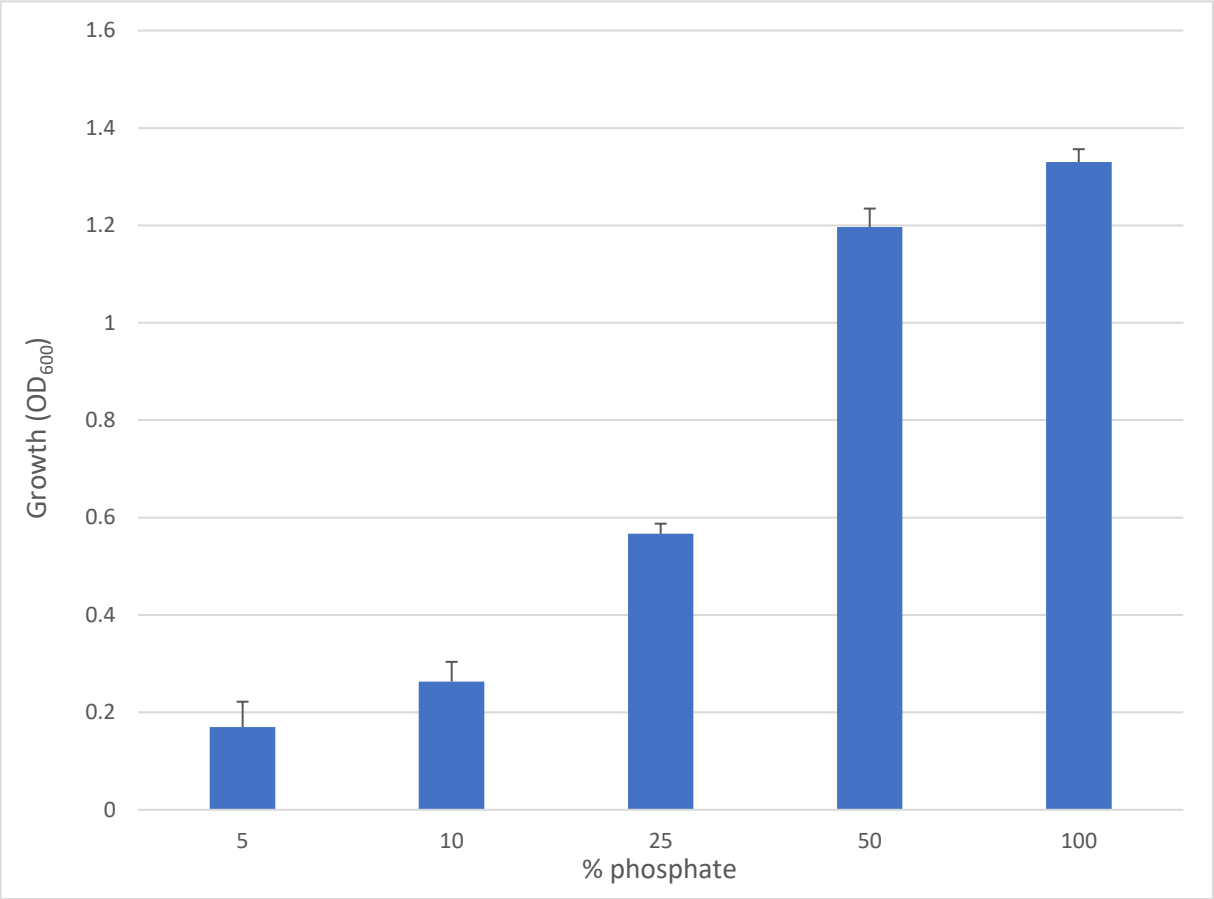


Figure 6.1 Growth of *E. coli* in M9 medium at a range of concentrations of phosphate. The OD_{600} represents the growth of *E. coli* BW25113 after 24 h in 15 ml tubes and 5 ml of medium (0.4% glucose M9 medium, with the indicated level of phosphate; 100% represents 70 mM). Results shown are the average of three replicates (n=3).

6.2.2 Phytate as a phosphate source for E. coli and role of appA

The growth of *E. coli* wild-type and an isogenic *appA* mutant (*appA* encodes the periplasmic acid phosphatase and has been shown to possess phytase activity; Golovan et al., 2000) were then compared in the phosphate-restricted M9 medium, in the presence of phytate. Growth was performed in a Bioscreen C apparatus using 100 deep-well plates (300 µl medium per well). The results (Figure. 6.2, 6.3) show that phytic acid can be used as a phosphate source to support growth. The presence of phytic acid significantly increased the growth of both wild-type and mutant at every concentration of phytic acid added ($p<0.05$). Growth increased as the concentration of phytic acid increased from 0 to 10 mM. Both wild-type and *appA* mutant increased by an average of 3-, 4- and 6-fold with 2.5, 5 and 10 mM of phytic, respectively (Figure. 6.2). With the addition of iron, both bacteria showed a slightly lower overall growth increase with only 2-, 3- and 4-fold increase with the same concentration of phytic acid added but was not significantly different than the non-iron medium ($p>0.05$). Although the mutant lacks acid phosphatase, there is no significant difference in growth with respect to the wild-type ($p>0.05$). Both bacteria showed a similar growth pattern but the mutant showed generally a slightly lower growth which was observed regardless of the presence of iron. The cultures displayed a lag phase at the beginning of growth of ~2 h, followed by a rapid growth phase up to the 5 to 8 h and then a growth plateau was established. At 10 mM phytic acid, both wild-type and mutant reinitiated growth at ~11 h for a further ~2 h before, reaching a second plateau. Despite a similar growth pattern, the wild-type showed an average of 20% and 11% more growth over the mutant in the iron-free and -containing M9 minimal medium, respectively. As seen from Figures 4.2 and 4.3, the addition of iron increased the growth of both wild-type and mutant, giving a slight advantage in growth in the presence of iron at 0-5 mM phytate, but a reduced growth at 10 mM phytate. On average, iron increases the OD_{600} by 10% compared to the non-iron counterpart at 0-5 mM phytate but was not significant when tested statistically ($p>0.05$).

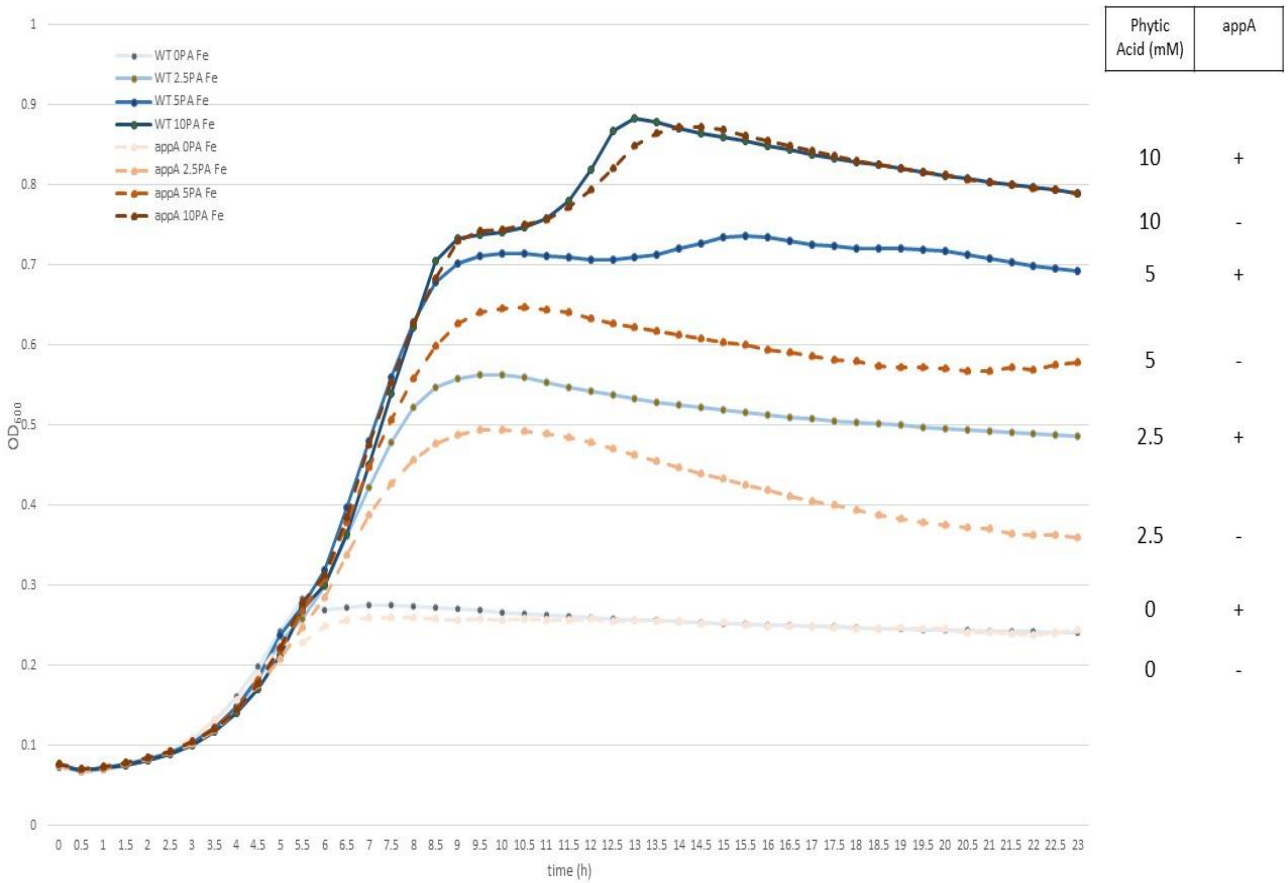


Figure 6.2 Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* with/without phytate, in the presence of iron. WT (solid lines, +), indicates the wild-type *E. coli*; and *appA* (dashed lines, -) indicates the mutant. The bacteria were grown in low phosphate (3.5 mM) M9 minimal medium in the presence of 10 µM ferric citrate and phytic acid at 0, 2.5, 5 and 10 mM. Constant shaking under aerobic conditions in a Bioscreen C system. The results shown are the average of triplicates. Precultures were grown overnight in M9 medium with 70 mM phosphate and 10 µM ferric citrate.

Thus in conclusion, it is clear that *E. coli* can utilise phytate as the major phosphate source, with growth boosted from 0.15 to 0.89 OD units by 10 mM phytate (nearly six-fold). In addition, acid phosphatase is not required for phytate utilisation but it does provide a minor increase in phytate-dependent growth and thus is required for maximum use under limiting conditions. This can be considered consistent with the findings of Golovan et al. (2000). Finally, provision of iron promotes growth in the presence or absence of low levels (5 mM or lower) of phytate by a similar degree, but at 10 mM phytate the addition of iron appears to cause a non-significant growth reduction ($p>0.05$).

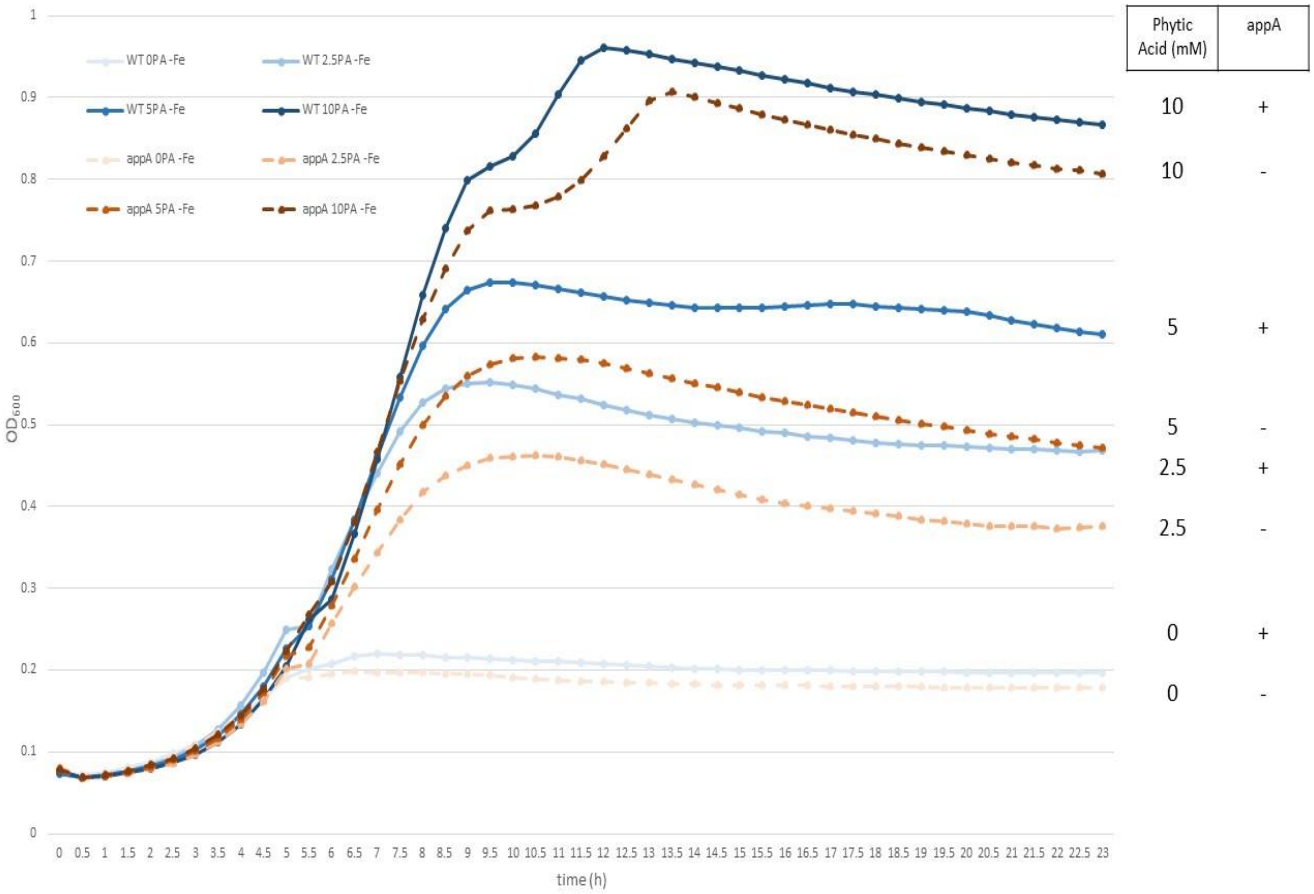


Figure 6.3 Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* with/without phytate, in the absence of iron. Details are in Figure 6.2 except for the absence of iron.

6.2.3 Phytate as a carbon source for E. coli and role of appA

The above results show that phytate can be used by *E. coli* as a phosphate source. However, it could also act as a carbon and energy source given that it is an ester of inositol (a sugar alcohol). This possibility was tested using M9 medium lacking glucose. There was essentially no growth in the absence of phytate and glucose with the OD remaining at ~0.07-0.08 (Figures 6.4 and 6.5). However, there was an increase in growth when phytic acid was provided, but phytic acid appears not to be a good carbon source for *E. coli* as shown by the relatively poor growth observed. When phytic acid was provided, both wild-type and mutant showed an average of 2-, 3- and 5-fold increase at 2.5, 5 and 10 mM phytic acid, respectively, in the non-iron media. When iron was provided, wild-type *E. coli* showed a higher growth than the mutant with a 2.5-, 5.2- and 8.5-fold increase compared to 2-, 3- and 5-fold increase at 2.5, 5 and 10 mM phytic acid, respectively. All bacteria showed a similar

growth pattern with rapid growth initially to reach a plateau by around 5 h. However, in 10 mM phytic acid, both wild-type and mutant continued to grow until they plateaued at 7 h. The wild-type showed a better growth than the mutant at all phytate concentrations, with the exception of no phytic acid where no growth was achieved. At all other concentrations, the wild-type showed an average of 25 and 33% higher growth than the mutant for the iron supplemented and non-iron supplemented conditions. In contrast to the phosphate limitation experiment above, the addition of iron did not increase the growth of either strain, indicating that under such extreme growth-restricted conditions, the growth limiting factor was the carbon/energy source rather than iron.

In conclusion, it appears that *E. coli* can utilise phytate as a carbon and energy source, but does so poorly, and it also appears that this capacity is strongly affected by *appA* status which suggests that acid phosphatase assists in converting phytate to a form that can be utilised for catabolism.

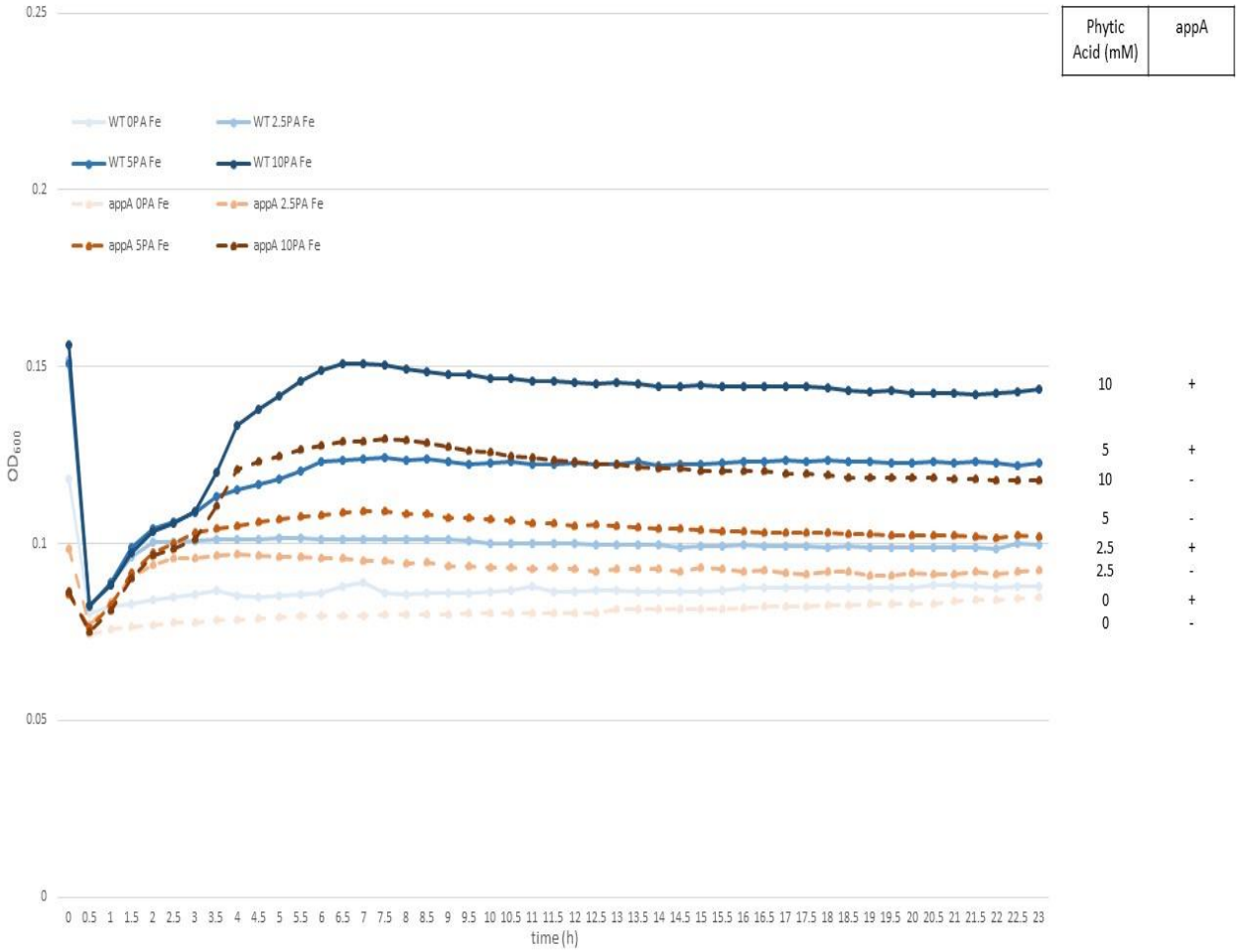


Figure 6.4 Carbon-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* with/without phytate, in the presence of iron. Details are in Figure. 6.2 except that glucose was excluded and phosphate levels were sufficient at 70 mM.

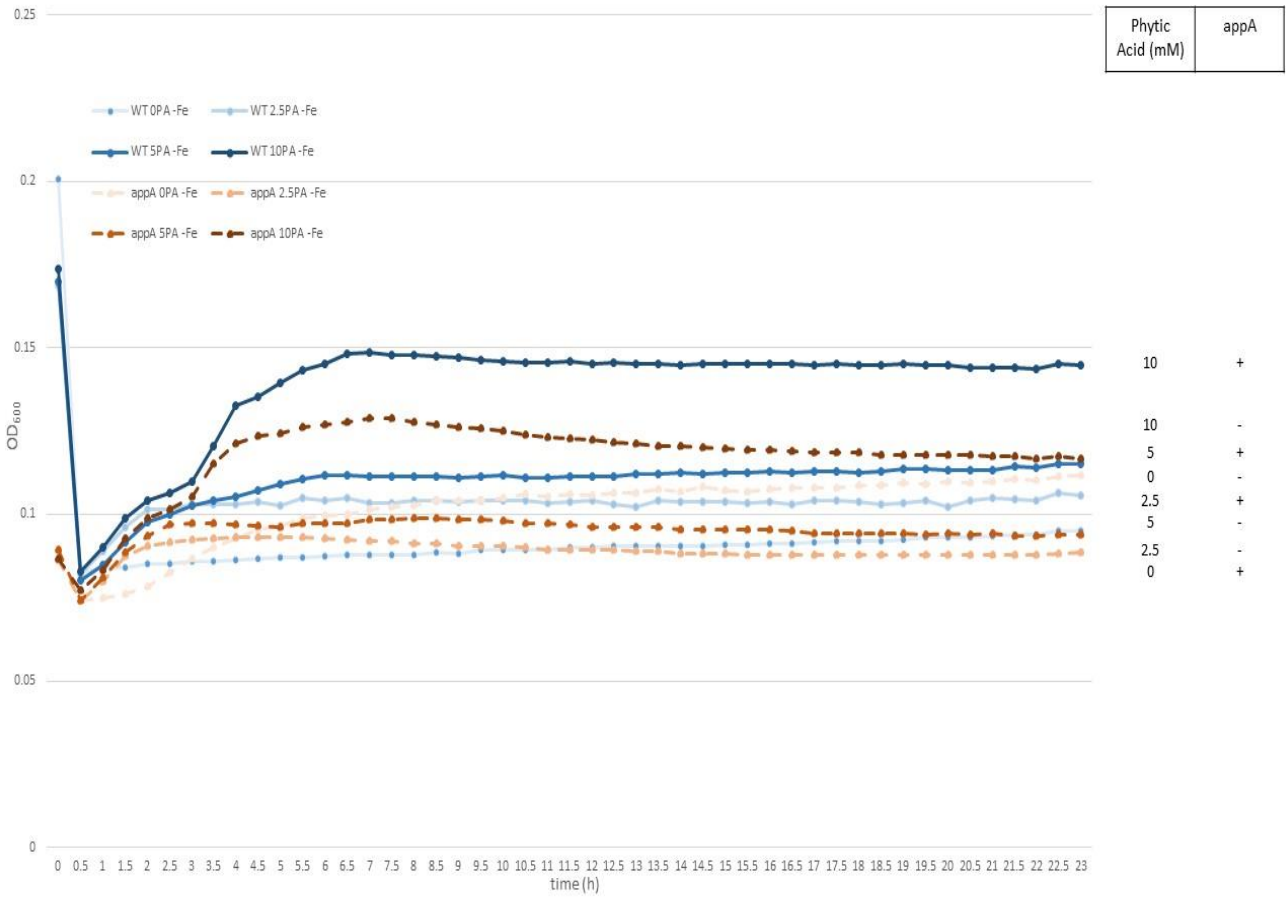


Figure 6.5 Carbon-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* with/without phytate, in the absence of iron. Details are in Figure 6.2 except for the absence of iron.

6.3 Role of acid glucose-1-phosphatase in utilisation of phytate by *E. coli.*

Glucose-1-phosphatase (G1Pase) is encoded by the *agp* gene in *E. coli.* Acid phosphatase and G1Pase are related proteins (53% amino acid sequence identity) (Dassa et al, 1990). G1Pase has the ability to hydrolyse phytic acid to lower inositol phosphates, thus this enzyme is included in this experiment to determine its role in the utilisation of phytic acid as a phosphate and carbon source.

6.3.1 Role of acid glucose-1-phosphatase in utilisation of phytate as a phosphate source

Previous work has reported that the *agp* gene of *E. coli*, encoding acid G1Pase, hydrolyses phytate (Cottrill et al., 2002). Thus, the potential role of this enzyme in utilisation of phytate as major phosphate source was explored using an *agp* mutant. Just like the *appA* mutant, the *agp* mutant showed a similar growth pattern as the wild-type and there is not significant difference

between them ($p>0.05$) (Figures 6.6 and 6.7). However, the mutant showed a reduced overall growth compared to the wild-type. Both mutant and wild-type had a ~2 h lag phase followed by a rapid growth phase up to 5-8 h and then a growth plateau was reached after 7-12 h. However, with phytic acid at 10 mM, both strains showed an apparent diauxic growth effect with a temporary growth reduction at 9-10 h. The addition of phytic acid again significantly increased the growth of both wild-type and mutant at all phytic acid concentration ($p<0.05$), with the latter having a lower growth than the former both in the presence and absence of iron. The wild-type increased 2.4-, 3.3- and 4.2-fold when 2.5, 5 and 10 mM phytic acid was provided while the mutant increased 2.2-, 3- and 4.4-fold, respectively. In the non-iron medium, both strains showed a 3-, 4-, and 6.5-fold increase with 2.5, 5 and 10 mM phytic acid. Although the wild-type and mutant had similar fold increases in growth, the wild-type gave on average an 11% and 17% higher growth than the mutant in the non-iron and iron supplemented medium respectively as shown by the higher OD_{600} reading. Similar to the results above (6.2.2), the addition of iron increased the growth of both strains by approximately 20% with the exception of 10 mM phytic where the non-iron medium gave a higher growth than the iron supplemented medium. The reason for this effect of iron at high phytate levels is unclear and surprising since a greater overall growth would be expected to result in a higher demand for iron and thus a greater impact for lack of iron. Indeed, the result suggests that the added iron may be toxic when levels of phytate are high. In summary, as for acid phosphatase, the data support a minor role for acid glucose-1-phosphatase in supporting growth *of E. coli* on phytate as the major phosphate source, and thus are consistent with the results of Cottrill et al. (2002).

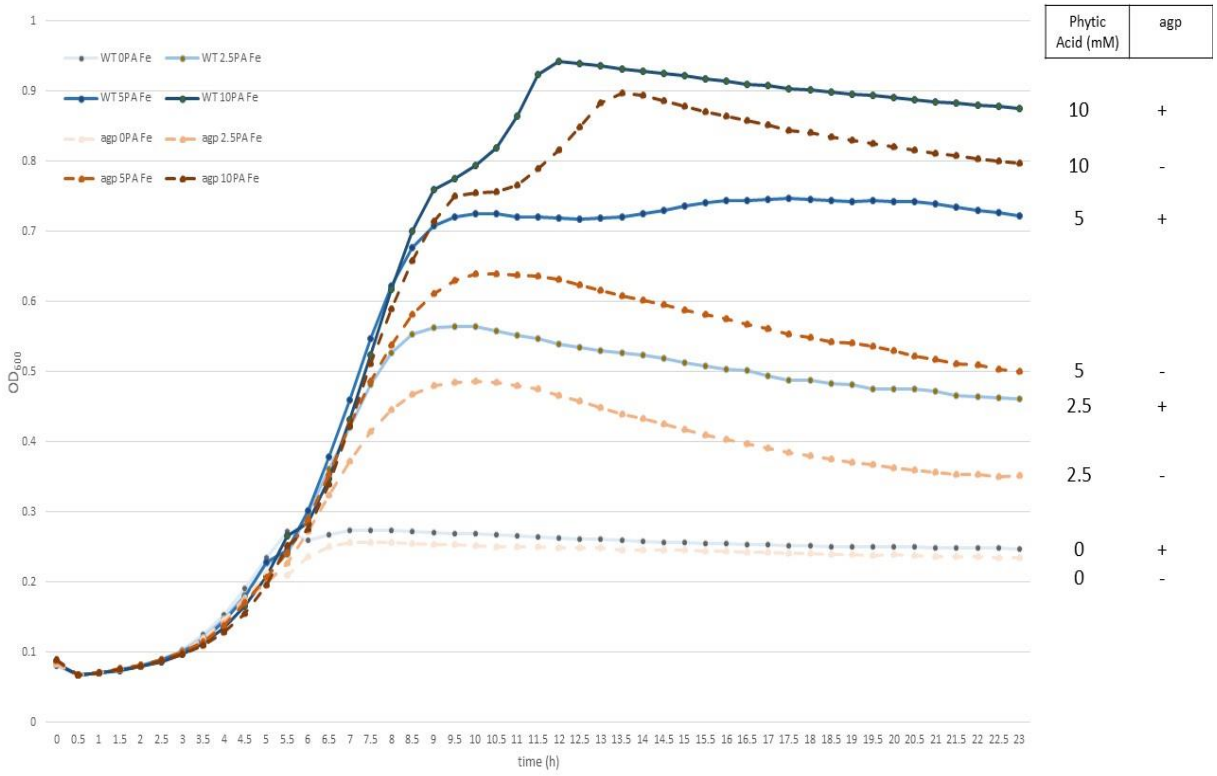


Figure 6.6 Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*agp* with/without phytate, in the presence of iron. Details are in Figure. 6.2 except that BW25113 Δ*agp* was employed.

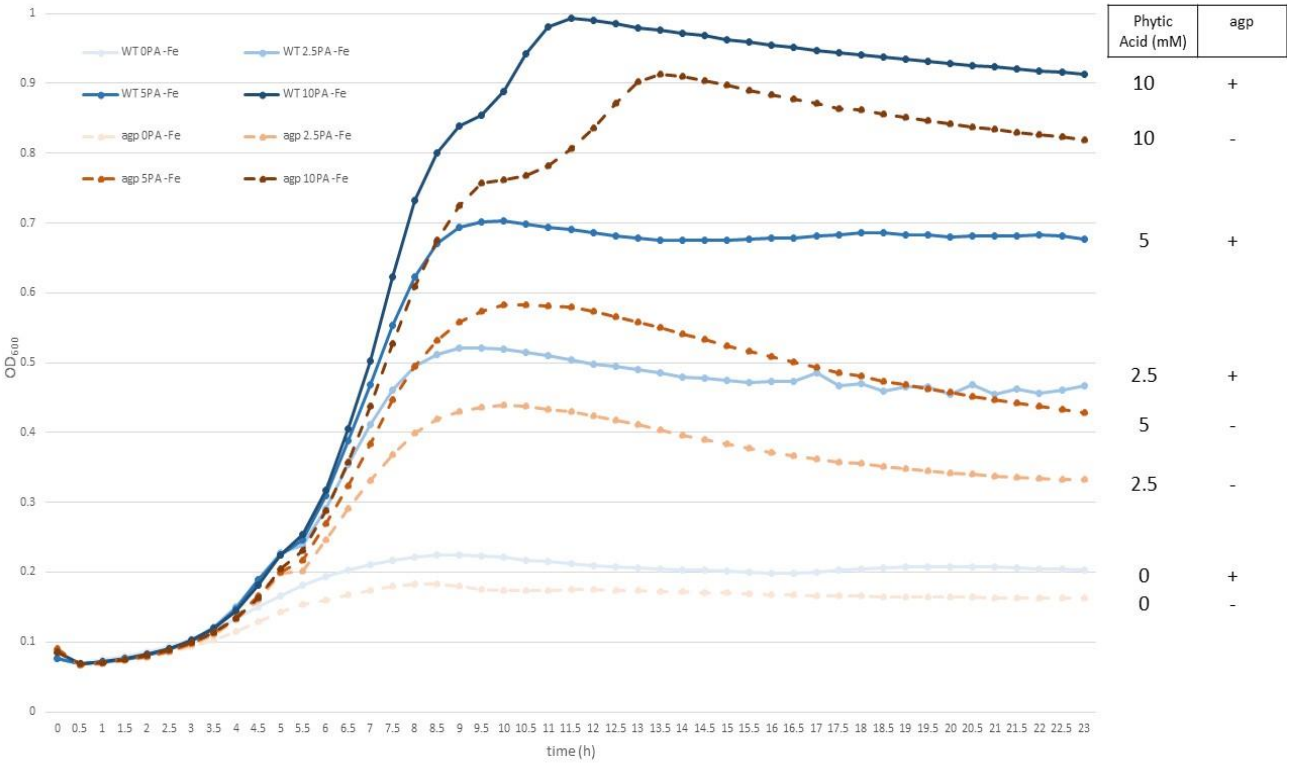


Figure 6.7 Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*agp* with/without phytate, in the absence of iron. Details are in Figure. 6.2 except for the absence of iron.

6.3.2 Role of acid glucose-1-phosphatase in the utilisation of phytate as sole carbon source by E. coli

As shown in Figure 6.8 and 6.9, similar to the previous results above (section 6.2.3), phytic acid is a poor carbon source for *E. coli*. Wild-type *E. coli* and the *agp* mutant showed virtually no growth when phytic acid was not provided (in the absence of glucose or any other carbon/energy source). The addition of phytic acid increased the growth of both strains in a quantitative manner. In the iron supplemented medium with 2.5 and 5mM phytic acid, both wild-type and mutant showed a gradual increase in growth with time to reach a plateau at around 6 h. At 10 mM, both strains showed a rapid increase until around 7 h where the wild-type continued to grow until the end of the experiment while the OD for the mutant gradually decreased. In the non-iron medium, with the exception of 0mM phytic acid, both strains showed the same growth pattern as in the iron supplemented medium. Without phytic acid, both bacteria gradually increased and reached a plateau around 11h. In the iron medium, the wild-type showed a 2.76-, 5.14- and 11-fold increase with increasing concentration of phytic acid from 2.5 to 10mM. The mutant on the other hand showed a 2.4-, 3.96- and 7.25-fold increase for the same concentrations. The wild-type did not differ much from the mutant at 0 and 2.5 mM phytic acid as seen by similar OD_{600} readings. But at 5 and 10 mM phytic acid, the wild-type had a 14% and 32% higher growth than the mutant. In the non-iron medium, the mutant showed on average 14.7% lower growth than the wild-type across all concentrations. The two strains showed similar growth with a 1.4- and 1.23-fold reduction when 2.5 mM phytic acid was added for the wild-type and mutant, respectively. At 5 and 10 mM phytic acid, the wild-type showed a 1.24- and 2.29-fold increase accordingly while the mutant had a 1.27- and 2.26-fold increase for the same concentration.

In conclusion, the results indicate that acid glucose-1 phosphatase supports growth on phytate as sole carbon/energy source when phytate is at 5- 10 mM, with the presence/absence of iron generally having little impact, presumably due to the weak growth achieved as a result of the severe energy/carbon limitation arising from reliance on phytate.

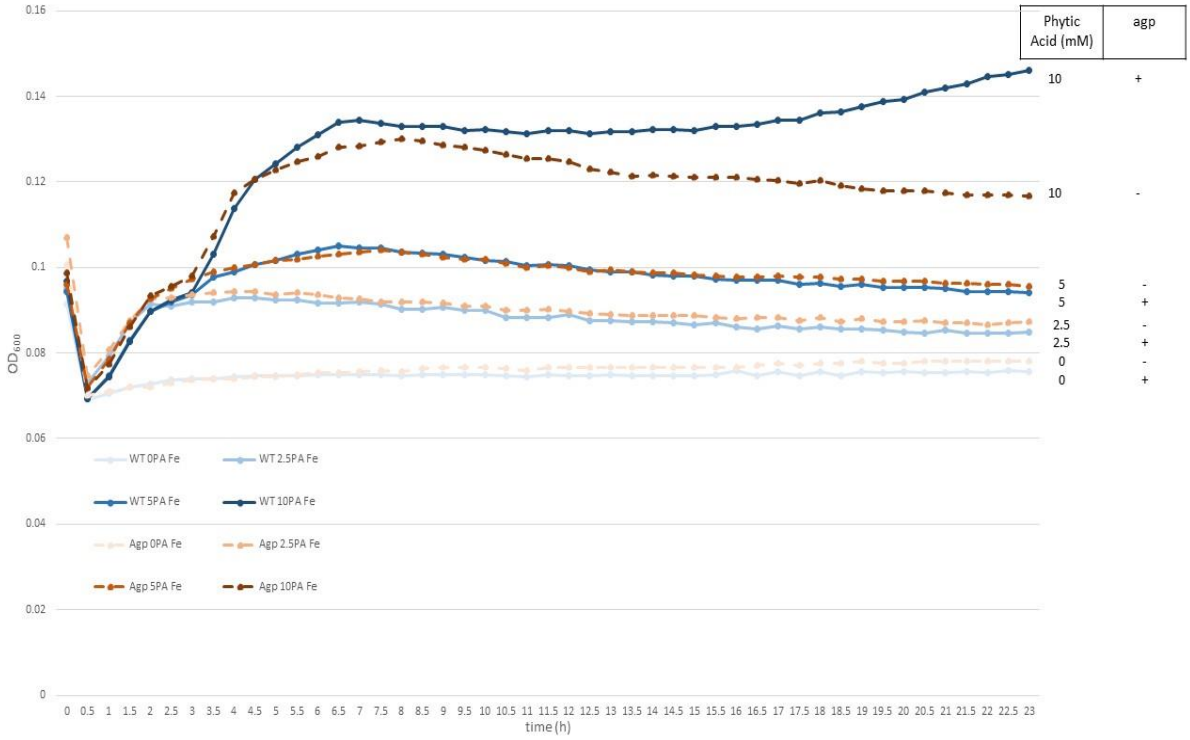


Figure 6.8 Carbon-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*agp* with/without phytate, in the presence of iron. Details are in Figure. 6.4 except that the *agp* mutant was employed.

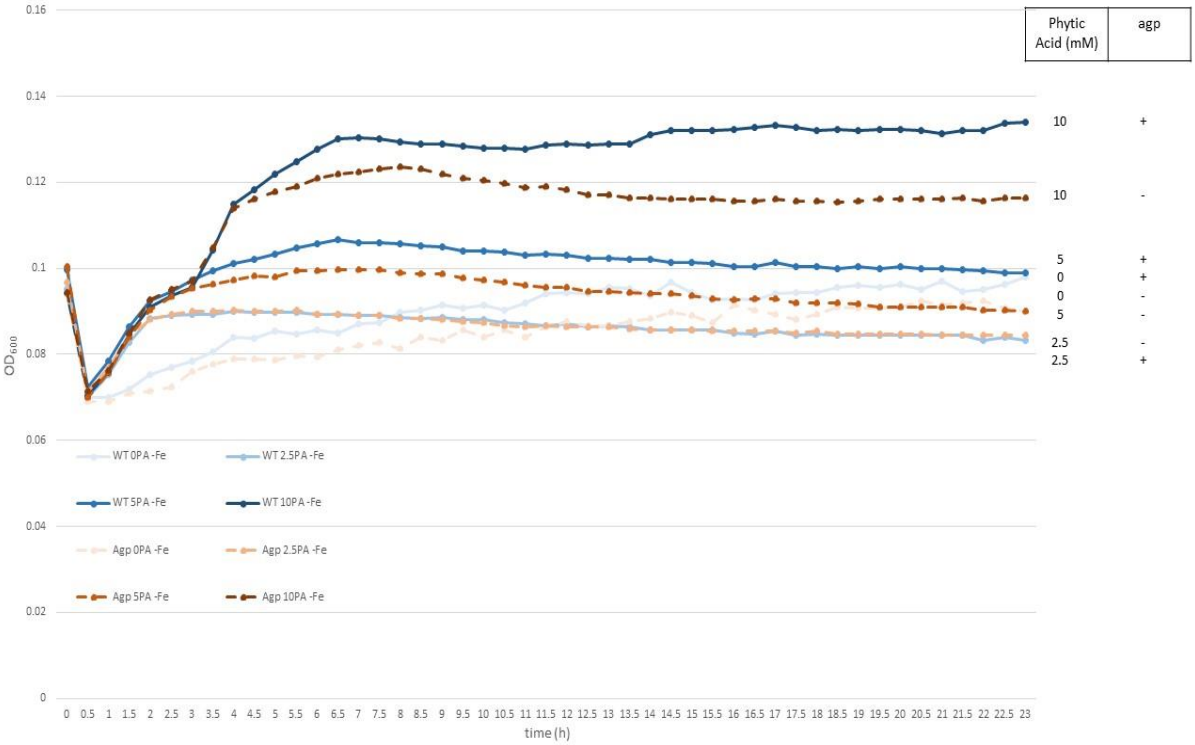


Figure 6.9 Carbon-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*agp* with/without phytate, in the absence of iron. Details are in Figure. 6.8 except for the absence of iron.

6.4 Role of alkaline phosphatase in utilisation of phytate by *E. coli*

6.4.1 Role of alkaline phosphatase in the utilisation of phytate as a phosphate source

Similar to the *appA* and *agp* mutant, the *phoA* mutant shared a similar growth pattern with the wild-type and there was no significant difference in growth between the two strains ($p>0.05$). However, the mutant has a longer lag phase of 6 h compared to the wild-type with only 4 h as seen by the shift in the growth curves in Figure 6.10 and 6.11, with the effect being more prominent when iron was added. The presence of phytic acid as a phosphate source significantly increased the growth of both strains at all concentrations ($p<0.05$). Surprisingly, the wild-type and *phoA* mutant showed very similar growth with a 4.3-, 5.9- and 7.4-fold increases when the concentration of phytic acid increased from 2.5 to 10 mM. The addition of iron slightly reduced the growth of both strains, resulting in only a 2-, 2.7- and 3.3-fold increase for the same phytic acid concentrations. Despite having the same growth over time, the wild-type outperformed the mutant by an average of 5.8% and 9.2% in the iron-free and iron-containing medium, respectively. The wild-type showed on average 0.03 and 0.06 higher OD_{600} reading than the mutant in the non-iron and iron medium but were not significant ($p>0.05$). The addition of ferric citrate did not significantly increase the growth of the bacteria in the presence of phytic acid. However, the presence of iron significantly increased the growth of both bacteria (more than 2-fold) when no phytic acid was added (Figure 6.10).

6.4.2 Role of alkaline phosphatase in utilisation of phytate as a carbon source

As seen in Figures 6.12 and 6.13, the addition of phytic acid increased the growth of both strains. In the iron supplemented medium, the wild-type and mutant showed similar maximal growth (highest OD achieved). In the non-iron medium, with the wild-type and mutant again showed similar maximal OD values. The supplementation of iron had little overall impact on growth. The results thus suggest little role for alkaline phosphatase in use of phytate as sole carbon and energy source.

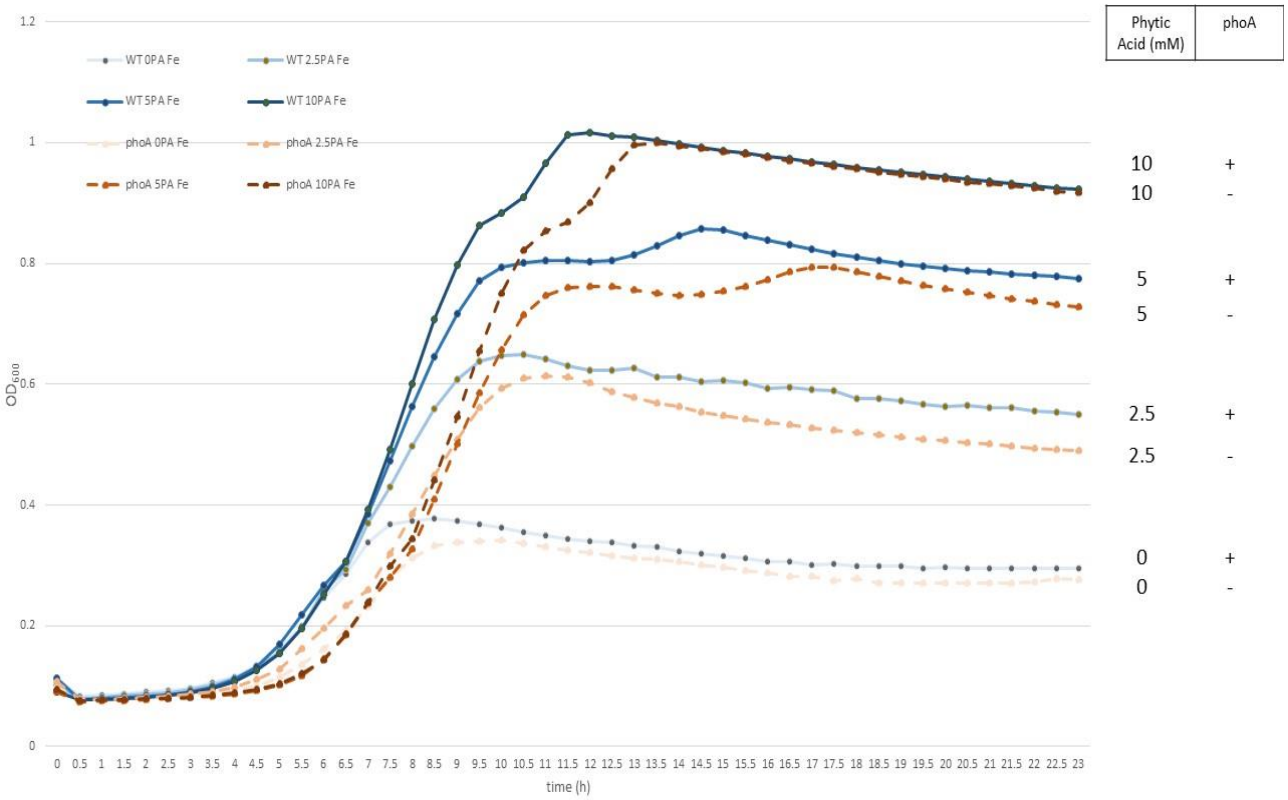


Figure 6.10 Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*phoA* with/without phytate, in the presence of iron. Details are in Figure 6.2 except for the use of the BW25113 Δ*phoA* mutant.

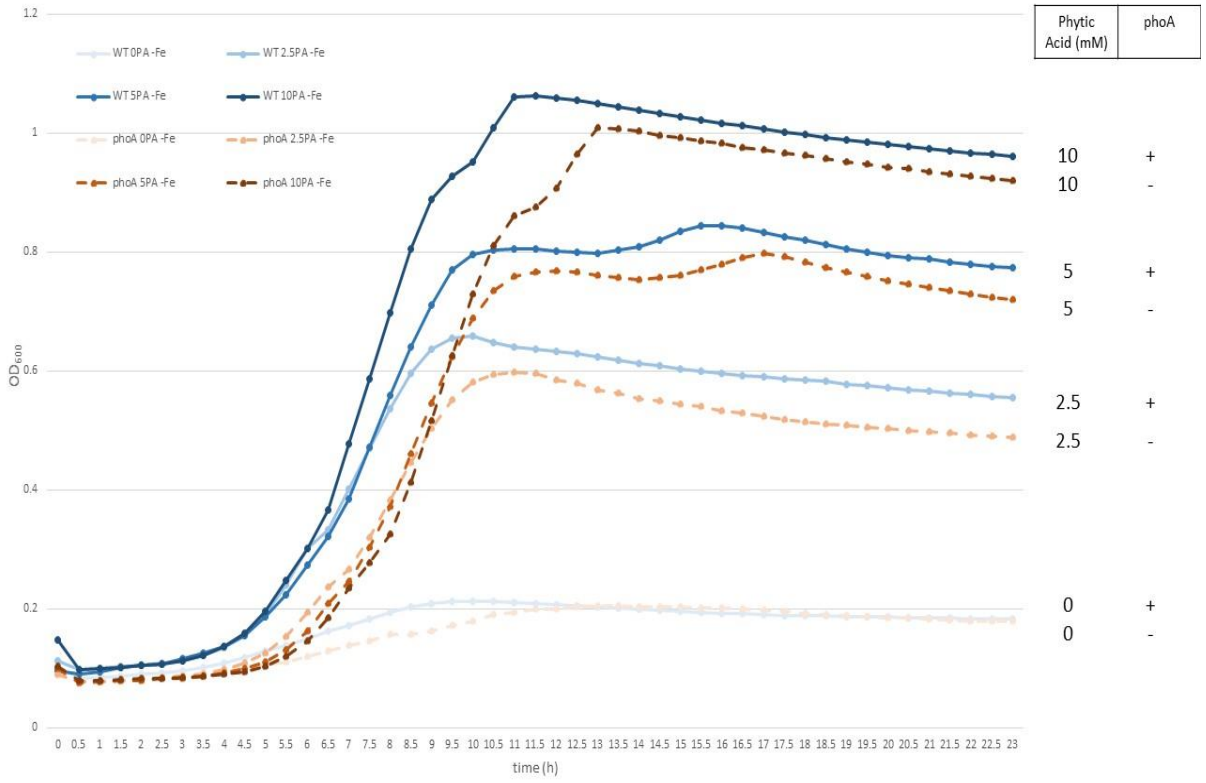


Figure 6.11 Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*phoA* with/without phytate, in the presence of iron. Details are in Figure. 6.10 except for the absence of iron.

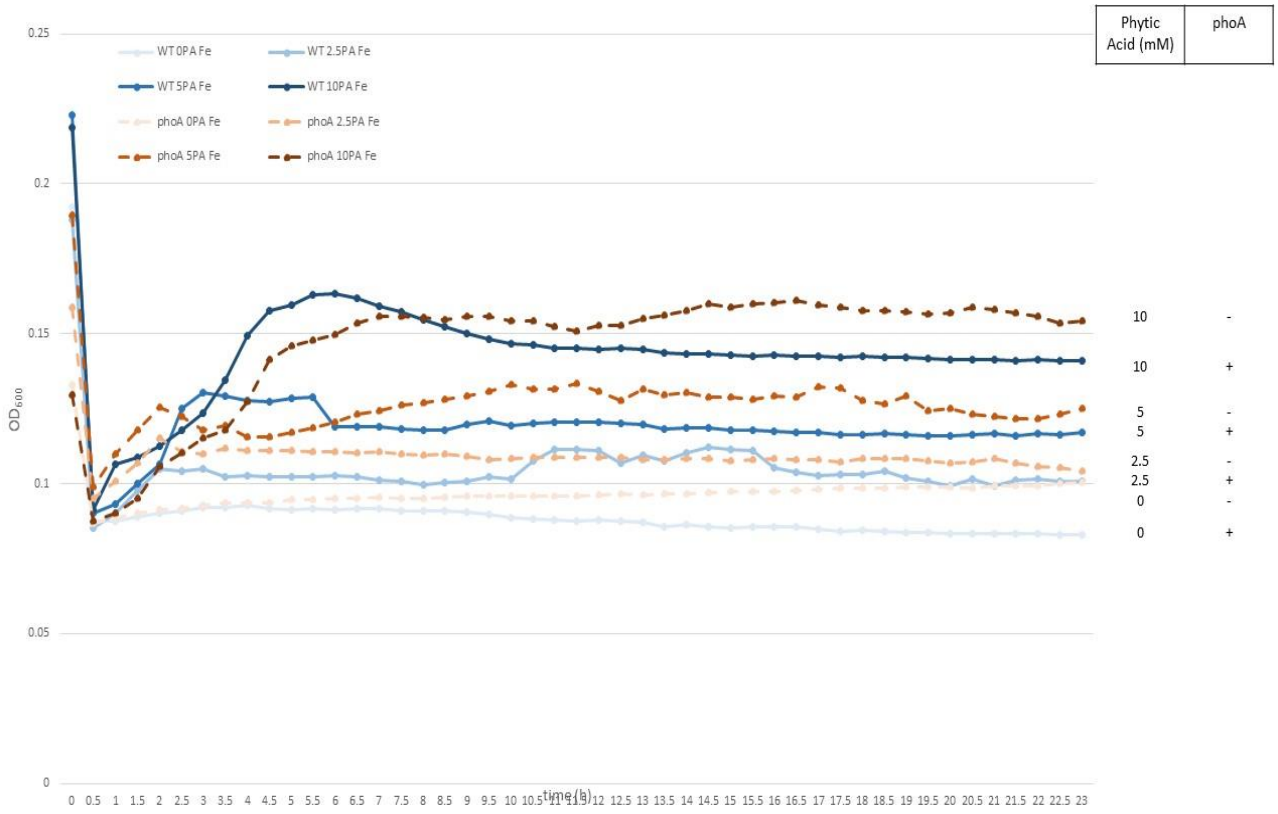


Figure 6.12 Carbon-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*phoA* with/without phytate, in the absence of iron. Details are as for Figure. 6.4 except for the use of the BW25113 Δ*phoA* mutant.

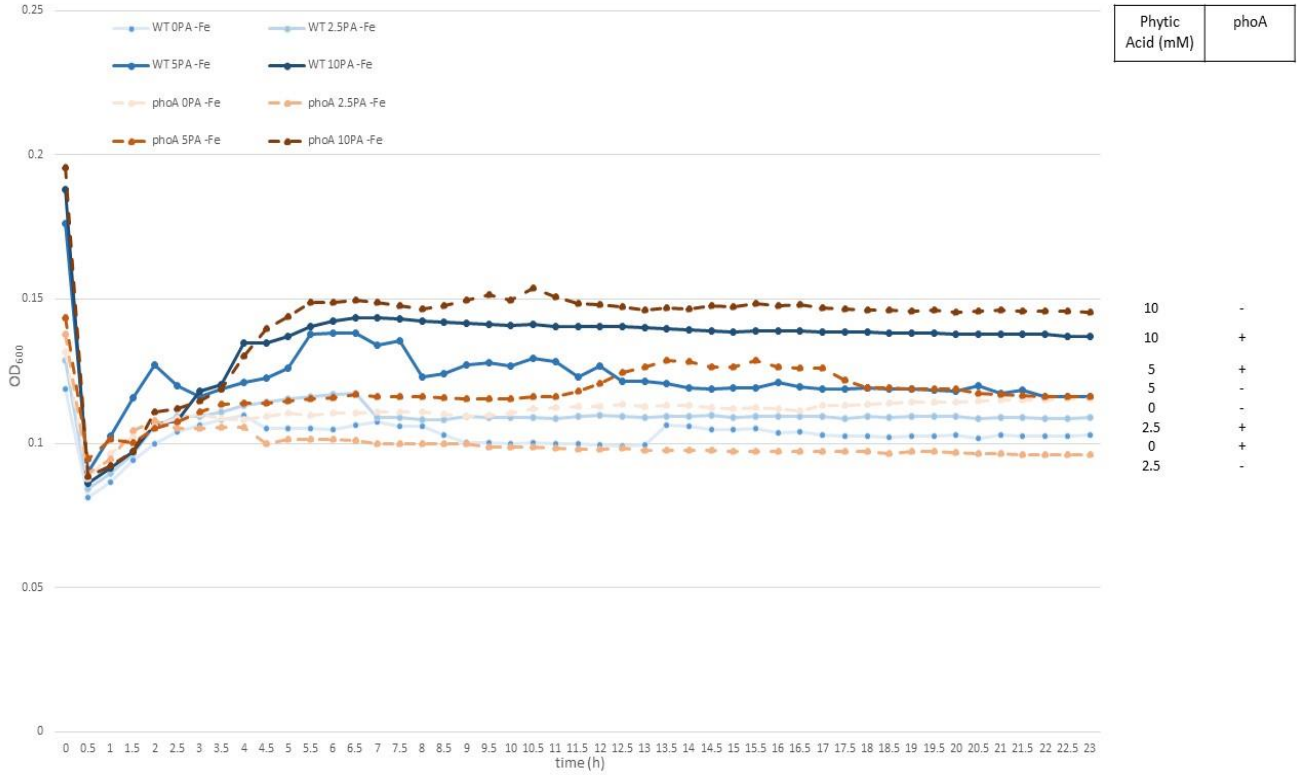


Figure 6.13 Carbon-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*phoA* with/without phytate, in the absence of iron. Details are as for Figure. 6.12 except for the absence of iron.

6.5 Effect of pH on phytate-dependent growth of *E. coli*

All three mutations tested appear to have similar impacts on growth of *E. coli* with phytate as the major phosphate source. However, two of the phosphatases studied (AppA and Agp) have a low pH preference whilst the other (PhoA) has highest activity under alkaline pH. Thus, the impact of pH on the utilisation of phytate as phosphate source was explored to determine whether the role of the phosphatases considered here in phytate-dependent growth is affected by pH. Indeed, it was expected that AppA and Agp would be more important at low pH, whereas PhoA would be less so. Three different pH values were used in this experiment: acidic (pH 5.5), neutral (pH 7) and basic (pH 8.5). Three different buffers: MES (acidic), HEPES (neutral) and TAPS (alkaline), each with the best pKa at the desired pH, were mixed to create a master mix of 100 mM which was then adjusted to the correct pH prior to use. The use of the master mix which contains all three buffers reduces the variability that may be caused by using different buffers for each of the three pH conditions. A final concentration of 10 mM of buffer was added to the medium to achieve the desired pH.

6.5.1 The roles of appA, agp and phoA on phytate-dependent growth under low phosphate conditions and low pH

When the pH of the medium was lowered to 5.5 through the addition of 1 ml of 100 mM pH 5.5 master mix to 9 ml medium (final concentration 10mM buffer), the wild-type showed a significantly stronger growth under all conditions tested than the *appA* and *agp* mutants in the presence of iron ($p<0.05$). The wild-type showed on average 18% and 22% higher growth than the *appA* and *agp* mutants. In the absence of iron, the *appA* mutant showed an average of 1.7% better growth than the wild-type while the wild-type was on average 7.4% higher than the *agp* mutant but they are not significantly different when tested statistically ($p>0.05$). The *phoA* mutant showed a similar growth to that of the wild-type in the medium with iron but showed a significantly higher (17.2%) growth ($p<0.05$) when iron was not provided (Figure. 6.14). The presence of phytic acid significantly increased the growth of the bacteria ($p<0.05$) but this effect was seen only when iron was not supplemented. The wild-type showed an average of 32.8% increase in growth when phytic acid was

added while the mutants had a slightly lower increment with only a 23.6% (*appA*), 22.7% (*agp*) and 22% (*phoA*) increase. The absence of the phosphatases may explain the lower increment for the mutants compared to the wild-type. Although the presence of iron significantly increases the growth of the bacteria, except the *phoA* mutant ($p<0.05$), the addition of phytic acid to the iron-containing medium on average lowered the growth of the wild-type by 1.1% while the mutants had a 4.2% (*appA*) and 13.5% (*agp*) reduction. Iron did not significantly increase the growth of the *phoA* mutant but it showed a 1.5% increase when both iron and phytic acid were present. The bacteria showed a significantly higher growth with phytic acid supplementation in the absence of iron but the concentration of phytic acid had little effect on growth, as 2.5 and 5 mM phytate showed comparable effects ($p>0.05$), suggesting pH has a bigger effect on growth than phosphate limitation under the conditions used. In addition, high growth was achieved at pH 5.5 under low phosphate conditions, which was considerably greater (2.3-fold) than that obtained under the previous conditions where pH was not controlled. This suggests that *E. coli* can utilise phosphate from the medium more efficiently at low pH. Iron had little notable effect on growth, except in the absence of phytate where all strains showed an increased growth with iron, particularly the wild-type (1.5-fold increase).

In summary, the enhanced growth observed at low pH prevents an assessment of the role of the three phosphatases in utilisation of phytate at pH 5.5 as a phosphate source. However, the relatively poor growth of the *appA* and *agp* mutants with respect to the wild-type and *phoA* mutant is consistent with the pH dependence of the corresponding phosphatases.

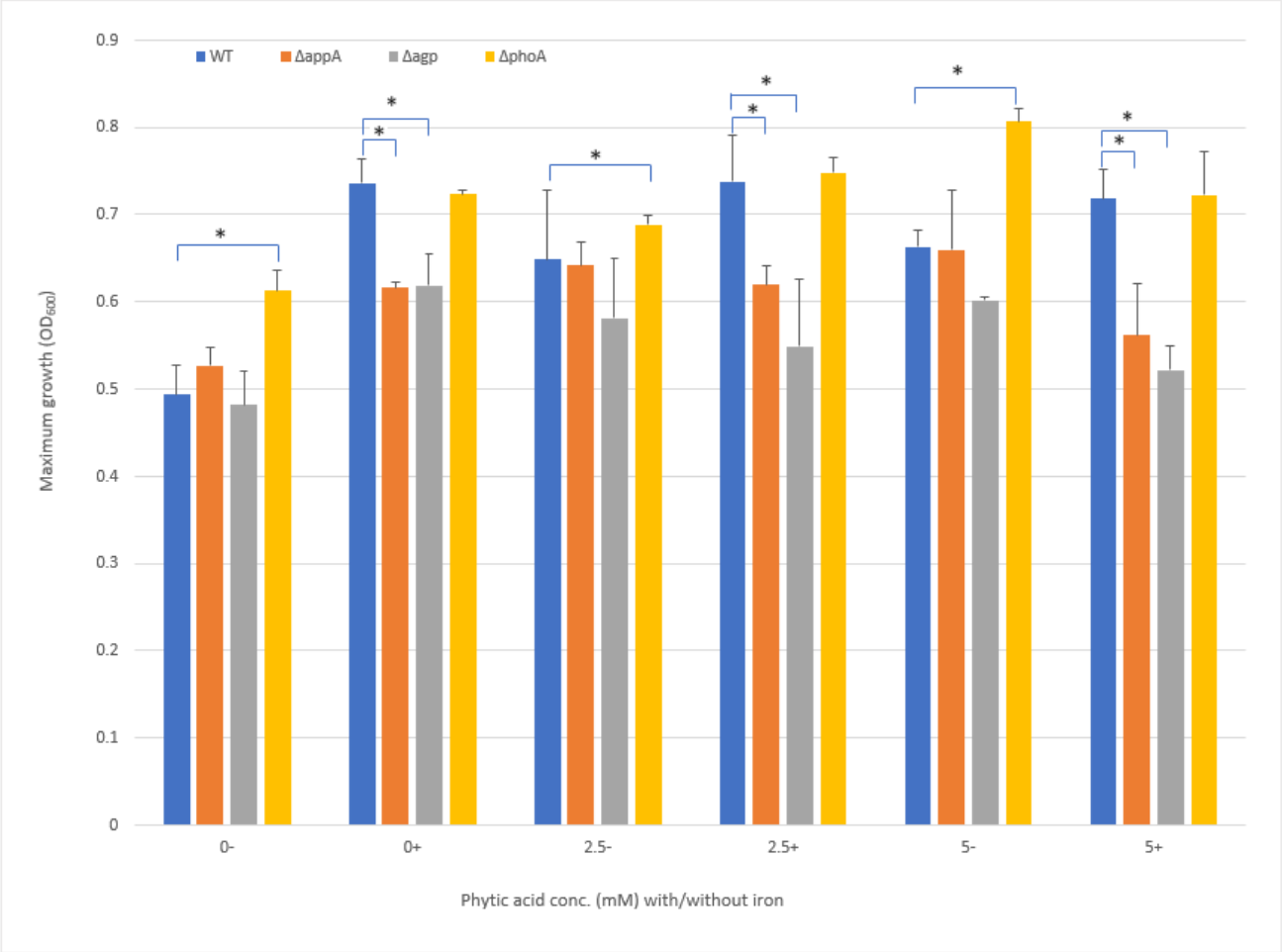


Figure 6.14 Effect of phytate (and iron) on growth of wild-type *E. coli* BW25113 (blue) and Δ*appA* (orange), Δ*agpA* (grey) and Δ*phoA* (yellow) mutants in low phosphate minimal medium at pH 5.5. The four strains were grown as before (Figure. 6.2) with and without 10 µM ferric citrate, in 0.4% glucose-containing M9 medium with low (3.5 mM) phosphate, but at pH 5.5. Various levels of phytate (0. 2.5 and 5 mM) were provided as a phosphate source. The results shown are the average of triplicates and indicate the maximum OD_{600} achieved as a representative indicator of overall growth. Asterisk (*) represents significant difference between the two bacteria. Full growth curves are shown in the Appendix.

6.5.2 The roles of appA, agp and phoA on phytate-dependent growth at neutral pH

The experiment in section 6.5.1 was repeated at pH 7 to determine whether the phosphatase mutants affect phytate-dependent, phosphate-limited growth at neutral pH and to determine whether the lack of phosphate limitation seen at pH 5.5 is reversed at pH 7. The neutral pH was obtained by adding of 1 ml of 100 mM pH 7 master mix to 9 ml medium (final concentration 10mM buffer). However, again good growth was observed without phytate (Figure. 6.14) indicating that severe phosphate restriction had not been obtained at pH 7.

The buffers used in this experiment do not contain any phosphate, indicating no external phosphate source present, thus further suggesting pH of the medium has a role in phosphate uptake by the bacteria.

Despite the high growth observed, a degree of phosphate limitation was obtained since addition of 2.5-10 mM phytate resulted in a significantly increased growth in both iron-free and iron supplemented medium ($p<0.05$). The bacteria showed an average of 27.8% increase in growth in the non-iron medium while the addition of iron showed an average of 21.4% increase when phytic acid was provided. The effect was not dependent on phytate concentration since just 2.5 mM phytate was sufficient to overcome the apparent phosphate limitation and achieve maximum growth (Figure. 6.15), and there is no significant difference between the phytic acid concentrations when tested ($p>0.05$). However, the *agp* mutant showed a significant increase in growth with increasing phytic acid concentration but this effect was seen only when iron was added ($p<0.05$). The *appA* and *agp* mutants generally displayed a lower growth than the wild-type under the conditions tested, by an average of 10.3 and 11.3% in the iron-free medium and 6.6 and 5.2% in the iron supplemented medium, respectively, but they are no significant differences in growth when tested statistically ($p>0.05$). In contrast, the *phoA* mutant showed average growth very similar to that of the wild-type (just 3% lower on average). Thus, as seen in Figure 6.14, the *appA* and *agp* phosphatases appear to have a role in supporting growth of *E. coli* under the conditions tested, whereas *phoA* does not. Interestingly, despite showing a higher OD_{600} reading than the mutants, the addition of phytic acid (in the absence of iron) on average only increased the growth of the wild-type by 25.4% while the growth of mutants were increased by 31.5% (*appA*), 26.1% (*agp*) and 28.4% (*phoA*). There was no notable impact of iron on the growth of the strains, indicating that phytate at up to 10 mM does cause iron restriction under the conditions tested. The addition of iron showed a lower growth increment with only an average 20.3% increase for the wild-type. The mutants on the other hand showed a slightly higher increase with 23.4% (*appA*), 20.4% (*agp*) and 21.6% (*phoA*), but there is no significant effect of the iron on growth ($p>0.05$).

In summary, the reduced growth of the *appA* and *agp* mutants is observed at pH 7 as well as pH 5.5, which suggests a role in phosphate acquisition at neutral pH for the corresponding phosphatases.

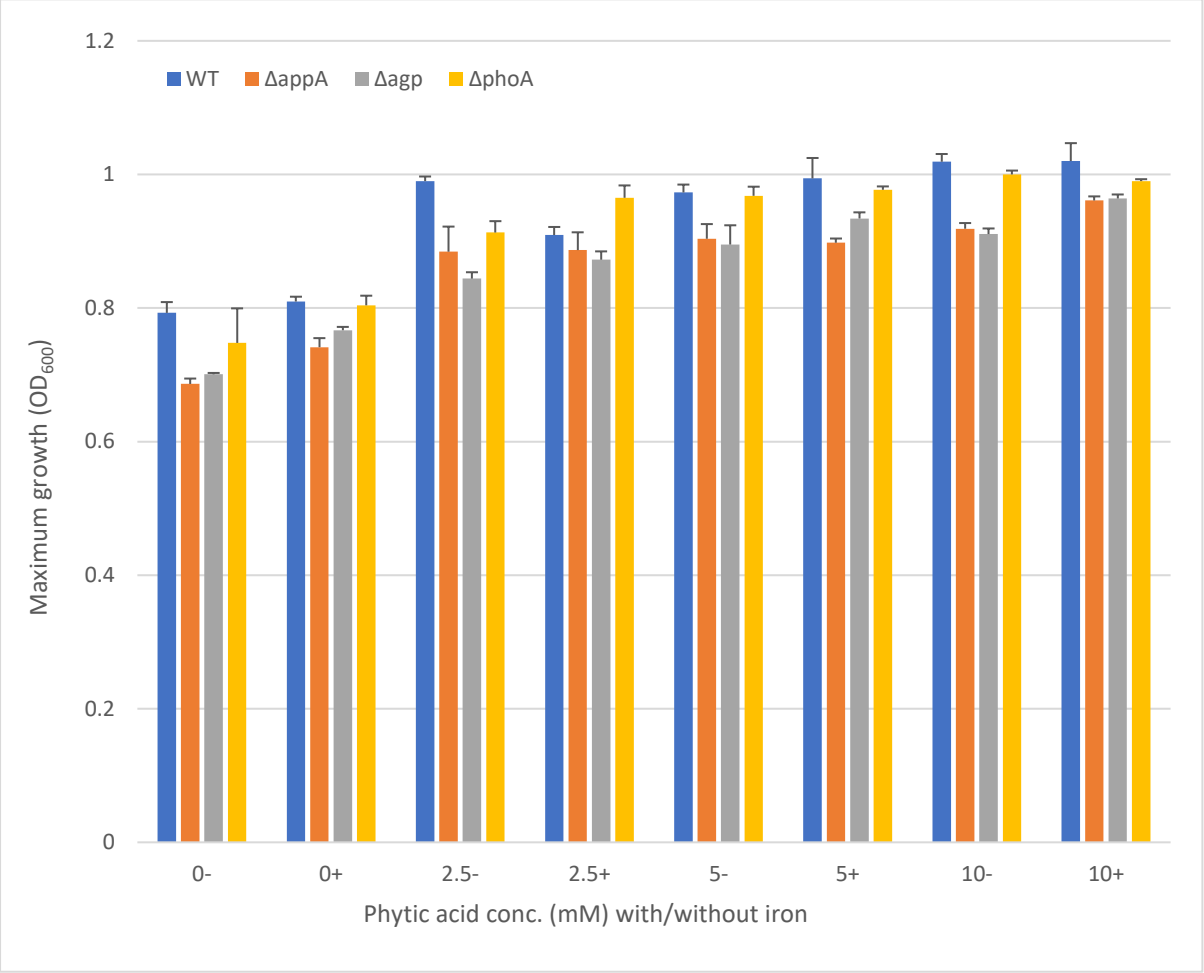


Figure 6.15 Effect of phytate (and iron) on growth of wild-type *E. coli* BW25113 (blue) and Δ*appA* (orange), Δ*agpA* (grey) and Δ*phoA* (yellow) mutants in low phosphate minimal medium at pH 7.0. Conditions were as in Figure. 6.14 except that the medium was buffered at pH 7. Full growth curves are shown in the Appendix.

6.5.3 The roles of appA, agp and phoA on phytate-dependent growth at alkaline pH

The effect of alkaline pH on the use of phytate as a phosphate source by the three phosphatase mutants was also investigated (Figure. 6.16). At pH 8.5, phytate was found to significantly enhance growth of all the bacteria tested ($p<0.05$), but this effect was not phytate-dependent (0.17 OD_{600} units). This indicates that the medium used was weakly phosphate deficient, as seen above at pH 7. concentrations above 2.5 mM did not show significantly greater growth ($p>0.05$). Phytate (2.5-

10 mM) enhanced growth by 37% on average in the absence of iron and only 13.7% with iron. This indicates that the medium used was weakly phosphate-deficient, as seen above pH 7. This suggests that the failure to observe phosphate limitation at pH 5.5 is due to the weaker overall growth (by 52-64%) obtained at acidic pH with respect to pH 7 and 8.5, which would reduce demand for phosphate. The *appA* and *agp* mutants displayed a lower overall growth (~12% on average with iron; ~7% on average without iron) than the wild-type, but this was not significantly different when tested statistically ($p>0.05$), which reflects the results seen above at lower pH. However, the *phoA* mutant displayed growth very similar to that of the wild-type (within 3% on average). At an alkaline pH, the presence of phytic acid greatly increases the growth of the bacteria compared to a neutral or acidic environment. The bacteria showed on average 36% increase in the presence of phytic acid (without iron) at pH 8.5 compared to 27.8% (pH 7) and 25.2% (pH 5.5). With the addition of iron, there is on average 13.7% increase at pH 8.5 and 21.4% increase at pH 7. At pH 5.5 however, the presence of both phytic acid and iron reduces the growth of bacteria on average 4.3%. Interestingly, provision of iron significantly enhanced growth of all bacteria in absence of phytate by an average of 36% ($p<0.05$). No such effect was seen in the presence of phytate (a slight 1% decrease in OD with iron, on average). This suggests that at alkaline pH phytic acid is able to increase availability of iron, presumably by acting as a mobilising (chelating) agent. This is in contrast to the reported effect of phytates as dietary inhibitors of iron absorption (Kumar et al, 2010), and indicates that the gut microbiota may benefit from dietary phytate through enhanced iron availability even though the host suffers from reduced iron availability.

The *appA* and *agp* mutants showed a greater growth difference with respect to the wild-type and *phoA* mutant when phytate was present (average of 12%) than when it was absent (7%). A similar effect was seen for at pH 7. This effect is consistent with a role for *appA* and *agp* in providing phosphate from phytate.

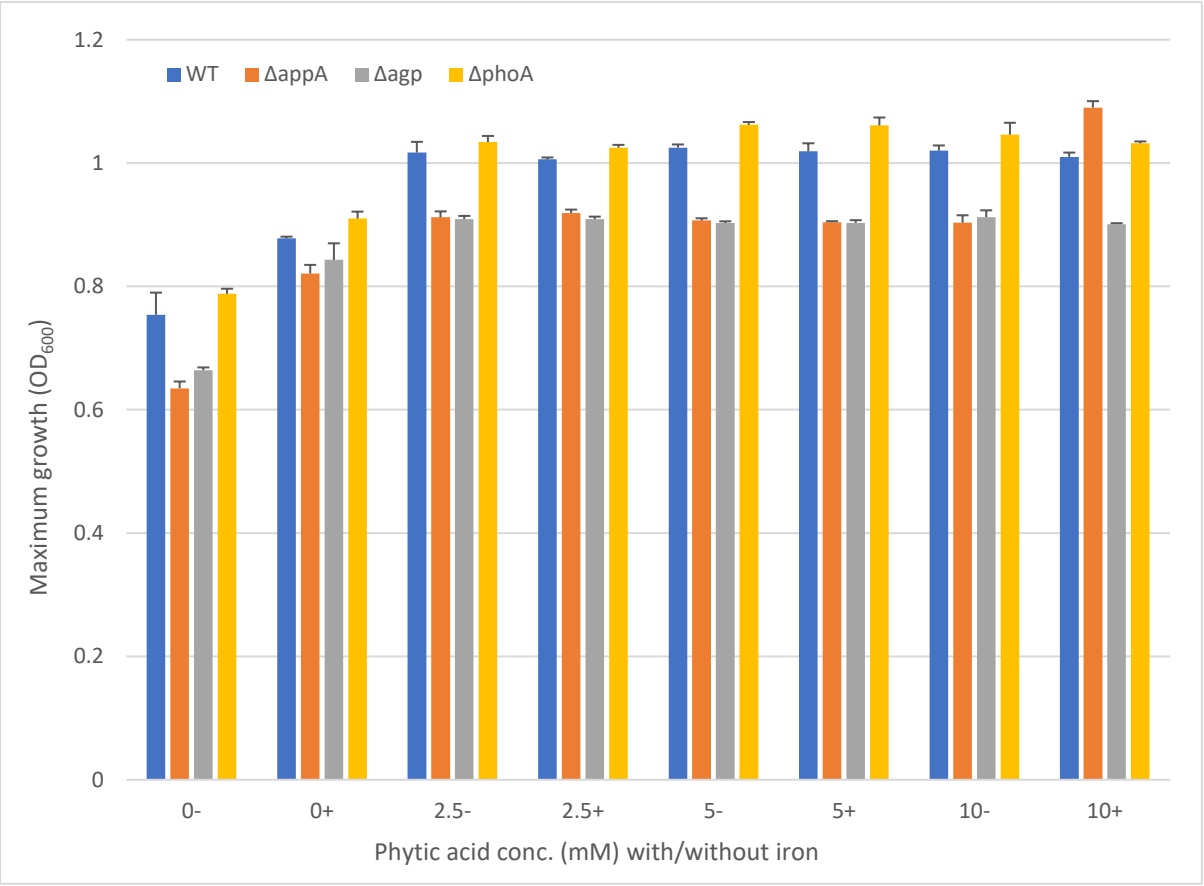


Figure 6.16 Effect of phytate (and iron) on growth of wild-type *E. coli* BW25113 (blue) and Δ*appA* (orange), Δ*agpA* (grey) and Δ*phoA* (yellow) mutants in low phosphate minimal medium at pH 8.5. Conditions were as in Figure. 6.14 except that the medium was buffered at pH 8.5. Full growth curves are shown in the Appendix.

6.6 The effect of combining *appA*, *agp* and *phoA* mutations on phytate-dependent, phosphate-limited growth

Since the three single phosphatase mutants showed either no reduction or only a limited reduction in growth with phytate as the major phosphate source, it was decided to combine the mutations to generate a series of double mutants (section 2.16.2.4). This would allow determination of whether multiple phosphatases support the use of phytate as phosphate source, and thus whether the three genes investigated provide a degree of redundancy in terms of their roles in phytate consumption. Three double mutants were therefore generated, as described in Methods (section 2.16), and tested for their growth in unbuffered, phosphate-limited (3.5 mM) M9 medium with glucose as carbon source.

6.6.1 The Δagp ΔphoA, ΔappA ΔphoA and ΔappA Δagp double mutants

As seen before (sections 6.2.2, 6.3.1, 6.4.1) in unbuffered phosphate-limited M9 medium, there was a clear growth enhancement caused by provision of phytate, and this was phytate concentration-dependent (Figure. 6.17). Thus, phosphate limitation had been achieved and phytate was able to act as an alternate source of phosphate. On average, the addition of 2.5, 5 and 10 mM phytic acid significantly increased the growth by 2.3-, 2.3- and 3.9-fold for all strains ($p<0.05$). With the exception of the Δ*appA* Δ*phoA* mutant (which showed similar growth to the wild-type), the mutants showed better growth compared to the wild-type with an average of 7.5% and 10.6% higher growth for the Δ*agp* Δ*phoA* and Δ*appA* Δ*agp* mutants, respectively, but this difference was not significant when tested statistically ($p>0.05$). However, there was no discernible impact of iron (Figure. 6.17) indicating that iron restriction had not been achieved on this occasion. Surprisingly, the three double mutants showed no major, consistent or significant growth impairment with respect to the obtained previously with the single mutants that suggested that the phosphatases under study had a wild-type (Figure. 6.17) either with phytate or in its absence. This is in contrast to the result minor role in phytate utilisation.

In summary, despite the lack of 2 phosphatases, the bacteria still displayed the ability to utilise phytate as a phosphate source. Although previous work has reported phytase activity associated with these enzymes (Greiner et al, 1993; Cottrill et al, 2002) and the results obtained with single mutants (above) suggested a potential contribution. A triple mutant whereby all 3 phosphatases were knocked out was used to confirm the presence of unknown phosphatases/phytases.

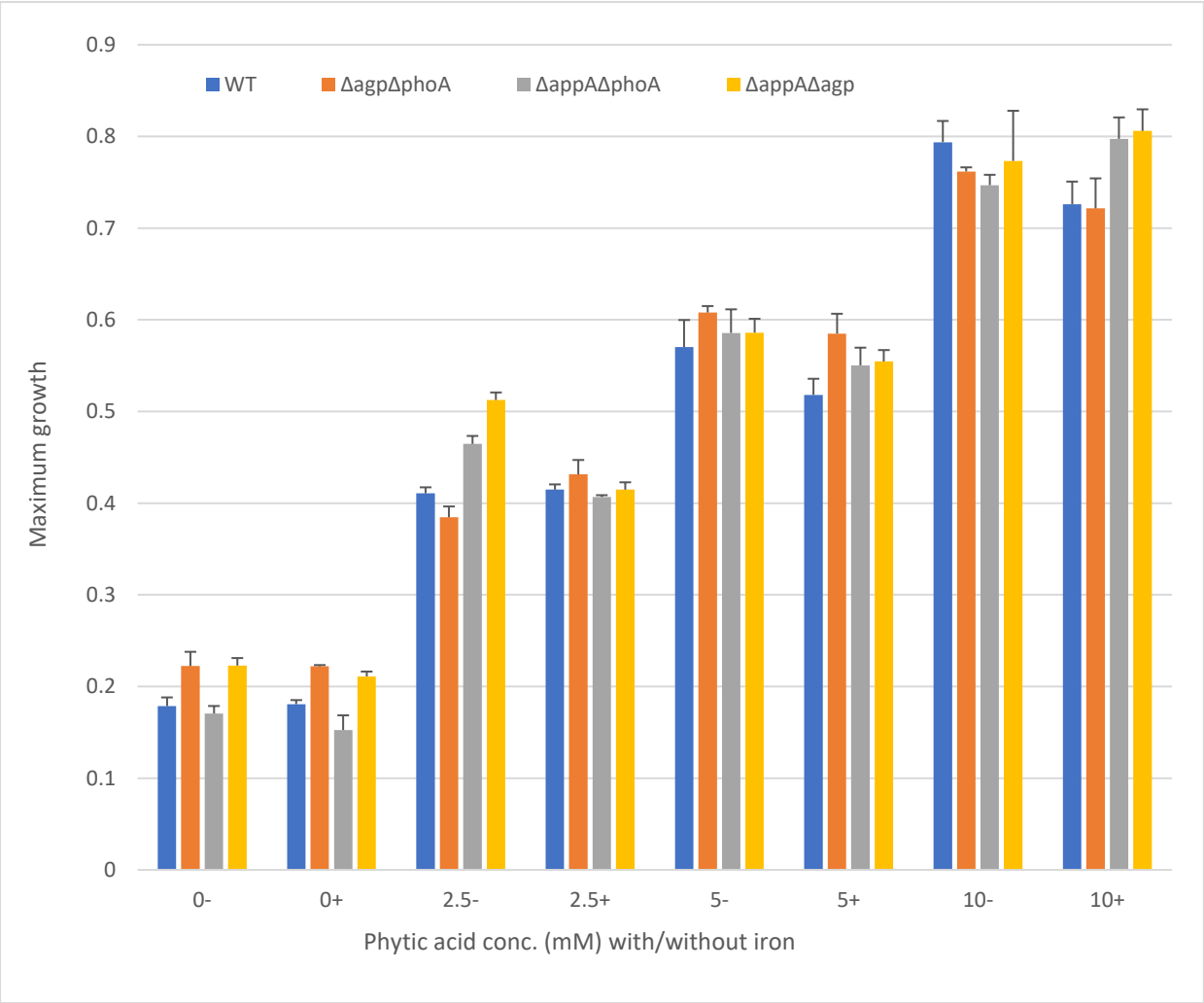


Figure 6.17 Effect of Δ*agp* Δ*phoA,* Δ*appA* Δ*phoA* and Δ*appA* Δ*agp* double mutations on phytate-dependent growth under phosphate limitation, unbuffered minimal medium, in presence/absence of iron. Conditions were as in Figure. 6.2 (0.4% glucose M9 medium, +/- 10 µM ferric citrate, aerobic, 37 ˚C, shaking, Bioscreen C, in triplicate) except double mutants rather than single mutants were employed. Full growth curves are shown in the Appendix.

6.6.2 The ΔappA ΔagpA ΔphoA triple mutant

In order to confirm that none of the phosphatases considered here have any major role in use of phytate as a phosphorus source, a triple mutant was generated (Methods section 2.16) and compared with the wild-type under the same conditions as those used above (Figure 6.17). The results (Figure 6.18) again clearly indicate that phytate can act as a phosphate source and significantly boosts phosphate-limited growth in a quantitative fashion with a 2.5-, 3.1- and 4.7-fold increases in growth achieved with 2.5, 5 and 10 mM phytate, respectively ($p<0.05$). The addition of iron significantly increased the growth of both wild-type and mutant ($p<0.05$). However, the wild-

type showed a more prominent growth increase than the wild-type by an average of 0.14 OD_{600} units while the mutant only increases 0.09 OD_{600} units. The greatest iron-enhancing effect on the growth of both bacteria was seen at 0 mM phytic acid, indicating that the presence of phytic acid has an inhibitory effect on iron-enhanced growth despite providing phosphate to support growth. In the absence of phytic acid, the wild-type showed a 2.1-fold increase while the mutant gave a 1.4-fold increase when iron was supplemented. When phytic acid was added, the wild-type showed a 1.3-, 1.3- and 1.1-fold higher growth with increasing concentration of phytic acid. The mutant, on the other hand, showed 1.2-, 1.1- and 1.1-fold increase when 2.5mM, 5mM and 10mM phytic acid are added. Most importantly, the triple mutant showed similar growth to that of the wild-type both with and without phytate supplementation (Figure. 6.18), which confirmed the results in section 6.6.1 indicating that none of the phytases considered here have any major role in utilisation of phytate for use as a phosphate source under the conditions tested here.

In summary, the above results show that the major extra cytoplasmic phosphatases of *E. coli* are not required for use of phytate as the major source of phosphate during growth. This suggests that phytate utilisation occurs through another pathway that has yet to be identified. In addition, the results indicate that a display of phytase activity *in vitro* does not necessarily correlate with any requirement for phytate utilisation during growth and thus suggests that phytase activity results

obtained *in vitro* should be confirmed through studies with a corresponding mutant before any conclusion can be drawn with regard to physiological relevance.

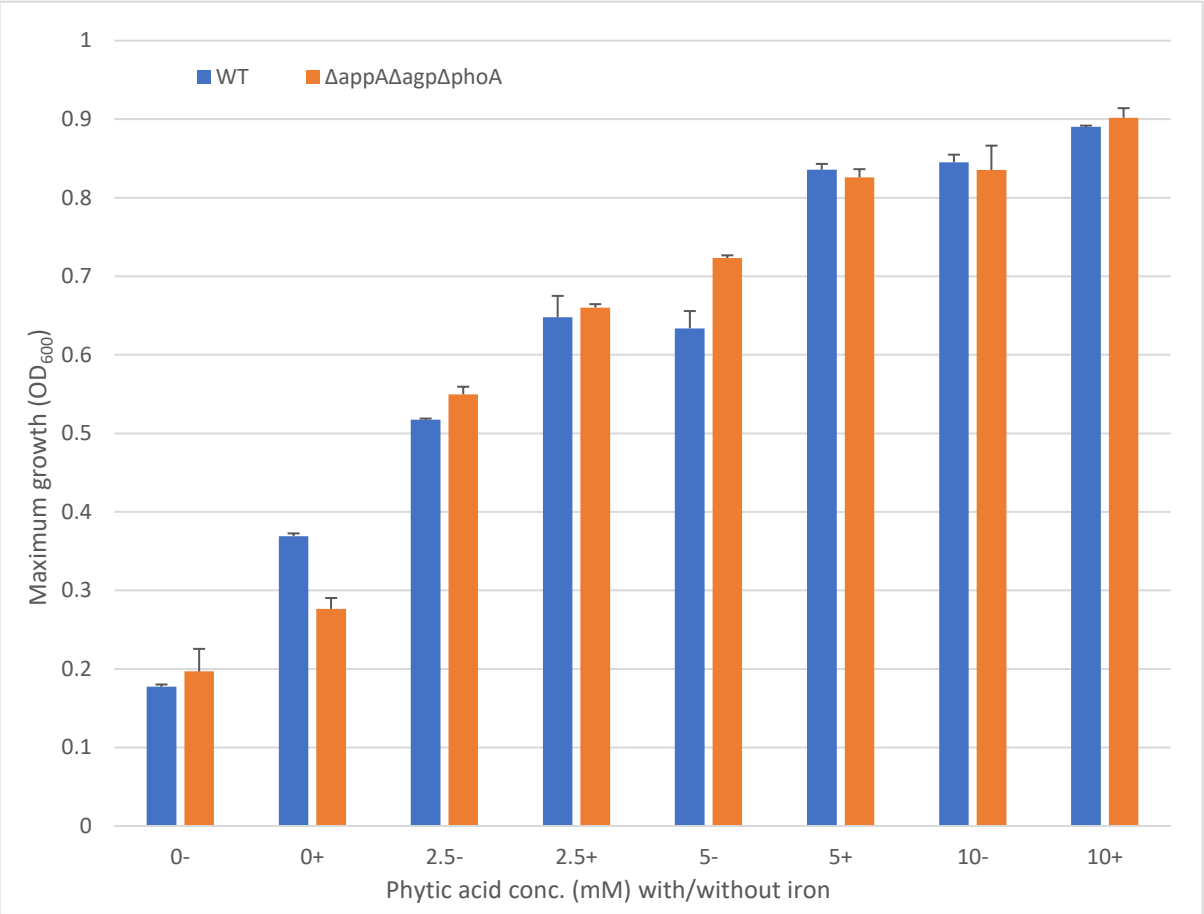
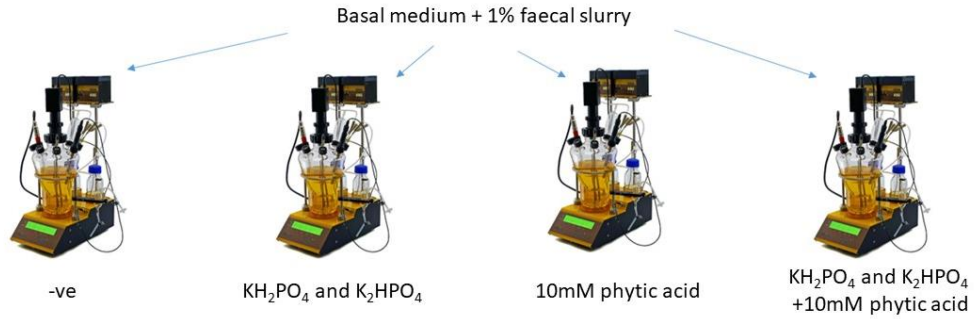


Figure 6.18 Effect of Δ*appA* Δ*agpA* Δ*phoA* triple mutation on phytate-dependent growth under phosphate limitation, unbuffered minimal medium, in the presence/absence of iron. Conditions were as in Figure. 6.17 (0.4% glucose M9 medium, +/- 10 µM ferric citrate, aerobic, 37 ˚C, shaking, Bioscreen C, in triplicate) except that the triple mutant was employed. Full growth curves are shown in the Appendix.

6.7 Impact of phytate as the sole phosphate or carbon source on the human gut microbiota

The results above (section 6.2- 6.6), obtained with *E. coli* grown aerobically, indicate that phytate can act as a good source of phosphate and that it does not apparently impair iron availability. However, it is unclear whether the human gut microbiota as a whole can also utilise phytate as its major or sole phosphate source, and whether the composition or activity of the microbiota might be altered when phytate acts as the major (or sole) source of phosphate (or carbon/energy). To investigate this question, batch cultures experiment were performed. The conditions employed are summarised in Figure 6.19.

A)



B)

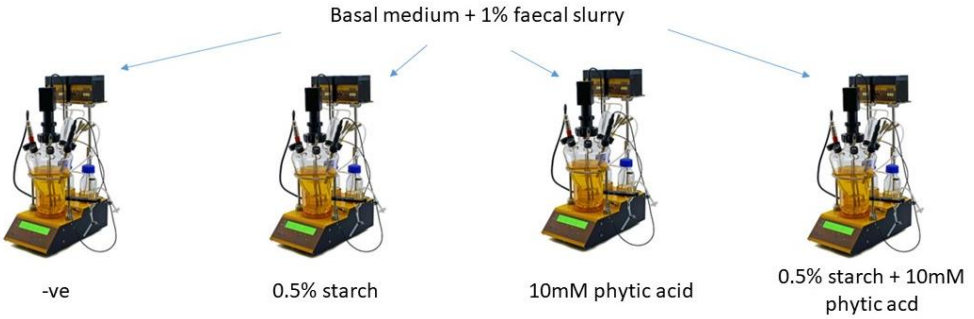


Figure 6.19 Experimental design. Diagram A represents the phosphate experiment set up while diagram B represents the carbon/energy experiment set up. Basal medium containing faecal inocula from 3 different donors were grown anaerobically for 48 h at a constant pH of 5.5, maintained through the addition of 1 M HCl or 1 M NaOH.

*6.7.1 Phytate as phosphate source for the human gut microbiota***.**

The medium used was normal basal medium with starch (0.5%) but without yeast extract or peptone, since preliminary phosphate assay results suggest that these provide phosphate (data not shown). The vitamins and minerals provided by yeast extract were replaced by addition of trace elements and vitamins (section 2.3.1.2). The phytic acid was provided at 10 mM (thus giving 60 mM phosphate levels) while the KH_2PO_4 and K_2HPO_4 were at 3.67 and 2.87 mM (level used in normal basal medium), respectively. The condition with potassium phosphate provided a state of phosphate limitation, whereas the condition without either potassium phosphate or phytate acted as a control to demonstrate the importance of a phosphate source for growth of the gut microbiota.

The addition of phosphate resulted in a higher growth compared to the phosphate-free negative control (Figure 6.20). In the absence of phosphate, the control showed a gradual decrease in cell numbers over time. The control showed a 2.4-, 2.9- and 4.04-fold decrease at t12, t24 and t48,

respectively. The bacterial counts lowered from an initial $9.7x10^6$ cells/ml medium (t0) to $3.92x10^6$ (t12), followed by $3.33x10^6$ after 24 h and finally $2.4x10^6$ at t48. When 6.5 mM potassium phosphate (KH_2PO_4 and K_2HPO_4) was added to the medium, the microbiota showed a decrease in number for the first 24 hours but then an increase by at 48 h. The bacteria had a decrease of 3.64- and 1.84-fold for t12 and t24 respectively, followed by a 1.39-fold increase at t48 cf. 0h. The bacteria decreases from $1.12x10^7$ cells/ml medium at t0 to $3.06x10^6$ at t12 and gradually increase to $6.05x10^6$ at t24 and finally $1.55x10^7$ at t48. The use of phytic acid as sole phosphate source showed a similar trend whereby the bacteria experienced a drop in number at t12 but this then gradually increased up to t48. There was a drop of 2.34-fold at t12, followed by a 1.5- and 6.07-fold increase for t24 and t48, accordingly. The bacterial counts dropped from an initial $1.43x10^7$ cells/ml medium to $6.10x10^6$ at t12 and gradually increased to $2.14x10^7$ (t24) and $8.65x10^7$ (t48). When both potassium phosphate and phytic acid were used to supplement the medium, the bacteria showed a slight decrease in the beginning followed by a major increase between t24 and t48. The bacterial numbers were 1.44- and 1.29-fold lower at t12 and t24, respectively, than at t0; this drop was followed by a 6.06-fold increase at t48 from t0. Bacterial counts showed an initial decrease from $1.24x10^7$ cells/ml medium (t0) to $8.61x10^6$ at t12, followed by a gradual increase to $9.63x10^6$ and then $7.51x10^7$ cells at t24 and t48, respectively. As seen in Figure 6.20, all conditions showed a decrease at t12 but the addition of 6.5 mM potassium phosphate allowed a gradual but modest increase in the bacterial counts until the end of the experiment. In contrast, the bacteria showed a continuous decrease in numbers when no phosphate source was provided (blue bars in Figure 6.20). Thus, it is clear that the presence of potassium phosphate increased the bacterial counts compared to the control. However, at t12, the phosphate-free control showed a 1.28-fold higher bacterial count than the potassium phosphate supplemented vessel ($3.92x10^6$ vs $3.06x10^6$). Supplementation with phytic acid increased the cell counts by 1.56-fold ($6.1x10^6$ vs $3.92x10^6$) at t12 and the addition of both potassium phosphate and phytic acid showed a 2.2-fold increase ($8.61x10^6$ vs $3.92x10^6$) at t12 compared to the control.

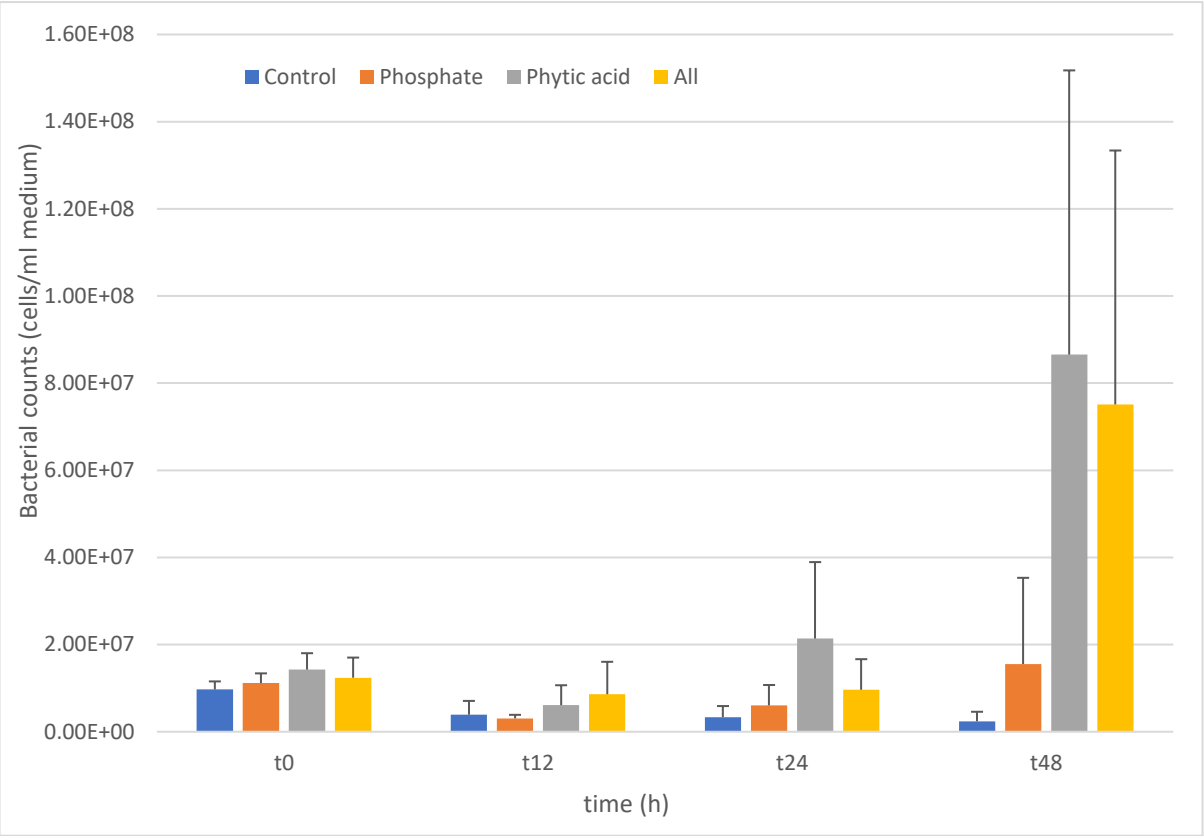


Figure 6.20 Effect of phytate as sole phosphate source on the growth of the human gut microflora in batch culture. Total bacterial counts were determined by Flow-FISH using Eub I-II-III probes. Four growth conditions using modified basal medium were used: no phosphate (blue); normal phosphate level (3.67 mM KH_2PO_4/2.87 mM K_2HPO_4; orange); 10 mM phytate, no potassium phosphate (grey); and 6.5 mM potassium phosphate with 10 mM phytate (yellow). Bacterial counts are expressed as number of cells/ml medium. Results are the average of three normal subjects and error bars represent ±SD.

However, the bacterial counts at t12 showed no significant difference when tested statistically ($p>0.05$). At t24, the presence of potassium phosphate increased the cell numbers by 1.8-fold compared to the control ($6.05x10^6$ vs $3.33x10^6$ cells/ml medium). The phytic acid on the other hand increased the bacteria by 6.42-fold ($2.14x10^7$ vs $3.33x10^6$ cells/ml medium) but this difference was not significant ($p=0.063$). The addition of both potassium phosphate and phytic acid showed a 2.89-fold increase ($9.63x10^6$ vs $3.33x10^6$ cells/ml medium) at t24 but was not significant ($p>0.05$). At t48 with potassium phosphate, an increase in the bacterial counts by 6.47-fold compared to the control ($1.55x10^7$ vs $2.4x10^6$ cells/ml medium) was obtained. Although the presence of phytic acid showed a greater increase in growth with a 36.1-fold compared to the control ($8.65x10^7$ vs $2.4x10^6$ cells/ml medium), it is not significant ($p=0.057$). A 31.3-fold increase in growth was seen with both potassium

phosphate and phytic acid compared to the control but was not significant ($7.51x10^7$ vs $2.4x10^6$ cells/ml medium) at t48.

In conclusion, the results indicate that (as expected) a source of phosphate is vital for growth of the microbiota. Importantly, the provision of phytate as sole source of phosphate allows its utilisation by the gut microbiota to support their growth, as compared to conditions where phosphate is absent. These results reflect those obtained for *E. coli*. This indicates that phytate can indeed support the growth of the gut microbiota as sole phosphate source. It would be of great interest to determine the effects of different levels of phytate on phosphate-limited growth of the microbiota, as it is unclear whether the level used is optimal; indeed, 10 mM phytate can reduces the growth of the gut microbiota when all nutrients are sufficient (yellow bar in Figure 6.20). When potassium phosphate and starch are added to the vessel as phosphate and carbon source, the presence of phytic acid reduces the growth of bacteria. Further, the differences in total growth observed between subjects was unexpectedly high (as indicated by the large error bars) suggesting that the impact of phytate on the microbiota is strongly subject-dependent (i.e. influenced by the gut microbiota community profile). Thus, the effect of phytate dependent growth on bacterial composition would be of great interest.

6.7.2 Phytate as carbon source for the human gut microbiota.

Normal basal medium (with yeast extract and peptone water) was used in this experiment with starch acting as a positive control. Phytic acid is a very poor source of carbon/energy for the gut microbiota, as shown by the poor growth and the comparable cell counts with the starch-free control when phytate is the sole carbon source (Figure 6.21). The presence of starch as a carbon source showed the highest growth among all treatments with the highest cell number occurring at t24. However, when both starch and phytic acid were added to the medium, the growth was lowered significantly ($p<0.05$) compared to starch alone. This suggests that 10 mM phytic acid has an inhibitory effect on the microbiota. It would be very interesting to determine the effect of

phytate concentration on microbiota inhibition and to determine whether there is a differential effect of phytate on the growth of different members of the gut microbiota.

There was an increase in bacterial numbers over time in the presence of starch, peaking at t24; the non-starch vessels peaked at t48 but showed very low levels of growth (Figure 6.21). Both control and the phytic acid supplemented growths showed a gradual increase until the end of the experiment while the starch condition showed an increase until t24 followed by a drop at t48. The control showed a 16-, 19.3- and 42.5-fold increase with time, increasing from $1.17x10^5$ cells/ml medium (t0) to $1.88x10^6$ (t12), $2.26x10^6$ (t24) and finally $4.98x10^6$ (t48). With starch, the bacterial counts increased 8.05-, 32.8- and 23.7-fold at t12, t24 and t48, respectively; levels increased from an initial count of $1.18x10^7$ cells/ml to $9.51x10^7$ at t12, then to $3.87x10^8$ (t24) followed by a drop to $2.8x10^8$ (t48). On the other hand, there was an increase of 5-, 10.8- and 17.3-fold with phytic acid; the bacteria number increased from $2.99x10^5$ cells/ml medium at t0 to $1.49x10^6$ at t12, then $3.22x10^6$ at t24 and finally $5.15x10^6$ at t48. When both phytic acid and starch were added, the bacterial counts increased 1.27-, 17.7- and 16.0-fold from t12 to t48 compared to t0; bacterial numbers were initially at $1.26x10^7$ cells/ml medium, gradually increasing to $1.6x10^7$ at t12 and then $2.23x10^8$ at t24, followed by a decrease to $2.01x10^8$ at t48.

At t12, the presence of starch as the sole carbon source gave a significantly ($p=0.05$) higher bacterial count (50.7-fold) compared to the control where no carbon source was present ($9.51x10^7$ vs $1.88x10^6$ cells/ml medium). This increase was lowered when phytic acid was added such that when both starch and phytic acid were present, the bacteria only showed an 8.5-fold increase compared to the control at t12 ($1.6x10^7$ vs $1.88x10^6$ cells/ml medium), but the increase were not significant when statistically tested. When only phytic acid was added, there was a 1.26-fold lower growth compared to the control (1.49-10^6 vs $1.88x10^6$ cells/ml medium).

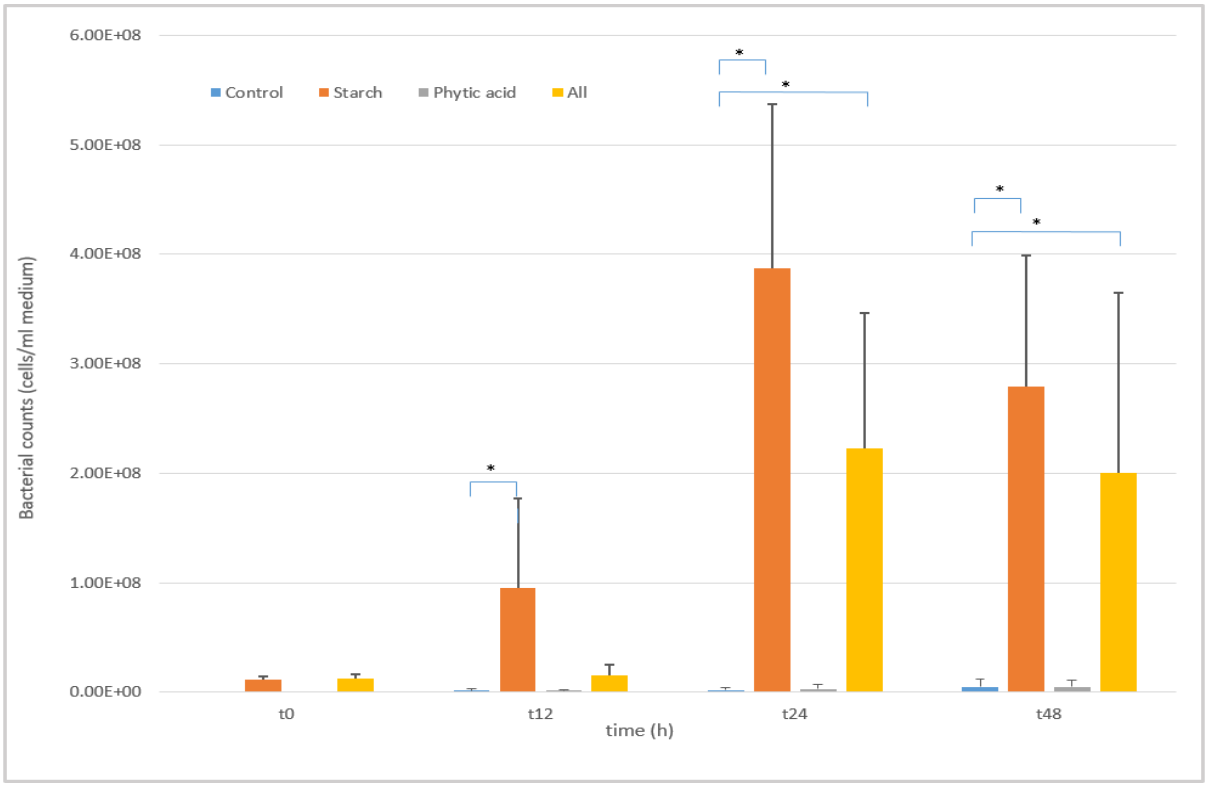


Figure 6.21 Effect of phytate as sole carbon source on the growth of the human gut microflora in batch culture. Details are as in Figure. 6.19 except that normal basal medium was used (phosphate levels at 6.5 mM, with yeast extract and peptone water), and the carbon source was varied between each of the four experimental conditions, as follows: blue (no starch), orange (0.5% starch), grey (10 mM phytic acid) and yellow (0.5% mM starch and 10 mM phytic acid). Asterisk (*) represents significant difference ($p<0.05$)

At t24, all the supplemented vessels had a higher growth compared to the control. The starch-only vessel showed the highest growth with a 171-fold increase ($3.87x10^8$ vs $2.26x10^6$ cells/ml medium) with respect to the control, followed by the combined starch and phytic acid condition where there was a 98.5-fold increase ($2.23x10^8$ vs $2.26x10^6$ cells/ml medium) compared to the control. The two starch-containing conditions showed a significantly higher growth compared to the control ($p<0.05$). The addition of phytic acid as sole carbon source only showed a 1.42-fold higher growth than the control ($3.22x10^6$ vs $2.26x10^6$ cells/ml medium), suggesting that phytate is a poor carbon source for the microbiota.

At t48, the starch-only condition still showed the highest growth with a 56.1-fold increase and a significantly higher growth ($p<0.05$) compared to the control ($2.8x10^8$ vs $4.98x10^6$). When phytate

was also included, the bacterial counts showed a slight decrease to 40.3-fold ($2.01x10^8$ vs $4.98x10^6$ cells/ml medium) but were still significantly higher than those in the control ($p<0.05$). Phytic acid as sole carbon source gave comparable results to the control with $5.15x10^6$ cells for the former and $4.98x10^6$ for the latter. At t24 and t48, the presence of starch showed significantly higher growth than for phytic acid alone ($p<0.05$), further indicating phytic acid as a poor carbon source for the bacteria.

In conclusion, similar to the results above obtained from the growth experiment with *E. coli*, phytic acid is a poor carbon source. The use of phytic acid as a carbon source showed comparable bacterial counts to the control. Thus it does not provide any carbon/energy source to support the growth of bacteria. When starch was present as a carbon source for the bacteria to utilise, the addition of phytic acid showed a reduction in growth of the bacteria, indicating its inhibitory effect, possibly through the binding of minerals, making them unavailable for the bacteria to utilise. The growth conditions used in Figures 6.20 and 6.21 are similar except that the medium in Figure 6.20 (phosphate-restricted medium) does not contain yeast extract or peptone. Both sets of data include conditions with adequate phosphate and carbon source, as well as phytic acid added to support the growth of the bacteria. However, the total bacterial counts in Figure 6.21 (with yeast extract and peptone) are 2.67-fold higher (containing ~$1.2x10^8$ more cells) than their counterparts in Figure 6.20. This indicates that yeast extract and peptone provide additional nutrients that enhance the growth of the gut microbiota and that cannot be replaced by vitamin and mineral supplements.

6.8 Discussion

As seen from the phytic acid utilisation studies, the growth of *E. coli* increases with increasing concentration of phytic acid due to the availability of higher concentrations of phosphate. Phytic acid can be used by *E. coli* as a phosphate source but it is a very poor carbon source. Although acid phosphatase encoded by the *appA* gene has been shown to be a phytase which is able to hydrolyse phytic acid, mutants lacking this gene showed growth comparable to the wild-type, indicating the presence of other enzymes which can also hydrolyse phytic acid. The homologous G1Pase has been

shown to liberate phosphate from glucose-1-phosphate to support growth, and thus might be able to hydrolyse phytic acid in the same manner. Alkaline phosphatase is another protein of interest which may have phytase properties. However, *agp* and *phoA* mutants also showed comparable growth to the wild-type. When double mutants were generated to give *E. coli* strain only possessing either the acid phosphatase (*appA)*, glucose-1-phosphatase (*agp)* or alkaline phosphatase (*phoA*), all the mutants also showed growth comparable to the wild-type. This result might indicate that all of these phosphatases are able to hydrolyse phytic acid to liberate free phosphate and thus support phytate-dependent growth. However, it might also suggest that other phosphatases which hydrolyse the phytic acid are present. The latter hypothesis appears to be true since a triple mutant which lacks of all the phosphatases of interest was generated and it showed better growth compared to the wild-type (Figure 6.18). The result thus suggest that other phosphatases that have the ability to hydrolyse phytic acid are present. Pradel and Boquet showed that the *phoA* and *agp* mutants is able to utilise glucose-1-phosphate as a phosphate source provided that both phosphoglucomutase (*pgm*) and the hexose-6-phosphate:phosphate antiporter (*uhpT*) are present (Pradel and Boquet, 1991). Although utilisation of phytic acid has not been shown, this might be a possible mechanism which can explain the results obtained from the triple mutant experiment.

The class B acid phosphatase, as encoded by the *aphA* gene, has been shown to have a relatively low activity towards phytic acid as it tends to prefer aryl phosphoesters and both 3'- and 5'- nucleotides (Thaller et al, 1997). Although it shows low activity towards phytic acid, it may help hydrolyse phytic acid in the absence of other phosphatases.

When the pH of the medium was adjusted to either acidic (5.5), neutral (7) or alkaline (8.5), the mutants showed a growth pattern similar to the wild-type, which suggests that pH has a bigger effect on growth than all other factors considered. However, the wild-type has a higher growth than the mutant as seen by the higher OD_{600} reading.

In the phosphate utilisation experiment using batch cultures, the addition of phytic acid was able to support growth of the gut bacteria in a low phosphate/phosphate-deficient medium. The vessel which contained phytic acid showed higher growth compared to the KH_2PO_4 and K_2HPO_4 vessel. This may be due to the higher concentration of phytic acid supplemented (10mM) as compared to KH_2PO_4 and K_2HPO_4. Thus, this can be seen as a limitation in the experimental design which can affect the growth differences observed between vessels. The concentration of phosphate provided as potassium phosphate or the phytic acid should be kept the same, so that the use of phytic acid as a phosphate source can be truly determined. However, when both phytic acid and phosphate (KH_2PO_4 and K_2HPO_4) were present, the microbiota showed a slight reduced growth, indicating that the phytic acid might have an inhibitory effect on growth, possibly by chelating cations, making them unavailable for the microbiota.

When phytic acid was added as a carbon source for the bacteria, the bacteria showed poor growth with numbers comparable to the control (no carbon source). Starch is a good carbon source for the microbiota to utilise, however. When phytic acid is added, the bacteria showed a reduced growth compared to the presence of starch alone. Similar to the phosphate experiment, phytic acid may inhibit the growth of bacteria through lowering the availability of minerals for the bacteria. Both the phosphate utilisation and carbon source experiment conducted using the gut bacteria gave results which are in agreement with the growth studies whereby phytic acid is a good phosphate source and a poor carbon source. One limitation with the phosphate experiment is the removal of peptone water and yeast extract due to their high phosphate content. Vitamin and mineral solutions were provided as supplements to compensate for the removal of yeast extract. However, the lack of a nitrogen source or alternative amino acid source may place certain bacterial groups at a disadvantage, thus reducing their growth.

In summary, phytic acid can be used as a phosphate source to support the growth of the gut microbiota but it is a very bad carbon source. Although the literature has shown that acid

phosphatase and acid glucose-1-phosphatase of *E. coli* have the ability to hydrolyse phytic acid (Greiner et al, 1993;Cottrill et al, 2002), the results from the experiments reported here show that these phosphatases are not required for phytate utilisation by *E. coli* and that there must be another phytate utilisation pathway that has yet to be discovered.

Chapter 7: Impact of zinc supplementation on the gut microbiota

7.1 Introduction

An estimated 17% of the global population has an inadequate zinc intake, with the highest prevalence occurring in the South Asia region with 29% of the population in that region facing deficient zinc intake. A high percentage of the population of countries in the African continent, and East and Southeast Asia are reported to have low zinc intake (Wessells and Brown, 2012). An adult human contains 2-3 g of zinc, of which 0.1% must be replenished daily. Numerous enzymes in the human body are zinc dependent, thus showing zinc's important role in various biological processes. In particular, zinc has a role in growth and development, being involved in proliferation, differentiation and apoptosis at the cellular level (Maret and Sandstead, 2006). Zinc supplementation or food fortification has been used to reduce zinc deficiency, however, the impact of such interventions on the gut microbiota has yet to be determined. The experiment below is designed to mimic zinc supplementation and its impact on the gut microbiota in a healthy human setting (i.e. employing faecal inocula from healthy donors and a nutritionally sufficient medium, except for Zn) rather than a malnourished person. The experiment was conducted using single stage batch cultures containing a modified gut model medium in which mucin, yeast extract and tryptone were removed to create a zinc-restricted medium. However, a vitamin and mineral solution were added to compensate for the lack of yeast extract, while peptone water was used to replace tryptone. The modified gut model medium contains only 3 µM zinc as compared to 19 µM in a normal gut model medium (measured using ICP-OES by another member of the lab). A 2 ml volume of faecal slurry from four healthy subjects (two male and two female) was inoculated into 198 ml of medium and the cultures were allowed to propagate for 48 h in a pH-controlled environment. The conditions used were:

control (no zinc);

77 µM $ZnSO_4$ (equivalent to 10 mg/d zinc);

192 µM $ZnSO_4$ (equivalent to 25 mg/d zinc); and

770 µM $ZnSO_4$ (equivalent to 100 mg/d zinc).

The total bacterial numbers were determined by Flow-Fish (section 2.4) and community profiles were compared at 0, 24 and 48 h by 16S rRNA-dependent community profiling (section 2.8).

7.1.1 Selection of zinc concentrations to be tested

As indicated above, modified gut model medium was used in this experiment which has a background zinc concentration of 3 µM compared to the normal gut model medium (~20 µM Zn). The low zinc concentration of the modified medium allows a better reflection of the impact of zinc supplementation on the gut microbiota. The addition of 77 µM Zn is equivalent to the intake of commercially available 10 mg zinc tablet per day (assuming a 2-litre colonic volume, Olsson et al, 1952). These tablets are readily available and accessible which can be bought in stores. A study in the US has shown multivitamin-mineral is the most common supplement consumed in adults (Bailey et al, 2013). The most common motivation leading to the consumption of supplements includes to improve or maintain health, supplement the diet and to boost immunity (Bailey et al, 2013). However, the zinc intake for the UK population is considered to meet the daily requirement (7 and 9.5 mg/day for women and men), thus there is a chance of an excessive zinc intake through supplementation. The impact of zinc supplementation on the gut microbiota of a nutritionally sufficient (as well as a zinc deficient individual) has yet to be establish, thus the use of batch culture and gut model are able to provide insights to these effects. The daily limit zinc for supplement intake is 25 mg (equivalent to 192 µM) which may be prescribed by healthcare providers to a severely zinc deficient or malnourished patient. However, the impact of zinc at such concentrations on the gut microbiota has not been determined, thus operating batch cultures and gut models at this concentration may provide insight into any such effects. Zinc at 770 µM is equivalent to 100 mg of zinc per day which is 4 times higher than the daily zinc allowance (25 mg). This concentration was selected to determine the effect of extremely high zinc intake on the gut microbiota which might be achieved through over-supplementation whereby consumption of multiple different supplements

with zinc as a common ingredient in them. These concentrations would be representative of the daily diet and lifestyle of individuals with unusually high zinc intake.

7.2 Effect of zinc of microbiota cell numbers in batch culture

As seen from Figure 7.1, the bacteria in all zinc regimes increased over time. As expected, no significant difference was seen at t0, with all regimes containing between $1.19x10^7$ to $1.37x10^7$ cells/ml medium. These differences are likely to represent experimental variability relating to the inoculation level and Flow-FISH analysis. Supplementation with zinc enhanced the growth of bacteria at t24, but at high concentration (770 µM) the presence of zinc suppressed the growth of bacteria, as seen in Figure 7.1.

When no zinc was added, the bacteria increased 4.07-, 14.2- and 20.5-fold at t12, t24 and t48, respectively. The bacteria increased from $1.33x10^7$ cells/ml medium at t0 to $5.44x10^7$ at t12, subsequently to $1.89x10^8$ at t24 and finally $2.73x10^8$ cells/ml at t48. With 77 µM Zn, the bacterial levels showed a bigger growth increase with a 3.58-, 19.7- and 23.2-fold increase from t12 to t48. The bacterial counts increased from an initial $1.19x10^7$ cells/ml medium to $4.25x10^7$ at t12 and $2.34x10^8$ at t24. The bacteria continued to increase and peaked at t48 with $2.75x10^8$ cells/ml. When the amount of supplemented zinc was increased to 192 µM, from t12-t48, the bacteria increased 3.6-, 17.5- and 22.3-fold, accordingly. At t0, the vessel contained $1.25x10^7$ cells/ml which subsequently increased to $4.47x10^7$ at t12, $2.2x10^8$ at t24 and reached a peak of $2.79x10^8$ at t48. With 770 µM zinc added to the medium, the bacteria showed a reduced growth compared to the control and had the lowest growth among all the regimes. The bacteria only showed a 3.53-, 8.73- and 14-fold increase for t12, t24 and t48, respectively. The bacterial counts increased from $1.37x10^7$ initially to $4.82x10^7$ at t12 and continue to increase to $1.19x10^8$ and peaked at $1.91x10^8$ cells/ml at t48. The reduced growth effect was only notable at 24 and 48 h, where it contrasts markedly with the slight increase in growth caused by 77 and 192 µM Zn at 24 h (Figure 7.1).

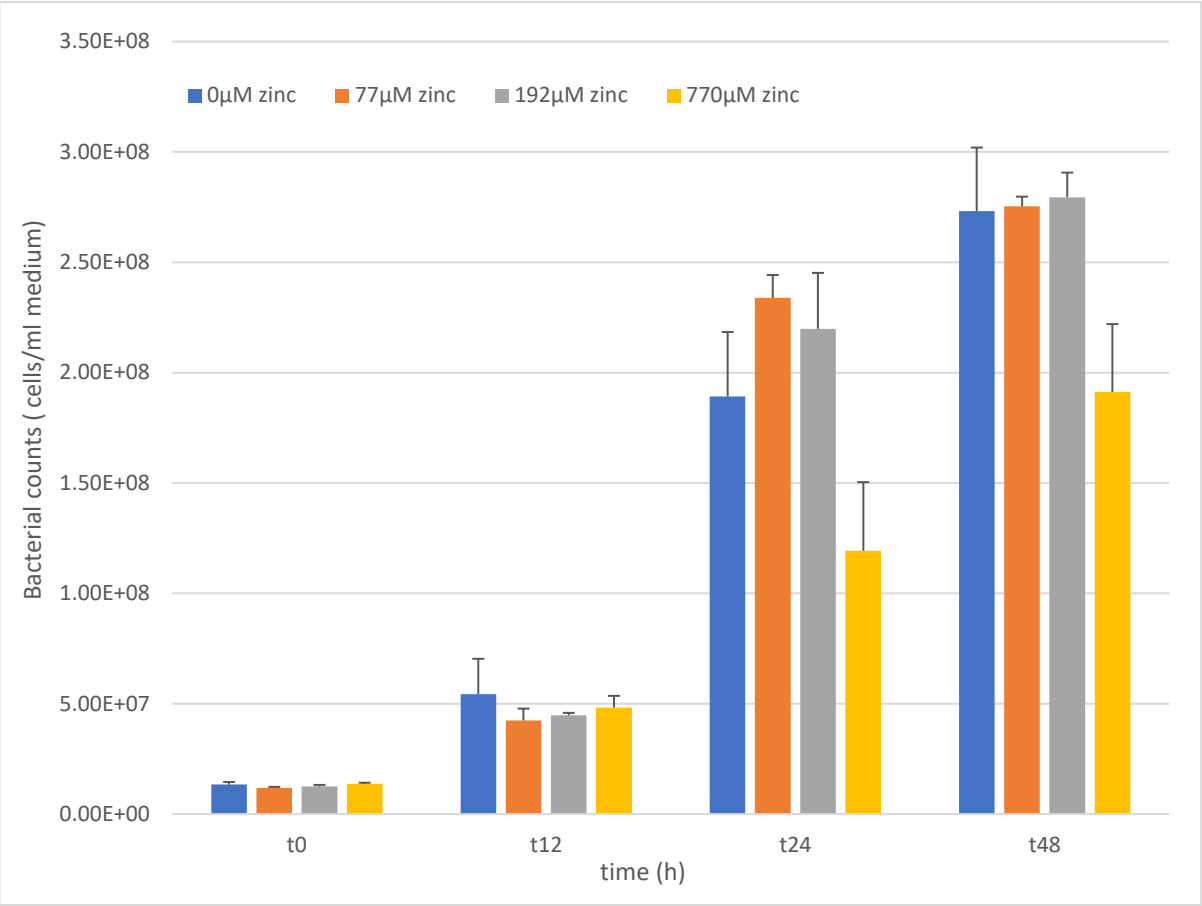


Figure 7.1 Effect of Zn regime on total bacterial counts. Total bacterial counts for each zinc regime were determined by Flow-FISH using Eub I-II-III probes. Each bar indicates a different regime: blue (no-zinc addition), orange (77 µM Zn addition), grey (192 µM Zn addition) and yellow (770 µM Zn addition). Bacterial counts are expressed as cells/ml medium. Results are average of 4 subjects and error bars represent SD. One-way Anova with Tukey Post Hoc has been employed for statistical analysis. No significant difference is seen ($p>0.05$).

As seen from tables 7.1 and 7.2, the presence of zinc has some effect on the total bacterial counts compared to the control. At t12, the control (0 µM Zn) showed higher growth than all media containing zinc (4.07- versus 3.5-3.6-fold increase, respectively), corresponding to a 1.28-, 1.22- and 1.13-fold higher growth with 77, 192 and 770 µM Zn, respectively. At t24, medium supplemented with 77 and 192 µM Zn showed 1.24- and 1.16-fold higher total bacterial counts than the control while the control is 1.59-fold higher than the medium containing 770 µM Zn. At t48, there was very little difference between the control and the growths with 77 and 192 µM Zn, but when 770 µM Zn was supplemented to the medium, the total numbers were 1.43-fold lower than the control.

In summary, the Flow-FISH results show that medium doses of Zn, matching those considered to be within normally dietary ranges, increase growth of the microbiota slightly (but not significantly) at

24 h. This effect is consistent with the essential nature of Zn as a micronutrient and would suggest that the reduced growth is a result of Zn insufficiency. However, high levels of Zn had the opposite impact on growth at both 24 and 48 h, indicating a growth inhibition effect. Such effects are consistent with the reported impact of high zinc doses used to treat infant diarrhoea in the third world and prophylactic deployment of Zn to prevent post weaning diarrhoea in pigs, although it is unclear at this stage which elements of the gut microflora have been impacted by the high Zn (Starke et al, 2013; Pieper et al, 2011; Wang et al, 2018).

Table 7.1 Total bacterial numbers at each time point and the fold difference against t0. Data are derived from Figure. 7.1 (see Figure 7.1 for details).

Zinc concentration (µM)	Bacterial counts (cells/ml medium)				Fold Difference		
	t0	t12	t24	t48	t12/t0	t24/t0	t48/t0
0	1.33E+07	5.44E+07	1.89E+08	2.73E+08	4.07	14.18	20.47
77	1.19E+07	4.25E+07	2.34E+08	2.75E+08	3.58	19.68	23.17
192	1.25E+07	4.47E+07	2.20E+08	2.79E+08	3.57	17.53	22.28
770	1.37E+07	4.82E+07	1.19E+08	1.91E+08	3.53	8.73	14.00

Table 7.2 Fold difference between different zinc regimes against the control (0 µM zinc). Data are derived from Figure. 7.1.

Zinc concentration	Fold Difference			
	t0	t12	t24	t48
77 µM/0 µM	-1.12	-1.28	1.24	1.01
192 µM/0 µM	-1.06	-1.22	1.16	1.02
770 µM/0 µM	1.02	-1.13	-1.59	-1.43

7.3 Impact of zinc on the composition on the gut microbiota in batch culture

7.3.1 Impact at phylum level

To determine whether the Zn regimes employed had any impact on the relative abundance of the gut microbiota population composition, particularly under conditions where Zn impacts total growth, 16S rRNA gene amplicon NGS analysis was performed. From the 48 samples (at 0, 24 and 48 h only) thus analysed, a total 1,345,598 reads were assigned to OTUs.

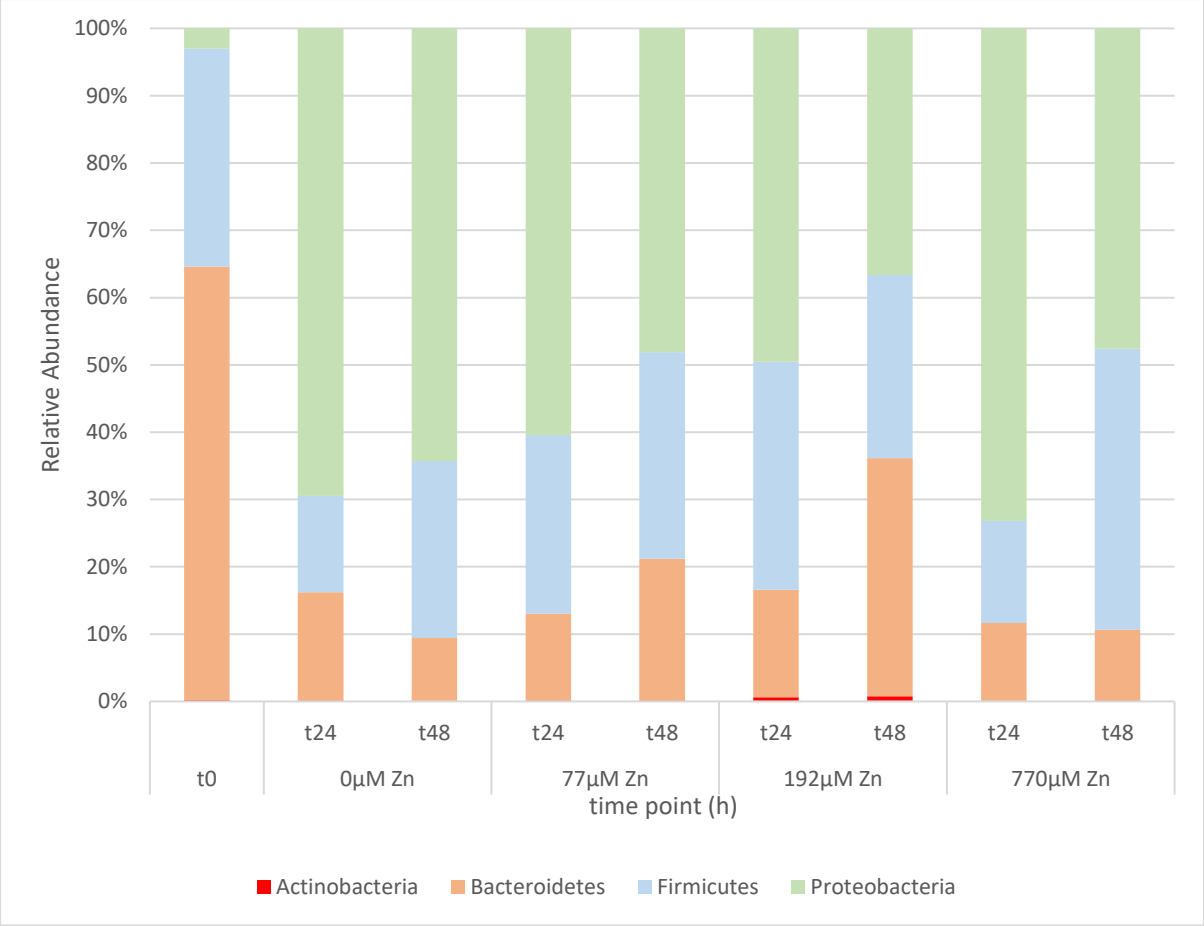


Figure 7.2 Composition of gut microbiota at phylum level. The gut microbiota is classified at phylum level for all the zinc regime (0, 77, 192 and 770 μM Zn) at both t24 and t48.

Table 7.3 Relative abundance of different bacterial groups at phylum level. The relative abundance of different bacterial groups at t0, t24 and t48 and they are expressed as a percentage of the total microbial community profile. The results are the average of 4 subjects. One-way Anova with Bonferroni Post Hoc was employed for statistical analysis. Values in bold indicates significant difference ($p<0.05$) with respect to t0 time point.

	t0	0μM Zn		77μM Zn		192μM Zn		770μM Zn	
		t24	t48	t24	t48	t24	t48	t24	t48
Actinobacteria	0.19	0.15	0.05	0.14	0.14	0.61	0.75	0.05	0.14
Bacteroidetes	61.90	**15.53**	**9.08**	**12.49**	**20.29**	**15.56**	**34.48**	**11.20**	**10.26**
Firmicutes	31.12	13.88	25.54	25.76	29.55	33.01	26.47	14.60	40.81
Proteobacteria	2.89	**67.27**	**62.38**	**58.63**	**46.36**	**48.26**	**35.70**	**70.60**	**46.49**

Table 7.4 Fold changes in abundance at phylum level with respect to t0. Fold changes of the bacteria at phylum level at t24 and t48 compared to t0. Data derived from table 7.3. One-way Anova with Bonferroni Post Hoc was employed for statistical analysis. Values in bold indicates significant difference ($p<0.05$) with respect to t0 time point.

	0μM Zn		77μM Zn		192μM Zn		770μM Zn	
	t24/t0	t48/t0	t24/t0	t48/t0	t24/t0	t48/t0	t24/t0	t48/t0
Actinobacteria	0.81	0.27	0.74	0.72	3.22	3.94	0.29	0.75

Bacteroidetes	**0.25**	**0.15**	**0.20**	**0.33**	**0.25**	**0.56**	**0.18**	**0.17**
Firmicutes	0.45	0.82	0.83	0.95	1.06	0.85	0.47	1.31
Proteobacteria	**23.27**	**21.57**	**20.28**	**16.03**	**16.69**	**12.35**	**24.42**	**16.08**

As seen from Figure 7.2, Bacteroidetes and Firmicutes dominated the entire population with a combined relative abundance of more than 90% (61.9% for Bacteroidetes, 31.1% for Firmicutes) at t0. Actinobacteria and Proteobacteria only accounted for roughly 3%. Regardless of the zinc regime, the microbiota showed similar trends throughout all the conditions tested. At t24 and t48, Proteobacteria showed a significant increase in all the regimes and became the dominant phylum ($p<0.05$), which was accompanied by a reduction of both Bacteroidetes and Firmicutes. the Bacteroidetes displayed a significant decrease in relative abundance with a change between 1.80- to 6.81-fold ($p<0.05$) across all regimes tested. The relative abundance of Firmicutes showed a decrease to a lesser extend with only a 1.05- to 2.24-fold change, but these effects were not significant when tested statistically ($p>0.05$). In the absence of zinc (control), Proteobacteria showed the greatest increase while both Bacteroidetes and Firmicutes had the biggest reduction in their relative abundance. Proteobacteria increased from 2.89 to 67.3% (23.3-fold) at t24 and dropped slightly to 62.4% (21.6-fold) at 48 h. Bacteroidetes showed a 3.99- (t24) and 6.81-fold (t48) decrease, reducing in abundance from 61.9 to 15.5% and 9.08%, respectively, while Firmicutes reduced from 31.1 to 13.9% (t24) and 25.5% (t48), showing a 2.24- and 1.22-fold decrease. Thus, the increase in Proteobacteria was at the expense of both Bacteroidetes and Firmicutes. Actinobacteria on the other hand showed a reduction between of 1.24- to 3.69-fold across all the regimes with the exception of 192 µM Zn (equivalent to 25 mg zinc/day) whereby a 3.22- and 3.94-fold increase was observed at t24 and t48, respectively, correspondingly increasing in abundance from 0.19 to 0.61% and 0.75%. In addition, at this concentration, Proteobacteria displayed the lowest increase among all the regimes tested with only a 16.7- and 12.4-fold increase at t24 and t48 compared to t0, indicating a reduced growth upon supplementation with 192 µM Zn.

Table 7.5 Changes in relative abundance between the zinc supplemented media compared to the zinc deficient medium at phylum level. The abundance data for the zinc supplemented growths (77, 192 and 770 µM) were compared with the control (0 µM) at both t24 and t48. One-way Anova with Bonferroni Post Hoc correction was employed for statistical analysis; no significant difference was seen when tested in this fashion ($p>0.05$).

	77µM Zn/0µM Zn		192µM Zn/0µM Zn		770µM Zn/0µM Zn	
	t24	t48	t24	t48	t24	t48
Actinobacteria	0.92	2.65	3.99	14.53	0.36	2.76
Bacteroidetes	0.80	2.23	1.00	3.80	0.72	1.13
Firmicutes	1.86	1.16	2.38	1.04	1.05	1.60
Proteobacteria	0.87	0.74	0.72	0.57	1.05	0.75

Firmicutes benefitted from the presence of zinc (as zinc supplementation) regardless of concentration employed relative to the control but the increases were not significant when tested statistically ($p>0.05$). Proteobacteria showed the opposite relationship whereby zinc reduced levels by 1.15- to 1.75-fold compared to the control; this effect was strongest in the presence of 192 µM Zn. The addition of 77 µM zinc to the medium caused both Actinobacteria and Bacteroidetes to decrease at t24 followed by an increase at t48. Both phyla showed a particularly notable increase in growth with 192 µM Zn whereby the abundance Actinobacteria was 3.99- and 14.5-fold higher than the control at t24 and t48, respectively, and Bacteroidetes showed a 3.80-fold higher abundance at t48 compared to the control (34.5 vs 9.08%). Interestingly, the change in Actinobacteria levels in response to zinc showed a strong negative correlation with those seen for the Proteobacteria (Figure 7.3), suggestive of a competitive relationship between these two phyla.

In summary, the community profiling results shows that the Proteobacteria increased in abundance during the course of the batch cultures. This increase was largely at the expense of the Bacteroidetes and Firmicutes phyla, indicating that the Proteobacteria outcompete other elements of the gut microbiota under the rapid growth conditions employed here. The presence of zinc generally reduced the degree of expansion for the Proteobacteria, particularly when provided at 192 µM (25 mg/day), and the Actinobacteria and Bacteroidetes gained a benefit at this concentration.

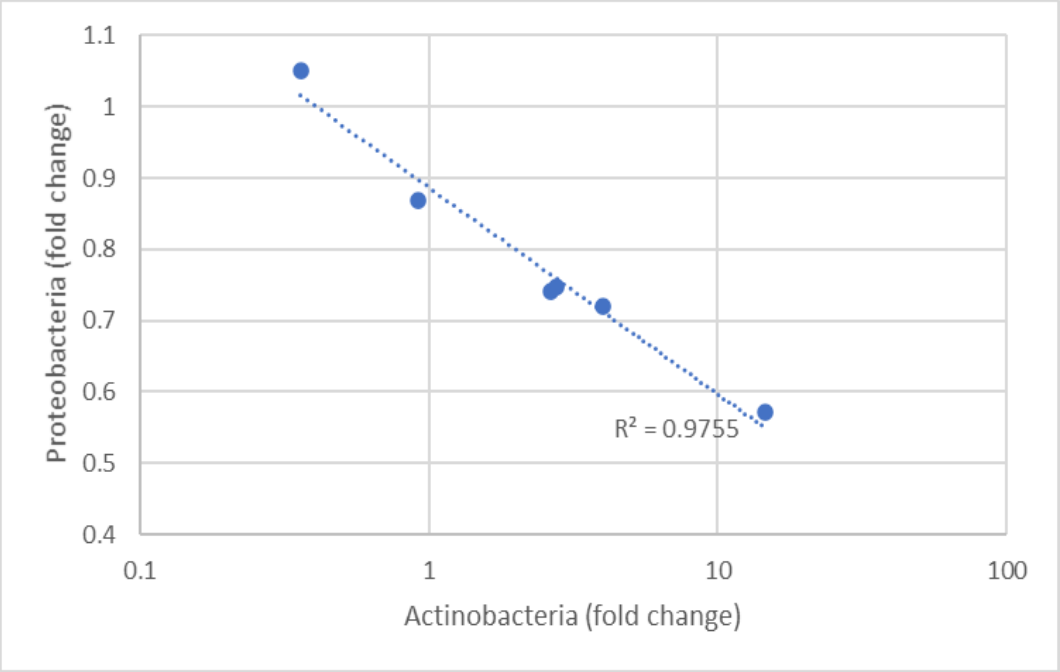


Figure 7.3 Relationship between change in Proteobacteria and Actinobacteria levels in response to zinc. Data are derived from Table 7.5. The logarithmic trendline shows a strong fit to the data points.

7.3.2 Impact of zinc on alpha and beta diversity

Alpha and beta diversity were determined using the Shannon Index (Hill et al, 2003) for the former and weighted UniFrac for the latter as both of these indices take into account the abundance for each species present in the samples rather than dominance (i.e. presence/absence of species). For alpha diversity, the higher the value of Shannon Index, the richer the species present in the sample, thus indicating a more diverse population of bacteria. Beta diversity, on the other hand, determines the variation in bacterial species between different samples; for example, the difference in the bacterial groups between the zinc regimes used in this study.

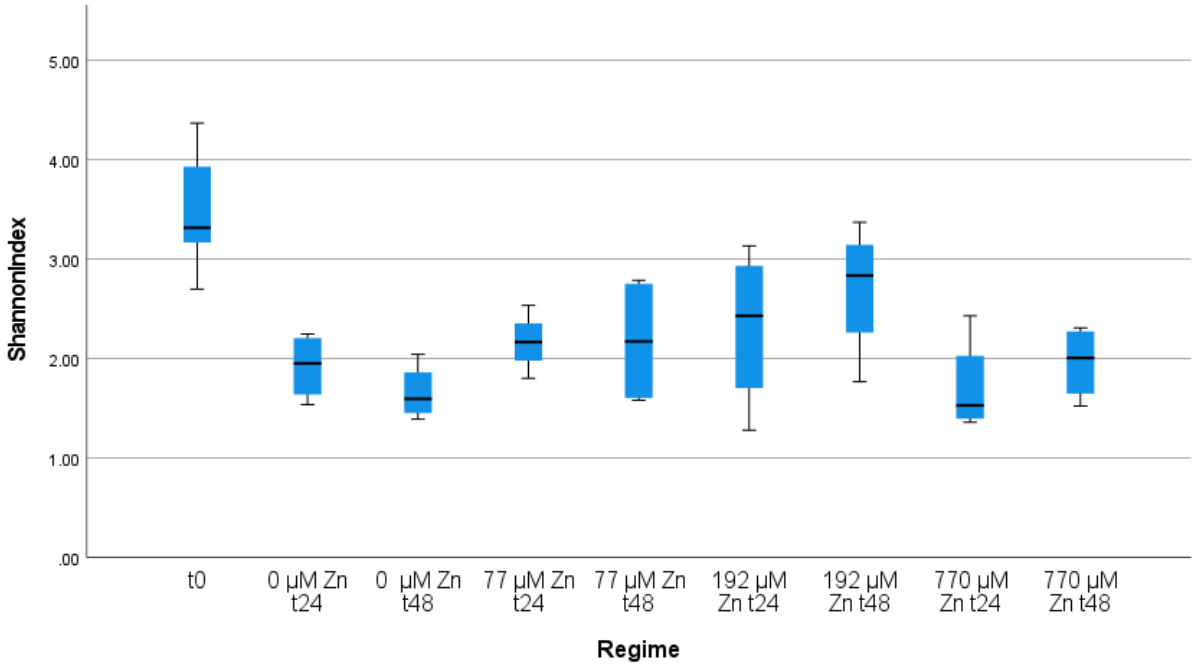


Figure 7.4 Alpha diversity measured by Shannon Index across all the regimes. A high the value in the Shannon Index indicates a high species richness in the sample tested. The Mann-Whitney U test was used for statistical analysis. No significant in diversity was is seen between the regimes tested ($p>0.05$).

As seen from Figure 7.4, all zinc regimes showed a lower diversity than that obtained at t0, as indicated by the lower reading on the Shannon Index scale. This indicates that the bacterial population suffers a loss in diversity over time regardless of the presence/absence of zinc or the zinc concentration employed. However, the presence of zinc at 77 and 192 µM Zn caused a modest increase in species richness, as shown by their higher reading compared to the control (0 µM Zn), indicating a positive effect on the diversity of the bacterial population. Zinc at 770 µM Zn showed a similar value as the control, indicating that zinc at both high and very low levels negatively affects the bacterial population in terms of richness.

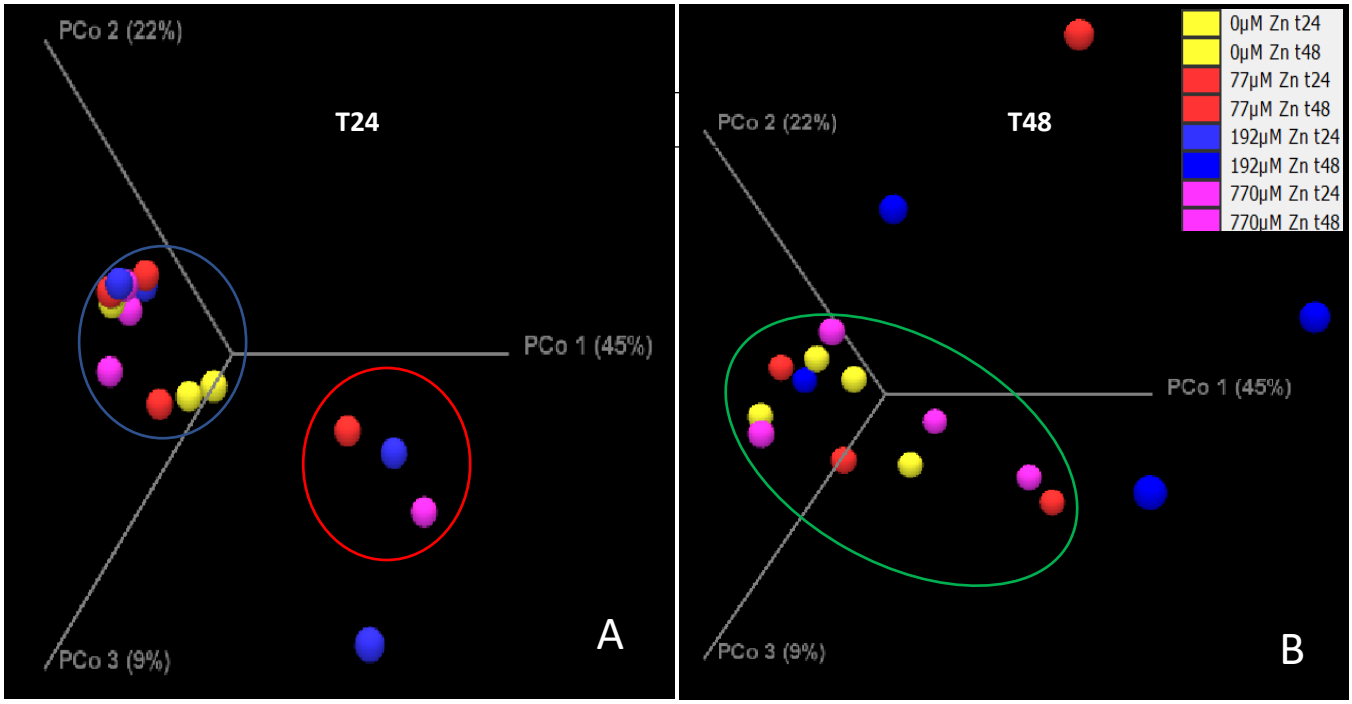


Figure 7.5 Beta diversity Principal Coordinate Analysis derived from weighted UniFrac in all samples. The beta diversity is grouped according to time point: **A**, t24 and **B**, t48. The zinc regimes are represented by the colours: 0 µM Zn (yellow); 77 µM Zn (red); 192 µM Zn (blue); 770 µM Zn (pink). For each axis, the precent of variation was reported in brackets.

As for beta diversity, there is no clear distinction between the bacterial population at t0 in all the zinc regimes tested (data not shown). However, the t24 and t48 data showed some clustering between the zinc regimes (Figure 7.5A & B). At t24 (Figure 7.5A), most of the bacteria clustered together with little difference between the zinc-containing regimes and the control (blue circle) but there are 4 outliers corresponding to 3 zinc regimes belonging to one of the subjects (red circle) and another 1 belonging to a different subject. Although the 3 zinc regimes (red circle) formed a separate cluster than the rest of the group, the results show that there was little difference between the regimes and the observed clustering is most likely due to individual variation. At t48 (Figure 7.5B), the results are a little more extreme whereby the 192 µM Zn regime showed further separation from the rest of the group. In addition, 77 µM Zn of one of the subject clustered differently than the others (being a outlier), indicating a considerable variation in the reaction towards 77 µM zinc for this donor and time point. Besides these 4 outliers, the rest of the populations seems to cluster close to each other, with 0 and 770 µM Zn displaying little difference.

7.3.3 Impact at species level

At lower taxonomic level, the impact of the distinct zinc regimes is summarised in Tables 7.6 and 7.7. Irrespective of the concentration of zinc, three species showed a significant increase in relative abundance over time in all the test conditions (*Enterococcus* sp and two Enterobacteriaceae species), whereas 9 species (3 from Bacteroidetes and 6 from Firmicutes) showed significant decreases (*Bacteroides* sp2, *Odoribacter* sp, Rikenellaceae sp, *Blautia* sp, *Faecalibacterium prausnitzii, Gemmiger formicilis*, *Lachnospira* sp, *Oscillospira* sp and Ruminococcaceae sp) (Table 7.6).

Table 7.6 Effect of zinc regime on relative abundance at the species level. The relative abundance of bacterial groups within the gut microbiota at species levels is indicated at t0, t24 and t48 , and 0 (orange), 77 (yellow), 192 (green) and 770 (grey) µM Zn. Data are expressed as a percentage of the total microbial community profile. The results are the average of 4 subjects. Only bacterial species present at >0.5% for at least one condition/time-point are shown. Values in bold indicates significant difference ($p<0.05$) with respect to t0. One-way Anova with Bonferroni Post Hoc was employed for statistical analysis. The t=0 time point data have been averaged for all 4 regimes. (sp: species, N/A: not identifiable).

Phylum	Order		t0	0µM		77µM		192µM		770µM	
				t24	t48	t24	t48	t24	t48	t24	t48
Bacteroidetes	Bacteroidales	*Bacteroides* sp1	1.20	0.35	0.47	0.30	1.25	0.50	2.24	0.27	0.54
		Bacteroides sp2	38.01	**8.34**	**6.13**	**7.07**	**5.32**	**8.38**	**12.12**	**5.83**	**4.24**
		Bacteroides coprophilus	1.38	0.80	0.04	0.71	0.43	0.67	0.17	0.86	0.03
		Bacteroides fragilis	0.32	0.10	0.03	0.09	0.13	0.41	5.77	0.02	0.10
		Bacteroides ovatus	1.84	0.92	0.78	0.83	2.03	2.60	9.91	0.68	2.55
		Bacteroides uniformis	5.23	1.47	0.89	1.08	9.51	1.00	3.52	1.16	1.82
		Odoribacter sp	1.00	**0.37**	**0.08**	**0.23**	**0.11**	**0.21**	**0.08**	**0.27**	**0.07**
		Parabacteroides sp	1.34	0.42	0.04	0.33	0.24	0.27	0.08	0.30	0.04
		Parabacteroides distasonis	0.93	0.31	0.08	0.22	0.52	0.20	0.16	0.21	0.29
		Prevotella sp	0.46	0.17	0.01	0.12	0.07	0.16	0.04	0.14	0.04
		Rikenellaceae s*p*	3.82	**1.19**	**0.37**	**0.82**	**0.31**	**0.48**	**0.20**	**0.77**	**0.35**
Firmicutes	Lactobacillales	*Enterococcus* sp	0.00	**7.55**	**8.77**	**13.53**	**15.97**	**13.83**	**6.53**	**6.18**	**21.99**
		Lactobacillus sp	0.00	0.02	2.23	0.03	0.91	0.03	0.13	0.06	2.46
		Streptococcus sp	0.10	0.03	0.01	0.52	0.21	7.60	3.44	0.37	0.54
	Clostridiales	*Acidaminococcus* sp	0.03	0.24	0.53	2.25	1.75	2.15	1.55	1.92	0.18
		Blautia sp	0.80	**0.18**	**0.07**	**0.31**	**0.09**	**0.32**	**0.15**	**0.22**	**0.03**
		Clostridiales sp	4.18	**0.72**	**0.27**	**0.70**	**0.30**	**0.58**	**0.34**	**0.59**	**0.15**
		Clostridium sp	0.29	0.05	8.11	1.36	3.81	0.30	3.98	0.05	9.19

		Clostridium bolteae	0.01	0.05	1.57	0.13	1.67	0.05	0.97	0.15	1.40
		Eubacterium biforme	0.61	0.19	0.03	0.32	0.16	0.41	0.09	0.35	0.01
		Faecalibacterium prausnitzii	6.15	**0.42**	**0.09**	**0.49**	**0.11**	**0.45**	**0.06**	**0.38**	**0.11**
		Gemmiger formicilis	1.07	**0.20**	**0.07**	**0.24**	**0.07**	**0.23**	**0.06**	**0.20**	**0.10**
		Lachnospiraceae sp	3.01	**0.43**	**0.46**	0.78	0.69	0.89	1.42	**0.57**	**0.18**
		Lachnospira sp	0.47	**0.04**	**0.02**	**0.06**	**0.01**	**0.05**	**0.01**	**0.05**	**0.00**
		Oscillospira sp	0.81	**0.25**	**0.05**	**0.23**	**0.07**	**0.16**	**0.21**	**0.14**	**0.07**
		Phascolarctobacterium sp	0.52	0.18	0.77	0.21	0.74	0.24	1.61	0.16	1.19
		Roseburia sp	0.47	**0.06**	**0.00**	0.08	**0.01**	0.06	**0.00**	**0.05**	**0.00**
		Roseburia faecis	0.50	0.12	0.05	0.09	0.02	0.05	0.02	0.12	0.03
		Ruminococcaceae sp	3.84	**1.18**	**0.23**	**1.25**	**0.39**	**1.03**	**0.23**	**0.82**	**0.33**
		Ruminococcus sp	1.21	0.29	0.06	0.34	0.15	0.36	0.11	0.20	0.03
		Ruminococcus bromii	1.54	0.42	0.13	0.71	0.19	0.54	0.18	0.54	0.03
Proteobacteria	Enterobacteriales	Enterobacteriaceae sp1	0.02	**6.78**	**6.58**	**6.31**	**4.59**	**4.54**	**3.31**	**7.60**	**4.30**
		Enterobacteriaceae sp2	0.12	**59.67**	**55.26**	**50.63**	**40.38**	**40.34**	**30.21**	**61.73**	**39.99**
	Burkholderiales	*Sutterella* sp	1.89	0.40	0.16	0.55	0.97	2.83	1.96	0.71	1.19
		N/A	3.74	3.14	2.94	2.95	3.65	2.55	2.60	3.51	2.29

Table 7.7 Fold changes in abundance at species level with respect to t0. Fold changes of the bacteria at species level at t24 and t48 compared to t0. Data derived from table 7.4. The colours indicate the different growth pattern (see Figure 7.3) that the bacteria displayed (yellow: pattern A; grey: pattern B; blue: pattern C; green: pattern D). One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. Values in bold indicates significant difference with respect to t0 ($p<0.05$)

Phylum	Order		0µM		77µM		192µM		770µM	
			t24/t0	t48/t0	t24/t0	t48/t0	t24/t0	t48/t0	t24/t0	t48/t0
Bacteroidetes	Bacteroidales	*Bacteroides* sp1	0.29	0.39	0.25	1.04	0.41	1.87	0.22	0.45
		Bacteroides sp2	**0.22**	**0.16**	**0.19**	**0.14**	**0.22**	**0.32**	**0.15**	**0.11**
		Bacteroides coprophilus	0.58	0.03	0.51	0.32	0.49	0.12	0.62	0.02
		Bacteroides fragilis	0.32	0.11	0.28	0.42	1.30	18.19	0.07	0.30
		Bacteroides ovatus	0.50	0.42	0.45	1.10	1.41	5.38	0.37	1.38
		Bacteroides uniformis	0.28	0.17	0.21	1.82	0.19	0.67	0.22	0.35
		Odoribacter sp	**0.37**	**0.08**	**0.23**	**0.11**	**0.21**	**0.08**	**0.27**	**0.07**
		Parabacteroides sp	0.31	0.03	0.25	0.18	0.20	0.06	0.22	0.03

		Parabacteroides distasonis	0.34	0.09	0.24	0.56	0.21	0.17	0.23	0.31
		Prevotella sp	0.37	0.03	0.25	0.14	0.34	0.08	0.31	0.09
		Rikenellaceae sp	**0.31**	**0.10**	**0.21**	**0.08**	**0.13**	**0.05**	**0.20**	**0.09**
Firmicutes	Lactobacillales	*Enterococcus* sp	**1725.22**	**2004.54**	**3091.99**	**3649.81**	**3160.51**	**1491.23**	**1413.17**	**5025.57**
		Lactobacillus sp	18.58	2558.79	30.43	1039.32	35.04	152.31	66.98	2823.40
		Streptococcus sp	0.28	0.13	5.09	2.02	73.90	33.43	3.59	5.26
	Clostridiales	*Acidaminococcus* sp	7.85	17.08	72.33	56.18	69.03	49.71	61.52	5.63
		Blautia sp	**0.22**	**0.09**	**0.39**	**0.11**	**0.39**	**0.19**	**0.27**	**0.04**
		Clostridiales sp	**0.17**	**0.06**	**0.17**	**0.07**	**0.14**	**0.08**	**0.14**	**0.04**
		Clostridium sp	0.16	28.06	4.70	13.17	1.02	13.76	0.17	31.77
		Clostridium bolteae	8.36	250.54	20.04	265.84	7.77	153.93	23.54	223.18
		Eubacterium biforme	0.32	0.04	0.52	0.26	0.67	0.15	0.58	0.02
		Faecalibacterium prausnitzii	**0.07**	**0.01**	**0.08**	**0.02**	**0.07**	**0.01**	**0.06**	**0.02**
		Gemmiger formicilis	**0.19**	**0.06**	**0.23**	**0.07**	**0.21**	**0.06**	**0.18**	**0.09**
		Lachnospiraceae sp	**0.14**	**0.15**	0.26	0.23	0.30	0.47	**0.19**	**0.06**
		Lachnospira sp	**0.09**	**0.04**	**0.13**	**0.02**	**0.10**	**0.03**	**0.11**	**0.01**
		Oscillospira sp	**0.30**	**0.07**	**0.28**	**0.09**	**0.20**	**0.26**	**0.18**	**0.08**
		Phascolarctobacterium sp	0.35	1.48	0.41	1.43	0.46	3.11	0.31	2.30
		Roseburia sp	**0.13**	**0.01**	0.17	**0.01**	0.12	**0.01**	**0.12**	**0.00**
		Roseburia faecis	0.23	0.10	0.18	0.04	0.09	0.04	0.23	0.06
		Ruminococcaceae sp	**0.31**	**0.06**	**0.33**	**0.10**	**0.27**	**0.06**	**0.21**	**0.09**
		Ruminococcus sp	0.24	0.05	0.28	0.13	0.30	0.09	0.17	0.03
		Ruminococcus bromii	0.27	0.09	0.46	0.12	0.35	0.12	0.35	0.02
Proteobacteria	Enterobacteriales	Enterobacteriaceae sp1	**369.27**	**358.43**	**343.39**	**249.77**	**247.31**	**180.26**	**413.81**	**234.33**
		Enterobacteriaceae sp2	**516.58**	**478.34**	**438.32**	**349.60**	**349.25**	**261.49**	**534.37**	**346.21**
	Burkholderiales	*Sutterella* sp	0.21	0.08	0.29	0.51	1.50	1.04	0.37	0.63
		N/A	0.84	0.78	0.79	0.98	0.68	0.69	0.94	0.61

In order to consider the impact of zinc regime on change in abundance at species level more clearly, the observed changes in abundance for each species were grouped according to the pattern of change in abundance seen over time. In this way, in the absence of zinc supplementation (0 µM Zn), four distinct change-in-abundance patterns were observed (Figure 7.6 and 7.7). The most common

pattern (growth pattern A; 21 species; Figure 7.6A) was characterised by a continuous decrease in relative abundance at t24 and t48 whereby a bigger reduction is seen in the latter time point compared to the former.

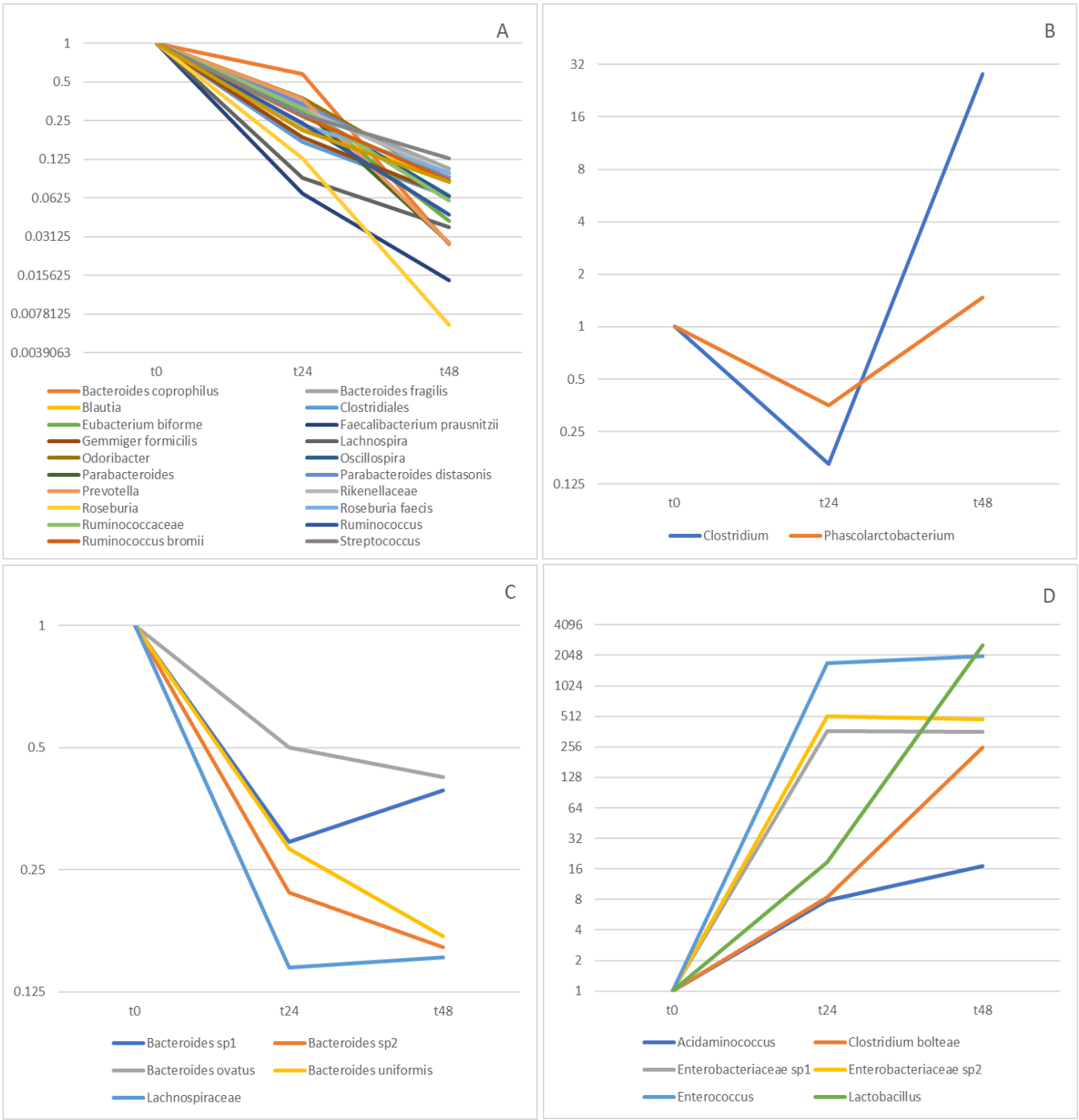


Figure 7.6 Change in abundance patterns of the most common bacterial species over time (0-48 h) during growth with 0 µM Zn supplementation. The data from Table 7.5 were plotted to determine similar patterns of change. In this way, all 34 species could be grouped into four distinct growth pattern types: **A**, decrease in abundance at both t24 and t48. **B**, slight decrease in abundance followed by major increase. **C**, major decrease followed by minor decrease or slight increase. **D**,

increase in abundance followed by little change of continued increase. The fold differences are expressed as $\log_2$.

This group includes *Roseburia* sp*, Faecalibacterium prausnitzii*, *Bacteroides coprophilus, Parabacteroides* sp and *Prevotella* sp which showed the greatest change (decrease) in their relative abundance among all the other bacterial groups exhibiting growth pattern A. *Roseburia* sp showed a significant decrease of 7.84- (t24) and 154-fold (t48), reducing its abundance from 0.47 to 0.06 and 0.003%, respectively. *F. prausnitzii* was amongst the most abundant species at t0 at 6.15% but its abundance dropped significantly to 0.42 and 0.09% at t24 and t48 ($p<0.05$), showing a 14.8- and 69.3-fold reduction. *B. coprophilus, Parabacteroides* sp and *Prevotella* sp showed similar decreases of between 1.72- to 3.22-fold at t24 and between 35.5- to 36.7-fold at t48, respectively, but neither species showed a significant decrease ($p>0.05$). Among the other bacteria that displayed growth pattern A, 7 bacterial groups showed a significant decrease (*Blautia* sp, *Gemmiger formicilis*, *Lachnospira* sp, *Oscillospira* sp, *Odoribacter* sp, Rikenellaceae sp and Ruminococcaceae sp) with a reduction between 2.67- to 11.2-fold at t24 and between 10.4- to 26.9-fold at t48 ($p<0.05$). The rest of the bacteria showed a decrease between 1.72- to 4.27-fold at t24 and between 7.9- to 36.7-fold, but these changes were not significant when tested statistically ($p>0.05$).

As seen from Figure 7.6B, *Clostridium* sp and *Phascolarctobacterium* sp were the only 2 bacterial groups that showed a drop at t24 followed by a recovery to achieve final abundancies at t48 that are greater than those seen at t0 (growth pattern B). *Clostridium* sp dropped from 0.29 to 0.05% (6.1-fold) at t24 but increased 28.1-fold at t48 to 8.11%. *Phascolarctobacterium* sp displayed an initial drop of 2.8-fold from 0.52 to 0.18% but increased to 0.77% (1.5-fold) at t48.

Bacteroides sp1 and sp2, *Bacteroides fragilis, Bacteroides ovatus*, *Bacteroides uniformis* and an unknown species belonging to the Lachnospiraceae all showed a drop in abundance at t24 followed by either a modest increase or drop at t48 with abundance remaining below that at t0, and an overall abundance change of less than 10-fold (growth pattern C; Figure 7.6C). *Bacteroides* sp2 was

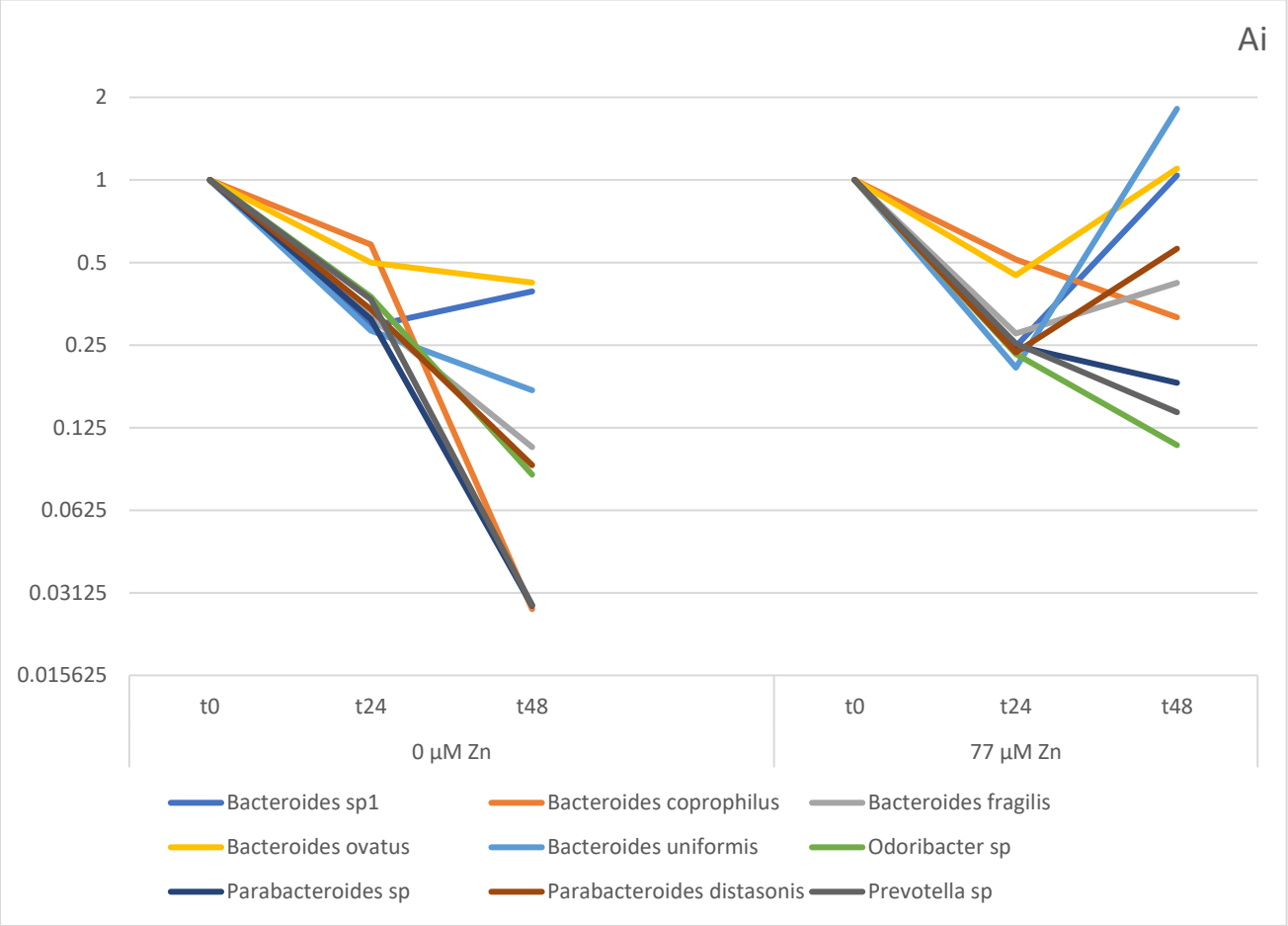
the most abundant species at t0 at 38.01% but decreased significantly at both t24 and t48 to 8.34% (4.6-fold) and 6.13% (6.2-fold), respectively ($p<0.05$). The remaining 5 growth pattern C species showed a decrease of between 2- and 9.42-fold but these were not significant ($p>0.05$).

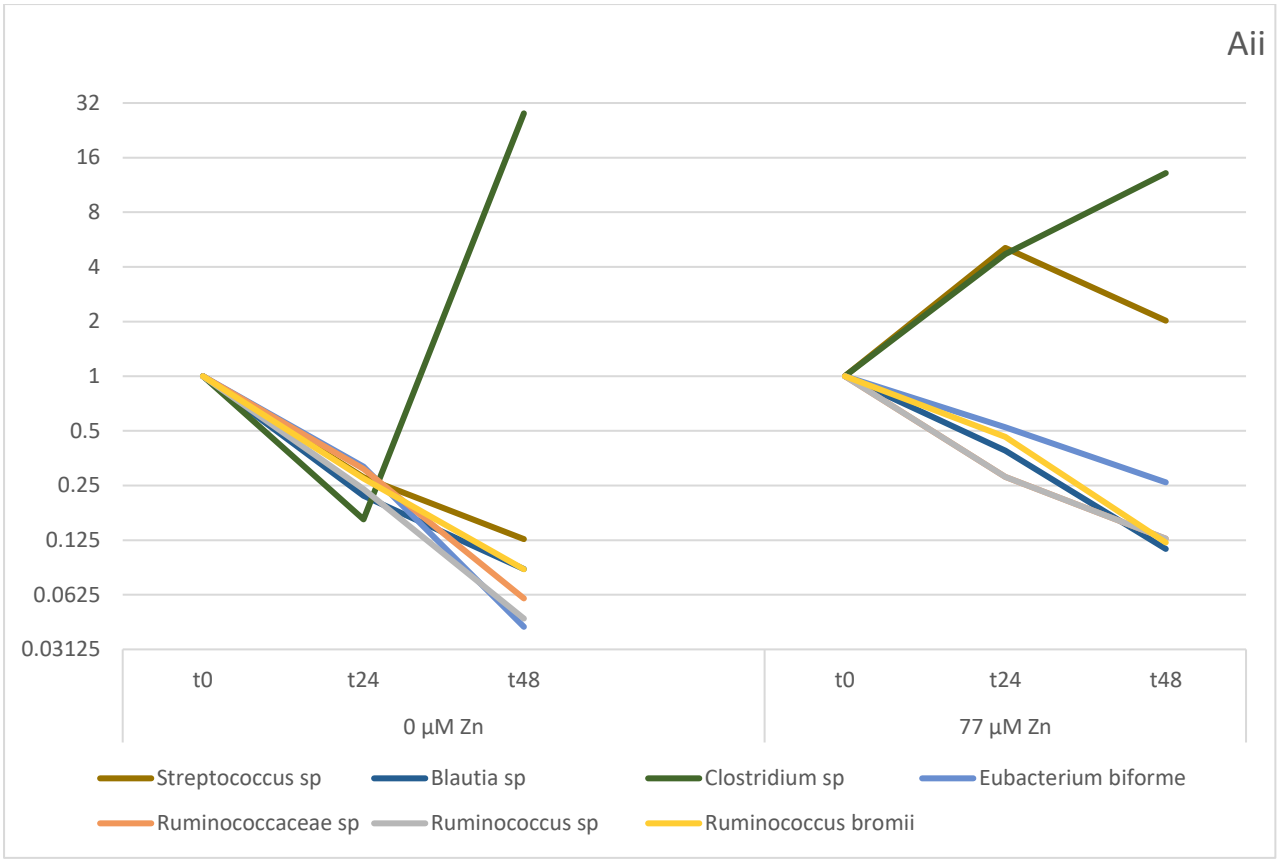
Acidaminococcus, *Lactobacillus*, *Clostridium bolteae*, *Enterococcus*, and Enterobacteriaceae sp1 and sp2 showed increases in abundance at both t24 and t48 with their relative abundance increased with respect to t0 (growth pattern D; Figure 7.6D). *Enterococcus*, and Enterobacteriaceae sp1 and sp2 showed significant increases at t24 and t48 ($p<0.05$) with *Enterococcus* increasing from 0% to 7.55% (1725.2-fold) and 8.77% (2000-fold); Enterobacteriaceae sp1 increased from 0.02 to 6.78% (369-fold) and 6.58% (358-fold); Enterobacteriaceae sp2 increased from 0.12 to 59.7% (517-fold) and 55.3% (478-fold), respectively. Although *Acidaminococcus*, *Lactobacillus* and *C. bolteae* showed an increase at both time point, these were not significant when tested statistically ($p>0.05$).

7.3.3.1 Impact of 77 µM zinc on the change-in-abundance patterns of the gut microbiota over time

To determine the impact of zinc regime on the gut microbiota at species level, the change in abundance patterns obtained over time were compared with those observed at 0 µM Zn. Half of the major bacterial groups (Figure. 7.7) showed a change in growth pattern in the presence of 77 µM Zn; these include 9 groups for the Bacteroidetes, 7 from the Firmicutes and 1 group from the Proteobacteria. This indicates that the addition of zinc had a bigger impact on bacteria belonging to the Bacteroidetes phylum than to groups from any of the other phyla. As seen from Figure 7.7Ai, *Bacteroides* sp1, *Bacteroides ovatus* and *Bacteroides uniformis* changed from growth pattern C (decrease at t24 with modest increase or decrease at t48) to growth pattern B (drop at t24 followed by recovery at t48). These species showed an initial decrease of between 2.23- and 4.83-fold at t24 followed by a modest increase of between 1.04- and 1.82-fold at t48; however, none of the changes were significant ($p>0.05$). *Bacteroides coprophilus, Bacteroides fragilis, Odoribacter* sp, *Parabacteroides* sp, *Parabacteroides distasonis* and *Prevotella* sp slightly benefitted from the addition of zinc whereby they changed from growth pattern A (continuous decrease over time) to growth pattern C (decrease at t24 followed by modest increase or decrease at t48) and experienced

a smaller decrease in their relative abundance as compared to that seen in the absence of zinc. This is seen for both *Bacteroides coprophilus* and *Prevotella* sp, particularly at t48 where they showed only a 3.17- and 7.03-fold (77 µM Zn) decrease as compared to 36.7- and 35.5-fold (0 µM Zn), respectively. Overall, this bacterial group showed a decrease between 1.95- to 4.31-fold at t24 and between 1.78-9.26-fold at t48, with *Odoribacter* sp being the only bacterial species that had a significant change in its relative abundance ($p<0.05$) compared to t0.

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Ruminococcus sp
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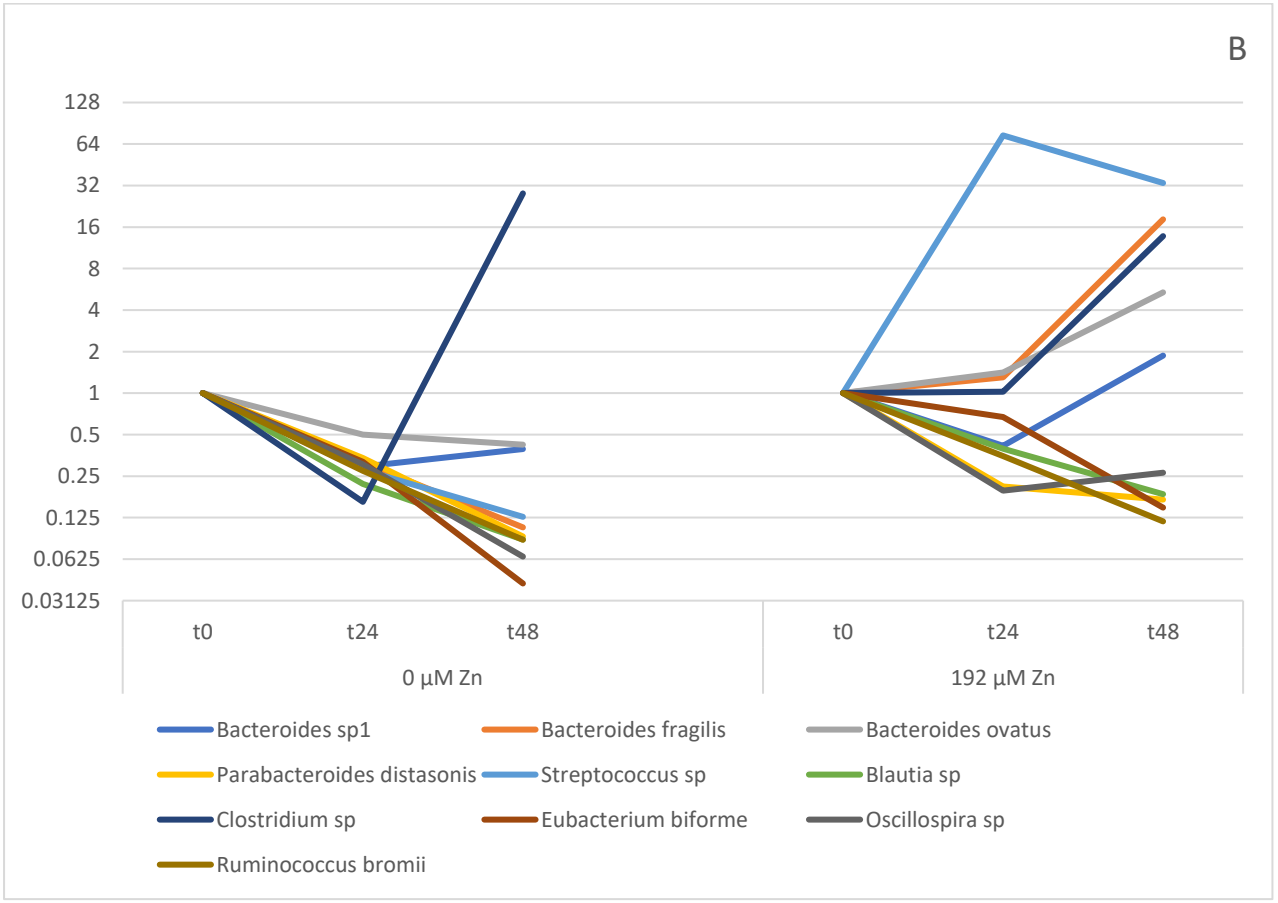
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Bacteroides fragilis
Bacteroides ovatus
Parabacteroides distasonis
Streptococcus sp
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Oscillospira sp
Ruminococcus bromii

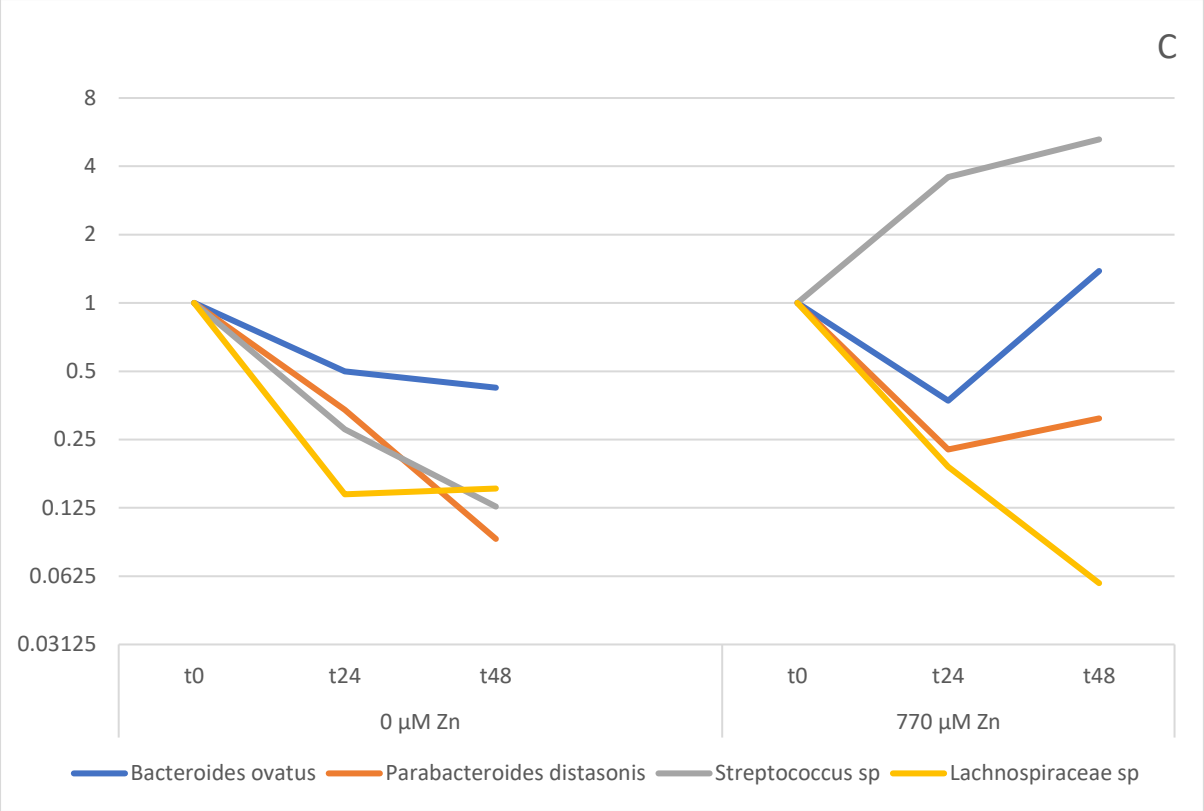


Figure 7.7 Change in growth pattern over time (0-48h) in the presence of zinc supplementation. The data from Table 7.5 were plotted to determine the effect of zinc on the growth pattern of the bacterial groups. Only bacterial groups that showed a change in growth are shown when different concentrations of zinc were added to the medium: **Ai,** 77 µM Zn for Bacteroidetes. **Aii,** 77 µM Zn for Firmicutes **B,** 192 µM Zn. **C,** 770 µM Zn. The original growth pattern is shown on the left of each graph while the changed pattern is shown on the right. The fold differences are expressed as $\log_2$.

As for the Firmicutes phylum (Figure 7.7Aii), *Streptococcus* sp and *Clostridium* sp displayed growth trend D (increase in abundance at t24 and t48) in the presence of 77 µM Zn. *Streptococcus* sp increased from 0.10 to 0.52% (5.09-fold) and 0.21% (2.02-fold) for t24 and t48 respectively while *Clostridium* sp increased from 0.29 to 1.36% (4.70-fold) and 3.81% (13.17-fold) for the same time point but neither species showed a significant difference when tested ($p>0.05$). The other 5 bacterial groups (*Blautia* sp, *Eubacterium biforme,* Ruminococcaceae sp, *Ruminococcus* sp and *Ruminococcus bromii*) also benefitted from the presence of zinc where they changed from growth pattern A to growth pattern C, but the effect was not as pronounced as seen for the Bacteroidetes. The bacterial groups showed a decrease of between 1.91- to 9.79-fold over time with only *Blautia* sp and Ruminococcaceae sp having a significant change in their relative abundance. *Blautia* sp decreased

2.57- and 8.94-fold, reducing in abundance from 0.80 to 0.31% (t24) and 0.09% (t48) while Ruminococcaceae sp reduced 3.08- and 9.79-fold, lowering in abundance from 3.84 to 1.25% (t24) and 0.39% (t48). *Sutterella* was the only species from the Proteobacteria phylum that showed a change in growth pattern, moving from growth pattern A to growth pattern C whereby it showed a 3.40- and 1.95-fold decrease at t24 and t48 as compared to 4.71- and 11.9-fold in the 0 µM Zn regime. Among all the bacterial groups, only 8 groups followed growth pattern A in the presence of 77 µM Zn whereas 21 groups followed this trend in the absence of zinc. Among those 8 groups, *Oscillospira* sp, *Faecalibacterium prausnitzii, Lachnospira* sp and Rikenellaceae sp showed a significant change in their relative abundance. *Oscillospira* sp decrease from 0.81 to 0.23% (3.5-fold) and 0.07% (11.6-fold) and *F. prausnitzii* dropped from 6.15 to 0.49% (12.4-fold) and 0.11% (55.8-fold) for t24 and t48, respectively ($p<0.05$). *Lachnospira* sp had a 7.77- and 40.4-fold reduction, reaching 0.06% (t24) and 0.01% (t48) from 0.47%. Rikenellaceae sp on the other hand dropped 4.68- and 12.2-fold, reducing in abundance from 3.82 to 0.82% (t24) and 0.31% (t48). Enterobacteriaceae sp1 and sp2, and *Enterococcus* showed a significant increase ($p<0.05$) at both t24 and t48 with the addition of 77 µM Zn slightly reducing the relative abundance of the Enterobacteriaceae species while enhancing the abundance of the *Enterococcus* sp. Enterobacteriaceae sp1 increased from 0.02 to 6.31% (343-fold) at t24 and 4.59% (250-fold) at t48 while Enterobacteriaceae sp2 increased from 0.12 to 50.6% (438-fold) and 40.4% (350-fold) for the same time point. *Enterococcus* sp increased 3090- and 3640-fold to 13.5% (t24) and 16.0% (t48). *Bacteroides* sp2, which was the most abundant bacteria at t0, showed a significant decrease to 7.07% (5.4-fold) and 5.32% (7.1-fold) at t24 and t48, respectively, similar to what is observed in the zinc deficient medium.

7.3.3.2 Impact of 192 µM zinc on the change-in-abundance patterns of the gut microbiota over time

The growth pattern of the bacterial groups in the presence of 192 µM Zn resembles that of 0 µM Zn but there were some notable changes (table 7.7; Figure 7.7B).

Bacteroides fragilis, Bacteroides ovatus, Streptococcus sp and *Sutterella* sp all moved to growth pattern D (increase in relative abundance at t24 and t48) and greatly benefitted from the addition of

zinc as shown by the highest relative abundance obtained among all the regimes tested. *Streptococcus* sp showed the greatest increase with 73.9- (t24) and 33.4-fold (t48) increases, increasing its abundance from 0.10 to 7.60% and 3.44%, respectively. The other bacterial species showed an increase between 1.30- to 18.2-fold over time but none of these bacterial groups showed a significant change when tested statistically ($p>0.05$). While these 4 bacterial species benefitted from zinc, supplementation with 192 µM Zn caused both Enterobacteriaceae sp1 and sp2 to have the lowest relative abundance among all the regimes at only 4.54% (t24) and 3.31% (t48) for the former and 40.3% (t24) and 30.2% (t48) for the latter, even though they were still significantly increased in abundance as compared to t0. Although *Bacteroides* sp2 also showed a significant decrease in its relative abundance, similar to observed in the other conditions, the presence of 192 µM Zn caused the lowest reduction as indicated by the highest relative abundance compared to the other conditions. *Bacteroides* sp2 was the most abundant species at t0 with a relative abundance of 38.0% and showed a 4.5- and 3.1-fold reduction, lowering its abundance to 8.38 and 12.1%, respectively, with 192 µM zinc. *Oscillospira* sp changed from growth pattern A (continuous decrease over time) to growth pattern C (decrease at t24 followed by modest increase or decrease at t48) and showed a similar pattern as *Bacteroides* sp2, with the lowest decrease in 192 µM zinc among all the regimes. The relative abundance showed a significant drop of 5.11- and 3.78-fold for t24 and t48, reducing its abundance from 0.81 to 0.20% and 0.26%, respectively. *Bacteroides coprophilus, Bacteroides uniformis, Odoribacter* sp, *Parabacteroides* sp, *Prevotella* sp, Ruminococcaceae sp and *Ruminococcus* sp returned to their original growth pattern A (growth pattern C for *Bacteroides uniformis*), as seen at 0 µM Zn, despite having a changed growth pattern with 77 µM Zn. These species showed a decrease of 2.06- to 5.21-fold at t24 and 1.49- to 17.2-fold at t48, but only *Odoribacter* sp showed a significant decrease of 4.76- (t24) and 12.9-fold (t48), reducing in abundance from 1.00 to 0.21% and 0.08% ($p<0.05$).

Blautia sp, *Eubacterium biforme, Ruminococcus bromii* and *Parabacteroides distasonis* maintained the growth pattern (growth pattern C) seen with 77 µM Zn, rather than reverting back to growth

pattern A as seen with 0 µM Zn. These bacteria showed a decrease in their relative abundance of 1.49- to 6.76-fold over a 48-hour time period with only *Blautia* sp showing a significant change in its relative abundance ($p<0.05$). In the 192 µM Zn supplemented medium, the majority of the bacterial groups (13) showed growth pattern A, an increase from the 8 groups in 77 µM Zn, but still lower than the 21 groups with this pattern in the 0 µM Zn condition. Among the 12 pattern A bacterial groups in 192 µM Zn, 6 species showed a significant decrease in their relative abundance, namely *Faecalibacterium prausnitzii, Gemmiger formicilis, Lachnospira* sp, *Odoribacter* sp, Rikenellaceae sp and Ruminococcaceae sp. *Faecalibacterium prausnitzii* showed the biggest decrease among these bacteria and also between all the regimes tested with 13.81- and 100.05-fold decrease for t24 and t48, having a relative abundance of only 0.45% and 0.06%, respectively ($p<0.05$). *Roseburia* showed only a significant decrease at t48 with a 97.1-fold decrease ($p<0.05$) but not at t24 when it showed a 8.00-fold reduction.

7.3.3.3 Impact of 770 µM zinc on the change-in-abundance patterns of the gut microbiota over time

As seen from Figure 7.7C, at high zinc concentration (770 µM Zn), the bacterial groups showed similar growth patterns as in the zinc deficient medium with the majority of the bacterial species displaying growth pattern A (continuous decrease over time) (19 groups at 770 µM Zn vs 21 groups at 0 µM Zn). Among these groups, 10 species showed a significant decrease between 3.70- to 235-fold over time with 2 species belonging to the Bacteroidetes phylum (*Odoribacter* sp and Rikenellaceae sp) and 8 from the Firmicutes phylum (*Blautia* sp, *Faecalibacterium prausnitzii, Gemmiger formicilis,* Lachnospiraceae sp, *Lachnospira* sp, *Oscillospira* sp, *Roseburia* sp and Ruminococcaceae sp) ($p<0.05$).

Roseburia sp had the highest decrease among these bacterial groups as well as among all the regimes tested with a 8.58- and 235-fold decrease at t24 and t48, reducing in abundance from 0.47 to 0.05% and 0.002%, respectively. *Lachnospira* sp showed the second highest decrease and, similar to *Roseburia* sp, the presence of high zinc caused the greatest reduction among all the regimes. Its relative abundance reduced from 0.47 to 0.05% (t24) and 0.004% (t48), a 9.40- and 112 -fold

reduction, accordingly. The rest of the bacterial groups that showed growth pattern A had a decrease between 1.61- to 14.6-fold at t24 and between 3.30- to 48.7-fold at t48. Although not significant, the presence of high zinc caused a few bacterial groups to experience the greatest reduction among all the conditions tested, namely *Bacteroides coprophilus, Bacteroides fragilis,* Clostridales sp, *Eubacterium biforme, Ruminococcus* sp and *Ruminococcus bromii.* Despite showing no changes in growth pattern throughout all regimes, *Bacteroides* sp2 also had the greatest decrease (6.52- and 8.96-fold), reducing its abundance from 38.0 to 5.83% (t24) and 4.24% (t48). With these bacterial groups showed a substantial decrease, *Enterococcus* sp and both Enterobacteriaceae sp1 and sp2 gained an advantage as shown by having their highest relative abundance levels among all the other regimes, with a combined abundance of over 60%. These latter 3 bacterial species showed a significant increase of 412- to 5030-fold over time with *Enterococcus* sp having the greatest fold change ($p<0.05$). Other bacterial groups that showed an increase (although not significant) in the presence of 770 μM Zn include *Lactobacillus* sp, *Streptococcus* sp, *Acidaminococcus* sp and *Clostridium bolteae* with *Lactobacillus* showing the biggest increase (67.0-fold, t24; and 2820-fold, t48) while the rest had an increase of 3.59-61.5-fold at t24 and 5.26- to 223-fold at t48. *Parabacteroides distasonis* and *Sutterella* gained some slight advantage in the presence of 770 μM Zn whereby their growth pattern changed from A to C, showing a smaller decrease over time when zinc was added. The former bacteria only decreased 4.42- (t24) and 3.23-fold (t48) as compared to 2.96- and 11.0-fold while the latter decreased 2.67- (t24) and 1.59-fold (t48) compared to 4.71- and 11.9-fold in the absence of zinc. *Bacteroides* sp1, *Clostridium* sp, *Eubacterium biforme* and *Ruminococcus bromii* returned to their original growth pattern, seen with 0 μM Zn, despite showing a different growth pattern in the presence of 77 and 192 μM Zn.

7.4 Changes in relative abundance with zinc

As seen from table 7.8, the addition of zinc generally caused Bacteroidetes species to decrease at t24 but subsequently increase at t48. As for Firmicutes, mixed results were obtained but most species showed an increase with low zinc (77 μM Zn) whereas the addition of 770 μM Zn caused a

decrease in their relative abundance. In the Proteobacteria phylum, *Sutterella* showed an increase in the presence of zinc regardless of concentration (Figure 7.8) while the Enterobacteriaceae generally showed a decrease across the regimes tested.

Table 7.8 Changes in relative abundance between the zinc supplemented media compared to the zinc deficient medium. The abundance data for the zinc supplemented growths (77, 192 and 770 µM) were compared with the control (0 µM) at both t24 and t48. Numbers with green background indicate an increase and numbers with red background indicate a decrease compared to the control. One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. Values in bold indicates significant difference ($p<0.05$) with respect to the control at the same time point.

Phylum	Order		77µM/0 µM Zn		192µM/0 µM Zn		770µM/0 µM Zn	
			t24	t48	t24	t48	t24	t48
Bacteroidetes	Bacteroidales	*Bacteroides* sp1	0.84	2.64	1.41	4.76	0.76	1.16
		Bacteroides sp2	0.85	0.87	1.00	1.98	0.70	0.69
		Bacteroides coprophilus	0.88	11.59	0.83	4.51	1.07	0.75
		Bacteroides fragilis	0.87	3.97	4.13	171.35	0.22	2.85
		Bacteroides ovatus	0.90	2.60	2.82	**12.72**	0.74	3.27
		Bacteroides uniformis	0.73	10.62	0.68	**3.94**	0.79	2.03
		Odoribacter sp	0.62	1.28	0.56	0.92	0.72	0.79
		Parabacteroides sp	0.80	6.51	0.65	2.07	0.72	1.13
		Parabacteroides distasonis	0.70	6.16	0.62	1.85	0.67	3.39
		Prevotella sp	0.69	5.05	0.93	2.80	0.84	3.26
		Rikenellaceae sp	0.68	0.85	0.41	0.54	0.65	0.95
Firmicutes	Lactobacillales	*Enterococcus* sp	1.79	1.82	1.83	0.74	0.82	2.51
		Lactobacillus sp	1.64	0.41	1.89	0.06	3.61	1.10
		Streptococcus sp	18.35	15.99	266.32	264.07	12.92	41.51
	Clostridiales	*Acidaminococcus* sp	9.22	3.29	8.80	2.91	7.84	0.33
		Blautia sp	1.77	1.29	1.80	2.14	1.23	0.41
		Clostridiales sp	0.98	1.11	0.81	1.29	0.82	0.57
		Clostridium sp	28.92	0.47	6.28	0.49	1.02	1.13
		Clostridium bolteae	2.40	1.06	0.93	0.61	2.82	0.89
		Eubacterium biforme	1.65	6.25	2.12	3.56	1.82	0.56
		Faecalibacterium prausnitzii	1.19	1.24	1.07	0.69	0.91	1.21
		Gemmiger formicilis	1.23	1.02	1.14	0.89	0.99	1.42
		Lachnospiraceae sp	1.80	1.50	2.06	3.09	1.32	0.38
		Lachnospira sp	1.44	0.67	1.13	0.69	1.19	0.24
		Oscillospira sp	0.94	1.32	0.64	4.06	0.58	1.23
		Phascolarctobacterium sp	1.17	0.97	1.32	2.10	0.89	1.56

		Roseburia sp	1.31	1.79	0.98	1.59	0.91	0.65
		Roseburia faecis	0.76	0.36	0.39	0.41	1.00	0.60
		Ruminococcaceae sp	1.05	1.71	0.87	1.01	0.69	1.44
		Ruminococcus sp	1.17	2.76	1.25	1.94	0.71	0.58
		Ruminococcus bromii	1.70	1.40	1.28	1.36	1.29	0.25
Proteobacteria	Enterobacteriales	Enterobacteriaceae sp1	0.93	0.70	0.67	0.50	1.12	0.65
		Enterobacteriaceae sp2	0.85	0.73	0.68	0.55	1.03	0.72
	Burkholderiales	*Sutterella* sp	1.38	6.10	7.06	12.36	1.76	7.49

At species level, most of the species in the Bacteroidetes phylum shared a similar response to zinc, except Rikenellaceae sp and *Odoribacter* sp where a modest decrease (1.05- to 2.47-fold) in the presence of zinc was observed. However, the presence of 77 µM Zn caused a 1.28-fold increase at t48 for *Odoribacter* sp. The rest of the species in this phylum showed overall increases in response to zinc with *Bacteroides fragilis* having the biggest increase (average of 30.6-fol)d while an average increase of 1.01- to 3.84-fold was seen for the remaining Bacteroidetes species. *Bacteroides coprophilus, Bacteroides uniformis, Parabacteroides* sp, *Parabacteroides distasonis* and *Prevotella* sp showed the greatest increase when 77 µM Zn was provided as compared to the other 2 concentration used. These species displayed on average increase of 2.87- to 6.24-fold. *Bacteroides* sp1*, Bacteroides* sp2*, B. fragilis* and *B. ovatus* gave the greatest increase in the presence of 192 µM Zn with *B. fragilis* showing the biggest increase (4.13-fold for t24 and 171-fold for t48) followed by *B. ovatus* with 2.82-fold for t24 and a significant increase of 12.7-fold at t48 ($p<0.05$). *Bacteroides* sp1 and sp2 had a more modest increase with an average of 3.09- and 1.49-fold, respectively. However, at 192 µM Zn *B. uniformis* showed a significant increase of 3.94-fold at t48 in the presence of zinc ($p<0.05$).

When the highest level of zinc (770 µM Zn) was used, roughly half of the Bacteroides species displayed an increase whilst the other half showed a decrease. *B. fragilis, B. ovatus, B. uniformis, P.*

distasonis and *Prevotella* sp showed, on average, a modest increase of 1.41- to 2.05-fold while the rest of the bacteria displayed a decrease of 1.04- to 1.44-fold. Although the presence of zinc generally supports the growth of members in the Bacteroidetes phylum, it is worth noting that the increase in abundance was mainly seen at t48 and only a few showed an increase at both time points.

For bacterial species belonging to the Firmicutes phylum, *Streptococcus* sp showed the biggest increase among all the bacterial groups with an average increase of 103-fold across all regimes. The highest increment occurred when 192 µM Zn was provided (266-fold at t24 and 264-fold at t48) while the other two concentration gave increases of 12.9- to 41.5-fold over time. *Acidaminococcus* sp, *Blautia* sp, *Eubacterium biforme,* Lachnospiraceae sp and *Ruminococcus bromii* shared similar a pattern with a general increase across all the regimes tested with the exception of t48 in 770 µM Zn where they showed a decrease. These species showed an average increase of between 1.21- to 5.40-fold across all the concentrations tested with *Acidaminococcus* sp having the greatest increase with its relative abundance being 5.4-fold enhanced by zinc, followed by *E. biforme* which showed the next highest zinc-stimulated average increase in relative abundance (2.7-fold). With the exception of *Blautia* sp and Lachnospiraceae sp, the supplementation by low zinc (77 µM Zn) favoured the growth of the other 3 species in this group with an average increase of 1.55- to 6.25-fold. *Blautia* sp and Lachnospiraceae sp on the other hand had the highest 192 µM zinc-enhanced growth with an average increase of 1.97- and 2.58-fold for the former and latter bacterial species, respectively. Although *Enterococcus* sp, *Clostridium* sp, *Phascolarctobacterium* sp and *Gemmiger formicilis* showed a decrease at some time points, they showed an increase overall in the presence of zinc with an average increase of 1.59-, 6.39-, 1.33- and 1.12-fold respectively. These 4 species also reacted differently to the different zinc concentration employed whereby their highest zinc-enhanced growth was obtained at 77 µM Zn (*Enterococcus* sp, 1.81-fold; *Clostridium* sp, 14.70-fold), 192 µM Zn (*Phascolarctobacterium* sp, 1.71-fold) and 770 µM Zn (*Gemmiger formicilis*, 1.20-fold). *Roseburia faecis* displayed a consistent but relatively modest decrease of 1.71-fold (on average) in all the

conditions tested with the maximum average decrease achieved at 192 µM Zn. As for bacterial groups that were diminished by zinc, the concentration of zinc played a greater role rather than the presence/absence of added zinc. *Lactobacillus* sp, *Clostridium bolteae, Faecalibacterium prausnitzii* and Ruminococcaceae sp showed a decrease in their relative abundance with 192 µM Zn but an increase in the other zinc concentration (77 and 770 µM Zn). These bacterial groups gave on average a modest reduction between 1.03- to 1.30-fold and an increase between 1.02- to 1.73-fold for the low zinc and between 1.06- 2.35-fold for the high zinc. *Blautia* sp, Clostridales sp, Lachnospiraceae sp, *Oscillospira* sp, *Roseburia* sp, *Ruminococcus* sp and *Ruminococcus bromii* showed a decrease in their relative abundance in the presence of 770 µM Zn and the low to medium zinc concentration enhanced their growth. The high zinc concentration resulted in an average 1.11- to 1.55-fold reduction while the low and medium zinc levels caused increases in their abundance of 1.04- to 2.58-fold, with most of the species favouring the low zinc condition.

In summary, the presence of zinc mostly benefitted the members in the Firmicutes phylum, particularly at low zinc concentration (77 µM Zn) whereby all bacterial groups (except *Roseburia faecis*) showed an increase in growth. However, at high zinc concentration (770 µM Zn), 9 bacterial groups showed a decrease in growth, indicating an inhibitory effect of zinc at high concentration.

For bacteria belonging to the Proteobacteria phylum, *Sutterella* sp showed an increase in its relative abundance in the presence of zinc with an average increase of 6.03-fold and the maximal zinc-stimulated growth occurring at 192 µM Zn (7.06-fold at t24 and 12.4-fold at t48). Enterobacteriaceae sp1 and sp2 showed the opposite effect with a decrease in the presence of zinc with an average reduction of 1.31- and 1.32-fold, respectively. Both species showed very similar fold changes in all the regimes with the highest reduction occurring at 192 µM Zn whereby Enterobacteriaceae sp1 decreased on average by 1.71-fold and Enterobacteriaceae sp2 decreased 1.64-fold. Even though the Enterobacteriaceae had the highest relative abundance across all the regimes tested, the presence of zinc had an inhibitory effect on their relative growth (particularly at 192 µM Zn), while the same concentration had a growth promoting effect on *Sutterella* sp.

7.5 Discussion

As mentioned before, the use of batch cultures provides a quick and convenient way to explore the effect of zinc on the gut microbiota under a range of conditions but do have a disadvantage whereby they tend to favour the faster growing bacteria as the fermentation operate of a short period of time (48-hours). Based on the results in both tables 7.6 and 7.8, time (table 7.6) has a bigger effect on the gut microbiota as compared to zinc (table 7.8) whereby more species showed a significant change in abundance in the former than the latter. The small amount of significant change in abundance in response to zinc (table 7.8) may be due to the large variation between the 4 different subjects. The high variability between the samples tested caused rendered the changes (due to zinc) insignificant. Besides this, some of the bacterial groups had small fold changes as shown by the similar relative abundance levels between the zinc regimes, again contributing to changes that are not statistically significant. As for statistical testing, Bonferroni correction was used as a Post Hoc test with one-way Anova rather than just Anova alone as the Bonferroni correction is able to reduce the chances of false positive (type I error) in the results, giving it a more accurate interpretation of the results obtained. One of the limitation of this experiment is that the gut microbiota inoculated in the batch culture were from zinc sufficient individuals rather than zinc deficient individual. Thus, the composition of the gut microbiota at t0 will not be expected to represent that of the zinc deficient population as the bacteria were likely in a zinc sufficient environment prior to being inoculated. Thus, the gut microbiota may not show drastic reaction towards the lower-level zinc supplements applied, but might be expected to respond to the 3 μM and high Zn levels.

Studies on the effect of zinc on the human gut microbiota are very limited and have mostly been conducted using animal models, particularly pigs. Zinc has been used as an antibacterial agent especially in the pig industry as it reduces the prevalence of diarrhoea and improves the overall wellbeing of weaned piglets (Starke et al, 2013; Pieper et al, 2011; Wang et al, 2018). Although the mechanism by which zinc improves piglet health has yet to be identified, farmers have been supplementing pig feeds with zinc at pharmacological doses (2000-3000 mg/kg) to treat or prevent

diarrhoea in piglets. Based on the results obtained from animal trials, the high zinc concentration causes a decrease in the number of Enterobacteriaceae in piglets (Starke et al, 2013; Wang et al, 2018) which is consistent with the findings obtained from the batch culture results produced here, even though Enterobacteriaceae are the most abundant bacteria in all regimes tested. Similar results were shown in a chick model whereby zinc-deficient chicks had a higher abundance of Enterobacteriaceae compared to zinc-replete counterparts, indicating the growth suppressing effect of zinc (Reed et al, 2015). Although *Enterococcus* species were shown to increase in the absence of zinc in the chick model (Reed et al, 2015), the results obtained from the batch culture resembles that of the piglet model whereby *Enterococcus* species increased with increasing concentration of zinc in piglets (Pieper et al, 2011). Results from piglet studies showed that the *Bacteroides-Prevotella–Porphyromonas* cluster are unaffected by high zinc concentration (Starke et al, 2013) which is different to what was observed in the study described herein whereby these species were either enhanced or diminished by high zinc concentration. However, individual species were not analysed in the Starke et al. (2013) study, which may explain the differences in findings. In the study presented in this chapter, each individual species was analysed which provides a more detailed impression of the effect of zinc on these species; however, combining the data for these species would indicate an overall increased growth in the presence of high zinc (in contrast to the findings of Starke et al., 2013).

It is also worth mentioning that the concentration and form of zinc used in the animal studies is different than that used in this experiment as the batch cultures were set up to mimic human gut microbiota and doses that are relevant in humans. Zinc oxide (ZnO) is commonly used in animal studies but zinc sulphate ($ZnSO_4$) was used in the batch cultures as zinc sulphate is water soluble, unlike zinc oxide. In addition, real-time PCR was used to analyse the composition of the piglet's microbiota for bacterial groups of interest, thus many elements of the gut microbiota were not considered.

As seen in Figure 7.3, the relative abundance of Actinobacteria showed an increase in the presence of zinc while Proteobacteria had a decrease and there is a strong relationship between these 2 phyla. However, the role of zinc is yet to be discovered whereby it can either be that the presence of zinc increases the abundance of Actinobacteria, which in turn inhibits the growth of Proteobacteria, or the presence of zinc inhibits the growth of Proteobacteria, giving Actinobacteria a chance to grow and develop, thus increasing its abundance. When zinc is added to the medium, it enhances the growth of many bacterial groups as shown in table 7.8. However, the mechanism by which zinc causes this enhanced growth is yet to be determined. Possibly, zinc could promote the growth of the corresponding bacterial species by diminishing the growth of others (such as Enterobacteriaceae), perhaps by interfering with iron uptake, or it could act as a direct growth promoter as a key micronutrient. The inhibitory effect of zinc on certain bacterial group has been shown in numerous studies, thus giving an advantage to other bacterial groups present in the same environment to grow. Zinc has to been shown to increase the production of the siderophore (pyoverdine) in *Pseudomonas aeruginosa* and this effect was more pronounced in an iron deficient medium (Hofte et al, 1993). In the presence of zinc, bacteria may produce siderophores to scavenge iron from the environment which can then be used for growth and development, outcompeting those bacteria that are unable to produce or utilise siderophores and further diminishing the concentration of iron available in the medium. A recent study showed that iron was accumulated under zinc excess and that the presence of zinc stress up-regulates the iron-uptake genes coupled by down-regulating iron Fe-storage genes at early stages (30 min) of exposure. The effect is then reversed by down-regulating Fe-uptake genes and up-regulating Fe-storage at later stages (60 min), causing an increased iron accumulation in the cell as bacteria required a higher demand of iron for survival under zinc stress (Xu et al, 2019). Both the observations by Hofte et al (1993) and Xu et al (2019) suggest that the competition for iron is enhanced by increasing zinc concentration. The modified gut model medium has sufficient levels of iron and heam which the bacteria can fully utilise and bacteria which has the ability to scavenge iron will have an advantage over the other bacteria present.

Ferrous iron uptake systems are considered to be the most important non-haem-iron pathways for the gut microbiota (Andrews et al, 2003), and such pathways are more likely to be blocked by free zinc than haem uptake pathways. This may explain why the *Bacteroides* species (haem utilisers) responded so well to zinc supplementation. However, this theory remains to be proven by conducting the same experiment but under conditions where the levels of haem and non-haem iron levels are adjusted.

As seen from table 7.6, the Enterobacteriaceae are the most abundant bacteria in all of the regimes tested. This may be due to the fact the doubling time for the corresponding species is relatively short, giving them an advantage in securing the nutrients available. For example, *E. coli*, which is a common gut-dwelling member of the Enterobacteriaceae family, has a doubling time of 15 min (Gibson et al, 2018) whereas that for *Bacteroides fragilis* in a batch culture is between 92-345 min (Dalland and Hofstad, 1974).

The use of batch cultures is useful in providing preliminary data for future work as it is relatively easy to set up and a wide range of different conditions can be tested in a single setting. However, the short time period (24-48 h) is a disadvantage to the slow growing bacteria, causing them to be outperformed by the fast growers, thus affecting their final abundance in the given sample. Continuous gut models represent an alternative approach which overcomes the issue associated with fast growth; thus, such systems will be utilised in studies described further below.

Chapter 8: Impact of zinc on gut microbiota using the three-stage gut model

8.1 Impact of zinc supplementation on zinc sufficient/deficient gut microbiota using the three-stage gut model

The gut model consists of 3 consecutively-linked vessels each representing a region of the colon with V1 representing the proximal colon, V2 representing the transverse colon and V3 representing the distal colon. The pH in each region also differs between vessels to mimic the pH in the corresponding region of the colon: pH 5.5 in V1, pH 6.1 in V2 and pH 6.8 in V3. The nutrient content and availability in each vessel is also different as the medium flows from the medium reservoir to V1 through a peristaltic pump which then flows to V2 and then V3 and finally to a waste collection bottle. As fresh medium flows to V1 first, bacteria in V1 have access to all of the nutrients available while bacteria in the subsequent vessels only have access to unused nutrient or breakdown products, which limits their nutrient utilisation compared to V1. The gut model is superior to the batch cultures as it mimics the entire colon, giving a better insight of the treatment/regimes that are being tested. In addition, it is a continuous culture whereby fresh medium is constantly fed to the vessels (similar to food intake in a human host) and the microbiota are allowed to reach equilibrium before measurements are taken. However, the disadvantage of the gut model is that it is labour intensive and time consuming compared to batch culture.

Thus, the gut model system, using biological triplicates, was used to explore the impact of zinc concentration on the microbiota. The experimental regime employed was designated 'Gut Model 1' (GM1) with modified GMM (mGMM) to enable control of zinc levels. Three distinct zinc regimes (or steady states) were explored: SS1, containing mGMM under normal zinc regime (19 μM); SS2, containing mGMM with only 3 μM zinc; and SS3, mGMM supplemented with 77 μM zinc to give a final concentration of 96 μM zinc. These conditions were designed allow the impact of zinc deficiency to be determined (SS2) in comparison to zinc sufficiency (SS1) and a regime resembling that of a zinc-supplemented diet (SS3). The experiment was also designed to test the impact of application zinc supplements to an individual previously on a zinc deficient diet. Effects of zinc deficiency and zinc supplementation on the gut microbiota have mostly been studied using animal

models (Starke et al, 2013; Pieper et al, 2011; Wang et al, 2018) and studies on humans have been very limited. The gut model is used in this study, rather than direct human dietary intervention studies, due to the ethical and practical issues associated with providing subjects with a very low zinc diet. Also, the gut model will offer a second approach, additional to the batch culture work above, to explore the impact of dietary zinc regime on the gut microbiota.

8.1.1 Impact of zinc regime on bacterial numbers

GM1 was inoculated with faecal slurry from distinct donors (2 females & 1 male; age 26-38; no antibiotic treatment within 6 months) thus allowing results to be produced in biological triplicate. The total bacterial count for each vessel was measured using Flow-FISH coupled with Eub I-II-III probes to ensure that all of the bacteria present are counted. As seen in Figure 8.1, the total bacterial count was the highest for V1 and lowest for V3. This is as expected as V1 received fresh medium whilst V3 would receive medium depleted of nutrients and raised in metabolic waste products. In SS1, the mGMM has a total zinc concentration of 19 µM Zn, resulting in V1 having a total bacterial count of 3.99×10^8 cells/ml, while V2 and V3 had 1.29×10^8 and 9.70×10^7 cells/ml, respectively. The low zinc regime (SS2), whereby no zinc was added to the medium, had a total zinc concentration of 3 µM Zn which resulted in reduced bacterial numbers in all 3 vessels with V3 showing the greatest decrease (2.62-fold), reducing its total count to only 3.70×10^7 cells/ml medium. V1 and V2 experienced minimal effects with only 1.05- and 1.17-fold decreases, respectively, bringing down their totals to 3.82×10^8 and 1.11×10^8 cells/ml. In SS3, where 77 µM Zn was added to the medium (total zinc concentration of 96 µM Zn), V1 and V2 showed a modest increase (with respect to SS1) to 4.54×10^8 (1.14-fold) and 2.00×10^8 cells/ml medium (1.54-fold), accordingly, but V3 showed a decrease (with respect to SS1) of 1.36-fold to 7.12×10^7 cells/ml medium; this may reflect higher growth in V1-V2 resulting in reduced nutrient flow into V3. Comparing SS3 with SS2 allows the impact of provision of supplementary levels of zinc to a previously zinc deficient regime to be considered. The results show that the provision of 77 µM zinc caused a clear increase in bacterial

numbers of 1.2-, 1.8 and 1.9-fold for V1, V2 and V3, respectively. This suggests that the microbiota in SS2 were zinc restricted, particularly in V2 and V3.

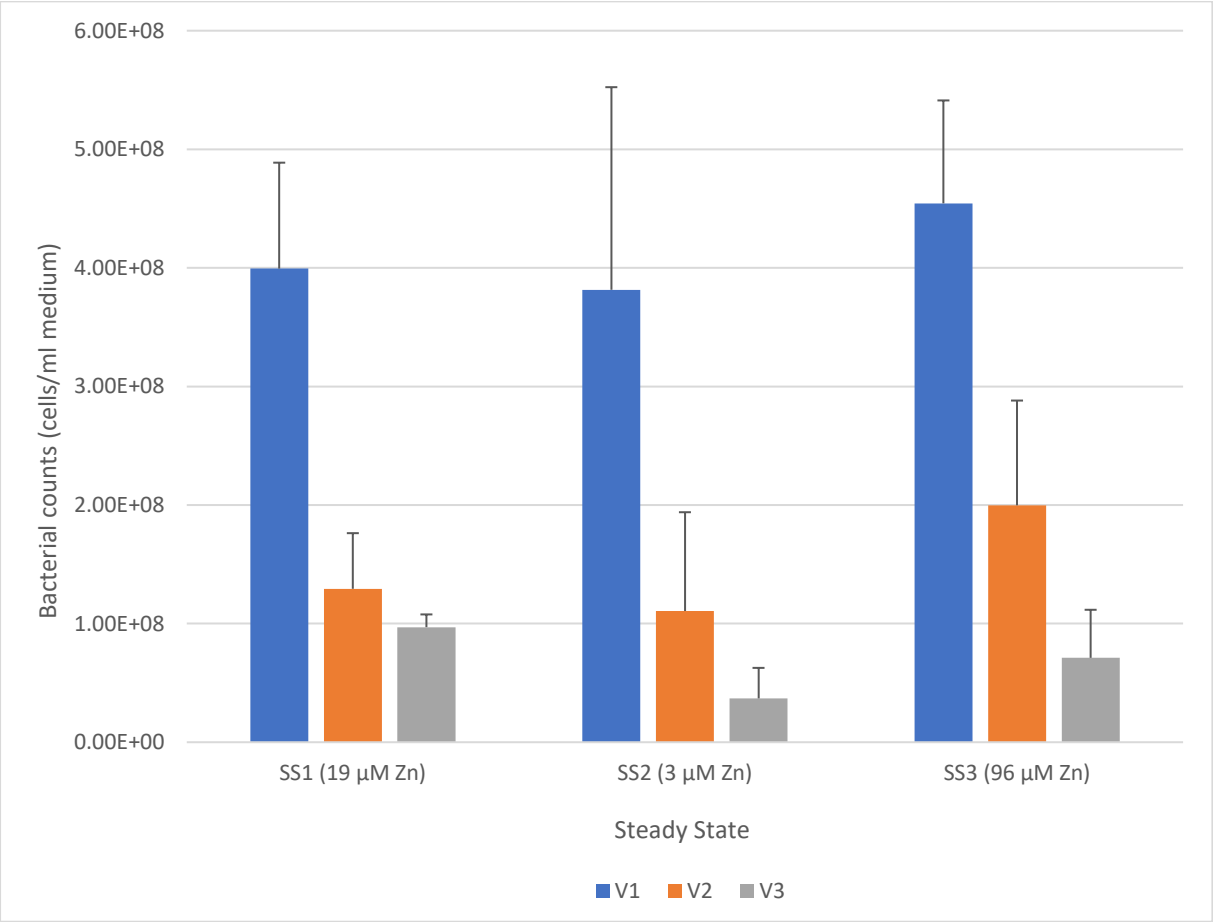


Figure 8.1 Total bacterial counts in each vessel for the 3 different steady states in GM1. Total bacterial counts for each zinc regime were determined by Flow-FISH using Eub I-II-III probes. Each bar indicates a different vessels representing a different region of the colon: blue (V1, proximal colon), orange (V2, transverse colon), grey (V3, distal colon). Bacterial counts are expressed as cells/ml medium. Results are average of 3 subjects and error bars represent SD. One-way Anova with Bonferroni Post Hoc correction has been employed for statistical analysis. No significant difference between conditions was seen ($p>0.05$).

Table 8.1 Total bacterial counts in each vessels of 3 different steady states in GM1. Data derived from Figure 8.1

		SS1	SS2	SS3
GM1	V1	3.99E+08	3.82E+08	4.54E+08
	V2	1.29E+08	1.11E+08	2.00E+08
	V3	9.70E+07	3.70E+07	7.12E+07

Table 8.2 Fold difference between the 3 different steady states in GM1. Data derived from Figure 8.1

		SS2/SS1	SS3/SS1	SS3/SS2
GM1	V1	0.96	1.14	1.19

	V2	0.86	1.54	1.81
	V3	0.38	0.73	1.93

8.1.2 Impact of zinc on the bacterial composition

8.1.2.1 Impact of zinc on bacterial composition at phylum level

The impact of zinc regime on bacterial composition was then determined by 16S rRNA gene amplicon NGS analysis. An unidentifiable bacterial species could not be categorised into any known phylum, thus there is a unidentifiable phylum shown in table 8.3. As seen from table 8.3, the majority of the bacteria detected in SS1 belong to the Bacteroidetes phylum, followed by the Firmicutes and Proteobacteria phyla with a combined relative abundance of roughly 90% in all of the vessels. Actinobacteria and Synergistetes made up less than 0.5% of the overall abundance in all 3 vessels. At SS2, which resembles a zinc deficient diet, 4 of the 5 phyla showed a modest change in their relative abundance while Synergistetes had a noticeable change of 16.3-fold on average across the 3 vessels. In the Synergistetes phylum, all 3 vessels showed an increase with V2 (transverse colon) showing the biggest increase (27.1-fold) followed by V3 (distal colon) and V1 (proximal colon) with 19.4- and 2.49-fold increases, respectively, but none of these changes were significant when tested statistically ($p>0.05$). The increase in Synergistetes was coupled with an overall decrease in the Actinobacteria, Bacteroidetes and Proteobacteria, with Actinobacteria showing the biggest relative drop with on average 1.53-fold reduction across all vessels. In the absence of zinc, the relative abundance of Bacteroidetes dropped from 77.9 to 67.0% (1.16-fold) in V1 and from 65.9 to 64.4% (1.02-fold) in V2, but V3 showed a 1.09-fold increase (from 51.4% to 55.8%). Firmicutes and Proteobacteria showed the same changes with V1 having an increase while the other 2 vessels showed a decrease. Firmicutes increased 1.72-fold (10.3 to 17.7%) and Proteobacteria increased 1.32-fold (2.61 to 3.45%). Firmicutes showed a 1.26- and 1.42-fold decrease in V2 and V3 while Proteobacteria decreased 1.71- and 1.39-fold, respectively.

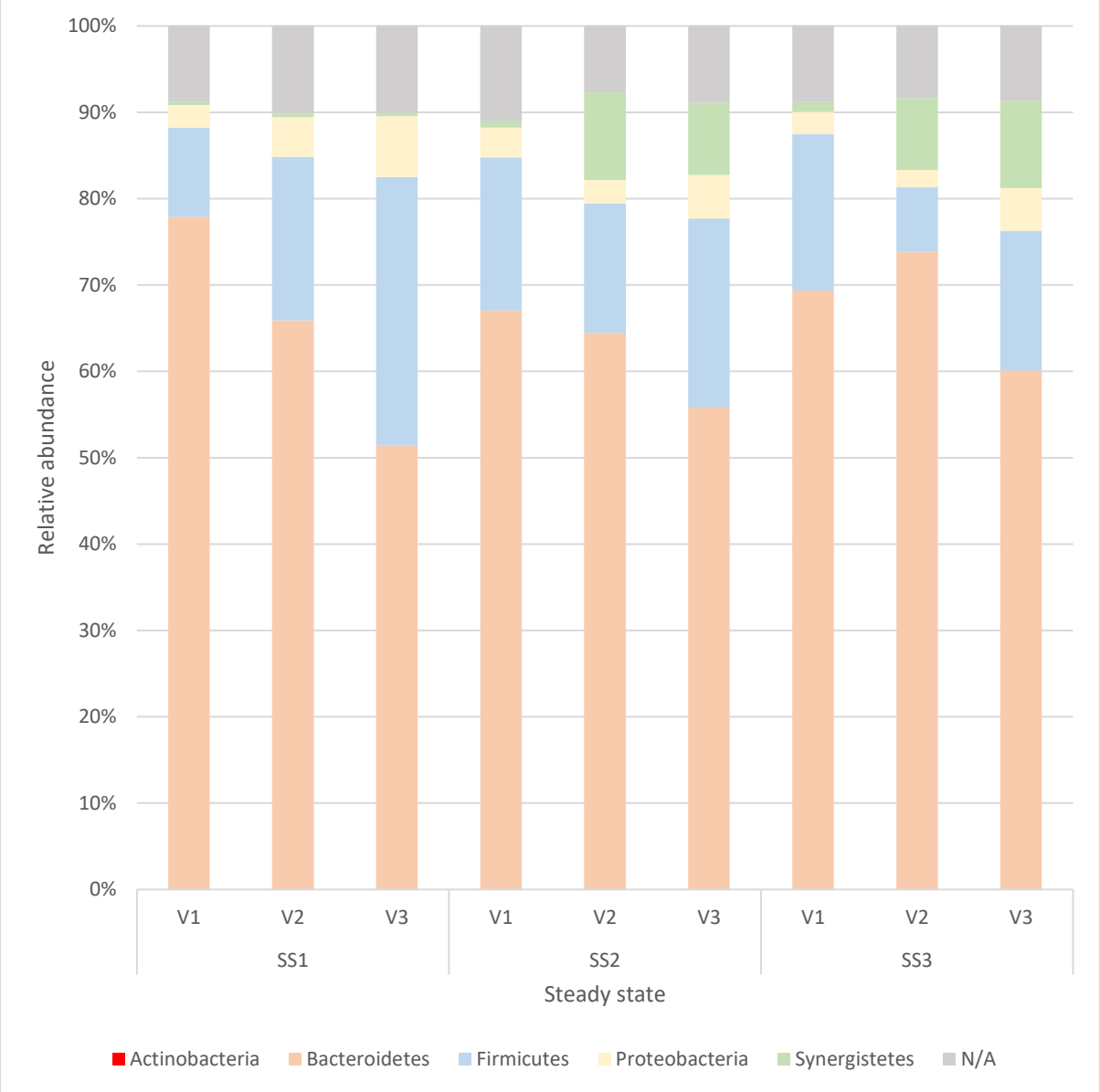


Figure 8.2 Composition of gut microbiota at phylum level. The gut microbiota is classified at phylum level for all vessels (V1, V2 and V3) at SS1, SS2 and SS3.

Table 8.3 Relative abundance of different bacterial groups at phylum level. The relative abundance of different bacterial groups in V1, V2 and V3 at SS1, SS2 and SS3. The bacterial phyla are expressed as a percentage of the total microbial community profile. The results are the average of 3 subjects. One-way Anova with Bonferroni Post Hoc correction has been employed for statistical analysis. No significant difference is seen between the steady states (N/A: not identifiable).

Phylum	SS1			SS2			SS3		
	V1	**V2**	**V3**	**V1**	**V2**	**V3**	**V1**	**V2**	**V3**
Actinobacteria	0.01	0.02	0.03	0.00	0.01	0.02	0.01	0.01	0.01
Bacteroidetes	77.89	65.88	51.39	67.04	64.41	55.79	69.37	73.83	60.11
Firmicutes	10.32	18.93	31.05	17.72	15.01	21.89	18.10	7.52	16.13
Proteobacteria	2.61	4.61	7.02	3.45	2.70	5.04	2.58	1.96	4.95
Synergistetes	0.33	0.37	0.43	0.83	10.13	8.37	1.13	8.35	10.10
N/A	8.83	10.16	10.02	10.95	7.71	8.87	8.81	8.33	8.68

Table 8.4 Fold changes in abundance at phylum level between steady states. Fold changes of the bacteria at phylum level between the 3 different steady states. Data derived from table 8.3. One-way Anova with Bonferroni Post Hoc correction has been employed for statistical analysis. No significant difference is seen between the steady states.

	SS2/SS1			SS3/SS1			SS3/SS2		
	V1	V2	V3	V1	V2	V3	V1	V2	V3
Actinobacteria	0.33	0.81	0.83	0.85	0.41	0.56	2.59	0.51	0.68
Bacteroidetes	0.86	0.98	1.09	0.89	1.12	1.17	1.03	1.15	1.08
Firmicutes	1.72	0.79	0.70	1.75	0.40	0.52	1.02	0.50	0.74
Proteobacteria	1.32	0.59	0.72	0.99	0.42	0.71	0.75	0.73	0.98
Synergistetes	2.49	27.08	19.40	3.40	22.32	23.42	1.36	0.82	1.21

In the presence of 77 µM Zn supplementation (SS3), Bacteroidetes and Firmicutes still made-up the majority of the bacterial microbiota but Synergistetes showed the biggest overall change with respect to SS1, with an average increase of 16.4-fold, contributing roughly 10% of the SS3 bacteria overall. Although the relative abundance of the Synergistetes increased in all 3 vessels, vessels resembling the middle (V2) and lower (V3) colon had a substantial increase of roughly 23-fold compared to V1 with only 3.40-fold. However, the increase was not significant when tested ($p>0.05$). The addition of 77 µM zinc increased the relative abundance of Bacteroidetes overall (with respect to SS1) but all 3 vessels showed only a modest fold change with V1 decreasing 1.12-fold while V2 and V3 increased 1.12- and 1.17-fold, respectively. Actinobacteria, Firmicutes and Proteobacteria on average showed a decrease in SS3 with respect to SS1 of 1.64-, 1.12- and 1.42-fold, accordingly. While Actinobacteria and Proteobacteria had a reduction across all 3 vessels, Firmicutes only showed a decrease in V2 (2.52-fold) and V3 (1.93-fold) with an increase in V1 (1.75-fold). Comparison of SS3 and SS2 resembles the effect of zinc supplementation on a zinc deficient gut microbiota. Bacteroidetes benefited from the addition of 77 µM Zn zinc whereas Proteobacteria displayed a negative effect. Bacteroidetes showed a modest increase of 1.03-, 1.15- and 1.08-fold in V1, V2 and V3 respectively reaching a relative abundance of 69.4, 73.8 and 60.1%. Proteobacteria decreased 1.34-, 1.38- and 1.02-fold for the same vessels to 2.58, 1.96 and 4.95%, accordingly. Actinobacteria, Firmicutes and Synergistetes responded in a similar manner whereby the presence

of zinc only increased the relative abundance of bacteria in the proximal colon (V1) between 1.02- to 2.59-fold, while the other 2 sections (V2, V3) showed a decrease of 1.02- to 2.00-fold.

In summary, the major impact of zinc regime was a dramatic increase in the abundance of Synergistetes under low zinc conditions, and this increase was retained when zinc levels were raised in SS3.

8.1.2.2 Impact of zinc on alpha and beta diversity

Shannon Index and weighted UniFrac were used to determine the alpha and beta diversity as both indices include abundance in their calculation rather than dominance (presence/absence of species). For alpha diversity, a high Shannon Index value indicates the presence of richer species diversity in a given sample, while beta diversity determines the variation of species between samples.

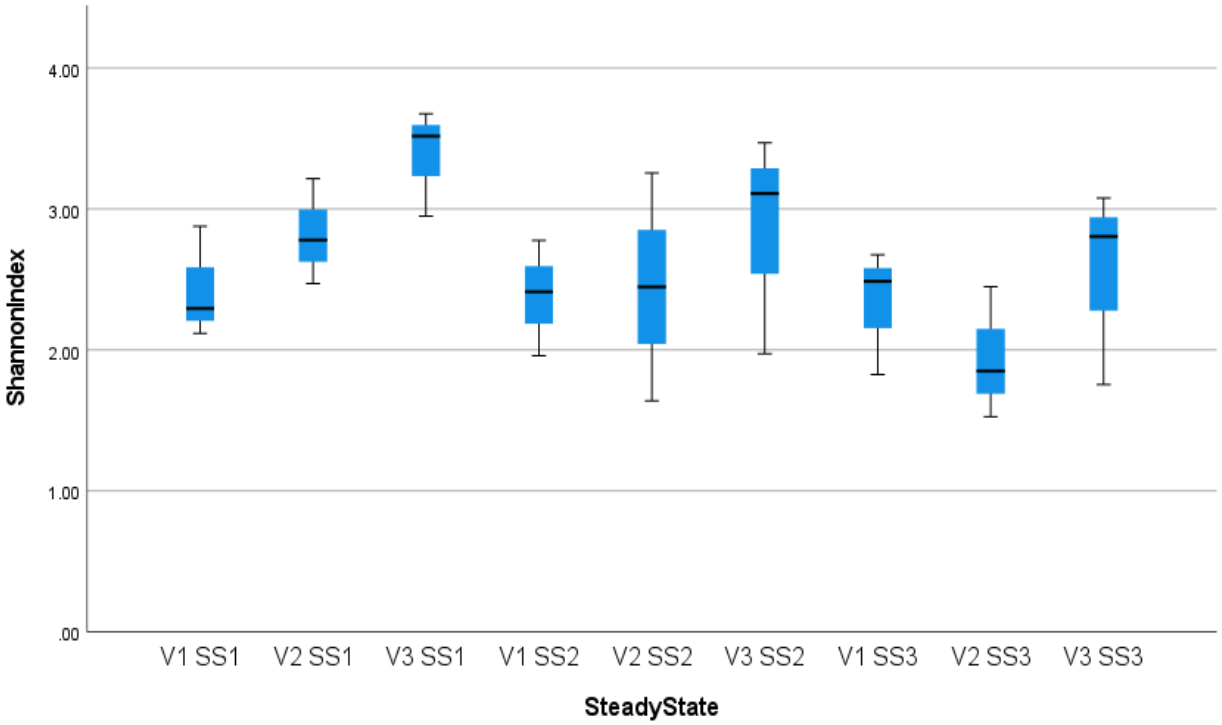


Figure 8.3 Alpha diversity measured by Shannon Index. The higher the value in Shannon Index indicates a higher species richness in the sample tested. Mann-Whitney U test were used for statistical analysis. No significant difference was seen between the regimes tested ($p>0.05$).

As seen from Figure 8.3, the diversity of the bacteria in SS1 increases v1 to V3 as indicated by the higher Shannon Index value. In SS2, the absence of zinc has negligible effect in V1 but reduced the diversity in V2 and V3, possibly due to zinc deficiency coupled with nutrient limitation in the later

part of the colon. When 77 µM Zn was added to the medium, this also had a negligible effect on the diversity in V1 as shown by a similar Shannon Index value but it reduced the diversity of the population in the other 2 vessels, especially V2. The results indicate that although zinc increases the total number of bacteria, it reduces the diversity of the bacteria present, particularly in the vessels corresponding to the transverse and distal colon.

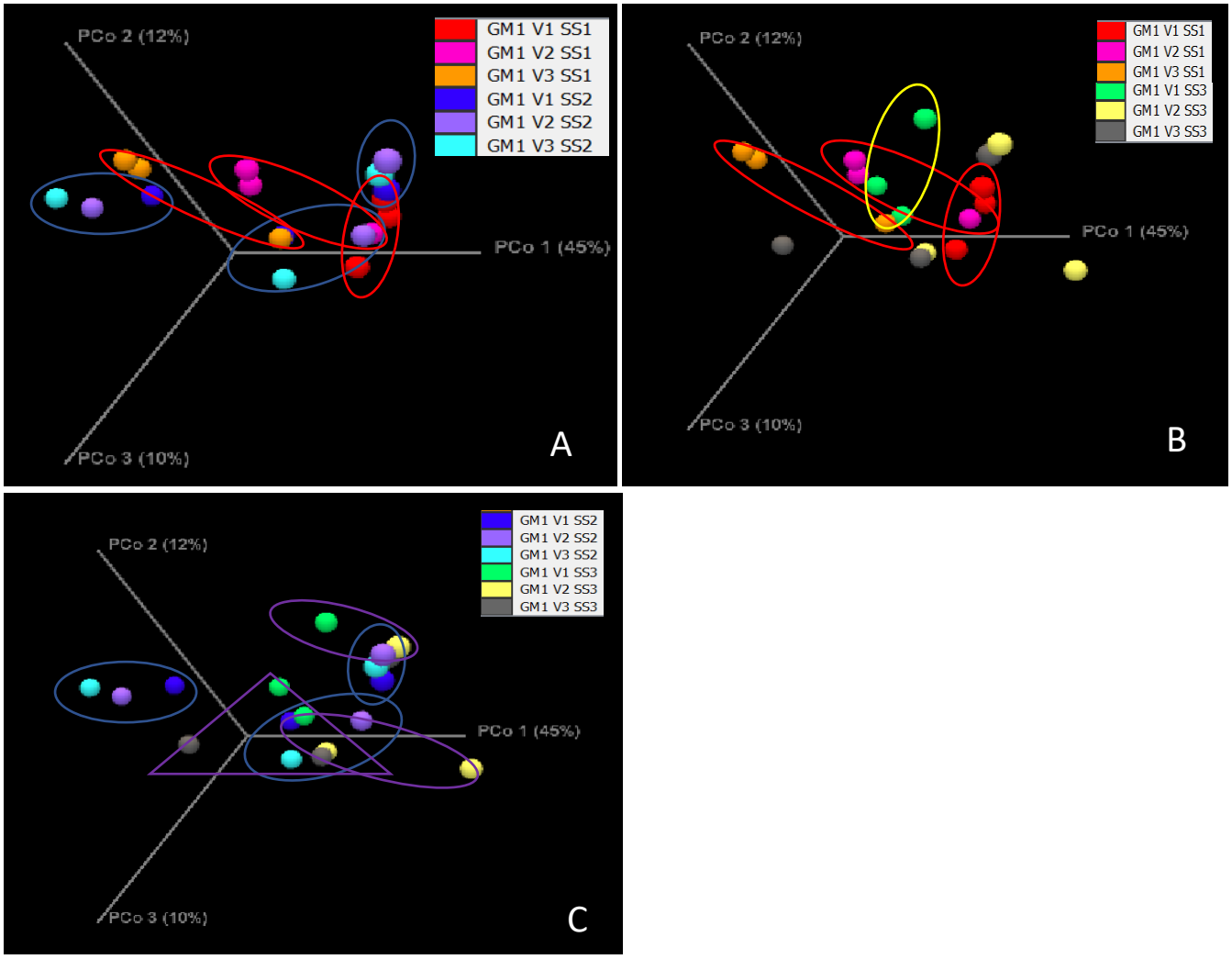


Figure 8.4 Beta diversity Principal Coordinate Analysis derived from weighted UniFrac in all samples. Each vessel at each steady state has a different colour: **SS1-** V1 (red), V2 (pink), V3 (orange); **SS2**- V1 (blue), V2 (purple), V3 (cyan); **SS3**- V1 (green), V2 (yellow), V3 (grey). The beta diversity is compared between 2 different steady states: **A**, SS1 with SS2; **B**, SS1 with SS3; **C**, SS2 with SS3. For each axis, the percent of variation is reported in brackets.

As seen from Figure 8.4, 3 beta diversity analyses were generated comparing the 3 different steady states. In Figure 8.4A (SS1 v SS2), the bacterial populations clustered according to their vessels in SS1

as shown by the red circles but the population from 1 of the subjects was slightly different than that of the other 2 in V2 (pink) and V3 (orange), causing it to be further apart. However, in SS2 where zinc levels are low, the populations formed 3 different clusters representing the 3 individual as shown by the blue circles. When 77 µM Zn was added to the medium (Figure 8.4B), V1 formed 2 separate clusters, indicating that zinc causes a variation to the composition of the gut microbiota. The V2 and V3 populations were further apart, showing that each subject shows a different response towards zinc and the interindividual variability outweighs that of the tested condition. Supplementing zinc to a previously zinc deficient condition also caused the bacteria to cluster according to individual as shown by the blue and purple circles (triangle) in Figure 8.4C.

8.1.2.3 Impact of zinc on bacteria composition at species level

The bacteria were compared at species level to give a better depth for the composition analysis compared to that that would be obtained at genus level or higher taxonomic level. The composition of the gut microbiota is presented at species level in table 7.13. Some of the names were at higher taxonomic level because their exact species were not in the reference database, thus their name were entered to the next higher taxonomic level.

Table 8.6 Effect of zinc regime on relative abundance at the species level. The relative abundance of bacterial groups within the gut microbiota at species levels is indicated SS1, SS2 and SS3. SS1 (yellow), SS2 (green) and SS3 (orange). Data are expressed as a percentage of the total microbial community profile. The results are the average of 3 subjects. Only bacterial species present at >0.5% in at least 1 vessels of any steady states is shown. One-way Anova with Bonferroni Post Hoc correction has been employed for statistical analysis.(sp: species, N/A: not identifiable).

Phylum	Order	Species	SS1			SS2			SS3		
			V1	V2	V3	V1	V2	V3	V1	V2	V3
Bacteroidetes	Bacteroidales	*Paraprevotella* sp	3.12	0.42	0.08	0.04	0.04	0.00	0.00	0.00	0.00
		Bacteroides sp1	2.98	2.46	2.20	2.41	1.34	1.72	2.44	2.09	2.18
		Bacteroides sp2	46.38	42.30	29.60	38.06	43.18	40.25	42.57	44.80	38.09
		Bacteroides ovatus	3.92	5.20	8.09	3.34	2.08	3.71	4.39	1.36	3.17
		Bacteroides fragilis	0.16	0.41	0.81	0.18	0.17	0.17	0.10	0.18	0.07
		Bacteroides uniformis	18.64	13.20	8.86	17.93	13.06	7.54	19.46	24.88	15.52
		Prevotella sp	1.69	1.33	0.88	4.23	3.23	1.44	0.05	0.03	0.02
		Rikenellaceae sp	0.72	0.21	0.22	0.54	0.74	0.35	0.12	0.26	0.50
Firmicutes	Clostridales	Clostridales sp	1.09	1.56	2.91	0.58	1.17	2.43	0.39	0.77	1.90
		Lachnospiraceae sp	0.85	1.51	2.82	0.73	1.24	2.41	1.04	0.78	2.27
		Dorea sp	0.04	0.16	0.32	0.26	0.57	0.78	0.11	0.18	0.20

		Lachnospira sp	0.14	0.59	0.89	0.05	0.08	0.25	0.04	0.08	0.29
		Ruminococcaceae sp1	1.48	2.91	4.26	3.93	2.47	2.30	4.15	0.91	1.39
		Ruminococcaceae sp2	0.80	1.38	2.46	0.83	0.93	3.23	0.29	0.75	2.08
		Oscillospira sp	0.62	1.27	1.77	0.35	0.47	1.24	0.26	0.50	1.47
		Ruminococcus bromii	3.67	7.07	10.59	9.64	5.80	4.97	10.58	2.27	3.37
		Dialister sp	0.09	0.18	0.33	0.21	0.01	0.52	0.12	0.04	0.33
		Megasphaera sp	0.04	0.07	0.17	0.09	0.26	0.59	0.02	0.02	0.38
		Acidaminococcus sp	0.07	0.04	0.08	0.12	0.23	0.21	0.05	0.11	0.08
		Faecalibacterium prausnitzii	0.05	0.05	0.03	0.10	0.01	0.26	0.44	0.08	0.19
		Clostridium bolteae	0.05	0.13	0.32	0.06	0.16	0.27	0.06	0.08	0.34
		Ruminococcus sp	0.13	0.23	0.18	0.08	0.10	0.10	0.01	0.00	0.05
		Ruminococcus gnavus	0.10	0.20	0.23	0.08	0.14	0.29	0.06	0.05	0.30
	Erysipelotrichales	*Eubacterium dolichum*	0.07	0.14	0.32	0.03	0.08	0.13	0.08	0.10	0.15
Proteobacteria	RF32	RF32 sp	2.13	3.91	5.43	2.88	2.37	2.42	1.99	1.59	1.95
	Burkholderiales	*Sutterella* sp	0.46	0.65	1.36	0.55	0.26	1.78	0.56	0.34	1.84
Synergistetes	Synergistales	*Pyramidobacter piscolens*	0.33	0.05	0.03	0.83	10.09	8.30	1.13	8.17	9.93
		N/A	8.83	10.16	10.02	10.95	7.71	8.87	8.81	8.33	8.68

Table 8.7 Fold changes in abundance at species level between steady states. Fold changes of the bacteria at species level between the 3 different steady states. Data derived from table 8.6. The colours indicate the different steady states comparisons: SS2/SS1 (green), SS3/SS1 (orange), SS3/SS2 (blue). Values in green indicates an increase in relative abundance and values in red indicates a decrease. One-way Anova with Bonferroni Post Hoc correction has been employed for statistical analysis. Bacteria in bold indicates significant difference at certain steady state ($p<0.05$)

Phylum	Order	Species	SS2/SS1			SS3/SS1			SS3/SS2		
			V1	V2	V3	V1	V2	V3	V1	V2	V3
Bacteroidetes	Bacteroidales	*Paraprevotella* sp	0.01	0.09	0.03	0.00	0.00	0.00	0.00	0.00	0.00
		Bacteroides sp1	0.81	0.54	0.78	0.82	0.85	0.99	1.01	1.56	1.27

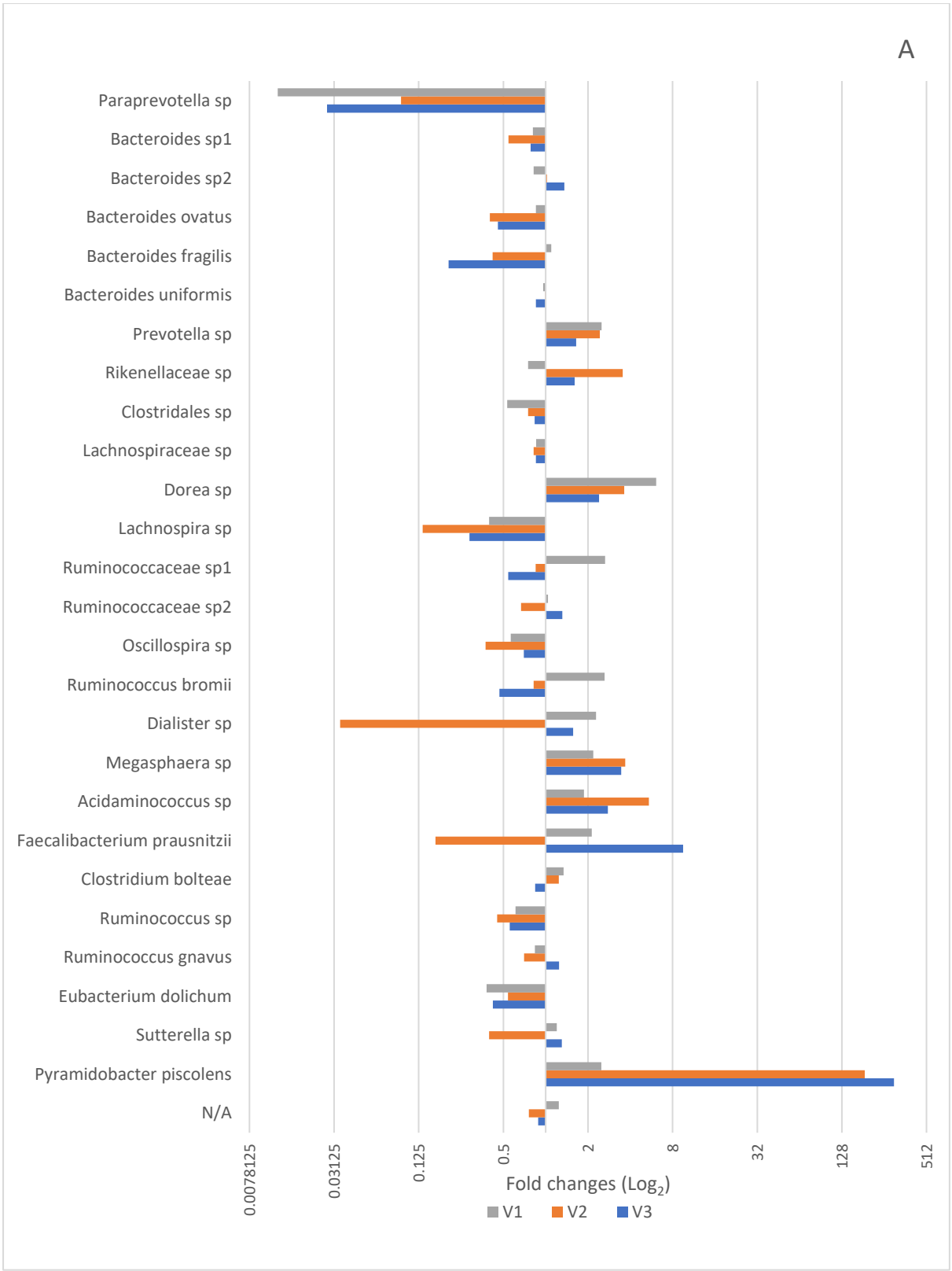
		Bacteroides sp2	0.82	1.02	1.36	0.92	1.06	1.29	1.12	1.04	0.95
		Bacteroides ovatus	0.85	0.40	0.46	1.12	0.26	0.39	1.32	0.65	0.86
		Bacteroides fragilis	1.10	0.42	0.20	0.61	0.44	0.08	0.56	1.04	0.39
		Bacteroides uniformis	0.96	0.99	0.85	1.04	1.88	1.75	1.09	1.90	2.06
		Prevotella sp	2.50	2.43	1.65	0.03	0.02	0.02	0.01	0.01	0.01
		Rikenellaceae sp	0.75	3.53	1.61	0.17	1.26	2.32	0.22	0.36	1.44
Firmicutes	Clostridales	Clostridales sp	0.53	0.75	0.83	0.35	0.49	0.65	0.66	0.66	0.78
		Lachnospiraceae sp	0.86	0.82	0.85	1.22	0.52	0.80	1.43	0.63	0.94
		Dorea sp	6.12	3.62	2.40	2.56	1.15	0.63	0.42	0.32	0.26
		***Lachnospira* sp**	**0.40**	**0.13**	**0.29**	**0.27**	**0.14**	**0.33**	0.69	1.07	1.14
		Ruminococcaceae sp1	2.65	0.85	0.54	2.80	0.31	0.33	1.06	0.37	0.60
		Ruminococcaceae sp2	1.04	0.67	1.31	0.36	0.54	0.84	0.34	0.81	0.64
		Oscillospira sp	0.56	0.37	0.70	0.42	0.39	0.83	0.74	1.05	1.18
		Ruminococcus bromii	2.63	0.82	0.47	2.89	0.32	0.32	1.10	0.39	0.68
		Dialister sp	2.28	0.03	1.57	1.30	0.22	0.98	0.57	6.37	0.63
		Megasphaera sp	2.18	3.68	3.45	0.53	0.27	2.21	0.24	0.07	0.64
		Acidaminococcus sp	1.87	5.42	2.77	0.71	2.48	1.11	0.38	0.46	0.40
		Faecalibacterium prausnitzii	2.13	0.16	9.51	9.21	1.75	6.99	4.32	10.62	0.74
		Clostridium bolteae	1.34	1.24	0.84	1.24	0.60	1.07	0.92	0.49	1.27
		Ruminococcus sp	0.61	0.45	0.56	0.07	0.01	0.28	0.12	0.02	0.51
		Ruminococcus gnavus	0.84	0.70	1.24	0.62	0.25	1.31	0.74	0.35	1.06
	Erysipelotrichales	*Eubacterium dolichum*	0.38	0.54	0.42	1.10	0.67	0.46	2.89	1.23	1.10
Proteobacteria	RF32	RF32 sp	1.35	0.61	0.44	0.93	0.41	0.36	0.69	0.67	0.81
	Burkholderiales	*Sutterella* sp	1.20	0.40	1.30	1.22	0.53	1.35	1.02	1.34	1.04
Synergistetes	Synergistales	*Pyramidobacter piscolens*	2.49	186.31	300.90	3.40	150.87	360.05	1.36	0.81	1.20
		N/A	1.24	0.76	0.89	1.00	0.82	0.87	0.80	1.08	0.98

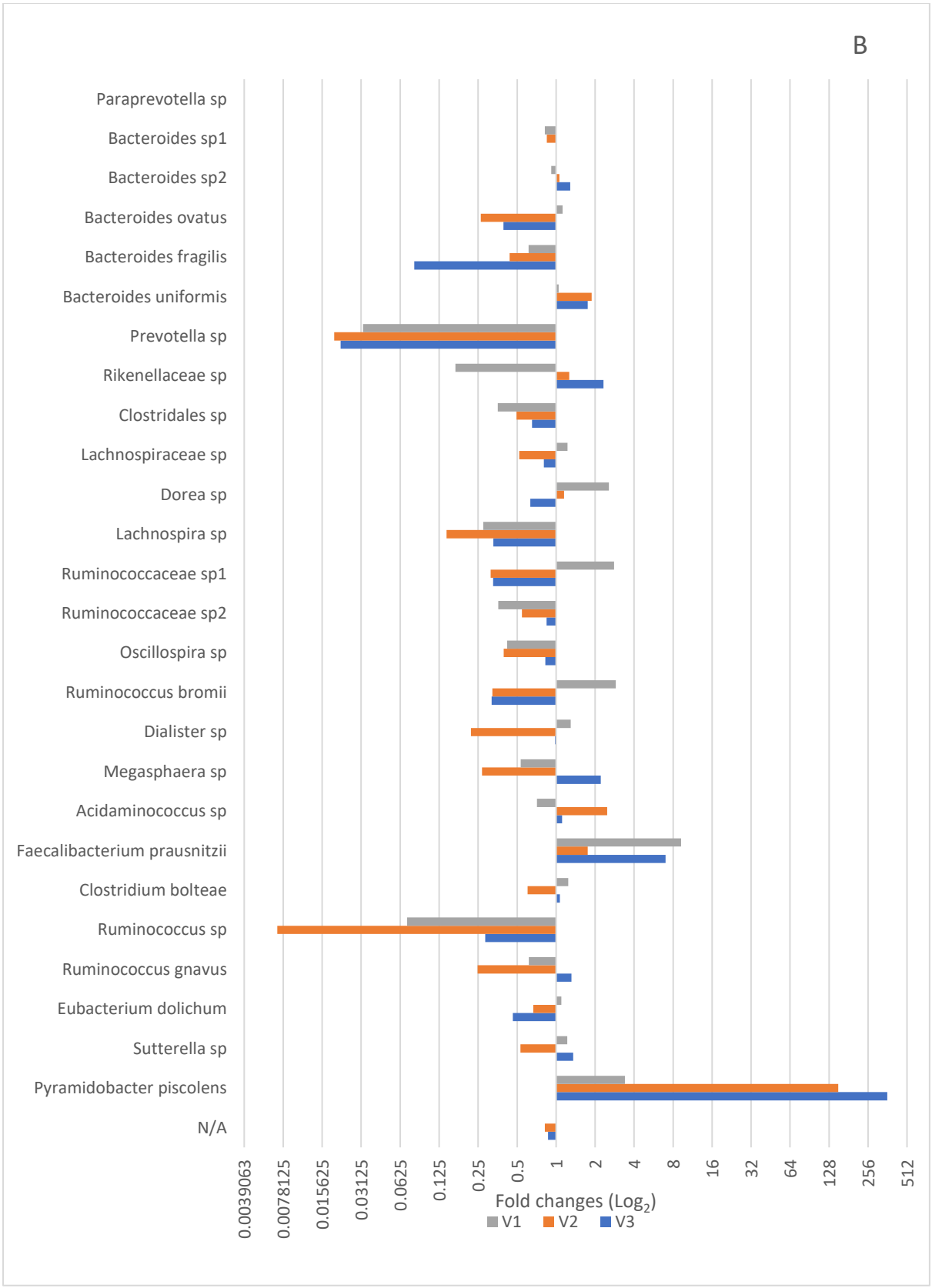
As seen from table 8.6, in SS1, the entire gut model is mainly dominated by Bacteroidetes, particularly *Bacteroides* sp2 and *Bacteroides uniformis* being the most abundant bacteria in all 3 vessels representing the 3 different regions of the colon. *Bacteroides* sp2 is most abundant in V1 (proximal colon) with a relative abundance of 46.4% followed by V2 (transverse colon) with 42.3% and V3 (distal colon) with 29.6%, while *B. uniformis* had a relative abundance of 18.6, 13.2 and 8.86% in the 3 vessels, respectively. The rest of the species in the Bacteroidetes phylum showed a relative abundance of 0.16 to 8.09% across the 3 vessels in SS1. The relative abundance of Bacteroidetes decreased along the gut model, indicating that these bacteria are more abundant in the upper part of the colon while Firmicutes, Protebobacteria and Synergistetes showed an increase in their abundance further down the colon. *Ruminococcus bromii* was the most abundant species in the Firmicutes phylum with a relative abundance of 3.67% (V1), 7.07% (V2) and 10.4% (V3) at SS1. Ruminococcaceae sp1, Clostridales sp and Lachnospiraceae sp were also among the most abundant species with relative abundances of 0.85 to 4.26% across the 3 vessels, while the rest of the species in the Firmicutes phylum had an abundance ranging from 0.04 to 2.46% in SS1. In the Proteobacteria phylum, the relative abundance of *Sutterella* sp at SS1 increased from V1 to V3, increasing from 0.46% (V1) to 0.65% (V2) and then 1.36% (V3). Enterobacteriaceae sp1 and sp2 had a relative abundance of less than 0.05% across all the steady states, unlike in the batch culture where they were the dominant species (see above). *Pyramidobacter piscolens* of the Synergistetes phylum was most abundant in V1 (proximal colon) at 0.33% and decreased in V2 and V3 to 0.05 and 0.03%, respectively.

8.1.2.3.1 Effect of zinc deficiency (SS2) on the composition of gut microbiota compared to SS1

In SS2, under zinc deficiency, Bacteroidetes were more negatively affected compared to the other phylum. Most of the species in this phylum showed a reduction in their relative abundance in all vessels with the V2 and V3 displaying a higher degree of reduction compared to V1, indicating that bacteria residing in the transverse and distal colon may be more affected by the absence of zinc than the bacteria in the proximal colon. *Prevotella* sp showed the highest increase of 2.50- (V1), 2.43- (V2)

and 1.65-fold (V3), increasing in abundance to 4.23, 3.23 and 1.44%, respectively, in the absence of zinc. *Paraprevotella* sp showed a substantial decrease of 22.4-fold on average, reducing its abundance to less than 0.05% in all vessels. *Bacteroides* sp1, *B. ovatus* and *B. uniformis* also showed a decrease across all vessels but with a more modest average reduction of 1.40-, 1.75- and 1.07-fold, respectively. Although *B. fragilis* showed a 1.10-fold increase in V1, the overall abundance of this species along the colon showed a 1.74-fold reduction with V3 having the largest decrease (4.90-fold). *Bacteroides* sp2 and Rikenellaceae sp are the other 2 Bacteroidetes species that showed an overall increase despite zinc being absent in the medium. *Bacteroides* sp2 had a slight decrease of 1.22-fold in V1 but increased 1.02- and 1.36-fold in V2 and V3, increasing its abundance to 38.1, 43.2 and 40.3%, respectively, thus becoming the most abundant species overall. However, this change was not significant when tested statistically ($p>0.05$). Rikenellaceae sp had a more modest overall level, increasing of 1.96-fold, reaching a relative abundance of 0.54 (V1), 0.74 (V2) and 0.35% (V3). Species from the Firmicutes phylum generally showed a positive response to the zinc deficient medium with 9 bacterial species displaying an increase overall while 7 showed a decrease in their relative abundance. *Dorea* sp, *Faecalibacterium prausnitzii, Acidaminococcus* sp and *Megasphaera* sp were among the species that showed an average increase of over 3-fold, with *Dorea* sp having the biggest increase among them (4.05-fold). These species increased between 1.87- to 6.12-fold (V1), 3.62- to 5.42-fold (V2) (except *Faecalibacterium prausnitzii* which decreased 6.07-fold in V2) and 2.40- to 9.51-fold (V3). Although these bacteria had the biggest increase, their relative abundance remained at less than 1% in all 3 vessels, thus they may be considered to have a minor impact on the overall composition of the gut microbiota. Ruminococcaceae sp1, *Ruminococcus bromii, Dialister* sp and *Clostridium bolteae* also showed an increased overall but with a more modest average increase of 1.14- to 1.35-fold.


A
Paraprevotella sp
Bacteroides sp1
Bacteroides sp2
Bacteroides ovatus
Bacteroides fragilis
Bacteroides uniformis
Prevotella sp
Rikenellaceae sp
Clostridales sp
Lachnospiraceae sp
Dorea sp
Lachnospira sp
Ruminococcaceae sp1
Ruminococcaceae sp2
Oscillospira sp
Ruminococcus bromii
Dialister sp
Megasphaera sp
Acidaminococcus sp
Faecalibacterium prausnitzii
Clostridium bolteae
Ruminococcus sp
Ruminococcus gnavus
Eubacterium dolichum
Sutterella sp
Pyramidobacter piscolens
N/A
0.0078125
0.03125
0.125
0.5
2
8
32
128
512
Fold changes (Log2)
V1
V2
V3


B
Paraprevotella sp
Bacteroides sp1
Bacteroides sp2
Bacteroides ovatus
Bacteroides fragilis
Bacteroides uniformis
Prevotella sp
Rikenellaceae sp
Clostridales sp
Lachnospiraceae sp
Dorea sp
Lachnospira sp
Ruminococcaceae sp1
Ruminococcaceae sp2
Oscillospira sp
Ruminococcus bromii
Dialister sp
Megasphaera sp
Acidaminococcus sp
Faecalibacterium prausnitzii
Clostridium bolteae
Ruminococcus sp
Ruminococcus gnavus
Eubacterium dolichum
Sutterella sp
Pyramidobacter piscolens
N/A
0.0039063
0.0078125
0.015625
0.03125
0.0625
0.125
0.25
0.5
1
2
4
8
16
32
64
128
256
512
Fold changes (Log2)
V1
V2
V3

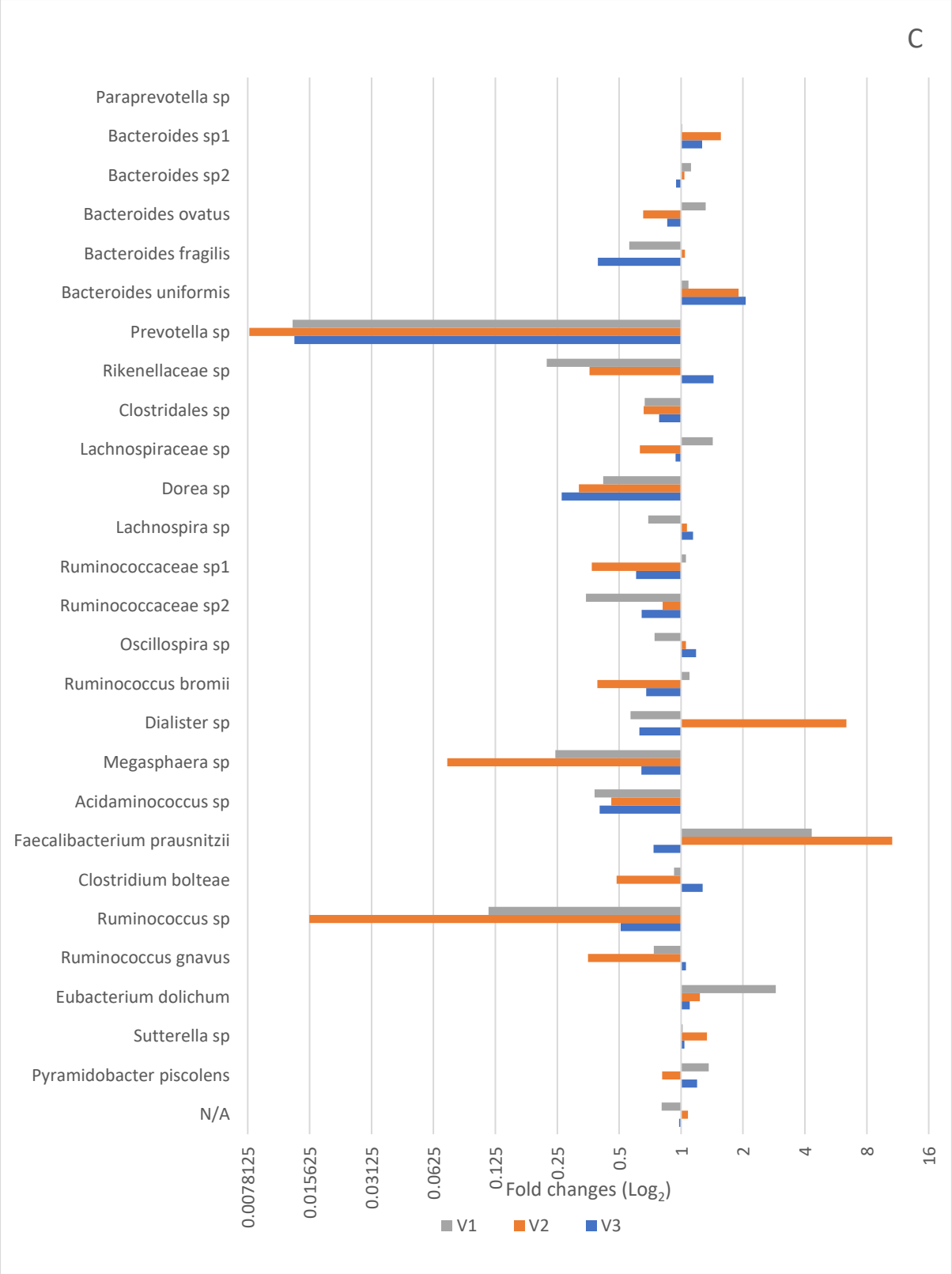


Figure 8.5 Fold changes in abundance at species level between different steady states. Fold changes of the bacteria at species level between SS2 and SS1 (**A**); SS3 and SS1 (**B**); SS3 and SS2 (**C**). Data derived from table 8.7. The fold changes are expressed as Log_2 fold.

Lachnospira sp had the biggest decrease among species in the Firmicutes phylum with a significant decrease of 3.68-fold (on average) across all the vessels. Clostridales sp, Lachnospiraceae sp, *Oscillospira* sp, *Ruminococcus* sp and *Eubacterium dolichum* also showed a decrease in all parts of the gut model with a roughly 2-fold difference between SS1 and SS2 (1.18- to 2.23-fold). Interestingly, in the absence of zinc, most of members in the Firmicutes phylum in V2 (transverse colon) showed a decrease in their relative abundance (12 species) compared to V1 (7 species) and V3 (9 species). *Sutterella* sp (Proteobacteria) showed a slight overall decrease in abundance (1.03-fold) mainly due to a decrease in V2 where it showed a 2.52-fold reduction, reducing abundance to 0.26 from 0.65%. V1 and V3 on the other hand showed an increase of 1.20- and 1.30-fold, raising its abundance to 0.55 and 1.78%, respectively. *Pyramidobacter piscolens* (Synergistetes) showed an increase in all 3 vessels with V2 and V3 showing a substantial 186- and 301-fold increase, respectively, increasing in abundance from 0.05 to 10.1% in V2 and from 0.03 to 8.30% in V3. V1 displayed a more moderate increase of 2.49-fold, representing an increase in abundance from 0.33 to 0.83%.

8.1.2.3.2 Effect of 77 µM Zn (SS3) on the composition of gut microbiota in zinc sufficient environment (SS1)

In SS3, 77 µM zinc was used to enhance an otherwise zinc deficient regime, to represent zinc supplementation. The results showed that in SS3 the levels of *Paraprevotella* sp dropped below the detection limit, possibly being outcompeted by the other bacterial species and excreted from the system, or due to sensitivity to zinc. *Prevotella* sp showed the biggest decrease (with respect to SS1) among the Bacteroidetes members with a decrease in all of the 3 vessels, with the biggest decrease occurring in V2 and V3 of 51.7- and 46.1-fold, respectively, corresponding to a reduction in relative abundance to 0.03% and 0.02%, while levels in V1 decreased from 1.69% to 0.05%, a 31.0-fold reduction. *Bacteroides* sp1, *B. ovatus* and *B. fragilis* showed a decrease (with respect to SS1) with an average overall reduction of 1.13-, 1.69- and 2.65-fold, respectively. The addition of 77 µM zinc caused *Bacteroides* sp2 and *B. uniformis* to show an increase in all vessels with the former species increasing slightly on average 1.09-fold while the latter increased on average 1.56-fold, making them

the most abundant bacteria. Although Rikenellaceae sp showed a 6.01-fold decrease in V1, in V2 and V3 it showed an increase of 1.26- and 2.32-fold respectively, thus giving an overall increase of 1.25-fold across all sections of the model. The presence of 77 µM Zn zinc increased the relative abundance of 3 Bacteroidetes species and decreased the abundance of 4 species overall, with respect to SS1.

For the Firmicutes phylum, the supplementation with 77 µM Zn exerted a negative effect overall, causing a reduction in relative abundance for 10 species, while 5 species showed an increase and 1 species remained unchanged, relative to SS1. Although *Megasphaera* sp reduced 1.88- and 3.73-fold in V1 and V2, it showed a 2.21-fold increase in V3, thus maintaining its relative abundance throughout the 3 vessels, showing no change in its abundance overall. Clostridales sp, *Lachnospira* sp, Ruminococcaceae sp2, *Oscillospira* sp and *Ruminococcus* sp showed a decrease in their relative abundance in all three vessels with respect to SS1. *Ruminococcus* sp exhibited the biggest decrease among these species with a 14.2-, 142- and 3.53-fold reduction in V1, V2 and V3, respectively, reducing its abundance to less than 0.05% in all 3 vessels. *Lachnospira* sp showed a significant decrease of 4.03-fold (on average) throughout all 3 vessels ($p<0.05$) while the rest of the Firmicutes species showed a reduction of 1.19- to 7.01-fold across the 3 vessels with respect to SS1, showing on average decrease of 1.71- to 4.03-fold. In summary, 77 µM zinc caused a decrease in the major Firmicutes species, mainly in V2 (transverse) and V3 (distal). Lachnospiraceae sp, Ruminococcaceae sp1, *Ruminococcus bromii, Dialister* sp and *Eubacterium dolichum* all showed an increase in V1 of 1.22- to 2.89-fold but a decrease in both V2 and V3 between 1.50- to 3.20-fold in V2 and between 1.02- to 3.15-fold in V3. *Faecalibacterium prausnitzii* was the only species that showed an increase in all vessels with 9.21- (V1), 1.75- (V2) and 6.99-fold (V3) increases, increasing its abundance to 0.44%, 0.08% and 0.19% respectively. *Dorea* sp, Ruminococcaceae sp1, *Ruminococcus bromii* and *Acidaminococcus* sp showed an overall increase with of 1.15- to 1.45-fold on average. *Sutterella* sp from the Proteobacteria phylum showed an increase in V1 (1.22-fold) and V3 (1.35-fold) but a 1.89-fold decrease in V2 while *Pyramidobacter piscolens* from the Synergistetes phylum showed an

average increase of 171-fold across all 3 vessels (with respect to SS1) with the highest increase occurring at V3 (360.05-fold).

8.1.2.3.3 Effect of 77 µM Zn supplementation on the gut microbiota in zinc deficient environment (SS3 vs SS2)

Comparing SS3 with SS2 resembles Zn supplementation of a zinc deficient host. The addition of 77 µM zinc increased the relative abundance of 8 bacterial species (3 from Bacteroidetes, 3 from Firmicutes, 1 from Proteobacteria, 1 from Synergistetes), with average increases of 1.03- to 5.23-fold, with *Faecalibacterium prausnitzii* showing the greatest increase amongst them. Members of the Bacteroidetes phylum benefited from the addition of zinc, particularly in V1 (proximal) and V2 (transverse) where the majority of the Bacteroidetes species showed an increase of 1.01- to 1.90-fold. *Bacteroides* sp1 and *B. uniformis* showed an increase in all 3 vessels with the former species increasing 1.01-(V1), 1.56- (V2) and 1.27-fold (V3) while the latter increased 1.09-, 1.90- and 2.06-fold, respectively. *Prevotella* sp, *B. ovatus, B. fragilis* and Rikenellaceae sp showed an overall decrease in the presence of zinc with *Prevotella* sp having the biggest reduction (88.1-fold on average) while the other 3 species showed a more modest decrease of 1.06- to 1.50-fold on average, but none of these differences were significant when tested statistically ($p>0.05$).

For members in the Firmicutes phylum, most of the major species showed a decrease upon zinc supplementation with the exception of *Dialister* sp, *Faecalibacterium prausnitzii* and *Eubacterium dolichum* which showed an average increase of 1.74- to 5.23-fold. Although Lachnospiraceae sp exhibited a decrease in both V2 (1.58-fold) and V3 (1.06-fold), in V1 there was an increase of 1.43-fold, thus maintaining the overall abundance of this species in the model despite some reduced/increased levels in specific vessels. Clostridales sp, *Dorea* sp, Ruminococcaceae sp2, *Megasphaera* sp, *Acidaminococcus* sp and *Ruminococcus* sp showed an overall reduction greater than 1.4-fold and they shared a similar pattern whereby their relative abundance was decreased in all 3 vessels particularly V1 and V2. *Ruminococcus* sp had the biggest overall reduction of 4.69-fold followed by *Megasphaera* sp with a 3.13-fold lower level; the remaining four species showed an average decrease of 1.42- to 3.00-fold. *Sutterella* sp and *Pyramidobacter piscolens* on the other hand

showed a slight overall increase with the supplementation of zinc, with an average increase of 1.13- and 1.12-fold respectively.

8.2 Impact of high zinc on gut microbiota using the three-stage gut model

As seen from the results from the batch culture (chapter 7), high levels of zinc have an impact on the total bacterial number and the microbiota composition. A gut model allows exploration of the effect of high zinc regimes on the gut microbiota in a manner more reflective of the conditions of the gut whereby its effect on the bacteria can be determined at the three sections of the colon. As mentioned before, 77 µM Zn is equivalent to a 10 mg zinc supplement tablet that can be easily obtained from any pharmacy or health supplement shop while 770 µM Zn is equivalent to 100 mg zinc per day which is 4 times the daily allowance (25 mg/d). The main reasons why supplements are taken by the public are to improve their overall health, to stay healthy, for bone health and to supplement the diet (Bailey et al, 2013; Gahche et al, 2017). Multivitamin-mineral is the most common supplement consumed by the public where it is used to 'maintain health' or 'supplement the diet' (Bailey et al, 2013). Although consumption of supplements is a common practice as it is believed to provide health benefit to the user, less than a quarter of all supplements are taken at the recommendation of healthcare providers (Kantor et al, 2016), thus the user might risk taking in certain nutrients over the daily allowance, particularly when multiple supplements are consumed daily as some nutrients may be available in different supplements. In order to address this problem, the gut model was used to identify the effect of high zinc (zinc over-supplementation) on the composition gut microbiota following a normally supplemented zinc diet. Thus, SS1 resembled a normal zinc diet (19 µM) condition, SS2 matched a normally zinc-supplemented diet (19+77 µM) and SS3 was designed to match over-supplementation (19+770 µM zinc).

8.2.1 Impact of zinc on the bacterial numbers in Gut Model 2 (GM 2)

The total bacterial number was determined using Flow-FISH coupled with Eub I-II-III probes to tag and count all the bacteria present, as described above. As seen from Figure 8.6, as before, the total bacterial counts decrease along the 3 vessels in the gut model and this pattern continues throughout

all the steady states. In SS1 whereby the medium had a total zinc concentration of 19 μM Zn, V1 (proximal colon) had a total of $4.05x10^8$ cells/ml medium but the total number reduced to $1.26x10^8$ and $1.11x10^8$ cells/ml medium as it moves along V2 (transverse colon) and V3 (distal colon), respectively. The total bacterial number increased slightly in V1 and V2, while V3 showed a decrease with increasing concentration of zinc from 77 μM (SS2) to 770 μM (SS3). In SS2, the addition of 77 μM Zn increased (total zinc concentration 96 μM Zn) the total bacteria in V1 and V2 by 1.06- and 1.16-fold (vs SS1), increasing its total number to $4.30x10^8$ and $1.46x10^8$ cells/ml medium, respectively, while V3 decreased 1.37-fold to $8.10x10^7$ cells/ml medium compared to SS1. When 770 μM Zn was added to the medium (789 μM Zn total zinc concentration), V1 and V2 showed a 1.19- and 1.24-fold increase compared to SS1, increasing its total bacteria to $4.81x10^8$ and $1.56x10^8$ cells/ml medium, respectively. V3 on the other hand had a 1.47-fold (vs SS1) decrease, reducing its total number to $7.53x10^7$ cells/ml medium. The 10-fold increase in zinc concentration (SS3 vs SS2) caused a 1.12- (V1) and 1.07-fold (V2) increase but V3 showed a 1.08-fold decrease with higher zinc concentration.

Table 8.8 Total bacterial counts in each vessels of 3 different steady states in GM1. Data derived from Figure 8.6

		SS1	SS2	SS3
GM2	V1	4.05E+08	4.30E+08	4.81E+08
	V2	1.26E+08	1.46E+08	1.56E+08
	V3	1.11E+08	8.10E+07	7.53E+07

Table 8.9 Fold difference between the 3 different steady states in GM1. Data derived from Figure 8.6

		SS2/SS1	SS3/SS1	SS3/SS2
GM2	V1	1.06	1.19	1.12
	V2	1.16	1.24	1.07
	V3	0.73	0.68	0.93

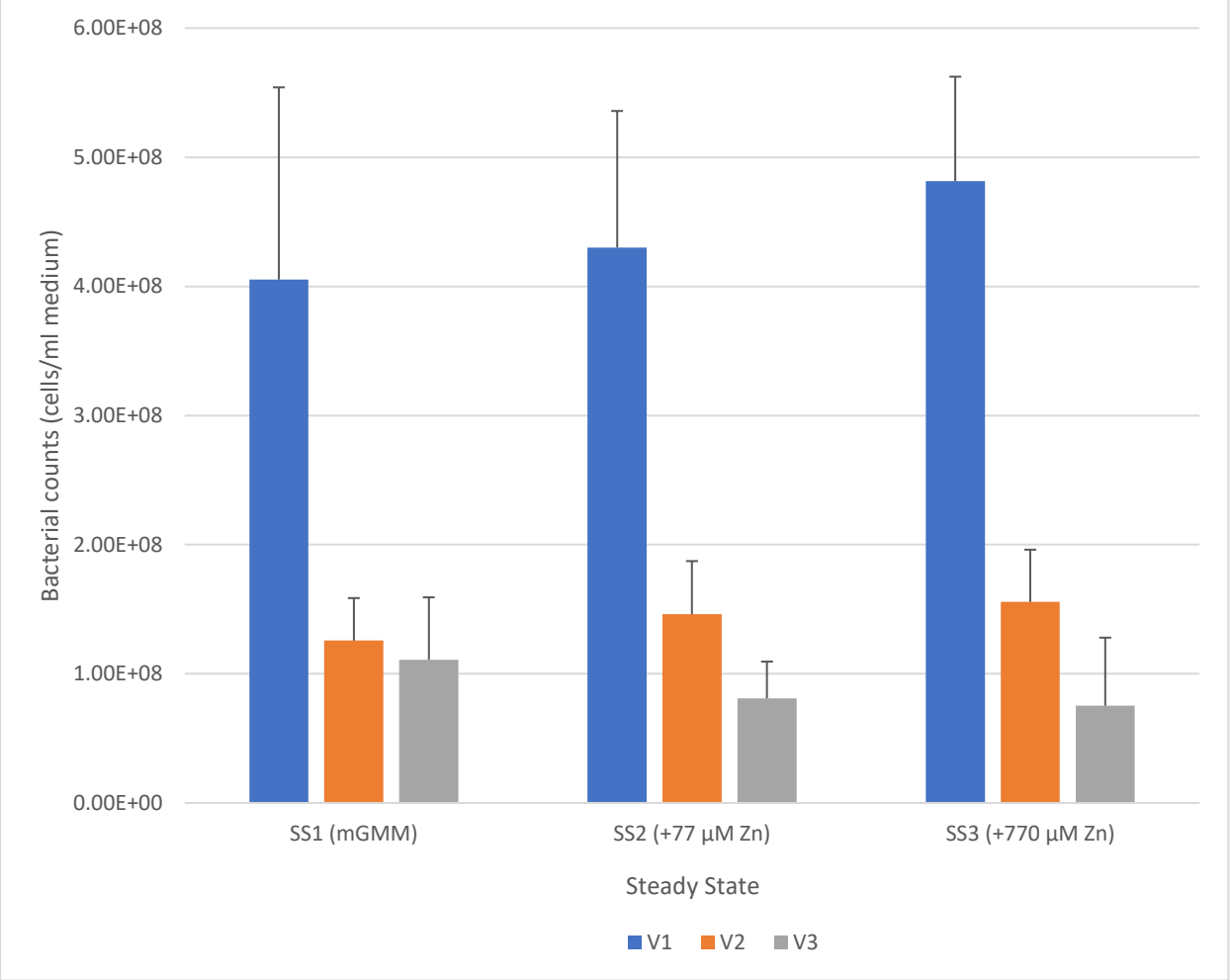


Figure 8.6 Total bacterial counts in each vessels of 3 different steady states in GM1. Total bacterial counts for each zinc regime were determined by Flow-FISH using Eub I-II-III probes. Each bar indicates a different vessels representing a different region of the colon: blue (V1, proximal colon), orange (V2, transverse colon), grey (V3, distal colon). Bacterial counts are expressed as cells/ml medium. Results are average of 3 subjects and error bars represent SD. One-way Anova with Bonferroni Post Hoc correction has been employed for statistical analysis. No significant difference was seen ($p>0.05$).

8.2.2 Impact of high zinc on the bacterial composition in Gut Model 2 (GM 2)

8.2.2.1 Impact of high zinc on the bacterial composition at phylum level

The bacterial composition was determined by 16S rRNA gene amplicon NGS analysis using an Illumina platform and compared to the reference database to determine the proportions of each type of bacteria present, as above.

As seen from Figure 8.7, Bacteroidetes was the most dominant phylum at SS1 with a relative abundance of 74.8% in V1 (proximal), 67.4% in V2 (transverse) and 77.9% in V3 (distal). Firmicutes were the second most abundant phylum but its abundance was 2-4 fold lower than Bacteroidetes

with a relative abundance of 24.1%, 30.1% and 19.7% in V1, V2 and V3, respectively. Actinobacteria, Proteobacteria and Synergistetes made up the remaining abundance with a relative abundance between 0.05 to 1.52% across all the vessels. With the addition of 77 µM Zn (SS2), only Actinobacteria showed an increase in all parts of the colon with a 3.14-, 1.58- and 3.27-fold increase (vs SS1) in the proximal (V1), transverse (V2) and distal (V3), increasing its abundance to 0.20, 0.15 and 0.16%, respectively, but such changes were not significant ($p>0.05$).

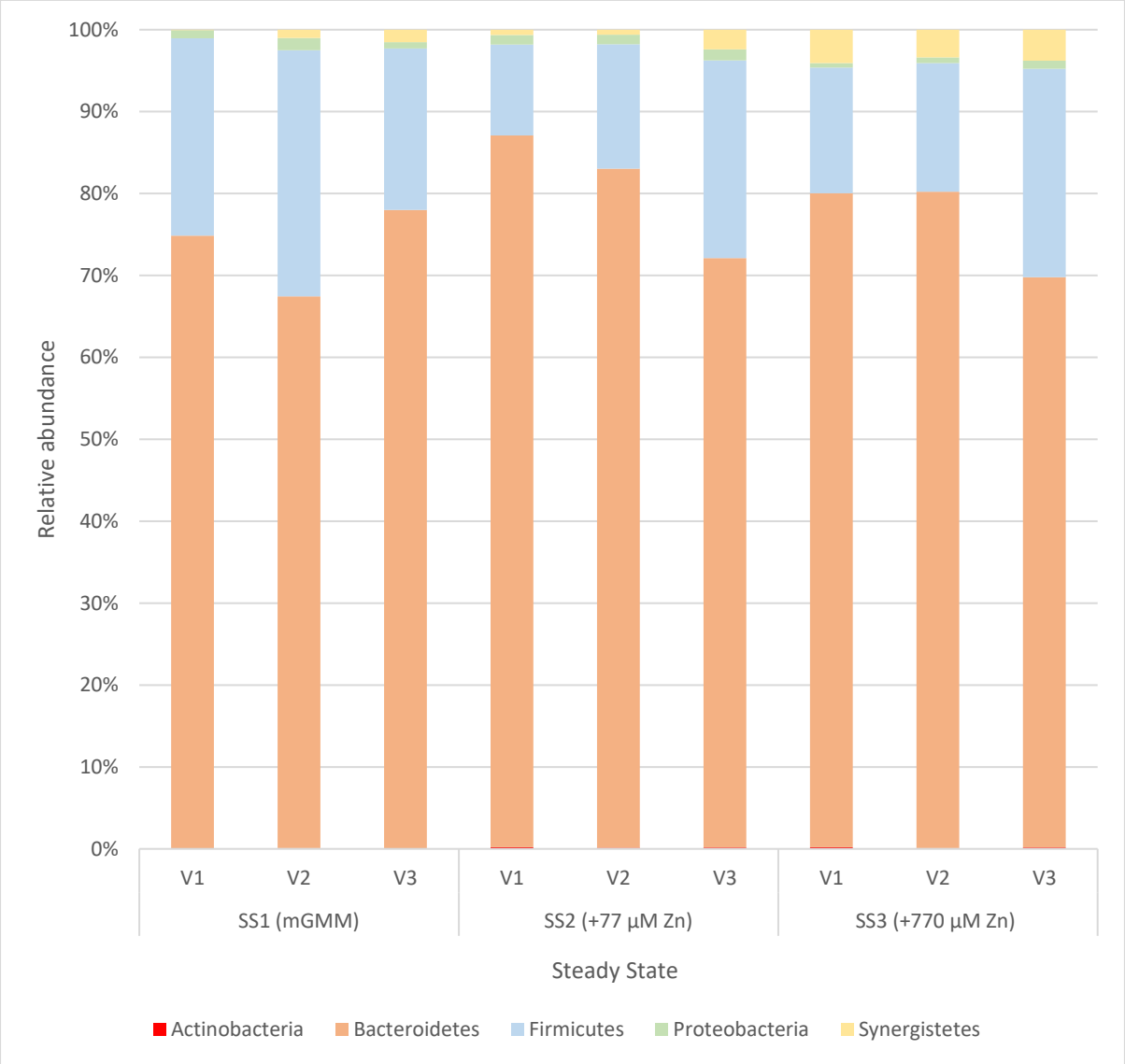


Figure 8.7 Composition of gut microbiota at phylum level. The gut microbiota is classified at phylum level for all vessels (V1, V2 and V3) at SS1, SS2 and SS3.

Table 8.10 Relative abundance of different bacterial groups at phylum level. The relative abundance of different bacterial groups in V1, V2 and V3 at SS1, SS2 and SS3. The bacterial phylum are expressed as a percentage of the total microbial community profile. The results are the average

of 3 subjects. One-way Anova with Bonferroni Post Hoc correction has been employed for statistical analysis. No significant difference is seen between the steady states.

Phylum	SS1			SS2			SS3		
	V1	V2	V3	V1	V2	V3	V1	V2	V3
Actinobacteria	0.06	0.10	0.05	0.20	0.15	0.16	0.22	0.10	0.17
Bacteroidetes	74.79	67.35	77.93	86.88	82.87	71.94	79.81	80.11	69.59
Firmicutes	24.11	30.05	19.72	11.10	15.19	24.14	15.34	15.70	25.47
Proteobacteria	0.97	1.48	0.77	1.18	1.19	1.34	0.56	0.73	0.95
Synergistetes	0.07	1.02	1.52	0.64	0.60	2.41	4.07	3.37	3.80

Table 8.11 Fold changes in abundance at phylum level between steady states. Fold changes of the bacteria at phylum level between the 3 different steady states. Data derived from table 8.10. One-way Anova with Bonferroni Post Hoc correction has been employed for statistical analysis. No significant difference is seen between the steady states.

Phylum	SS2/SS1			SS3/SS1			SS3/SS2		
	V1	V2	V3	V1	V2	V3	V1	V2	V3
Actinobacteria	3.14	1.58	3.27	3.45	1.08	3.39	1.10	0.68	1.04
Bacteroidetes	1.16	1.23	0.92	1.07	1.19	0.89	0.92	0.97	0.97
Firmicutes	0.46	0.51	1.22	0.64	0.52	1.29	1.38	1.03	1.06
Proteobacteria	1.22	0.80	1.73	0.58	0.49	1.23	0.48	0.61	0.71
Synergistetes	8.94	0.59	1.59	56.82	3.29	2.50	6.36	5.61	1.58

Bacteroidetes, which was still the most abundant phylum at SS2, showed a non-significant ($p>0.05$) increase of 1.16- and 1.23-fold in V1 and V2, increasing its abundance to 86.9% and 82.8%, respectively, but V3 showed a slight (1.08-fold) decrease, reducing in abundance to 71.9% in the presence of 77 µM Zn. Compared to Bacteroidetes, Firmicutes showed the opposing pattern whereby V1 and V2 had a 2.17- and 1.98-fold decrease in the presence of 77 µM Zn (vs SS1), reducing its abundance to 11.1% and 15.2%, respectively, while V3 showed a 1.22-fold increase, increasing its abundance to 24.1%, but these changes were not significant ($p>0.05$). Both Proteobacteria and Synergistetes showed similar patterns in which the addition of 77 µM Zn increased their relative abundance by 1.22- and 8.94-fold in V1 and by 1.73- and 1.59-fold in V3, respectively, while V2 showed a 1.24- and 1.70-fold decrease for the former and latter phyla, accordingly, but none of the changes were significant when tested ($p>0.05$). When 770 µM Zn (SS3) was applied to the medium, again none of the phyla showed a significant change ($p>0.05$) but the high zinc increased the abundance of both Actinobacteria and Synergistetes in all the vessels

compared to SS1, with Actinobacteria increasing 3.45-, 1.08- and 3.39-fold for V1, V2 and V3 while Synergistetes increased 56.8-, 3.29- and 2.50-fold for the same vessels. The addition of zinc caused Bacteroidetes to show a 1.07- and 1.19-fold increase in V1 and V2 compared to SS1, increasing in relative abundance to 79.8 and 80.1,% respectively, but in V3 a 1.12-fold decrease was experienced, reducing the abundance to 65.6%. Both Firmicutes and Proteobacteria share the same pattern whereby the high zinc caused a decrease in the first 2 vessels and an increase in V3 as compared to SS1. The relative abundance of Firmicutes decreased to 15.3% (1.57-fold) and 15.7% (1.91-fold) in V1 and V2, respectively, while V3 showed an increased to 25.5% (1.29-fold). Proteobacteria on the other hand showed a decrease of 1.72- and 2.04-fold for V1 and V2, reducing in abundance to 0.56 and 0.73%, respectively, while increasing the abundance of V3 to 0.95% (1.23-fold). When zinc concentration was increased from 77 µM Zn (SS2) to 770 µM Zn (SS3), the 10-fold increase in concentration benefited both Firmicutes and Synergistetes as they showed an increase in all 3 vessels while a negative affect was observed for Bacteroidetes and Proteobacteria as showed a decrease across all vessels. Firmicutes showed an increase of 1.38- (V1), 1.03- (V2) and 1.06-fold (V3) while the abundance of Synergistetes increased 6.36- (V1), 5.61- (V2) and 1.58-fold (V3) in SS3 compared to SS2. V1 of Bacteroidetes decreased 1.09-fold while both V2 and V3 decreased 1.03-fold. Proteobacteria on the other hand showed a 2.10- (V1), 1.64- (V2) and 1.41-fold (V3) reduction when 770 µM Zn was added as compared to 77 µM Zn. The high zinc regime (SS3) increased the abundance of Actinobacteria by 1.10- and 1.04-fold in V1 and V3, but V2 showed a 1.46-fold decrease.

8.2.2.2 Impact of high zinc on alpha and beta diversity

Ab before, Shannon Index and weighted UniFrac were used to determine the alpha and beta diversity.

As seen from Figure 8.8, the diversity of the bacterial microbiota increases from V1 to V3 and this pattern is seen across all steady states. Although not significant, the increased concentration of zinc decreases the diversity of the bacteria as shown by the lowered Shannon Index in SS2 and SS3 as

compared to SS1 (for V1 and 2). Comparing SS2 with SS3, a tenfold increase in zinc increases the diversity of the bacteria in V2 while both V1 and V3 showed a decrease in their diversity with the increasing zinc concentration.

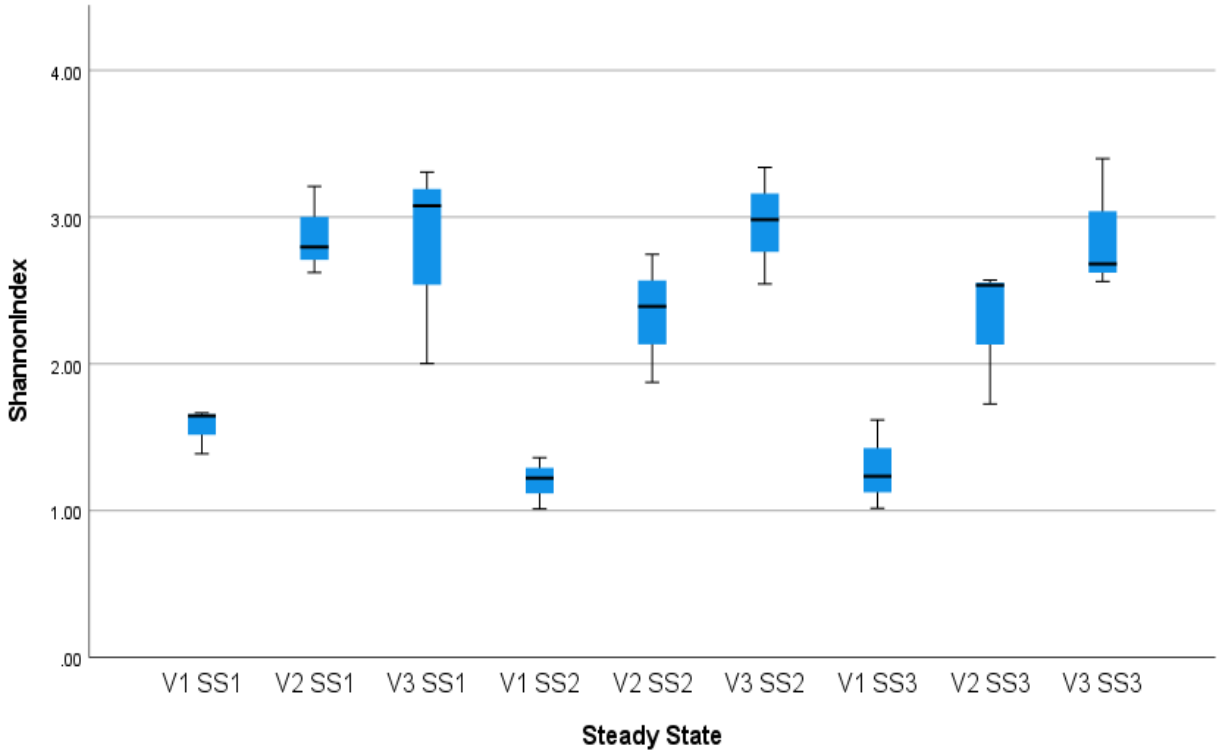


Figure 8.8 Alpha diversity measured by Shannon Index. The higher the value in Shannon Index indicates a higher species richness in the sample tested. Mann-Whitney U test were used for statistical analysis. No significant difference is seen between the regimes tested ($p>0.05$).

As seen from Figure 8.9, an increased zinc concentration (77 µM Zn or 770 µM Zn) did not have an effect on the beta diversity of the gut microbiota as no separate zinc clusters were seen with the exception of subject 1 (blue) whereby 2 separate but very close clusters are shown in the zinc regime (SS2 and SS3) as compared to SS1 (green circles within the blue circles) (Figure 8.9A; 8.9B). However, the 2 zinc regimes (77 µM Zn and 770 µM Zn) did not show any difference between them (Figure 8.9 C). Subject 2 (yellow) and subject 3 (red) also did not show any difference between the regimes tested, indicating the distinct nature of gut microbiota of the idividual subjects recruited have a bigger impact on the clustering than the zinc regime applied. Thus, the bacterial populations in

Figure 8.9 appears to be clustered based on the individual variation between the human subjects rather than the effect of zinc.

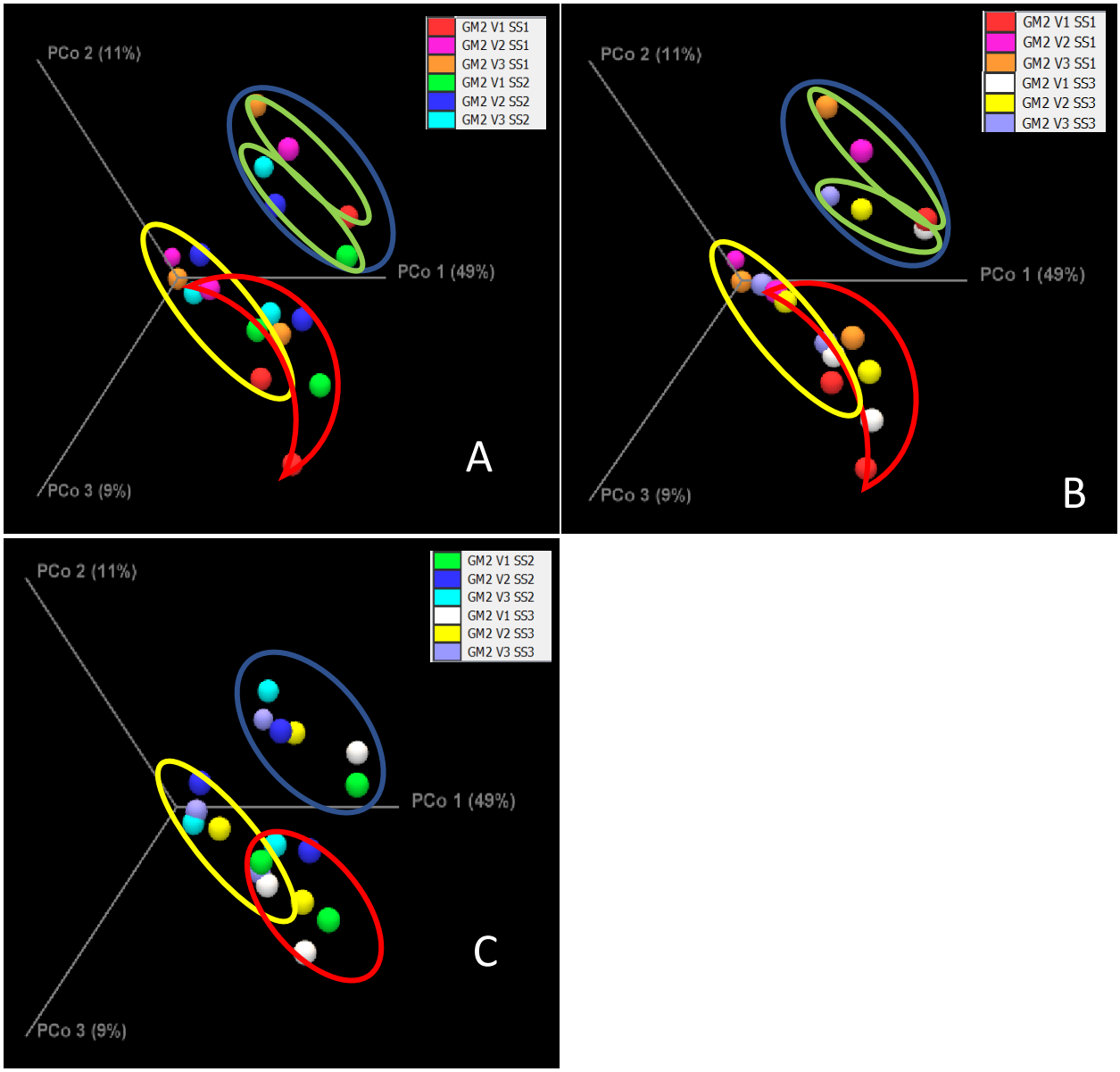


Figure 8.9 Beta diversity Principal Coordinate Analysis derived from weighted UniFrac in all samples. Each vessels at each steady state has a different colour: **SS1-** V1 (red), V2 (pink), V3 (orange); **SS2**- V1 (green), V2 (blue), V3 (cyan); **SS3**- V1 (white), V2 (yellow), V3 (purple). The beta diversity is compared between 2 different steady states: **A**, SS1 with SS2; **B**, SS1 with SS3; **C**, SS2 with SS3. For each axis, the precent of variation was reported in brackets.

8.2.2.3 Impact of high zinc on bacteria composition at species level

The NGS data were considered at 94% similarity on the Greengenes database (downloaded using CLC workbench) instead of the usual 99% as running at 99% similarity resulted in up to 30% of unidentified bacteria at species level while running at 94% showed almost no unidentifiable species.

However, it gave poorer separation of known species. For example, *Bacteroides* will be grouped as *Bacteroides* sp instead of *Bacteroides* sp1 and *Bacteroides* sp2 which was seen in the batch culture and GM1. However, the abundance of unidentifiable species was too high, thus the results at lower similarity were utilised.

Table 8.12 Effect of high zinc regime on relative abundance at the species level. The relative abundance of bacterial groups within the gut microbiota at species levels is indicated SS1, SS2 and SS3. SS1 (yellow), SS2 (green) and SS3 (orange). Data are expressed as a percentage of the total microbial community profile. The results are the average of 3 subjects. Only bacterial species present at >0.5% in at least 1 vessel of any steady states is shown. One-way Anova with Bonferroni Post Hoc correction has been employed for statistical analysis. (sp: species, N/A: not identifiable).

Phylum	Order	Species	SS1			SS2			SS3		
			V1	V2	V3	V1	V2	V3	V1	V2	V3
Actinobacteria	Coriobacteriales	Coriobacteriaceae sp	0.00	0.04	0.00	0.17	0.11	0.13	0.17	0.09	0.14
Bacteroidetes	Bacteroidales	*Bacteroides* sp	2.99	9.12	9.58	3.32	11.98	15.56	0.72	6.32	11.98
		Bacteroides fragilis	0.01	0.07	0.09	0.20	0.21	0.21	0.00	0.12	0.12
		Bacteroides ovatus	0.64	6.33	10.39	0.29	6.74	4.31	0.19	6.62	5.34
		Bacteroides uniformis	0.08	16.64	17.90	0.30	7.31	7.04	0.03	5.17	5.66
		Odoribacter sp	0.00	0.05	0.06	0.00	0.04	0.57	0.00	0.86	0.73
		Parabacteroides sp	0.00	0.22	0.32	0.00	0.04	0.08	0.00	0.06	0.07
		Parabacteroides distasonis	0.06	0.10	0.12	0.22	0.09	0.09	0.03	0.03	0.02
		Prevotella sp	71.01	33.99	38.15	82.53	56.03	43.14	78.81	60.47	43.82
		Rikenellaceae sp	0.00	0.33	0.41	0.01	0.14	0.20	0.00	0.15	1.36
		Alistipes indistinctus	0.00	0.31	0.70	0.00	0.24	0.57	0.00	0.18	0.36
Firmicutes	Clostridiales	*Acidaminococcus* sp	1.47	1.01	0.24	0.58	0.40	0.30	0.64	0.31	0.31
		Christensenellaceae sp	0.00	0.16	0.77	0.00	0.13	1.21	0.00	0.14	0.70
		Clostridiales sp	2.05	2.57	1.72	1.03	1.64	2.60	0.37	0.63	1.20
		Coprococcus sp	0.07	0.11	0.18	0.02	0.08	0.10	0.09	0.11	0.09
		Dialister sp	1.66	1.01	0.56	0.79	0.74	1.06	0.80	0.41	0.79
		Dorea sp	0.53	2.00	0.58	0.13	0.67	1.15	0.08	0.08	0.13
		Faecalibacterium prausnitzii	0.42	0.34	0.51	1.43	0.84	0.94	3.54	2.58	4.57
		Lachnospiraceae sp	0.71	1.39	1.26	0.28	0.73	1.34	0.91	1.14	2.25
		Lachnospira sp	0.19	1.26	0.32	0.10	0.19	0.20	0.04	0.11	0.16
		Megasphaera sp	1.12	1.48	0.49	0.77	0.59	0.94	0.65	0.54	0.79
		Oscillospira sp	0.05	1.47	1.61	0.04	1.93	2.91	0.14	1.15	2.45
		Roseburia faecis	0.02	0.01	0.00	0.34	0.51	0.55	0.39	0.43	1.06

		Ruminococcus gnavus	0.20	0.40	0.07	0.05	0.14	0.20	0.17	0.11	0.31
		Ruminococcaceae sp	1.11	11.89	4.12	0.55	2.85	5.37	0.44	1.80	3.86
		Ruminococcus sp	13.98	4.01	5.73	4.76	3.20	4.47	6.74	5.57	5.62
		Veillonellaceae sp	0.19	0.20	0.25	0.13	0.07	0.09	0.22	0.18	0.25
Proteobacteria	RF32	RF32 sp	0.02	0.07	0.12	0.02	0.59	0.30	0.02	0.24	0.20
	Burkholderiales	*Sutterella* sp	0.91	1.13	0.59	1.14	0.53	0.73	0.51	0.43	0.48
Synergistetes	Synergistales	*Pyramidobacter* sp	0.07	0.93	1.39	0.64	0.54	2.19	4.07	3.32	3.67
N/A			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 8.13 Fold changes in abundance at species level between steady states. Fold changes of the bacteria at species level between the 3 different steady states. Data derived from table 8.12. The colours indicate the different steady states comparisons: SS2/SS1 (green), SS3/SS1 (orange), SS3/SS2 (blue). Values in green indicates an increase in relative abundance and values in red indicates a decrease. Values in blue indicates undetectable abundance in the vessels, fold difference were unable to calculate. One-way Anova with Bonferroni Post Hoc correction has been employed for statistical analysis. Bacteria in bold indicates significant difference at certain steady state ($p<0.05$).

Phylum	Order	Species	SS2/SS1			SS3/SS1			SS3/SS2		
			V1	V2	V3	V1	V2	V3	V1	V2	V3
Actinobacteria	Coriobacteriales	Coriobacteriaceae sp	34.26	3.13	41.28	36.12	2.53	43.47	1.05	0.81	1.05
Bacteroidetes	Bacteroidales	*Bacteroides* sp	1.11	1.31	1.62	0.24	0.69	1.25	0.22	0.53	0.77
		Bacteroides fragilis	29.39	3.00	2.42	0.00	1.69	1.36	0.00	0.56	0.56
		Bacteroides ovatus	0.45	1.06	0.41	0.29	1.05	0.51	0.65	0.98	1.24
		Bacteroides uniformis	3.75	0.44	0.39	0.37	0.31	0.32	0.10	0.71	0.80
		Odoribacter sp	0.00	0.81	9.42	0.00	18.09	12.06	0.00	22.33	1.28
		Parabacteroides sp	0.00	0.16	0.26	0.00	0.26	0.22	0.00	1.60	0.84
		Parabacteroides distasonis	3.76	0.94	0.78	0.46	0.35	0.16	0.12	0.37	0.21
		Prevotella sp	1.16	1.65	1.13	1.11	1.78	1.15	0.95	1.08	1.02
		Rikenellaceae sp	11.81	0.41	0.49	4.46	0.45	3.34	0.38	1.10	6.78
		Alistipes indistinctus	0.00	0.78	0.82	0.00	0.58	0.51	0.00	0.74	0.63
Firmicutes	Clostridales	*Acidaminococcus* sp	0.40	0.39	1.25	0.43	0.30	1.31	1.09	0.77	1.05
		Christensenellaceae sp	0.98	0.85	1.57	0.00	0.91	0.91	0.00	1.07	0.58
		Clostridiales sp	0.50	0.64	1.51	0.18	0.25	0.70	0.36	0.39	0.46
		Coprococcus sp	0.32	0.71	0.55	1.44	1.04	0.49	4.53	1.46	0.88

		Dialister sp	0.48	0.73	1.91	0.48	0.40	1.42	1.01	0.55	0.74
		Dorea sp	0.24	0.34	2.00	0.15	0.04	0.22	0.62	0.13	0.11
		Faecalibacterium prausnitzii	3.36	2.42	1.85	**8.34**	**7.47**	**9.00**	**2.48**	**3.08**	**4.87**
		Lachnospiraceae sp	0.39	0.53	1.06	1.28	0.82	1.78	3.29	1.56	1.69
		Lachnospira sp	0.50	0.15	0.63	0.22	0.08	0.50	0.43	0.57	0.80
		Megasphaera sp	0.69	0.40	1.93	0.58	0.36	1.63	0.84	0.91	0.85
		Oscillospira sp	0.67	1.31	1.81	2.53	0.78	1.52	3.78	0.60	0.84
		Roseburia faecis	19.64	60.30	113.83	**22.36**	**50.10**	**217.50**	1.14	0.83	1.91
		Ruminococcus gnavus	0.24	0.35	2.90	0.82	0.28	4.49	3.46	0.81	1.55
		Ruminococcaceae sp	0.50	0.24	1.30	0.40	0.15	0.94	0.81	0.63	0.72
		Ruminococcus sp	0.34	0.80	0.78	0.48	1.39	0.98	1.42	1.74	1.26
		Veillonellaceae sp	0.71	0.35	0.34	1.18	0.94	1.01	1.66	2.66	2.98
Proteobacteria	RF32	RF32 sp	0.98	8.75	2.61	0.98	3.52	1.69	0.99	0.40	0.65
	Burkholderiales	*Sutterella* sp	1.25	0.47	1.23	0.56	0.38	0.82	0.44	0.81	0.66
Synergistetes	Synergistales	*Pyramidobacter* sp	8.94	0.58	1.57	56.82	3.55	2.64	6.36	6.15	1.67

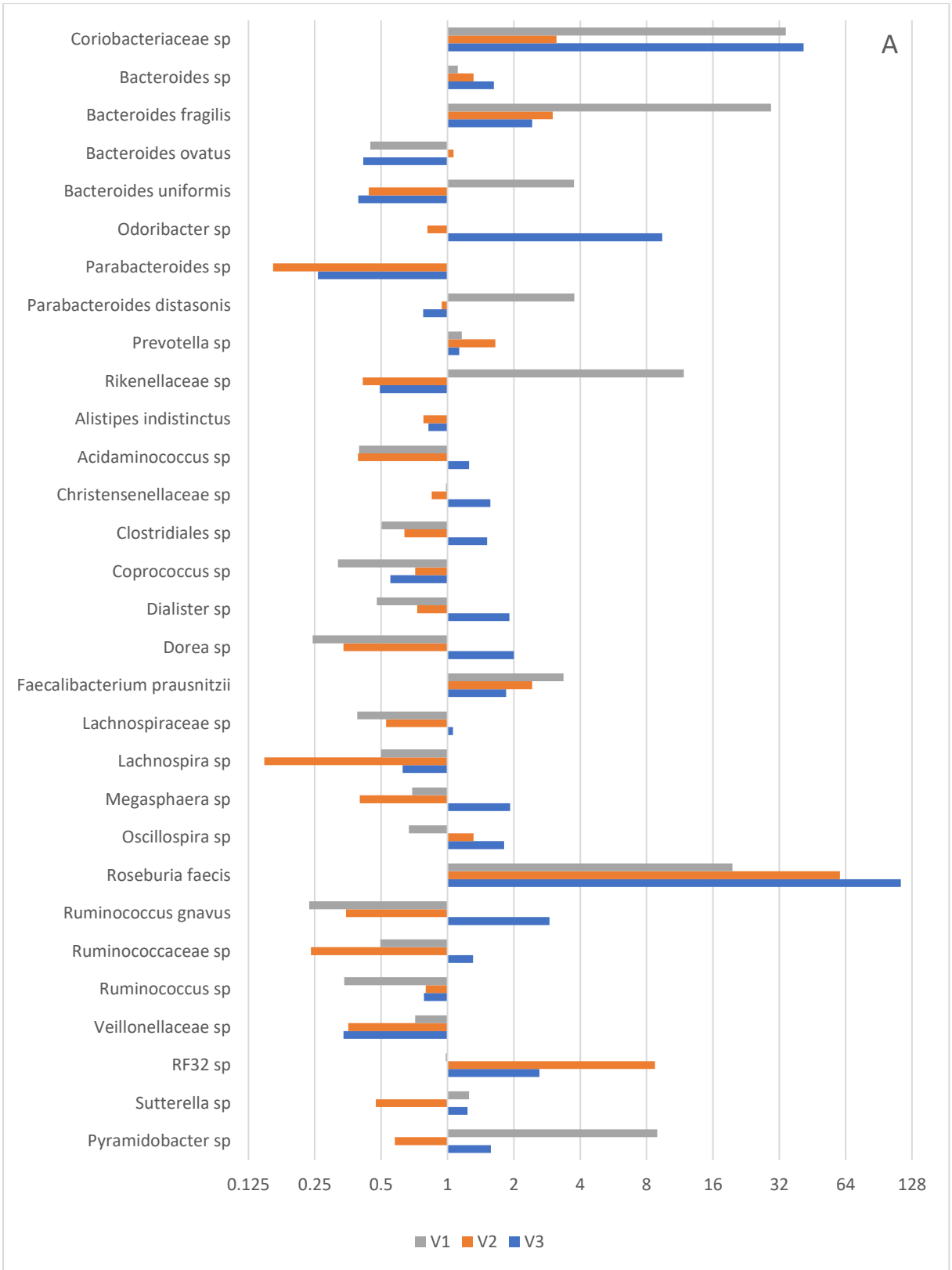
As seen from table 8.12, *Prevotella* sp, *Bacteroides uniformis*, *Ruminococcus* sp and *Bacteroides* sp were the most abundant bacteria in SS1 with an average relative abundance of 47.7, 11.5, 7.91 and 7.23%, respectively, across all 3 vessels. In SS1, the Bacteroidetes phylum was mainly dominated by the abovementioned species while the rest of the members in this phylum had an average abundance between 0.05 to 5.79% across all the vessels. *Ruminococcus* sp is the dominant bacteria species in the Firmicutes phylum followed by Ruminococcaceae sp with, an average abundance of 5.70%, and the remaining members in the Firmicutes phylum had an average abundance between 0.01 to 2.12%. Coriobacteriaceae sp of the Actinobacteria phylum and *Pyramidobacter* sp of the Synergistetes phylum had an average abundance of 0.01% and 0.80%, respectively, in SS1. RF32 sp and *Sutterella* sp from the Proteobacteria phylum showed an average abundance of 0.07% and 0.88% across all 3 vessels in the gut model.

8.2.2.3.1 Effect of 77 µM Zn (SS2) on the composition of gut microbiota compared to SS1

When 77 µM Zn was supplemented into the medium (SS2), the composition of the gut microbiota

showed some changes in response but none of the bacterial groups showed significant changes in SS2 ($p>0.05$). In the presence of 77 µM Zn, in V1 (proximal colon), the majority of the members in the Firmicutes phylum showed a decrease in abundance (14 out of 16 species) while most of Bacteroidetes, Actinobacteria, Proteobacteria and Synergistetes showed an increase in their relative abundance. Most of the bacteria species, irrespective of their phylum, showed a decrease in V2 (transverse colon) (21 out of 30 species) and an increase in their relative abundance in V3 (distal colon) (20 out of 30 speceis). Coriobacteriaceae sp showed an increase in all vessels in the presence of 77 µM Zn with V1, V2 and V3 increasing 34.3-, 3.13- and 41.3-fold, reaching an abundance of 0.17, 0.11 and 0.13%, respectively. Bacteroidetes also benefitted from the addition of zinc whereby 7 out of its 10 members showed an increase in abundance overall. *Bacteroides fragilis* showed the greatest increase among all the members with a 29.4- (V1), 3.00- (V2) and 2.42-fold (V3) increase (vs SS1), increasing its abundance to 3.32, 12.0 and 15.6%, respectively. Although showing a modest increase, *Bacteroides* sp and *Prevotella* sp shared a similar pattern with *B. fragilis* whereby the presence of zinc increases their abundance in all 3 vessels with the former species having an average increase of 1.35-fold and the latter increased 1.31-fold (on average). *Bacteroides uniformis*, *Parabacteroides* sp, *Parabacteroides distasonis*, Rikenellaceae *s*p and *Alistipes indistinctus* showed similar patterns in which they a decrease of between 1.06- to 6.19-fold across both V2 and V3 was observed. In V1 however, *B. uniformis, P. distasonis* and Rikenellaceae sp showed an increase of 3.75-, 3.76- and 11.8-fold respectively while the other 2 bacteria were undetected in SS2, thus their differences could not be determined. With the addition of 77 µM Zn, the abundance of *B. ovatus* increased slightly to 6.74% (1.06-fold) in V2 while in V1 and V3 levels decreased 2.24- and 2.41-fold, reducing abundance to 0.29 and 4.31%, respectively. *Odoribacter* sp on the other hand showed a 1.23-fold decrease in V2 and a 9.42-fold increase in V3, reaching an abundance of 0.04 and 0.57%, respectively, but its abundance in V1 was undetected in SS2. The bacteria in the Firmicutes phylum can be categorised into 4 groups based on their reaction towards 77 µM Zn. The first group consists of *Roseburia faecis* and *Faecalibacterium prausnitzii* which were the only 2 species that showed an

increase in all vessels, indicating a stimulatory effect in all parts of the colon, with the former species showing on average 64.6-fold increase and the latter increasing on average 2.54-fold as compared to SS1.


A
Coriobacteriaceae sp
Bacteroides sp
Bacteroides fragilis
Bacteroides ovatus
Bacteroides uniformis
Odoribacter sp
Parabacteroides sp
Parabacteroides distasonis
Prevotella sp
Rikenellaceae sp
Alistipes indistinctus
Acidaminococcus sp
Christensenellaceae sp
Clostridiales sp
Coprococcus sp
Dialister sp
Dorea sp
Faecalibacterium prausnitzii
Lachnospiraceae sp
Lachnospira sp
Megasphaera sp
Oscillospira sp
Roseburia faecis
Ruminococcus gnavus
Ruminococcaceae sp
Ruminococcus sp
Veillonellaceae sp
RF32 sp
Sutterella sp
Pyramidobacter sp
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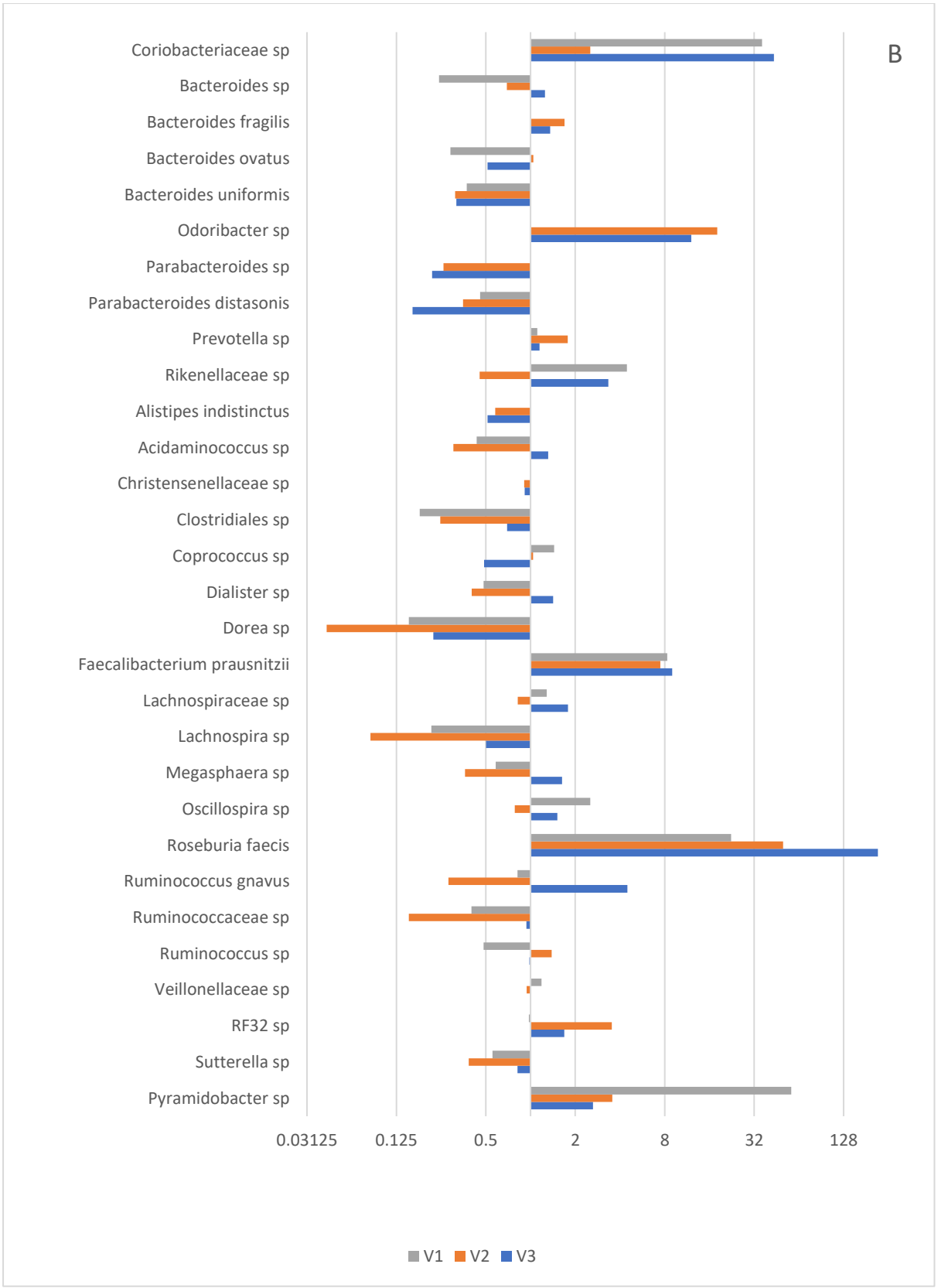
0.25
0.5
1
2
4
8
16
32
64
128
V1
V2
V3


B
Coriobacteriaceae sp
Bacteroides sp
Bacteroides fragilis
Bacteroides ovatus
Bacteroides uniformis
Odoribacter sp
Parabacteroides sp
Parabacteroides distasonis
Prevotella sp
Rikenellaceae sp
Alistipes indistinctus
Acidaminococcus sp
Christensenellaceae sp
Clostridiales sp
Coprococcus sp
Dialister sp
Dorea sp
Faecalibacterium prausnitzii
Lachnospiraceae sp
Lachnospira sp
Megasphaera sp
Oscillospira sp
Roseburia faecis
Ruminococcus gnavus
Ruminococcaceae sp
Ruminococcus sp
Veillonellaceae sp
RF32 sp
Sutterella sp
Pyramidobacter sp
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0.5
2
8
32
128
V1
V2
V3

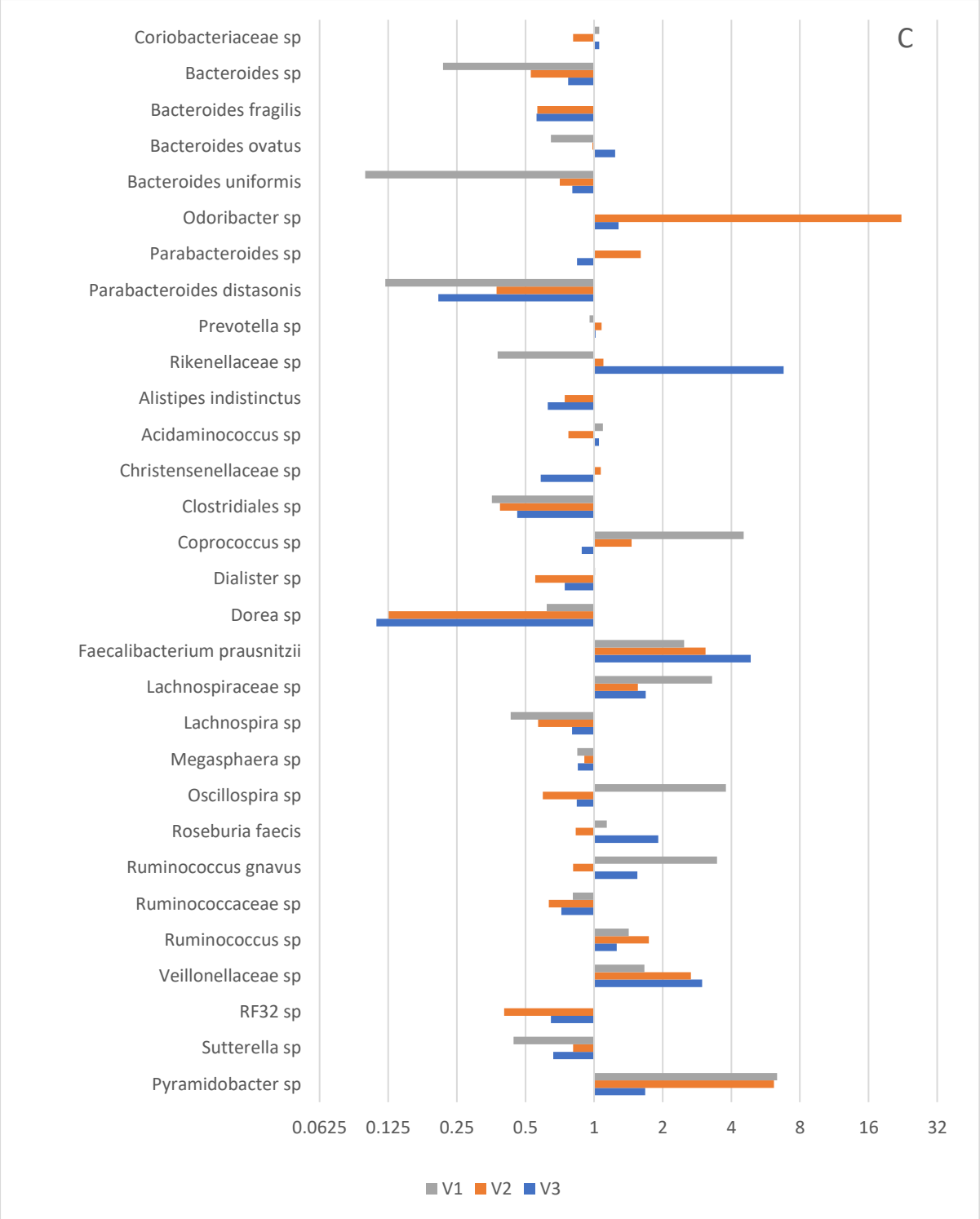

Figure 8.10 Fold changes in abundance at species level between different steady states. Fold changes of the bacteria at species level between SS2 and SS1 (**A**); SS3 and SS1 (**B**); SS3 and SS2 (**C**). Data derived from table 8.13. The fold changes are expressed as Log_2 fold.

Oscillospira sp was the only species in the second group which showed an increase in both V2 and V3

(of 1.31- and 1.81-fold, respectively), but showed a 1.50-fold decrease in V1 with 77 µM Zn. The

majority of the members of the Firmicutes phylum (9 out of 16 species) belong to the third group: *Clostridales* sp, Christensenellaceae sp, Lachnospiraceae sp, *Ruminococcus gnavus*, *Dorea* sp, *Ruminococcaceae* sp, *Acidaminococcus* sp, *Dialister* sp and *Megasphaera* sp whereby a decrease in both V1 and V2 occurred, with an increase in V3 when 77 µM Zn was included in the medium. These species showed a modest decrease of between 1.02- to 4.25-fold across V1 and V2, while in V3 an increase of between 1.06- to 2.90-fold was exhibited. The fourth group consists of *Lachnospira* sp, *Coprococcus* sp, *Ruminococcus* sp and Veillonellaceae sp whereby the presence of 77 µM Zn causes a reduction in all vessels, indicating an inhibitory effect of zinc on these specoes in all parts of the colon. *Lachnospira* sp showed the biggest decrease in its abundance of 2.00- (V1), 6.79- (V2) and 1.60-fold (V3) compared to SS1 while the remaining 3 species showed an average decrease of 1.89-, 1.56- and 2.14-fold, respectively. Unknown bacteria belonging to the RF32 order showed a decrease of 1.02-fold in V1, while in V2 and V3 there was a 8.75- and 2.61-fold increase in the presence of zinc. *Sutterella* sp and *Pyramidobacter* sp both showed an increase of 1.25- and 8.94-fold in V1, 1.23- and 1.57-fold in V3 respectively but a decrease of 2.11- and 1.73-fold in V2 when 77 µM zinc was applied.

8.2.2.3.2 Effect of 770 µM Zn (SS3) on the composition of gut microbiota compared to SS1

When 770 µM Zn was added to the medium (SS3), most of the species showed a decrease in V1 and V2 with 15 bacterial groups showing a decrease in the proximal region (V1) and 19 groups displaying a decrease in the transverse region (V2). In the distal region (V3), 17 bacterial groups showed an increase in the abundance when high zinc was present. The presence of 770 µM Zn increased the abundance of Coriobacteriaceae sp by 36.1- (V1), 2.53- (V2) and 43.5-fold (V3) as compared to SS1. Most of the members in the Bacteroidetes phylum showed a decrease in the high zinc regime, with only 4 bacterial groups showing an increase, however, none of the changes were significant when tested statistically ($p>0.05$). *Odoribacter* sp showed the greatest increase with an average increase of 10.1-fold in across all 3 vessels, followed by Rikenellaceae sp with 2.75-fold (on average). The relative abundance of *B. fragilis* and *Prevotella* sp were increased by an average of 1.02- and 1.35-

fold, respectively, across all the vessels. Although these 4 species showed an increase in their abundance overall, only *Prevotella* sp showed an increase in all 3 vessels. *B. fragilis* and *Odoribacter* sp showed an increase in both V2 and V3 but their abundance was undetected in V1, thus the changes could not be calculated. Rikenellaceae sp on the other hand showed an increase in V1 and V3 but a decrease in V2. *Parabacteroides* sp, *Bacteroides* sp, *B. ovatus, B. uniformis, P. distasonis* and *A. indistinctus* all showed a decrease in their abundance with *Parabacteroides* sp experiencing the greatest decrease with an average 6.29-fold reduction across all the vessels. The remaining species showed a more modest average decrease of between 1.37- to 3.10-fold. In terms of their reaction towards the high zinc regime, *B. uniformis* and *P. distasonis* showed a decrease in all 3 vessels. The relative abundance of *Parabacteroides* sp and *A. indistinctus* showed a decrease in both V2 and V3, but these species were not detected in V1, thus the fold changes in V1 were not calculated. The presence of 770 µM Zn decreased the abundance of *Bacteroides* sp in the proximal (V1) and transverse (V2) region but the distal region (V3) showed an increase. *B. ovatus* on the other hand showed a decrease in V1 and V3 in the presence of high zinc but V2 showed an increased abundance.

In the Firmicutes phylum, *Roseburia faecis* and *Faecalibacterium prausnitzii* were the only 2 bacteria that show an increase in all parts of the colon as indicated by the increased abundance across all vessels. *R. faecis* showed the greatest increase with a significant increase of 96.7-fold (on average) compared to SS1 ($p<0.05$) while *F. prausnitzii* also showed a significant increase of 8.27-fold (on average) in the presence of 770 µM Zn ($p<0.05$). Similar to the 77 µM Zn regime, members in the Firmicutes phylum could be categorised into 4 different groups based on their reaction towards the high zinc regime but none of the species showed a significant change ($p>0.05$). In the first group, the presence of 770 µM Zn decreased the abundance of *Dorea* sp, Clostridales sp, *Lachnospira* sp and Ruminococcaceae sp in all 3 vessels with *Dorea* sp showing the biggest decrease of 7.21-fold (on average) compared to SS1 while the other 3 bacteria had an average reduction of 2.68-, 3.75- and 2.01-fold respectively. Christensenellaceae sp can technically be considered in the first group as it

showed a decrease in both V2 and V3 but its abundance was undetected in V1, thus the changes could not be determined in the proximal region. The second group consist of *Ruminococcus gnavus, Acidaminococcus* sp, *Dialister* sp and *Megasphaera* sp whereby the presence of high zinc caused a decrease in their relative abundance in the proximal (V1) and transverse (V2) region but an increase in the distal (V3) region. These 4 species showed a modest decrease of 1.23- to 3.57-fold in V1 and V2, and an increase of 1.31- to 4.49-fold in V3. The third group of bacteria consists of Lachnospiraceae sp, *Oscillospira* sp and Veillonellaceae sp which showed an increase in abundance of 1.01- to 2.53-fold in V1 and V3, as compared to SS1, but in V2 they displayed a decrease between 1.06- to 1.28-fold. The fourth group comprises species that showed a pattern not shared by any other bacteria. *Coprococcus* sp and *Ruminococcus* sp were the only 2 species in this group whereby the former bacteria showed an increase of 1.44- and 1.04-fold in V1 and V2 but a decrease of 2.06-fold in V3 while the relative abundance of the latter bacteria was reduced by 2.07- and 1.02-fold in V1 and V3 but V2 had an increase of 1.39-fold in the presence of 770 µM Zn as compared to SS1. *Pyramidobacter* sp of the Synergistetes phylum showed an average increase of 21-fold across all 3 vessels while *Sutterella* sp of the Proteobacteria phylum gave an average reduction of 1.71-fold across all the vessels compared to SS1. RF32 sp on the other hand showed a 1.03-fold reduction in V1, while V2 and V3 showed an increase of 3.52- and 1.69-fold, respectively.

8.2.2.3.3 Effect of ten-fold increased zinc on the composition of gut microbiota (SS3 vs SS2)

Comparing SS2 and SS3 whereby the concentration of zinc was increased from 77 µM to 770 µM, the ten-fold increase in zinc caused 12 bacterial groups to show an overall increase in relative abundance (i.e. an average increase across 3 vessels) while the other 18 bacterial groups showed a decrease. The bacteria species were grouped based on their reaction towards the increased concentration of zinc as similarities in their growth patterns could be discerned. The first group of bacteria includes Lachnospiraceae sp, *Faecalibacterium prausnitzii, Ruminococcus* sp, Veillonellaceae sp and *Pyramidobacter* sp which showed an increase in all 3 vessels with an average increase of 1.47- to 4.73-fold. Of these 5 species, *F. prausnitzii* showed a significant increase of 3.48-fold (on

average) following the increase of zinc concentration ($p<0.05$) while the other 4 species showed a non-significant increase ($p>0.05$). *Odoribacter* sp, *Prevotella* sp and Rikenellaceae sp (second group) also showed an overall increase in their relative abundance in SS3 compared to SS2 in V2 and V3, showing an increase of 1.02- to 22.3-fold. Fold changes in V1 for *Odoribacter* sp were unavailable as its abundance was undetected in both SS2 and SS3. Unlike in V2 and V3, V1 of *Prevotella* sp and Rikenellaceae sp showed a decrease of 1.05- and 2.65-fold, respectively. The third group consists of Coriobacteriaceae sp, *Ruminococcus gnavus*, *Roseburia faecis* and *Acidaminococcus* sp which all showed an increase in the proximal (V1) and distal (V3) region of the colon of 1.05- to 3.46-fold but the transverse (V2) region showed a decrease of 1.20- to 1.30-fold. Despite showing a decrease in V2, the abundance of *R. gnavus* and *R. faecis* were increased overall while Coriobacteriaceae sp and *Acidaminococcus* sp showed a decrease in their abundance overall. The relative abundance of *Bacteroides fragilis*, *Alistipes indistinctus*, *Oscillospira* sp and *Dialister* sp (fourth group) was increased in V1 (proximal), while in V2 (transverse) and V3 (distal) a decrease was seen. V2 and V3 showed a decrease of 1.19- to 1.81-fold. *B. fragilis* and *A. indistinctus* were undected in the V1 region of SS3, thus the degree of change could not be calculated. *Oscillospira* sp and *Dialister* sp showed an increase of 3.78- and 1.01-fold, respectively, as compared to SS2. The increased zinc in SS3 decreased the abundance of *Parabacteroides* sp and *Coprococcus* sp (fifth group) in V3 by 1.19- and 1.13-fold respectively. *Parabacteroides* sp was undetectable in V1 while in V2 it showed a 1.60-fold increase with zinc. *Coprococcus* sp on the other hand showed an increase of 4.53- and 1.46-fold in V1 and V2, respectively. The last group consists of bacteria that show a decrease in all 3 vessels when the zinc concentration was increased by tenfold: *Bacteroides* sp, *Bacteroides uniformis*, *Parabacteroides distasonis,* Clostridales sp, *Dorea* sp, *Lachnospira* sp, Ruminococcaceae sp, *Dialister* sp, *Megasphaera* sp and *Sutterella* sp. Among these bacteria, *P. distasonis* showed the greatest decrease with an average reduction of 4.27-fold across all 3 vessels, followed by *Dorea* sp and Clostridales sp with 3.50- and 2.49-fold (on average) decreases, accordingly. The remaining species showed more modest decreases of 1.16- to 1.98-fold (on average) as compared to SS2.

8.3 Differences between the gut model medium (GMM) and modified gut model medium (mGMM) in the composition of gut microbiota using the three-stage gut model

The gut model medium (GMM) which mimics the content of the human colon provides nutrients needed for the gut microbiota to grow and develop and is useful for a wide range of *in vitro* studies involving the gut microbiota. However, the presence of mucin and yeast extract in the medium contributed 19 µM of zinc which may have affected the outcome of attempts to explore the impact of a low zinc regime. Thus, a modified version was utilised whereby mucin and yeast extract were excluded from the medium in an effort to lower the zinc content, reducing the background zinc concentration to 3 µM. A vitamin and mineral solution was used to supplement the medium, in place of yeast extract, to provide the microbiota with the necessary nutrients for growth. Although the modified gut model medium (mGMM) was successful in lowering the zinc content to enable zinc related studies to be conducted, its effect on the gut microbiota compared to the GMM was not clear. Thus, a gut model was set up to compare the two media to determine the effect on the gut microbiota to ensure that the bacteria were not heavily affected by the changes in the ingredients of the medium. For comparison between the two models, zinc levels were kept the same at 19 µM whereby additional 16 µM were added to the mGMM.

8.3.1 Impact of the medium on the bacterial numbers in Gut Model 3 (GM 3)

The total bacterial count for each vessel was measured using Flow-FISH coupled with Eub I-II-III universal probes. As seen from Figure 8.11, the total bacteria count in the mGMM (SS2) is lower than the GMM (SS1), particularly in V2 and V3. V1 for both media showed negligible difference, with the GMM giving a 1.06-fold lower count. V2 and V3 with mGMM gave 2.14- and 1.81-fold lower levels than GMM, having only 1.75×10^8 (vs 3.75×10^8) and 9.42×10^7 (vs 1.71×10^8) cells/ml medium, respectively. Although mGMM showed lower bacterial counts than the GMM, this was not significant ($p>0.05$).

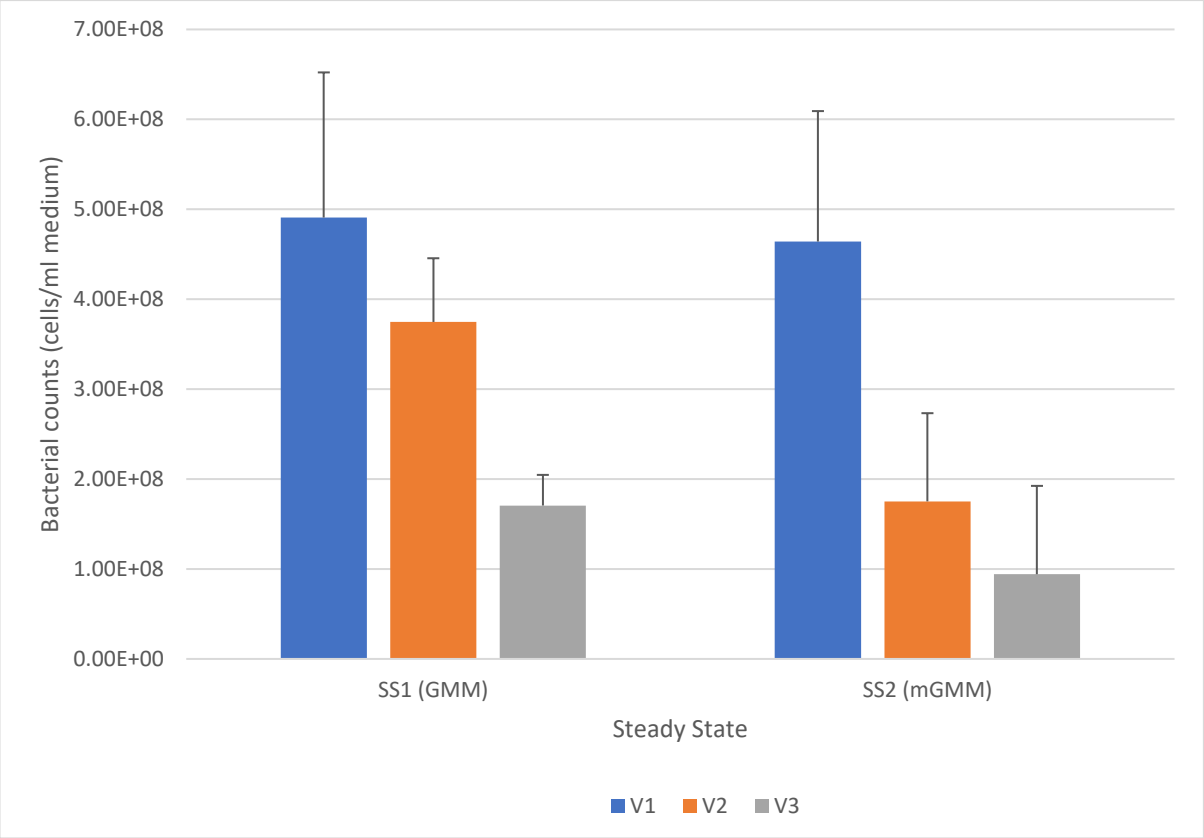

Figure 8.11 Total bacterial counts in each vessels of 2 different steady states in GM3. Total bacterial counts for each zinc regime were determined by Flow-FISH using Eub I-II-III probes. Each bar indicates a different vessels representing a different region of the colon: blue (V1, proximal colon), orange (V2, transverse colon), grey (V3, distal colon). Bacterial counts are expressed as cells/ml medium. Results are average of 3 subjects and error bars represent SD. One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. No significant difference is seen ($p>0.05$).

Table 8.14 Total bacterial counts and fold difference in each vessels of 2 different steady states in GM3. Data derived from Figure 8.11

	Vessels	Steady State		Fold Changes
		SS1	SS2	SS2/SS1
GM3	V1	4.91E+08	4.64E+08	0.95
	V2	3.75E+08	1.75E+08	0.47
	V3	1.71E+08	9.42E+07	0.55

8.3.2 Impact of the medium on the bacterial composition in Gut Model 3 (GM 3)

8.3.2.1 Impact of the medium on the bacterial composition at phylum level

The bacterial composition was determined by 16S rRNA gene amplicon NGS analysis whereby the samples are sequenced on the Illumina platform and subsequently compared to the reference

database to determine the type of bacteria present. Once the bacteria were determined, they were grouped according to phylum and species to enable further analysis (see Methods for details).

As seen from Figure 8.12, the gut microbiota was mainly dominated by Bacteroidetes and Firmicutes with Actinobacteria, Proteobacteria and Synergistetes contributing to the remaining abundance. In SS1 (GMM), the abundance of Bacteroidetes and Firmicutes are roughly the same in all vessels except V2. Bacteroidetes had a relative abundance of 48.7 and 48.3% in V1 and V3 while Firmicutes had 48.5 and 47.6% abundance in the same vessel. In V2, Bacteroidetes showed an abundance of 56.9% while Firmicutes only had 40.0%. Actinobacteria, Proteobacteria and Synergistetes had an average abundance of 0.51, 1.99 and 0.82% across all the vessels, respectively. In SS2, the mGMM tended to favour the growth of Bacteroidetes and Proteobacteria as seen by the increase in their relative abundance. Bacteroidetes showed an increase of 1.30- and 1.14-fold in V1 and V2, increasing in abundance to 63.3% and 64.7%, respectively, while V3 showed a slight decrease of 1.03-fold to 46.9%. The relative abundance of Proteobacteria on the other hand increased to 10.1 (V1), 4.97 (V2) and 4.94% (V3), showing a 5.43-, 2.69- and 2.18-fold increase, respectively. Firmicutes decreased 1.85-, 1.34- and 1.03-fold, reducing in abundance to 26.2, 29.8 and 46.2% in V1, V2 and V3, respectively. Actinobacteria showed a decrease in abundance of 3.62- and 1.30-fold in V1 and V3, a reduction in abundance to 0.25 and 0.38%, accordingly, while levels in V2 increased 1.26-fold to 0.16% in SS2. The relative abundance of Synergistetes increased to 0.08 and 1.65% in V1 and V3, showing a 78.4- and 1.22-fold increase from SS1 but decreased to 0.36% (3.15-fold) in V2. Although there was a difference between the relative abundance of the bacteria between both steady states, these were not significant when tested ($p>0.05$).

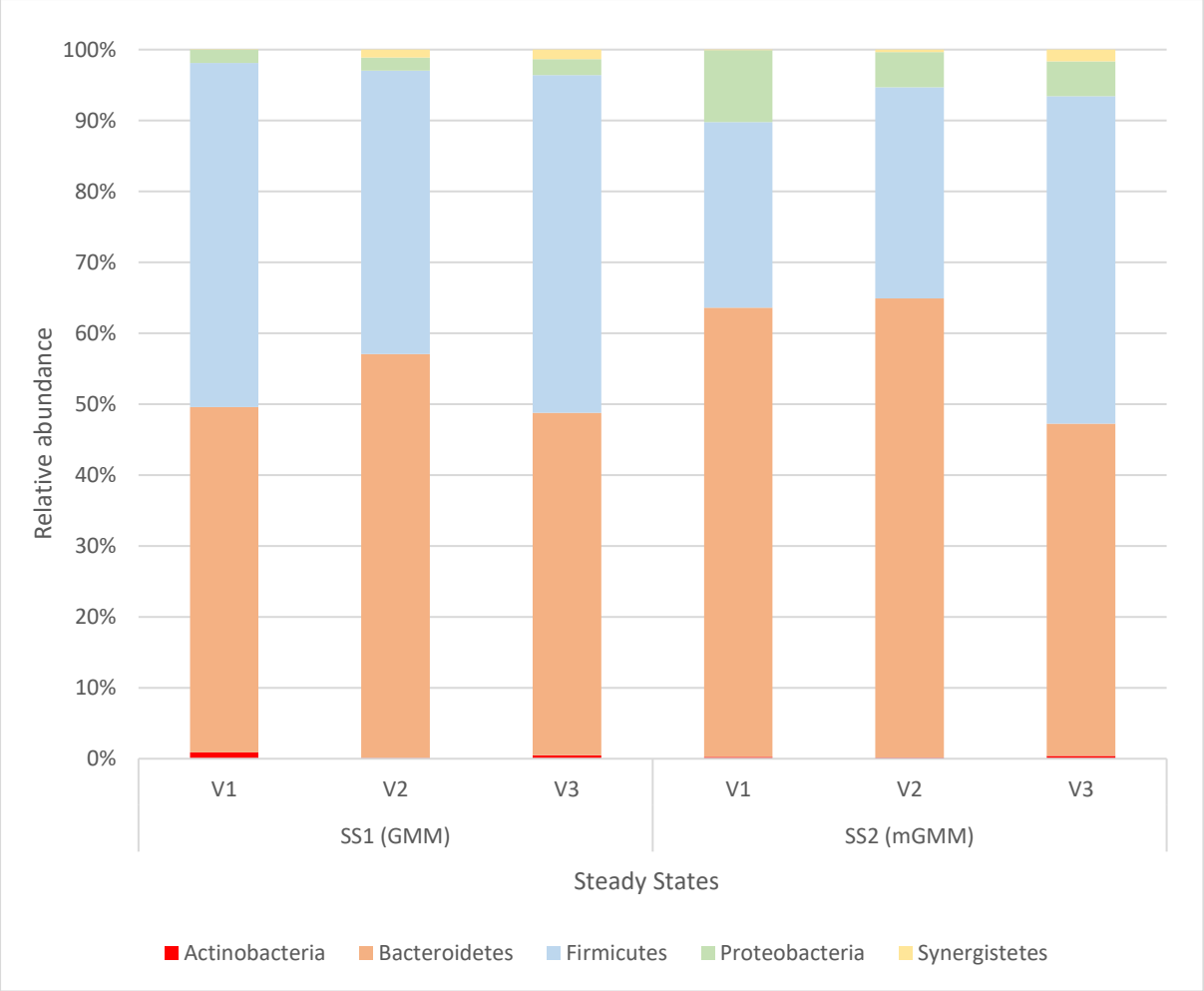

Figure 8.12 Composition of gut microbiota at phylum level. The gut microbiota is classified at phylum level for all vessels (V1, V2 and V3) at SS1 and SS2

Table 8.15 Relative abundance and fold changes of different bacterial groups at phylum level. The relative abundance of different bacterial groups in V1, V2 and V3 at SS1 and SS2. The bacterial phylum are expressed as a percentage of the total microbial community profile. The results are the average of 3 subjects. One-way Anova with Bonferroni Post Hoc correction has been employed for statistical analysis. No significant difference is seen between the steady states.

Phylum	Steady State						Fold changes		
	SS1			SS2			SS2/SS1		
	V1	V2	V3	V1	V2	V3	V1	V2	V3
Actinobacteria	0.90	0.13	0.49	0.25	0.16	0.38	0.28	1.26	0.77
Bacteroidetes	48.68	56.91	48.28	63.33	64.73	46.88	1.30	1.14	0.97
Firmicutes	48.49	39.95	47.57	26.20	29.77	46.15	0.54	0.75	0.97
Proteobacteria	1.87	1.84	2.26	10.13	4.97	4.94	5.43	2.69	2.18
Synergistetes	0.00	1.12	1.35	0.08	0.36	1.65	78.40	0.32	1.22

8.3.2.2 Impact of the medium on alpha and beta diversity

Shannon Index and weighted UniFrac were used to determine the alpha and beta diversity as both indices take into consideration of the abundance of bacteria in their calculation rather than dominance (presence/absence of species). For alpha diversity, the higher the value of Shannon Index indicates a richer species present in a given sample while beta diversity determines the variation of species between samples by clustering.

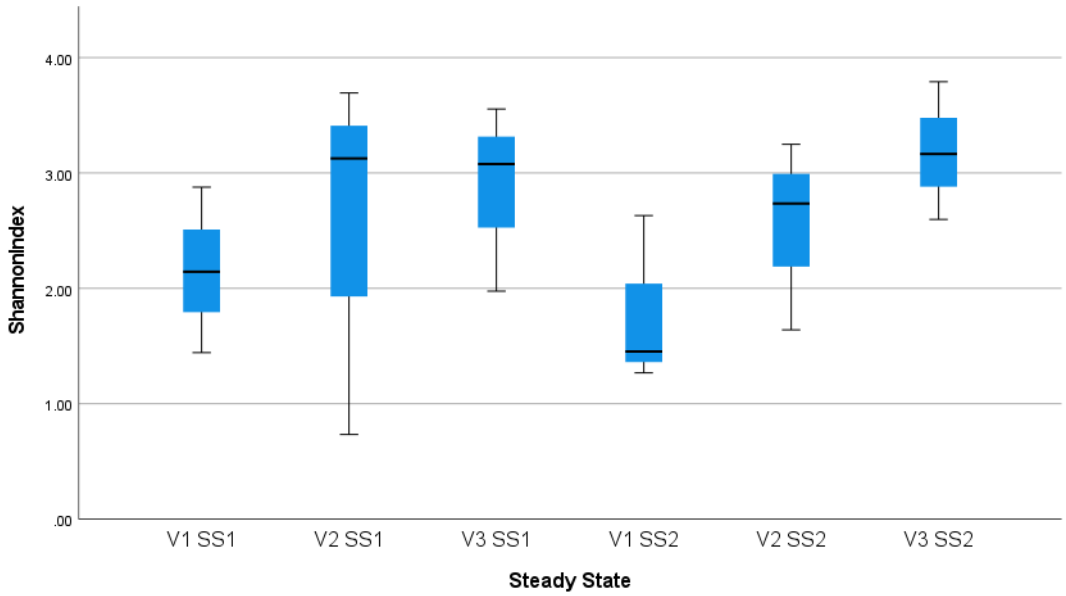

Figure 8.13 Alpha diversity measured by Shannon Index. The higher the value in Shannon Index indicates a higher species richness in the sample tested. Mann-Whitney U test were used for statistical analysis. No significant difference is seen between the regimes tested ($p>0.05$).

As seen from Figure 8.13, the diversity of the gut microbiota in SS1 showed an increase from V1 to V2 and V3 with the latter 2 vessels showing a negligible difference between them. In mGMM (SS2), V1 and V2 showed a lower diversity compared to GMM while V3 had a slightly more diverse composition in SS2 compared to SS1.

When beta diversity was considered, the population in GMM (SS1) showed a slightly different clustering pattern than that in mGMM (SS2), as seen in Figure 8.14. Clusters of GMM populations are indicated with triangles and those in mGMM are indicated with circles, while the 3 different colours indicated the 3 different subjects recruited for the experiment.

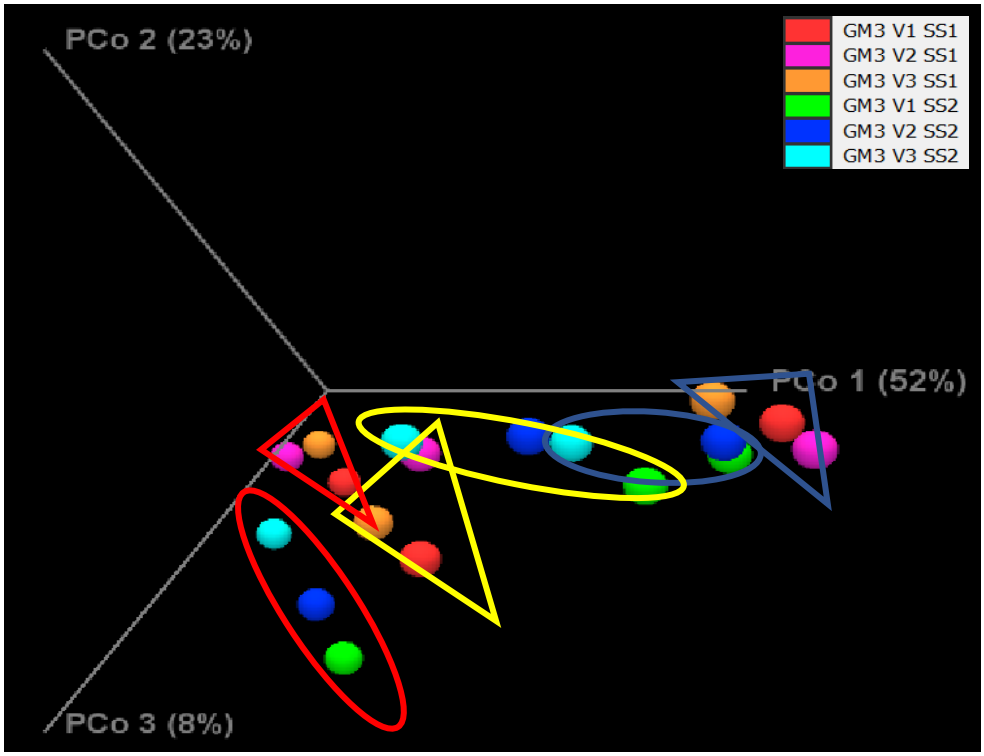

Figure 8.14 Beta diversity Principal Coordinate Analysis derived from weighted UniFrac in all samples. Each vessels at each steady state has a different colour: **SS1-** V1 (red), V2 (pink), V3 (orange); **SS2**- V1 (green), V2 (blue), V3 (cyan). For each axis, the precent of variation was reported in brackets.

Subject 3 (red) showed 2 very distinct clusters corresponding to the 2 different media. The composition of the gut microbiota in subject 1 (blue) were quite similar in V1 and V2 as seen by their close proximity while in V3 the two corresponding clusters were more distinct. In subject 2 (yellow), the microbiota in V1 clearly clustered distinctly between the 2 media, but in V2 and V3 the difference was not as great (Figure 8.14).

8.3.2.3 Impact of the medium on bacteria composition at species level

Similar to GM 2, the NGS data were analysed at 94% similarity using the Greengenes database instead of at 99% since use of 99% similarity resulted in 24-36% of species groups as unidentified at species level while running at 94% showed almost no unidentifiable species, although it gave poorer separation of known species. For example, *Bacteroides* sp, observed using the 94% identity cut-off, was identified as two species, *Bacteroides* sp1 and *Bacteroides* sp2 at 99% identity.

Table 8.16 Relative abundance of the gut microbiota between the 2 different medium at the species level. The relative abundance of bacterial groups within the gut microbiota at species levels is indicated SS1 and SS2. Data are expressed as a percentage of the total microbial community profile. The results are the average of 3 subjects. Only bacterial species present at >0.5% in at least 1 vessels of any steady states is shown. One-way Anova with Bonferroni Post Hoc correction has been employed for statistical analysis. Bacteria in bold indicates significant difference between the 2 steady state ($p<0.05$). (sp: species, N/A: not identifiable).

Phylum	Order	Species	SS1			SS2		
			V1	V2	V3	V1	V2	V3
Actinobacteria	Coriobacteriales	Coriobacteriaceae sp	0.79	0.06	0.33	0.15	0.10	0.24
		Collinsella sp	0.09	0.06	0.13	0.08	0.06	0.12
Bacteroidetes	Bacteroidales	*Bacteroides* sp	0.66	4.51	6.65	3.27	4.21	5.51
		Bacteroides ovatus	0.00	3.35	7.71	0.01	7.17	10.01
		Bacteroides uniformis	0.00	4.60	1.31	0.06	3.27	2.21
		Prevotella sp	48.00	43.41	31.58	59.92	49.40	27.95
		Rikenellaceae sp	0.00	0.37	0.28	0.00	0.30	0.61
Firmicutes	Clostridales	*Acidaminococcus* sp	2.98	3.53	5.44	0.56	0.46	1.07
		***Blautia* sp**	0.79	0.45	0.66	0.08	0.08	0.08
		Clostridiales sp	2.05	1.87	2.50	0.98	1.16	1.06
		Coprococcus sp	0.13	0.09	0.06	0.17	0.27	0.36
		Dialister sp	0.93	0.16	0.28	0.43	0.38	0.81
		Faecalibacterium prausnitzii	10.65	7.38	6.50	6.86	5.42	7.56
		Lachnospiraceae sp	6.03	2.82	3.28	0.65	1.08	1.82
		Lachnospira sp	0.31	0.42	0.27	0.23	0.35	0.97
		Megasphaera sp	0.82	0.58	1.30	0.51	0.48	1.38
		Oscillospira sp	0.17	0.44	1.18	0.09	1.12	2.22
		Roseburia faecis	0.43	0.70	0.23	0.01	0.01	0.02
		Ruminococcaceae sp	4.12	5.88	6.53	2.12	5.29	11.47
		Ruminococcus sp	16.24	13.34	16.88	12.87	12.05	15.63
		Veillonellaceae sp	1.18	0.75	0.66	0.29	0.44	0.31
	Erysipelotrichales	*Eubacterium biforme*	0.63	0.57	0.64	0.00	0.00	0.01
Proteobacteria	RF32	RF32 sp	0.10	0.15	0.23	8.49	3.78	2.16

	Burkholderiales	*Sutterella* sp	1.71	1.67	1.99	1.63	1.18	2.72
Synergistetes	Synergistales	*Pyramidobacter* sp	0.00	1.12	1.35	0.08	0.34	1.58
N/A			0.03	0.03	0.01	0.00	0.00	0.00

Table 8.17 Fold changes in abundance at species level between steady states. Fold changes of the bacteria at species level between the 2 different steady states. Data derived from table 8.16. Values in green indicates an increase in relative abundance and values in red indicates a decrease. One-way Anova with Bonferroni Post Hoc has been employed for statistical analysis. Species in bold indicate significant differences between the 2 steady states ($p<0.05$).

Phylum	Order	Species	V1	V2	V3
Actinobacteria	Coriobacteriales	Coriobacteriaceae sp	0.20	1.61	0.73
		Collinsella sp	0.95	0.93	0.89
Bacteroidetes	Bacteroidales	*Bacteroides* sp	4.92	0.93	0.83
		Bacteroides ovatus	4.02	2.14	1.30
		Bacteroides uniformis	17.76	0.71	1.68
		Prevotella sp	1.25	1.14	0.89
		Rikenellaceae sp	0.00	0.81	2.17
Firmicutes	Clostridales	*Acidaminococcus* sp	0.19	0.13	0.20
		***Blautia* sp**	0.10	0.18	0.12
		Clostridiales sp	0.48	0.62	0.42
		Coprococcus sp	1.31	3.15	6.21
		Dialister sp	0.47	2.44	2.91
		Faecalibacterium prausnitzii	0.64	0.74	1.16
		Lachnospiraceae sp	0.11	0.38	0.55
		Lachnospira sp	0.74	0.83	3.58
		Megasphaera sp	0.62	0.82	1.06
		Oscillospira sp	0.51	2.54	1.87
		Roseburia faecis	0.03	0.02	0.07
		Ruminococcaceae sp	0.51	0.90	1.76
		Ruminococcus sp	0.79	0.90	0.93
		Veillonellaceae sp	0.24	0.59	0.48
	Erysipelotrichales	*Eubacterium biforme*	0.01	0.00	0.01
Proteobacteria	RF32	RF32 sp	85.52	24.94	9.32
	Burkholderiales	*Sutterella* sp	0.95	0.71	1.37
Synergistetes	Synergistales	*Pyramidobacter* sp	78.40	0.31	1.18

As seen from table 8.16, *Prevotella* sp, *Ruminococcus* sp and *Faecalibacterium prausnitzii* were among the most abundant bacteria in SS1, with an average abundance of 41.0, 15.5 and 8.18% across all vessels, respectively. Although these bacteria still remained as the most abundant bacteria in SS2, the switch to mGMM caused an increase in the abundance of *Prevotella* sp to 45.8% (on average; 1.11-fold) while decreased the abundance of *Ruminococcus* sp and *F. prausnitzii* by 1.14- and 1.24-fold (on average), respectively, reducing their average abundance to 13.5 and 6.62%. With mGMM there were a few notable changes in the composition of the gut microbiota indicated the gut microbiota respond distinctly to the two media types. *Bacteroides ovatus, Coprococcus* sp, and an unknown bacteria from the RF32 order showed an increase across all 3 vessels with RF32 sp having the biggest average increment of 39.92-fold followed by *Coprococcus* sp (3.55-fold) and *B. ovatus* (2.49-fold). Both *Oscillospira* sp and *Dialister* sp showed an increase in their relative abundance overall with an average increase of 1.64- and 1.94-fold, respectively, across all vessels, but in mGMM these was a decrease in V1 and an increase in the latter two vessels. *Prevotella* sp showed the opposite pattern whereby it had an increase of 1.25- and 1.14-fold in V1 and V2 while V3 showed a decrease of 1.13-fold compared to SS1. The relative abundance of Rikenellaceae sp, *Lachnospira* sp, Ruminococcaceae sp, *Faecalibacterium prausnitzii*, *Megasphaera* sp and *Sutterella* sp all showed a decrease in V1 and V2 between 1.05- to 1.95-fold while V3 had an increase between 1.06- to 2.17-fold compared to SS1. Despite showing a decrease in V1 and V2, *Lachnospira* sp, Ruminococcaceae sp and *Sutterella* sp exhibited an increase in their overall abundance due their higher levels in V3, while the other 3 remaining bacterial groups showed a decrease in their overall abundance. *Bacteroides uniformis* and *Pyramidobacter* sp showed an increase in both V1 and V3, while in V2 there was a decrease in abundance compared to SS1. *Bacteroides uniformis* showed an increase of 17.8- (V1) and 1.68-fold (V3), while in V2 there was a decrease of 1.41-fold. *Pyramidobacter* sp, on the other hand, increased 78.40 and 1.18-fold in V1 and decreased of 3.27-fold in V2. *Bacteroides* sp, which had an average increase of 2.23-fold across all vessels, showed an increase in V1 but a decrease in both V2 and V3, compared to SS1.

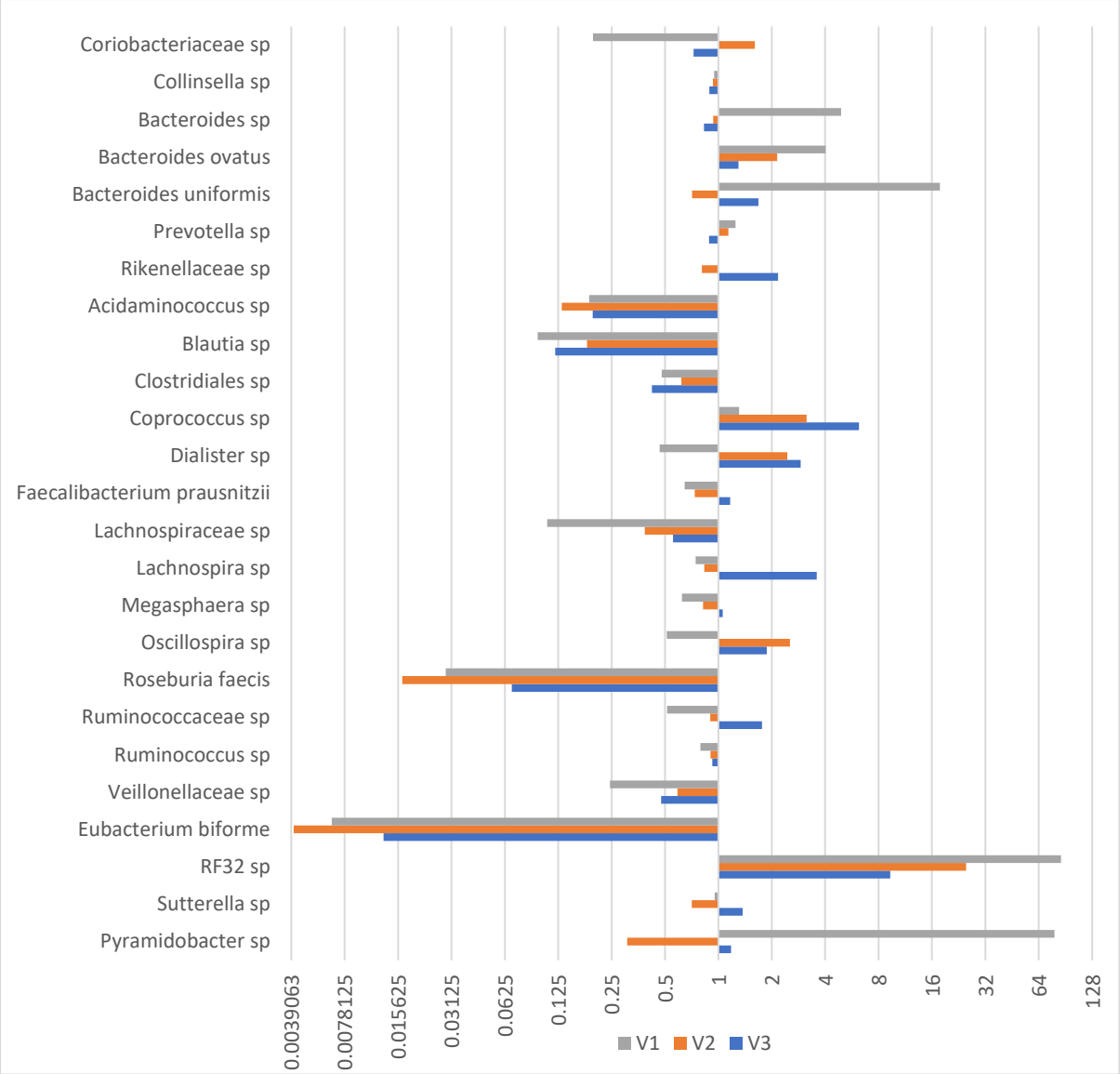

Figure 8.15 Fold changes in abundance at species level between different steady states. Fold changes of the bacteria at species level between SS2 and SS1. Data derived from table 8.17. The fold changes are expressed as Log_2 fold.

The relative abundance of Coriobacteriaceae sp was increased by 1.61-fold in V2 while V1 and V3 showed a decrease of 5.09- and 1.38-fold, respectively, compared to SS1. *Collinsella* sp, Clostridales sp, Lachnospiraceae sp, *Blautia* sp, *Roseburia faecis, Ruminococcus* sp, Veillonellaceae sp, *Acidaminococcus* sp and *Eubacterium biforme* showed a decrease in abundance in all 3 vessels. Among these bacteria, Lachnospiraceae sp and *Blautia* sp showed a significant reduction between SS1 and SS2, with an average decrease of 2.86- and 7.55-fold, respectively ($p<0.05$). Although not significant, *E. biforme* had the greatest decrease of 127-fold (on average) followed by *Roseburia*

faecis (26.3-fold on average). The remaining species showed a decrease of 1.06- to 7.65-fold across all 3 vessels as compared to SS1.

8.4 Discussion

The use of continuous-culture gut models has an advantage over the batch culture system whereby the cultures continue to grow for a longer period, thus allowing the slower growing bacteria to establish, giving a better reflection of the composition of the gut microbiota. As an example, the use of the gut model greatly reduced the presence of Enterobacteriaceae to below 0.05% (data not shown) in all vessels unlike the use of batch culture where their relative abundance made-up roughly two-thirds of the entire gut microbiota. Three different gut models were set up to test the impact of zinc on the gut microbiota under different circumstances. Gut model 1 (GM1) was used to test the impact of zinc deficiency on the gut microbiota as well as the impact of zinc supplementation in zinc deficient individuals. Gut model 2 (GM2) was set up to determine the effect of normal zinc supplementation and zinc over-supplementation on healthy zinc sufficient individuals. Gut model 3 (GM3) was used to determine the difference between the normal gut model medium (GMM) and the modified gut model medium (mGMM) whereby the latter medium can achieve an extremely low zinc concentration (3 µM) compared to the former (19 µM). mGMM was used throughout the experiment but 16 µM zinc was added to achieve a concentration of 19 µM (as would normally be present in the GMM) with the exception of SS2 of GM1 whereby zinc deficient conditions were utilised; thus no zinc was added and the concentration were kept at 3 µM. As seen from the results in table 8.17, most of the bacterial species showed a decrease when switching from the GMM to mGMM probably due to the removal of mucin which is commonly found in the gut. The removal of yeast extract is compensated with the addition of vitamin and mineral solution and tryptone is replaced with peptone, which would have minimal effect on the gut microbiota but a further experiment is needed to confirm this. mGMM provides a better control on the concentration of zinc which allows the impact of low Zn levels to be stretched to an extreme would not be ethical if conducted in human intervention studies.

In GM3, most of the members in the Firmicutes phylum showed a decrease in their abundance in the mGMM compared to GMM (table 8.17). Firmicutes are more abundant in the mucus layer in the human colon and bacteria such as *Eubacterium, Faecalibacterium prausnitzii*, Lachnospiraceae*, Roseburia* and Ruminococcaceae are enriched in the mucosal (Paone and Cani, 2020; Ouwerkerk, de Vos and Belzer, 2013; Van den Abbeele et al, 2013). The removal of mucin could thus have a bigger impact on the Firmicutes than the other phyla which is indeed shown by their reduced abundance in SS2 (mGMM). The abovementioned bacteria also showed a decrease in their relative abundance in mGMM which could be explained by the absence of mucin that have an impact on their growth and development, resulting in a lower abundance as compared to the GMM. A potential solution to this issue could be to add mucin back to mGMM following dialysis against metal chelators in order to remove iron and zinc from the mucin utilised.

Comparing the SS1 of GM1 and GM2, both gut models should present a similar microbiota composition as both employ mGMM. Both models showed similar composition at the phylum level with Bacteroidetes being the dominant phylum followed by Firmicutes (table 8.3 & 8.10). However, at species level, a difference is more obvious, particularly in the Bacteroidetes phylum. SS1 of GM1 was mainly *Bacteroides*-dominated, while SS1 of GM2 was mainly *Prevotella*-dominated. Similar results have been reported in another study whereby the authors stated that such effects may be due to changes in the initial fermentation conditions such as anaerobiosis, pH or inoculation duration (Dostal et al, 2012). The 3 gut models were operated in parallel and inoculation with the gut microbiota was performed on the same day starting with GM3, followed by GM2 and GM1. Although the inoculations were performed as quickly as possible, the delay between the inoculations may give rise to the observed differences in microbiota composition in the models. Although GM1 and GM2 showed different bacterial compositions, both gut models assumed the different enterotypes of gut microbiota suggested by Arumugam and Wu whereby GM1 leaned towards the *Bacteroides* enterotype while GM2 leaned towards the *Prevotella* enterotype (Arumugam et al, 2011; Wu et al, 2011). In running the gut models, it is worth noting that stool samples from healthy

individuals were used, thus the composition of bacteria may be different than that of a zinc deficient individuals and the bacterial inocula will not be acclimatised to a low zinc regime, and thus may react differently to the low zinc regime in the model. Stool samples from zinc deficient individual can be used in future experiments to determine whether the composition of gut microbiota differs from a zinc sufficient individual in the gut model, which can provide a better understanding and explanation to the results obtained.

As seen from tables 8.7, 8.13 and 8.17, only a few bacteria species showed a significant change towards the zinc regimes tested. This was the case despite the huge fold changes observed and this presumably reflects the large variation in the abundance of species obtained between the 3 different subjects which causes these difference to be insignificant when tested. This is the limitation of a small sample size and to overcome this, more subjects should be recruited to the experiment, thus increasing the sample size which will reduce the variation. However, running more gut models would be time consuming and costly.

Interestingly, *Pyramidobacter piscolens* is a bacterial species is found in the mouth and is isolated from odontogenic abscesses (Downes et al, 2009). The species is described as a non-motile, non-pigmented, obligately anaerobic, Gram-negative bacilli that has a distinctive fishy odour when cultured (Downes et al, 2009). *P. piscolens* is found in the mouth where the resting pH does not fall below 6.3 and maintained near neutrality by the saliva (Baliga, Muglikar and Kale, 2013), thus this species may be expected to grow well in V2 and V3 where the pH is similar to that in the mouth. In addition, the absence of zinc causes the bacteria to grow substantially as zinc is able to inhibit or reduce the growth of the bacteria. Zinc is a component in toothpaste, mouth rinses and dental materials as zinc is effective against oral diseases (Uwitonze et al, 2020; Lynch, 2011). Zinc inhibits bacterial growth by targeting the glycolytic enzymes, inhibits acid production in dental plaque and inhibits the formation of plaque (Uwitonze et al, 2020), proving its role in maintaining good oral health. Since the bacterium is isolated from abscess, the lack of zinc may increase its growth as seen

in SS2 and the addition of zinc in SS3 slightly reduces its abundance in GM1. Although zinc causes an increase of the bacteria in GM2, the presence of zinc causes the bacteria to be at a much lower abundance as compared to GM1.

The presence of zinc mainly affects members in the Firmicutes phylum whereby more bacteria showed a decrease in their relative abundance as compared to the other phyla. Although the exact mechanism of this observation is yet to be discovered, it can be speculated that Firmicutes are more sensitive to zinc as compared to the other phyla. The impact of zinc on the overall composition of the gut microbiota and the possibly effect on health will be discuss further in the next chapter.

Chapter 9. Discussion

The experiments were designed to study the impact of the nutrients, iron and zinc, on the gut microbiota. In chapters 3 to 5, the experiments explored the impact of iron (haem/$FeSO_4$) on the gut microbiota using two different approaches, Hungate tubes and single vessel batch cultures. The Hungate tubes were explored as a quick and simple method for preliminary study, but the lack of pH regulation limited their usefulness. The addition of buffers (particularly 300 mM MES) enabled pH to be maintained within the desired range, but batch cultures were used as the preferred method due to the dynamic pH control provided. The batch culture system was set up to study the effect of different forms of iron (haem and $FeSO_4$) on the gut microbiota. NGS data revealed that certain species showed no preference towards the presence/absence of iron but some species preferred one form or the other. In chapter 6, experiments were set up to study the utilisation of phytic acid (a major dietary inhibitor of zinc absorption) as a phosphate and carbon source. Mutants of *E. coli* were produced whereby the three known phytases were knocked out but the bacteria were still able to utilise phytic acid, thus indicating the presence of unknown phytases or mechanisms in degrading phytic acid. A batch culture was also set up to determine the ability of the gut microbiota to utilise phytic acid as a phosphate and carbon source, and results showed that phytic acid is a good phosphate source but a poor carbon source for the gut microbiota. Chapters 7 and 8 explored the impact of zinc on the gut microbiota using a single stage batch culture and a three-stage continuous gut model. Zinc sulphate was chosen as the zinc source as it is available as a zinc supplement and three different levels of zinc were tested: low (77 µM), medium (192 µM) and high (770 µM). Although zinc did not drastically affect the gut microbiota, it had an impact towards certain bacterial groups.

9.1 Impact of reduced inoculum levels.

In vitro gut culture experiments require the inoculation of faecal samples from volunteers into the growth medium (gut model medium). Faecal samples are usually mixed with PBS to create a 10% w/v mixture prior to inoculation. The mixture is typically inoculated at 10% of the final volume of the

medium (i.e. 1 ml mixture to 9 ml medium) to give a final concentration of 1% faecal matter and the culture is allowed to run for a fixed period of time (24, 48 or 72 h) with sampling at specific time points. As mentioned before, the Hungate tube provides a quick and easy method for preliminary studies despite having the disadvantage of not being pH regulated. From Figure 3.1, the total bacteria number showed minimal growth after 24 h with a maximum growth increase of just 1.1-fold. This may be due to the high initial inoculation concentration of faecal matter as similar growth responses have been obtained in batch culture using the same protocol (Khalil et al, 2014, Costabile et al, 2014; Beards, Tuohy and Gibson, 2010). Therefore, the inoculation concentration for the batch culture experiment (chapters 5 and 7) was been lowered to 0.1% final concentration to allow more scope for the microbiota to grow. Results from Figures 5.1 and 7.1 show that by lowering the concentration of faecal matter to 0.1%, the total bacterial count had increased by 8.73- to 43.8-fold, thus indicating considerably more bacterial growth as compared to the 1% faecal inoculum. The increased growth allows a greater response of the microbiota towards the iron and zinc regimes explored, which should enable a better understanding of the impact the iron and zinc have on the gut microbiota.

9.2 The batch and continuous culture systems.

The use of single vessel batch cultures provides a convenient approach for conducting *in vitro* experiments related to the gut microbiota as they are relatively easy to set up, allow the testing of numerous conditions simultaneously and the experiments can be run according to the desired time frame. However, the use of single vessel batch cultures has a disadvantage as the number and/or relative abundance of Enterobacteriaceae can rise to levels that are much greater than seen *in vivo*, and this which might obscure the effect of the tested regime on the composition of other elements the gut microbiota. Several studies which were conducted using single stage batch cultures also showed an increased in Enterobacteriaceae, and in some cases the Enterobacteriaceae became the dominant bacterial group (Takagi et al, 2016; Ahmadi et al, 2019; Kristek et al, 2019; Wiese et al, 2018; Ding et al, 2019). Similar results were obtained in both the iron and zinc regime experiments,

whereby the number of Enterobacteriaceae showed a huge increase, particularly in the latter regime. In some of the tested condition, the Enterobacteriaceae accounted for nearly two-thirds of the entire bacterial composition, which may have masked some of the effects of zinc on other elements of the gut microbiota. However, this occurrence can be avoided or overcome through the use of the three-stage continuous gut model whereby sampling is done at each steady state, unlike the single vessel batch culture where samples are at various times including the growth end point. The relative abundance of Enterobacteriaceae can be very low as seen from other studies conducted using the three-stage continuous gut model (Healy et al, 2017; Bahrami et al, 2011). A similar observation was obtained from the gut model experiment where Enterobacteriaceae showed an increase at t24 but were undetectable during the subsequent steady states (data not shown), thus showing its competitive advantages over short incubation periods. Single vessel batch cultures are usually run for 24 to 72 h which might give Enterobacteriaceae a competitive advantage in acquiring nutrients from the medium, leading to a poorer growth of the other slower growing bacteria present. The three-stage continuous gut model is operated for a longer period of time and fresh medium is constantly provided to the culture, ensuring a constant supply of nutrients to support the growth and development of the microbiota.

The single vessel batch culture is useful in conducting preliminary experiments whereby the results can be obtained rapidly and changes could be made to further improve the experimental design. However, the disadvantage is that the results may be biased towards bacteria that are able to replicate at a faster rate or are able to better compete for nutrients, leading to a reduced composition for the slower growing bacteria. The three-stage continuous gut model is an established model which better reflects what is happening in the large intestine as it represents the three section of the colon, each with its specific conditions. However, the continuous gut model is more labour intensive, requiring constant monitoring throughout the whole study period and operates at a high cost as the medium needs to be replenished regularly.

9.3 Major factors affecting the gut microbiota

The human gastrointestinal tract is one of the largest interfaces (250-400 m^2) between the environment, antigens and the host in the human body. An estimated 60 tonnes of food pass through the gastrointestinal tract over the typical lifespan of human along with an abundance of microorganisms from the environment. The microorganisms colonising the gut have co-evolved with the host over thousands of years to form a mutually beneficial relationship (Thursby and Juge, 2017). It is estimated that over 10^{14} microorganisms inhabit the human gastrointestinal tract with bacterial cells being 10 times more than the human cells with over 100 times the genomic content than the human genome (Thursby and Juge, 2017). The human microbiota is mainly dominated by Firmicutes, Bacteroidetes and Actinobacteria with lower abundance of Verrucomicrobia and Proteobacteria (Scott et al, 2012). The composition of the gut microbiota is affected by the mode of delivery upon birth while diet, supplementation and medication play a role in modulating the gut microbiota later. Infants delivered via Caesarean section or vaginally harvest a different inoculum for gut colonisation. Infants delivered vaginally have a higher abundance of *Bifidobacterium*, *Bacteroides* and *Lactobacillus* while Clostriaceae, *Veillonella* and *Klebsiella* were higher in infants delivered via Caesarean section (Rutayisire et al, 2016). Diet has a huge impact on the composition of gut microbiota. Infants who were breast-fed or formula-fed showed different composition whereby breast-fed infants had a more uniform and stable population mainly dominated by *Bifidobacterium* (Guaraldi and Salvatori, 2012). Formula-fed infants have a more diverse bacterial population with higher counts of *Clostridium, Streptococcus, Bacillus subtilis, Veillonella parvula, Lactobacillus acidophilus, Bacteroides vulgatus* and *Enterococcus faecalis* (Guaraldi and Salvatori, 2012). Upon weaning, the microbiota will move towards a more adult-like composition whereby the macronutrients and micronutrients of the diet will have an impact. Diets rich in fibre can cause an increased abundance of Bacteroidetes (particularly *Prevotella* and *Xylanibacter*) coupled with a decrease in Firmicutes (De Filippo et al, 2010). The use of supplementation such as prebiotics can increase certain groups of the gut microbiota. For example, the intake of inulin (10 g/d) for 16 days

was shown to increase the abundance of *Bifidobacterium adolescentis and Bifidobacterium bifidum* as well as *Faecalibacterium prausnitzii* (Ramirez-Farias et al, 2009). The consumption of galacto-oligosaccharides (GOS) also showed an increase in *Bifidobacterium* in a dose dependent manner (Davis et al, 2010).

Antibiotics can have a huge impact on the gut microbiota as these substances are designed to kill bacteria. The use of antibiotics can lead to a loss of diversity, expansions of certain bacterial groups, a shift in metabolic capacity and reduced colonisation resistance against pathogens (Lange et al, 2016; Modi, Collins and Relman, 2014). In addition to the disruption to the gut microbiota, the use of antibiotics allows the growth of opportunistic pathogens such as *Salmonella typhimurium* and *Clostridium difficile* as well as the development of antibiotic resistance in bacteria which have a negative effect on the host (Lange et al, 2016; Modi, Collins and Relman, 2014).

9.4 Impact of iron on the gut microbiota

The batch cultures were set up to determine the effect of different forms of iron on the gut microbiota. Bacteria only require 0.3 – 1.8 μM of iron for optimal growth (Kim et al, 2009) while the gut model medium has a background concentration of 28 μM iron which is more than sufficient to support growth of bacteria. Thus, this experiment was not designed to study the effect of iron supplementation on the gut microbiota but rather the effect of provision of different forms (haem/non-haem) of iron. As seen from table 5.8, *Clostridium butyricum* showed the greatest increase among all the bacteria identified in the haem-only and $FeSO_4$-only regimes but when both forms of iron are present, this species showed a decrease in its abundance as compared to the control, suggesting it grows well in moderate levels of iron. However, the concentration of haem was higher than that of $FeSO_4$ (77 vs 18 μM), thus it is not clear whether the enhanced growth was due to the higher concentration of iron present or the preference towards haem, further experiment with similar levels of iron is needed for confirmation. The growth enhancing effect of iron was shown in a hydrogen production experiment whereby the addition of 65 μM $FeSO_4$ caused a 1.6-fold increase in *C. butyricum* growth rate and a 1.4-fold increase in final cell levels (Chen et al, 2005). *C. butyricum*, a

butyrate producer, has been used as a probiotic in humans (Imase et al 2008; Sun et al, 2018); *C. butyricum* CBM588 reduces the occurrence of soft stools and diarrhoea as well as the presence of *Clostridium difficile* toxin A following *H. pylori* eradication therapy (Imase et al, 2008). *C. butyricum* supplementation also improved the overall IBS-D symptoms, particularly the quality of life and bowel habit of the patients as determined by questionnaires (Sun et al, 2018).

Another member of the Firmicutes phylum that showed an increase in abundance with iron is *Lactobacillus*. *Lactobacillus* showed an increase in all the iron-supplemented media with a greater increase at t24 than t48. *Lactobacillus* has been used as a probiotic which can impart some general benefits to the host (Hill et al, 2014) and has been used in the management of gastrointestinal diseases (Ritchie and Ramanuk, 2012). *Lactobacillus* as a probiotic has also been shown to increase iron absorption (Hoppe et al, 2015; Hoppe, Önning and Hulthén, 2017) possibly through the production of a ferric reducing compound (*p*-hydroxyphenyllactic acid) which has been isolated from *Lactobacillus fermentum* and enables the reduction of ferric iron to ferrous iron which subsequently increases iron absorption through the DMT-1 of enterocytes (González et al, 2017). Iron is usually absorbed in the small intestine, however the consumption of prebiotic also increases iron absorption indicating that iron absorption occurs in the colon (Paganini et al, 2015). An increase in fractional iron absorption (from ferrous fumarate and ferric sodium EDTA supplements) is positively correlated with an increase of *Lactobacillus*/*Pediococcus*/*Leuconostoc (as stimulated by GOS provision), giving a positive health effect to the host* (Paganini et al, 2015). *However, consumption of iron supplementation alone has been shown to reduce the level of Lactobacillus* (Paganini et al, 2015; Zimmermann et al, 2010). Results from table 5.8 indicate an opposing effect whereby the abundance of *Lactobacillus* was enhanced by iron supplementation. This difference may be due to the difference in subjects utilised as well as the experimental design whereby the human study was done in children from a remote rural area with limited food choices while the subjects in the batch culture experiment were adults living in urban conditions. Future studies involving adults and iron supplementation is needed to confirm this effect.

The presence of iron, particularly haem, caused a sharp decline in the abundance of *Faecalibacterium prausnitzii*. Similar results were shown in a continuous culture whereby the addition of 658 μM of $FeSO_4$ and 77 μM of haem also caused a reduction in their abundance (Dostal et al, 2013). In an iron replacement therapy study involving IBD patients together with iron deficient control subjects, an increased iron concentration (faecal iron content) reduced the abundance of *F. prausnitzii, Ruminococcus bromii* and *Collinsella aerofaciens* which agrees with results in table 5.8 (Lee et al, 2016). Subjects showed a reduction in these bacterial groups upon oral iron supplementation (300 mg $FeSO_4$) but not when receiving 300 mg iron sucrose infusion intravenously, indicating an excess iron in the gut can negatively impact the residing bacteria (Lee et al, 2016). Even though both methods of iron administration showed improved iron levels in the subjects (IBD and control), oral iron supplementation caused a negative effect on the gut microbiota (Lee et al, 2016). *F. prausnitzii* is considered beneficial in the gut due to its butyrate-producing ability which feeds the colonocytes (Ferreira-Halder, Faria and Andrade, 2017). In addition to being a butyrate producer, *F. prausnitzii* is able to produce anti-inflammatory metabolites which benefits the host and has thus received attention in its relationship with diseases (Ferreira-Halder, Faria and Andrade, 2017) whereby a reduced or lower abundance is detected in patients with IBD (Sokol et al, 2009; Machiels et al, 2014), diabetes (Qin et al, 2012; Karlsson, 2013; Navab-Moghadam et al, 2017) and colorectal cancer (Balamurugan et al, 2008; Wu et al, 2013).

The presence of iron decreases the abundance of *Bifidobacterium*; this has been shown in numerous experiments, both *in vitro* (Dostal et al, 2013; Kortman et al, 2016) and in human studies (Jaeggi et al, 2014). Similar to *Lactobacillus, Bifidobacterium* is a probiotic which can confer health benefits to the host directly (Hill et al, 2014; Flach et al, 2018) or indirectly by stimulating the growth of other bacteria. Co-culturing *Bifidobacterium* and *F. prausnitzii* has been shown to increase the amount of butyrate in the gut and the levels of butyrate can be enhanced through the addition of prebiotics (Rios-Covian et al, 2015; Kim et al, 2020). Acetate that is produced as a metabolic end product by *Bifidobacterium* is used by *F. prausnitzii* as a substrate for butyrate production. However, the

amount produced varies depending on the species or strains of *Bifidobacterium* as well as the carbon source available (Kim et al, 2020). The presence of iron which reduces the abundance of these two beneficial bacteria in the gut microbiota may thus have a negative health effect on the host.

Bacteroides is a common inhabitant of the human gut; indeed 30 out of the 56 species isolated and identified can be found in the human intestine (Wang et al, 2020). *Bacteroides* have a requirement for haem or inorganic iron with PPIX for growth and development (Rocha et al, 2019), and all of the *Bacteroides* species showed an increase in their abundance in the presence of iron with the greatest growth occurring when both $FeSO_4$ and haem were added to the medium (table 5.8). *Bacteroides* can be a friend or a foe to the host whereby it contributes to numerous beneficial activities or acts as an opportunistic pathogen sitting in the gut waiting to attack the host. In the gut, *Bacteroides* encodes a cytochrome *bd* oxidase which can reduce levels of intracellular oxygen which encourage the growth of strict anaerobes, thus benefiting the gut microbiota (Wexler and Goodman, 2017). The other beneficial activities performed by *Bacteroides* includes breakdown of plant- and host-derived polysaccharides, maturation of the immune system, bile acid metabolism and energy harvesting (Wang et al, 2020; Sun et al, 2019; Wexler and Goodman, 2017; Rocha and Smith, 2013). *Bacteroides fragilis* is probably the only opportunistic pathogen among all the *Bacteroides* species found in the gut. However, not all *Bacteroides fragilis* are pathogenic as this behavioue is strain specific. *B. fragilis* can be classified into nontoxigenic (NTBF) or enterotoxigenic (ETBF) strain whereby the former is a beneficial commensal which antagonise ETBF via interspecific competition and the latter is a pathogen (Sun et al, 2019; Wexler and Goodman, 2017). *B. fragilis* has a distinctive bacterial capsule that enables it to escape complement-mediated killing and phagocytic killing as well as triggering abscess formation in the human host that can causes intestinal obstruction if left untreated or bacteraemia upon escape into the circulation (Wexler, 2007). Besides this, *B. fragilis* also produces an enterotoxin – *B. fragilis* enterotoxin (BFT) - which is a zinc metalloprotease and has been shown as a cause for diarrhoea (Sears et al, 2008; Nguyen et al, 2005; Pathela et al, 2005) as well as

inducing IBD and colorectal cancer (Boleij et al, 2015; Sun et al, 2019). Although it is not clear which strain is enhanced by iron, an increase in iron can increase the risk of infection in the host if ETBF is enhanced.

From table 5.8, the presence of haem had a bigger effect on the Firmicutes as compared to the Bacteroidetes whereby few members of the Firmicutes showed a reduced abundance in the presence of haem. Most members of the Bacteroidetes, particularly *Bacteroides*, displayed an increase in abundance presumably because they are known to require haem due to their inability to synthesise haem (Rocha et al, 2019). *Dorea formicigenerans, Mitsuokella multacida, Ruminococcaceae, Ruminococcus, Veillonella dispar* all showed a bigger decrease when haem was added to the medium whether as sole iron source or together with $FeSO_4$. When both $FeSO_4$ and haem were added to the medium, these bacteria showed a higher abundance (compared to haem alone), indicating that the presence of $FeSO_4$ alleviates some of the inhibitory effects of haem. Since *Bacteroides* has a requirement for haem, it may have a more developed system in acquiring haem which allows it to outcompete the other bacteria present in the same community. Haem acquiring systems have been identified in bacteria, mainly pathogens, whereby they can scavenge haem from the host (Contreras et al, 2014; Runyen-Janecky, 2013). These bacteria either secrete haemophores (IsdX1,IsdX2, HasA, HxuA) which can binds to haemoglobin/haptoglobin or possesses outer membrane receptor (e.g. HmuY, HmuR, HutA, HemR) which can sequester and deliver haem from host haemoprotein (Contreras et al, 2014; Runyen-Janecky, 2013). *B. fragilis* encodes a unique 44-kDa outer membrane protein (HupA) which is involved haem binding and shares features with HutA and HemR (Otto et al, 1996). However, HupA can only be found in *B. fragilis* and not other *Bacteroides* species (Otto et al, 1996). New haem acquisition systems may be present in the commensal *Bacteroides* which have yet to be identified and that allows them to scavenge haem from the environment for growth and development. Besides this, haem can be bactericidal towards bacteria possibly through the generation of radicals which damages DNA, lipids and proteins (Choby and Skaar, 2016). *Bacteroides* contains multiple iron storage and iron detoxifying ferritin-like

proteins which can protect them from radicals attack (Rocha and Smith, 2013). Ferritin and bacterioferritin found in the bacteria limit the toxicity of cellular iron by sequestering iron into a hollow shell while the Dps (DNA-binding protein from starved cells) protein catalyses the oxidation of ferrous iron using hydrogen peroxide, producing water instead of radicals (Rocha and Smith, 2013; Andrews, 2010). These protective mechanism found in *Bacteroides* may increase its tolerance to the toxic effects of exogenous haem.

9.5 Impact of zinc on the gut microbiota

Oral zinc supplementation has been deployed to reduce the incidence of diarrhoea in children in low- and middle-income settings. The use of zinc treatment reduces the episode duration, stool frequency, stool output and length of hospitalisation which can greatly benefit children who receive such treatment (Lamberti et al, 2015). In addition, children who received zinc supplementation show improved growth in terms of height and weight and this effect is more pronounced in stunted children (Brown et al, 2002; Rivera et al, 1998; Umeta et al, 2000; Roy et al, 1997). Although serum zinc level is not a good indicator of overall zinc status, children that received zinc supplementation showed an increase in serum zinc level, thus indicating a positive response to the zinc supplement taken (Roy et al, 1997; Brown et al, 2002; Umeta et al, 2000).

Studies involving zinc supplementation tend to focus on the health and nutrition aspect, and generally do not consider the relationship between zinc supplementation and the gut microbiota, thus studies on this area are very limited. In the farming industry, zinc is supplemented to pig feed to reduce the incidence of post-weaning diarrhoea (Wang et a, 2019). The addition of zinc (usually insoluble zinc oxide) reduces the abundance of Enterobacteriaceae (Starke et al, 2013; Wang et al, 2018), *Campylobacter coli* (Bratz et al, 2013) as well as the total counts of lactic acid bacteria and lactobacilli but increases the numbers of coliforms in the colon (Hojberg et al, 2005; Shen et al, 2014). Zinc supplementation has improved the animal growth performance and reduces the incidence of diarrhoea which benefits the farmer from an economic perspective. However, the doses

used in the pig industry higher (2-3g/kg feed) than can be utilised in humans, thus the results from the animal studies may not replicate the changes that zinc have on the human gut microbiota.

As for the effect of zinc supplementation on the gut microbiota, two different approaches were used here to study its effect, single vessel batch cultures and the three-stage continuous gut model. Conditions of the batch cultures were set to mimic the proximal colon as the modified gut model medium is nutrient rich which is more likely to be encountered by the bacteria in the upper colon than the lower colon. Results obtained from table 7.5 are similar to those of table 8.4 (GM 1) but not table 8.11 (GM 2). The presence of 77 µM Zn increased the abundance of Firmicutes while reducing the abundance of Actinobacteria, Bacteroidetes and Proteobacteria (table 7.5, table 8.4). However, results from table 8.11 showed opposing results whereby Firmicutes showed a decrease while Actinobacteria, Bacteroidetes and Proteobacteria displayed an increase in abundance. Discrepancies in results may be partly due to the methods of data processing whereby the assigned OTUs determined in table 7.5 and 8.4 were considered at 99% similarity, using the Greengenes database, resulting in a 3 to 9% level of unidentifiable species, but the OTUs identified in table 8.11 were determined at just 94% similarity, which is below the levels required for species identification. Most microorganisms required 0.1-10 µM of zinc for optimal growth *in vitro* (Atmaca et al, 1998). The modified gut model medium has a background zinc concentration of 3 µM which is sufficient to support the growth of most bacteria. Comparing with the normal gut model medium which has a background concentration of 19 µM, the modified version is sufficiently low in zinc to enable the study of the effect of zinc supplementation on the gut microbiota. Zinc is involved in numerous cellular processes but at high concentration is detrimental to bacteria whereby 7.4 – 12.4 mM of zinc sulphate ($ZnSO_4$) can achieve total inhibition of the common pathogens associated with diarrhoea (Surjawidjaja, Hidayat and Lesmana, 2004). Although zinc sulphate was used here to supplement the modified gut model medium, the concentrations used were only up to 770 µM which is not at a sufficiently high concentration to completely inhibit bacterial growth.

Supplementing with zinc encouraged the growth of Bacteroidetes but reduced the abundance of Firmicutes. A few bacterial groups showed an increased abundance including *Bacteroides*, *Faecalibacterium prausnitzii* and *Roseburia faecis* when zinc was added to the medium. As mentioned before, *Bacteroides* has several iron storage proteins and possibly multiple strategies for iron uptake due to its requirement for iron. Under zinc stress, genes involving in iron uptake and storage have been shown to be upregulated (Xu et al, 2019), which may explain the increase in growth of *Bacteroides* when zinc is supplemented. Since no additional iron was added to the medium, the bacteria were left to compete for a limited pool of iron whereby bacteria possessing more effective iron-scavenging systems may display an advantage. However, the addition of zinc (regardless of concentration) in the batch culture had an inhibitory effect in *Bacteroides* during early growth; a decrease in abundance at t24 was observed, which was followed by an increase at t48 (table 7.8), indicating that the adaptability of *Bacteroides* to zinc is time dependent. The increase of *Bacteroides* may confer a health benefit to the host via stimulating the immune system, providing resistance towards colonisation of pathogens (*Bacteroides fragilis*) and modulating the imbalance between anti-oxidant and pro-oxidant mechanisms (Wang et al, 2020).

Besides this, zinc stimulated the growth of the butyrate producers such as *Faecalibacterium prausnitzii* and *Roseburia faecis* whereby they showed a non-significant increase at 77 μM and a significant increase at high zinc concentration (770 μM) (table 8.13). The increase in these butyrate producers would benefit the host as the presence of butyrate inhibits inflammation and carcinogenesis, decrease oxidative stress and increases colonic barrier function (Hamer et al, 2008). *F. prausnitzii* showed a decrease in its abundance in the iron experiment even though the iron levels were the same between the iron and zinc experiment, indicating that zinc is able to counter the inhibitory effect of iron on *F. prausnitzii*.

The bacteria in both GM 1 and GM 2 mainly belonged to the Bacteroidetes phylum and regardless of the zinc regime, there was little change to the relative abundance at phylum level with Bacteroidetes

being the major phylum at SS1, SS2 and SS3 in both GM 1 and GM 2. The Bacteroidetes phylum was dominated by two main genera - *Bacteroides* and *Prevotella*, and their combined relative abundance accounts for more than half of the total microbiota. Despite similar media being used in GM 1 and GM 2, the former (table 8.6) presented a *Bacteroides*-dominated microbiota while the latter (table 8.12) leaned towards a *Prevotella*-dominated profile. Such an observation has been reported previously whereby 2 parallel continuous cultures presented different microbiota profiles, possibly due to initial inoculation conditions (Dostal et al, 2013). These two genera are usually antagonistic and are anticorrelated in microbiome studies (Ley, 2016). Such an effect is shown in mice whereby bi-colonisation of both *Bacteroides thetaiotaomicro* with *Prevotella copri* resulted in lower levels of both bacteria compared to when they are mono-colonisers (Kovatcheva-Datchary et al, 2015) and the same effect is also seen in humans whereby an increase of *Prevotella* is usually paired with a decrease in *Bacteroides* (Wu et al, 2011). Similar effects can be found in the three-stage gut model where an increase in *Bacteroides* correlates with reduced relative abundance of *Prevotella* and because of this, there are little changes to the overall relative abundance of the Bacteroidetes phylum. *Bacteroides* and *Prevotella* may be competing for the same niche in the gut, causing them to be antagonistic with each other (Kovatcheva-Datchary et al, 2015). In GM 1, the absence of zinc caused the relative abundance of *Prevotella* to drop while the abundance of *Bacteroides* showed an increase, particularly *Bacteroides* sp2 at SS2 (table 8.7). The ability of *Bacteroides* to outcompete *Prevotella* has been shown in mice whereby bi-colonisation of both bacteria in mice resulted in higher counts of *Bacteroides* (Kovatcheva-Datchary et al, 2015). Even when zinc is added to the medium at SS3, the high number of *Bacteroides* established at SS2 appears to limit *Prevotella* abundance, resulting in even lower *Prevotella* counts (relative abundance below 0.05%; table 8.6).

In GM3, the difference between the standard gut model medium (GMM) and the modified version (mGMM) is that the latter had: yeast extract removed and replaced with a vitamin and mineral solution; replacement of peptone water with tryptone; and had mucin excluded. These changes were made to ensure that background zinc levels were as low as possible to better enable the study

of the effect of zinc supplementation on the gut microbiota. Perhaps the biggest difference between these two media was the presence of mucin, as the other parameters are kept as similar to each other as possible. Mucins are a family of complex, large, glycosylated proteins with O-glycosylation being the main modification whereby it allows the creation of a glycan coat that protects mucins from degradation by endogenous proteases and also aids in its solubility in water and its gel forming properties (Paone and Cani, 2020). In addition to protecting the intestinal cells against biological, chemical and mechanical attacks, mucins provide nutrients and attachment sites for the gut microbiota. In GM3, by comparing the composition of the gut microbiota between the two steady states, GMM (SS1) showed a higher relative abundance of Firmicutes than Bacteroidetes at phylum level while the mGMM (SS2) tended to favour Bacteroidetes over Firmicutes (table 8.16). Firmicutes are generally found in higher abundance in the mucus layer in humans which may explain the difference seen between the two media (Ouwerkerk, de Vos and Belzer, 2013; Paone and Cani, 2020). In addition, members belonging to Lachnospiraceae and Ruminococcaceae as well as *F. prausnitzii* have been suggested to be enriched in the mucus layer (Ouwerkerk, de Vos and Belzer, 2013; Paone and Cani, 2020). This is in agreement with the results obtained here whereby *Ruminococcus bromii*, *Ruminococcus,* Ruminococcaceae sp1, Ruminococcaceae sp2, Lachnospiraceae and *F. prausnitzii* showed a higher relative abundance in GMM compared to mGMM. In terms of total bacteria counts, the removal of mucins which act as a nutrient source to support the growth and development of the gut microbiota, caused reduced total bacterial counts in SS2 compared to SS1 (table 8.15). This effect was more pronounced in V2 and V3 than V1. Although the removal of mucin in mGMM caused the reduction of certain bacterial species as compared to the normal GMM, this step was deemed necessary in order lower the zinc level as far as possible so that the background zinc did not contribute greatly to the zinc pool in the medium during zinc supplementation.

9.6 Phytate as a source of phosphate

Phytate (*myo*-inositol hexakisphosphate) consists of an inositol ring with six phosphate groups and is the primary phosphorus store in plants, comprising of 50-85% of total phosphorus (Gupta, Gangoliya and Singh, 2013). Phytate accumulates rapidly during the ripening period in seeds which is important for effective germination as large amount of phosphorus is released when digested by phytase upon germination and incorporated into ATP (Gupta, Gangoliya and Singh, 2013; Dersjant-Li et al, 2014; Wodzinski and Ullah, 1996). Plants possess intrinsic phytases which are capable of degrading phytate but monogastric animals (humans, poultry and pigs) cannot metabolise phytate due to the lack or insufficient levels of phytase (Gupta, Gangoliya and Singh, 2013; Wodzinski and Ullah, 1996). Despite being an important component in plants, phytic acid can be seen as an antinutrient in animals. Phytate is a highly negatively charged ion which forms stable complexes with minerals including zinc, iron, calcium, copper, manganese and magnesium, thus reducing their bioavailability as these complexes are nonabsorbable in the gastrointestinal tract (Greiner and Konietzny, 2006; Dersjant-Li et al, 2014; Schlemmer et al, 2009; Vohra and Satyanarayana, 2003).

Phytases are enzymes that have the ability to hydrolyse phosphate group(s) from phytate yielding a series of lower inositol phosphates and free phosphate (Angel et al, 2002; Selle and Ravindran, 2008; Vohra and Satyanarayana, 2003). These enzymes can be divided into two groups (3-phytase or 6-phytase) depending on their site of action on phytate. 3-phytases initiate the dephosphorylation of phytate at the 3 position, producing 1,2,4,5,6-pentakisphosphate and a free phosphate while 6-phytases dephosphorylate phytate at position 6, yielding 1,2,3,4,5-pentakisphophate and a free phosphate (Angel et al, 2002; Yao et al, 2012). 3-phytases are usually from fungal or bacterial sources, while 6-phytases are found in oil seeds and grains of higher plants (Yao et al, 2012). Although both phytases have the same function, 3-phytases do not always completely dephosphorylate phytate although 6-phytases do (Angel et al, 2002). Phytases can also be categorised into different groups (HAPs, PAPs, PTP and BPPs) based on their catalytic action as well as their pH optima (acid and alkaline phytases). Acid phytases includes histidine acid phosphatases

(HAPs), purple acid phosphatases (PAPs) and protein tyrosine phosphatase (PTP)-like inositol polyphosphatases, while β-propeller phytases (BPPs) from *Bacillus* are examples of alkaline phytases (Yao et al, 2012; Kim et al, 1998; Kerovuo et al, 1998). Enzymes in the HAPs class share a conserved active site motif, RHGXRXP, and hydrolyse phytate via a two-step mechanism: i) a nucleophilic attack on the phosphorous atom via histidine in the active site; and ii) subsequent hydrolysis of the resulting phosphor-histidine intermediate (Yao et al, 2011; Shin et al, 2001; Mullaney and Ullah, 2003). Enzymes in the PAPs class have a characteristic purple colour due to a charge transfer transition at 560 nm from tyrosinate to Fe(III). These enzymes contain an Fe(III)-Zn(II) centre in their active site that mediates the hydrolysis process via a Fe(III)-coordinated hydroxide ion that attacks the phosphorus atom of the substrate directly (Klabunde et al, 1996). Enzymes in the BPPs class resembles a propeller with six blades and have two phosphate binding sites: a 'cleavage site' where hydrolysis of substrate occurs and an adjacent 'affinity site' which increases the binding affinity for molecules containing neighbouring phosphate groups such as phytate. The enzymic reaction is most likely to proceed via a direct attack on the phosphorus atom of the substrate by a water nucleophile (Shin et al, 2001; Mullaney and Ullah, 2003).

Phytases from microbial sources have been shown to have beneficial effects on both animals and plants. The ability of *E. coli* to hydrolyse phytic acid has been shown in numerous studies due to the presence of phosphatase/phytase enzymes. The supplementation of *E. coli* phytases into animal feed has shown beneficial results in chickens (Ravindran et al, 2006; Augspurger and Baker, 2004) and pigs (Jendza et al, 2005; Veum et al, 2006). Chickens which have phytases added in their feed showed a higher body weight when fed with either a basal diet or a phosphate deficient diet (Augspurger and Baker, 2004), showing a role in phosphate utilisation from phytic acid. The chickens also showed higher Tibia ash weight compared to the chicken with inorganic phosphate supplemented feed, suggesting the presence of phytase yielded better results than for phosphate supplementation (Augspurger and Baker, 2004). A later study also showed that phosphorus digestibility is increased with increasing concentration of phytase, which complements the results

obtained by Augspurger and Baker, showing that phytases are able to liberate phosphate from phytic acid for utilisation in the animal (Ravindran et al, 2006). In studies using pigs, when *E. coli* phytases were supplemented in the feed, phosphorus absorption increased with increasing concentration of the enzyme as shown by the low phosphorus content in the faecal matter (Veum et al, 2006). In addition to increased phosphorus absorption, iron and zinc absorption also increase with increasing levels of phytase, due to the breakdown of phytate (Veum et al, 2006). Other evidence that phytases increase phosphorus absorption in pigs includes the increase in plasma phosphorus content when phytases are supplemented in the feed (Jendza et al, 2005), showing the ability of *E. coli* phytase to liberate phosphate from phytic acid for utilisation by the host.

In plants, organic phosphorus has an important role in the phosphorus cycle of agricultural soil. However, organic phosphorus exists predominantly as phytate (60% of organic phosphorus in soil) which is poorly utilised in plants and must be dephosphorylated prior to assimilation in plants (Singh and Satyanarayana, 2011). Bacteria present in soil have the ability to breakdown phytate due to their deployment of phytases such that the released phosphorus can then be utilise by plants to support their growth (Patel et al, 2010; Unno et al, 2005; Hameeda et al, 2006). Bacterial groups isolated from various composts that possess phytase activities, such as *Pseudomonas*, *Klebsiella, Bacillus* and *Enterobacter*, have been shown to have growth promoting effect in pearl millet, causing a significant increase in the plant dry weight as compared to the uninoculated control (Hameeda et al, 2006). Bacteria isolated from soil belonging to the *Burkholderia* genus showed a promoting effect in Lotus seedlings accompanied by increased phosphorus uptake (Unno et al, 2005). Inoculation of members of the Enterobacteriaceae family such as *Pantoea*, *Citrobacter*, *Klebsiella* and *Enterobacter* on pigeon pea caused an increased phosphorus accumulation in the shoot as compared to the uninoculated control, indicating their ability to degrade phytate for utilisation of the releases phosphate in plants (Patel et al, 2010).

The above studies thus show the beneficial impact of bacteria phytase activity in enabling animals and plants to utilise phytate as a phosphate source, but there is limited research on phytate utilisation by bacteria. There is certain evidence of bacterial phytate utilisation as a carbon and phosphate source but this is limited to isolating bacterial from soil samples for their phytase activity (Richardson and Hadobas, 1997; Unno et al, 2005). Results from the experiment in chapter 6 showed that *E. coli* is able to utilise phytate as a phosphate source and the growth increases with increasing concentration of phytic acid supplemented to a phosphate limiting medium. The three genes of interest, *appA*, *agp* and *phoA*, had been shown to encode enzymes that hydrolyse phosphate monoesters (Golovan et al, 2000; Cottrill et al, 2002; Hayakawa et al, 1991). *appA*, which encodes the acid phosphatase, is the most extensively studied enzyme among the three with various modification being done on the enzyme to improves its thermostability (Zhu et al, 2010; Yao et al, 2013) and resistance to trypsin (Wang et al, 2018) to support its use as a supplement to animal feed. The *agp* gene, which encodes glucose-1-phosphatase, and *phoA*, which encodes the alkaline phosphatase, have received limited attention despite their phytase activity and have not been shown to have any industrial application. As seen from the results in chapter 6, although *E. coli* is able to utilise phytate, the mutants showed similar growth to the wild-type with phytate as phosphate source indicating that these 3 enzymes are not the only phytases that *E. coli* has. Indeed, a triple mutant with these enzymes knocked out also showed comparable phytate dependent growth to the wild type, indicating the presence of other phytases or phosphatases in *E. coli.* Surprisingly, the combined lack of these enzymes did not affect *E. coli* in acquiring phosphate from phytic acid to support its growth and this suggests that there is a yet to be discovered mechanism for phytate utilisation. It is possible that *E. coli* is able to scavenge phosphate through expression of another phytase or directly stripping a phosphate group off phytic acid via an outer membrane receptor, but this remains to be explore.

Although *E. coli* is able to use phytic acid, the phosphate groups liberated are not known as the resulting inositol phosphate(s) were not analyse in this project. It would be interesting to determine

the number of phosphate groups released as well as the site(s) of attack to further uncover the mechanism involved, which can be a project in the future. The M9 medium used in this experiment is not entirely phosphate free as phosphate is essential for growth, thus it contains a background concentration of 3.5 mM. An early study showed that nitrifying bacteria are able to grow with a minimal phosphorus level between 0.1-0.65 µM (Meiklejohn, 1952). Assuming *E. coli* has the same requirement, the background phosphate content is sufficient to support the growth of the bacteria. Interestingly, when buffers were used to maintain the pH at 5.5, 7 and 8.5 (section 6.5), both the mutant and wild-type showed similar growth regardless of the concentration of phytic acid concentration. Bacteria in medium containing no phytic acid and the medium containing that 10 mM showed similar growth, indicating that the bacteria were not utilising phosphate from phytate but were instead using the background phosphate to support growth. This observation suggests that an unknown mechanism is triggered at these pH levels whereby *E. coli* is able to scavenge phosphate from its environment to support growth and not through the use of phytase to degrade phytic acid for phosphate. Although the bacteria in the non-phytic acid supplemented medium showed a reduced growth than in the supplemented medium, the bacteria performed better in the pH regulated medium as compared to the non-regulated medium, indicating that activation of a system that enhances the growth of the bacteria under these conditions. One possible explanation is through the upregulation of the phosphate uptake system that is present in *E. coli*, the Pst and Pit systems. Pst is an ABC transporter which is the predominant phosphate uptake system. Pit is considered to be a low affinity symporter that operates in a phosphate-rich environment and can transport divalent cations such as zinc that form complexes with phosphate. The Pit system can be considered a metal transport system rather than a phosphate system as it is regulated by the presence of zinc (Hsieh and Wanner, 2010; Zheng et al, 2016).

Bacteria have been used in wastewater treatment plants to remove excess phosphate in the wastewater which can cause environmental eutrophication. Phosphate has been shown to accumulate in bacterial cells and shotgun metagenomics revealed that genes in both of the Pst and

Pit system show an increase in abundance at pH 5.5 vs pH 8.5 (Weerasekara et al, 2016). Although the bacteria are able to accumulate phosphate at both pH levels, the acidic environment gave the bacteria an advantage over the higher pH. Another study using sludge microorganisms also showed a higher phosphate uptake in bacteria at pH 5.5 as compared to pH 7.5 (McGrath et al, 2001). This may explain the possible enhanced growth in *E. coli* when the pH is regulated through the addition of buffers despite no phytic acid is supplemented to the medium. The phosphate uptake system is upregulated which allows the available phosphate to be scavenged from the medium for growth and development.

Since the growth studies show that *E. coli* has the ability to degrade phytic acid and utilise it to support its growth, it would be interesting to explore the gut microbiota as a whole and its ability to utilise phytic acid. The ability of the gut microbiota to hydrolyse phytic acid has been shown in studies comparing germ free and conventional mice (Yoshida et al, 1982) and rats (Wise and Gilburt, 1982). In germ free mice, phosphorus retention is low and a large portion is excreted in the urine. In conventional mice, excretion is lower and the mice retain higher amounts of phosphorus in a phytic acid supplemented diet, showing that the gut microbiota has a role in hydrolysing the phytic acid and the retention of phosphorus in the animal (Yoshida et al, 1982). Similar findings have been obtained by Wise and Gilburt (1982) using rats. This shows that the gut microbiota has a role in hydrolysing phytic acid which allows the animal to absorb the hydrolysed product. Bacteria with the ability to degrade phytic acid have been isolated from faecal samples using culture techniques either directly from faeces (Markiewicz et al, 2013) or single stage continuous culture (Steer et al, 2004). *Bacteroides* spp., *E. coli*, *Clostridium* spp., and *Staphylococcus* spp. are among the bacteria isolated from the phytic acid-enriched medium (Steer et al, 2004). Markiewicz and colleagues also found that coliforms are the most effective phytate degraders followed by Proteobacteria-*Bacteroides* cultures (Markiewicz et al, 2013). Results from both experiments showed that certain bacterial groups in the gut are able to degrade and utilise phytic acid. The batch culture experiment was set up to investigate the ability of gut microbiota to utilise phytic acid rather than just degrading it. Results

from Figure 6.20 showed that the presence of phytic acid enhanced the growth of the gut microbiota compared to the control or the phosphate only group. In addition, a molecule of phytate can liberate 6 phosphate groups once hydrolysed which then can be utilise by the bacteria. It would be interesting to determine whether same growth would be obtained if the concentration of phosphate and phytic acid were matched in future experiments. From Figure 6.21, phytic acid appears to be a very poor carbon source for the bacteria as the growth in the phytic acid supplemented medium is comparable to the control. Despite early experiment showing that phytic acid can be used a carbon source (Richardson and Hadobas, 1997; Unno et al, 2005), the results obtained from the batch culture suggested otherwise. The addition of phytic acid to the starch containing medium caused a reduced growth as compared to the starch only medium, suggesting that phytic acid acts as an antinutrient which limits the availability of minerals in the medium. In summary, phytic acid is a good source of phosphate but a poor carbon source for bacteria. *E. coli* may possess other phytases that would be interesting to be discovered in the future and its mechanism in acquiring phosphate from phytic acid would also be an interesting topic.

9.7 Future experiments

Many studies on iron and the gut microbiota have been conducted using *in vitro* batch cultures/gut models, animal models and human studies, mainly looking at iron supplementation and its effect on the host and the gut microbiota. However, the iron forms used were mainly non-haem iron sources. In future work, it would be interesting to explore the effect of haem-iron on the gut microbiota using *in vitro* cultures whereby haem-iron is concentration-matched with non-haem iron (for equivalent comparison) and faecal matter from both iron sufficient and iron deficient individuals is used to inoculate the cultures. This will mimic situation whereby individuals increase their iron intake through diet or supplements. In human studies, having two different diets with similar nutritional compositions but different forms of iron (e.g. beef vs white fish; or with/without haem dietary supplements) would be an interesting way to explore the impact of haem-iron on the gut microbiota.

As for the impact of zinc on the gut microbiota, previous studies were mainly conducted in animals where there may be an economic impact on farmers. Zinc (alone or with vitamin A) has been provided as a supplement to children in areas where malaria is prevalent. Zinc supplementation reduces clinical malaria episodes as well as diarrhoea in children, which is a beneficial and promising effect (Owusu-Agyei et al, 2013; Yakoob et al, 2011; Muller et al, 2001; Zeba et al, 2008). Future experiments involving zinc supplementation in children or adults should include collection of faecal samples as part of the project to consider the impact of zinc on the gut microbiota.

As for the ability of gut microbiota to breakdown and utilise phytic acid, it would be interesting to determine the bacterial composition via NGS to identify the impact on species composition. A modified medium will be needed to ensure that phosphate present in the medium is as low as possible. It would also be interesting to see the difference in the capacity of the gut microbiota to utilise phytic acid using faecal samples from vegetarian/vegan individuals as compared to omnivorous individuals (i.e. those on high and low phytate diets).

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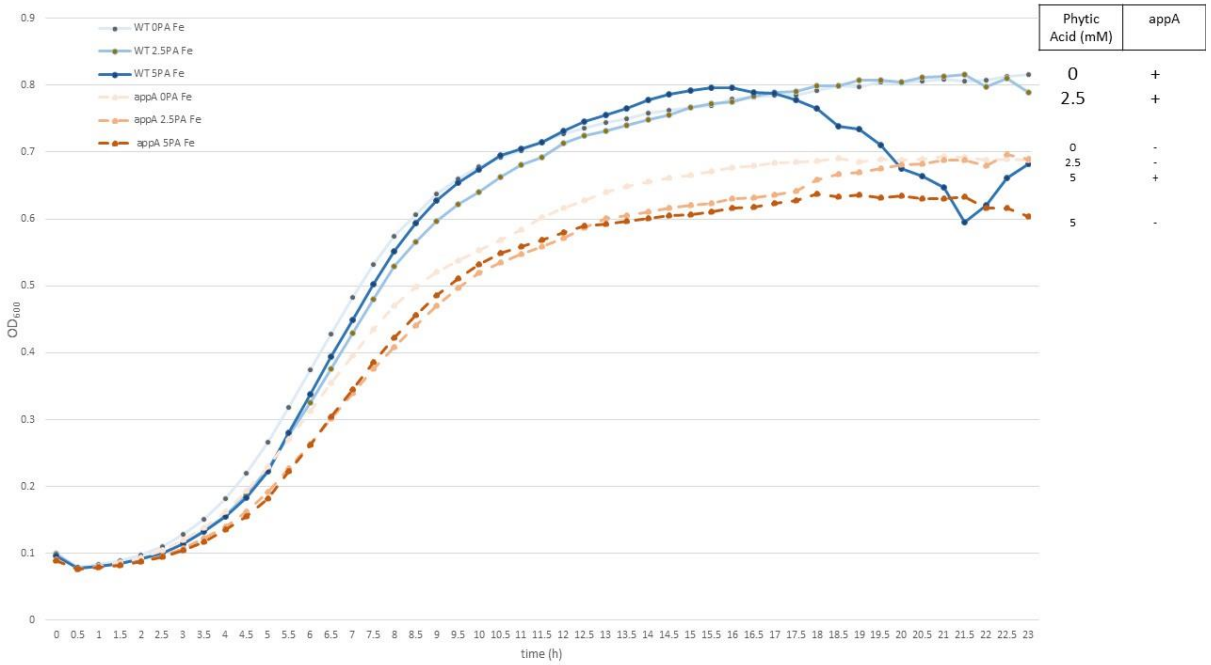
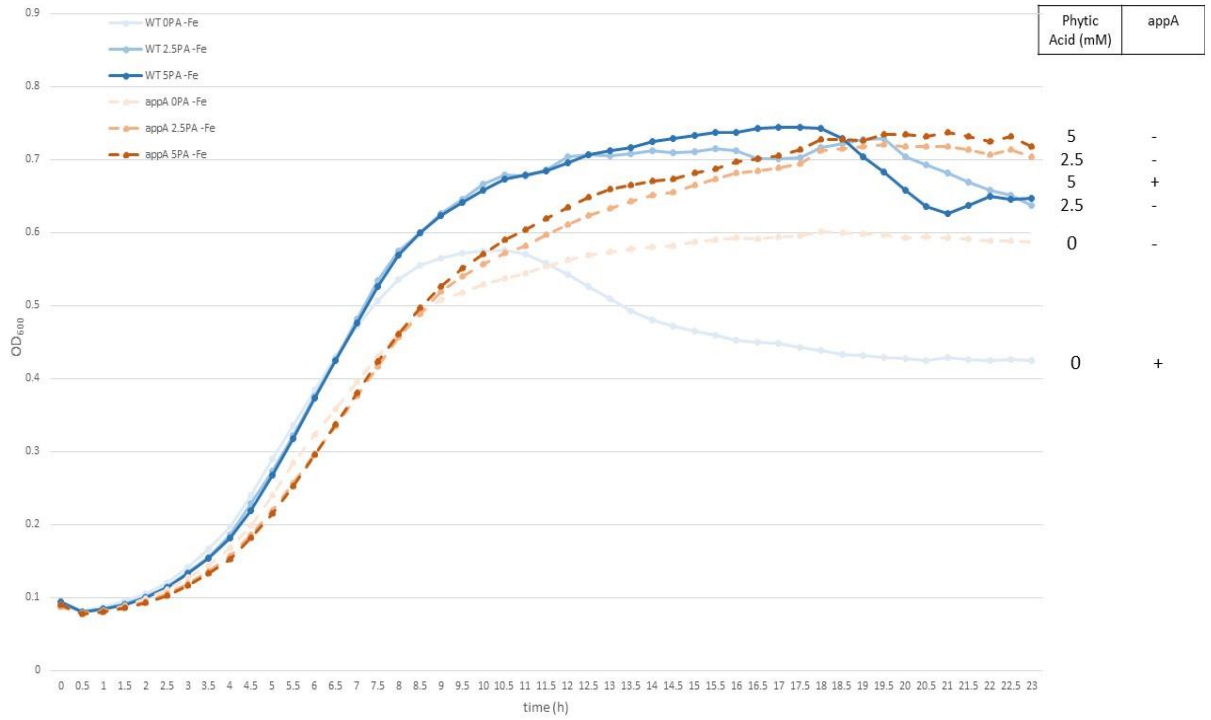
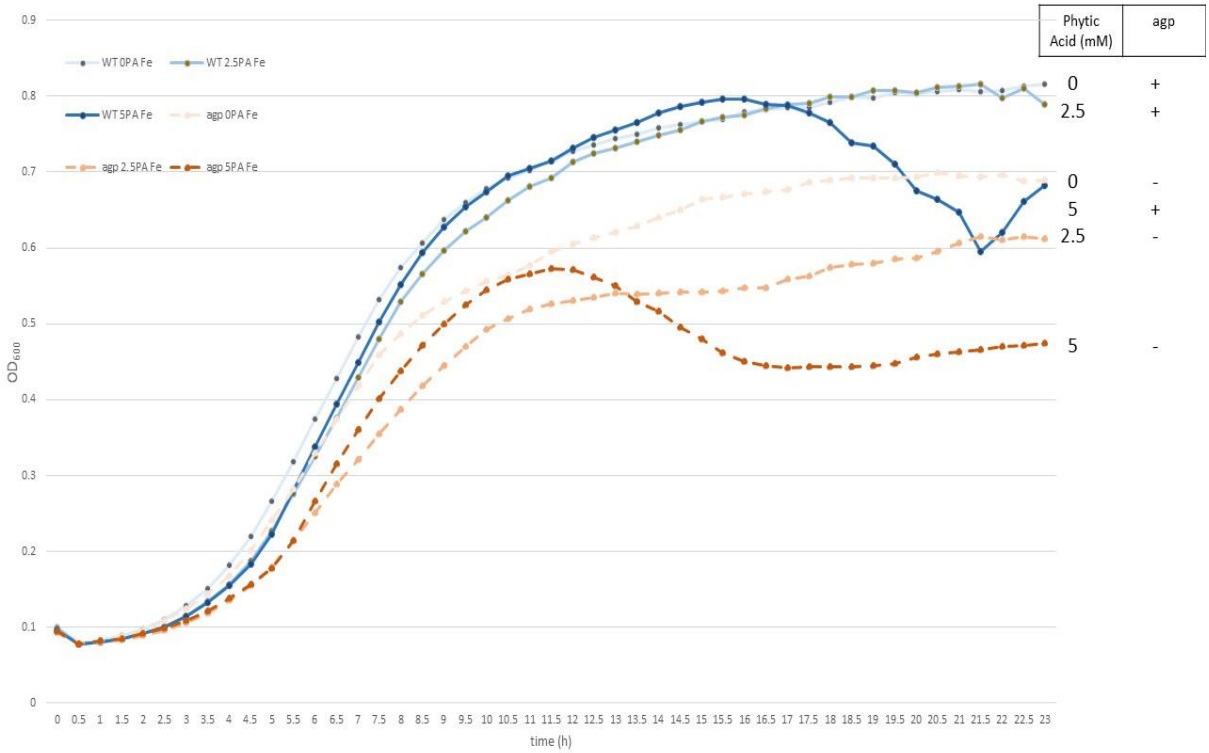
Appendix



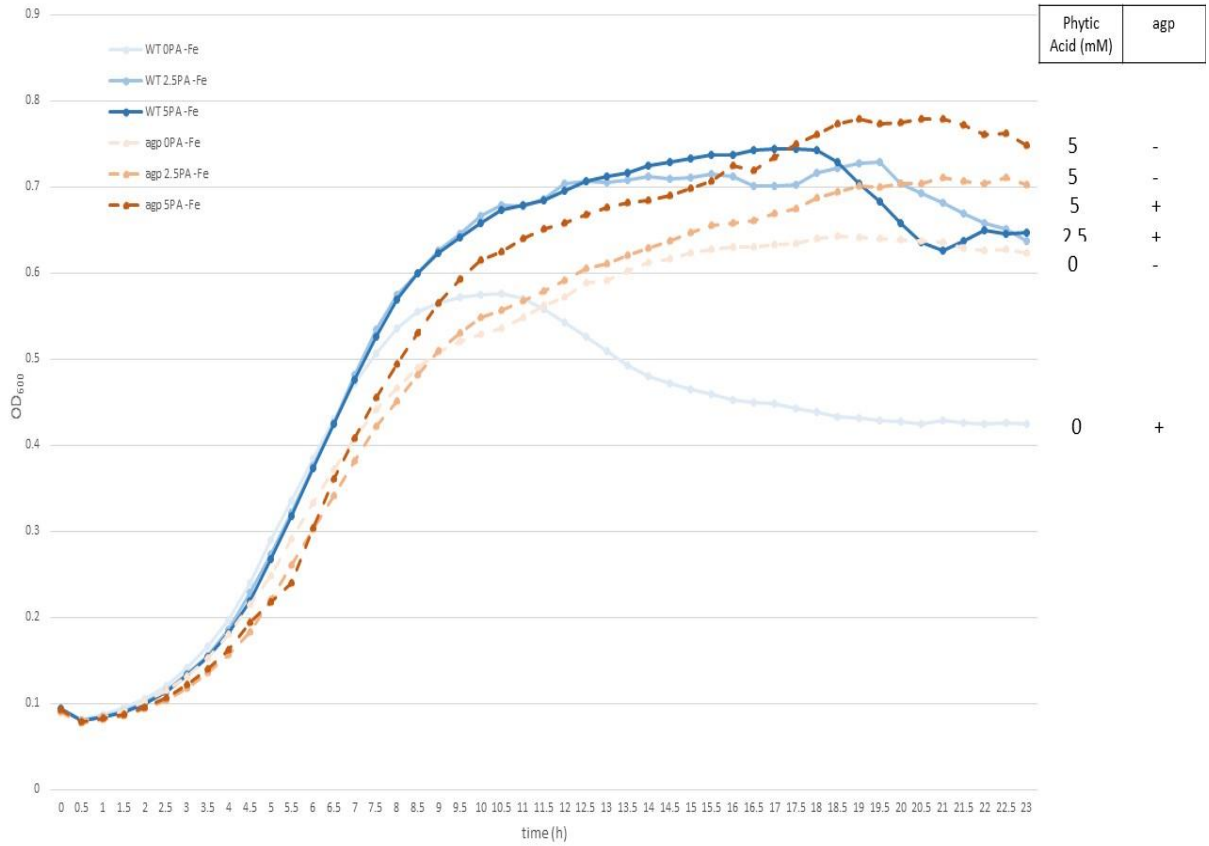
Suppl 1. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* with/without phytate, in the presence of iron. WT (solid lines, +), indicates the wild-type *E. coli*; and mutant (dashed lines, -) indicates the mutant. The bacteria were grown in low phosphate (3.5 mM) M9 minimal medium at pH 5.5 in the presence of 10 µM ferric citrate and phytic acid at 0, 2.5 and 5 mM. Constant shaking under aerobic conditions in a Bioscreen C system. The results shown are average of triplicates. Precultures were grown overnight in M9 medium with 70 mM phosphate and 10 µM ferric citrate.



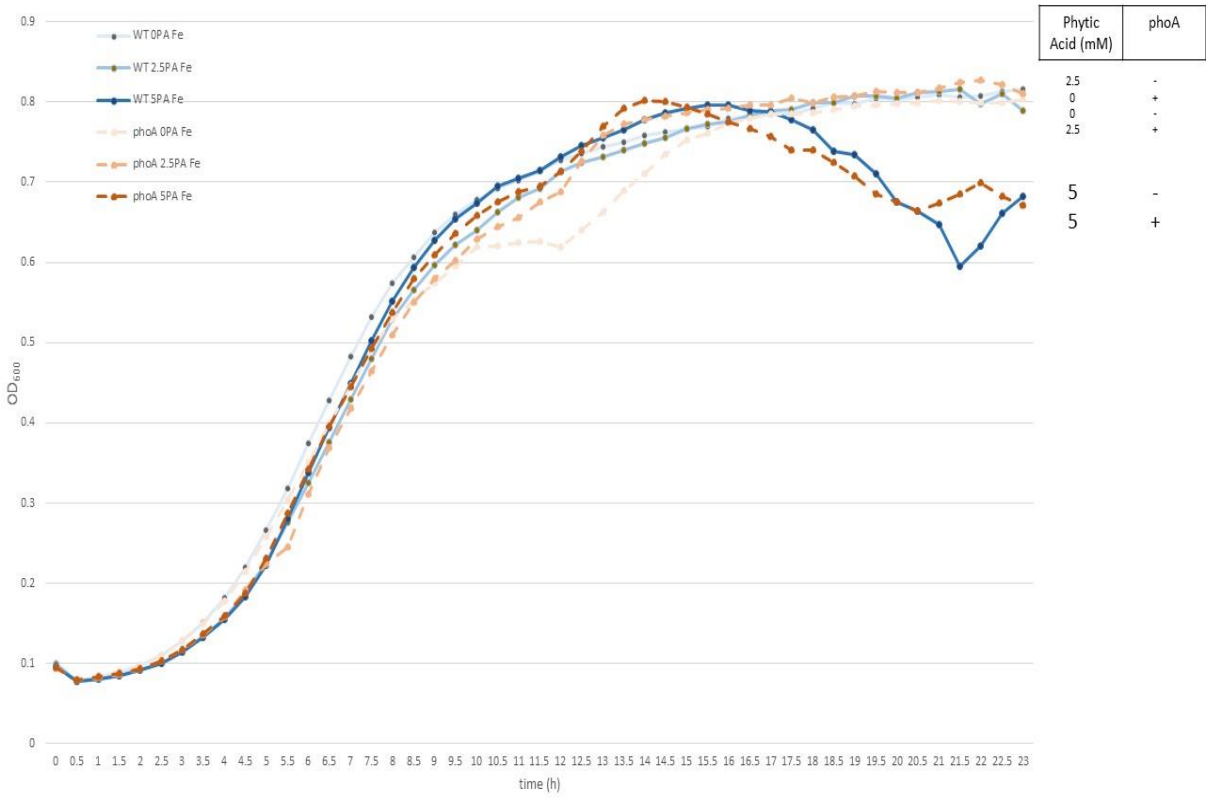
Suppl 2. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.



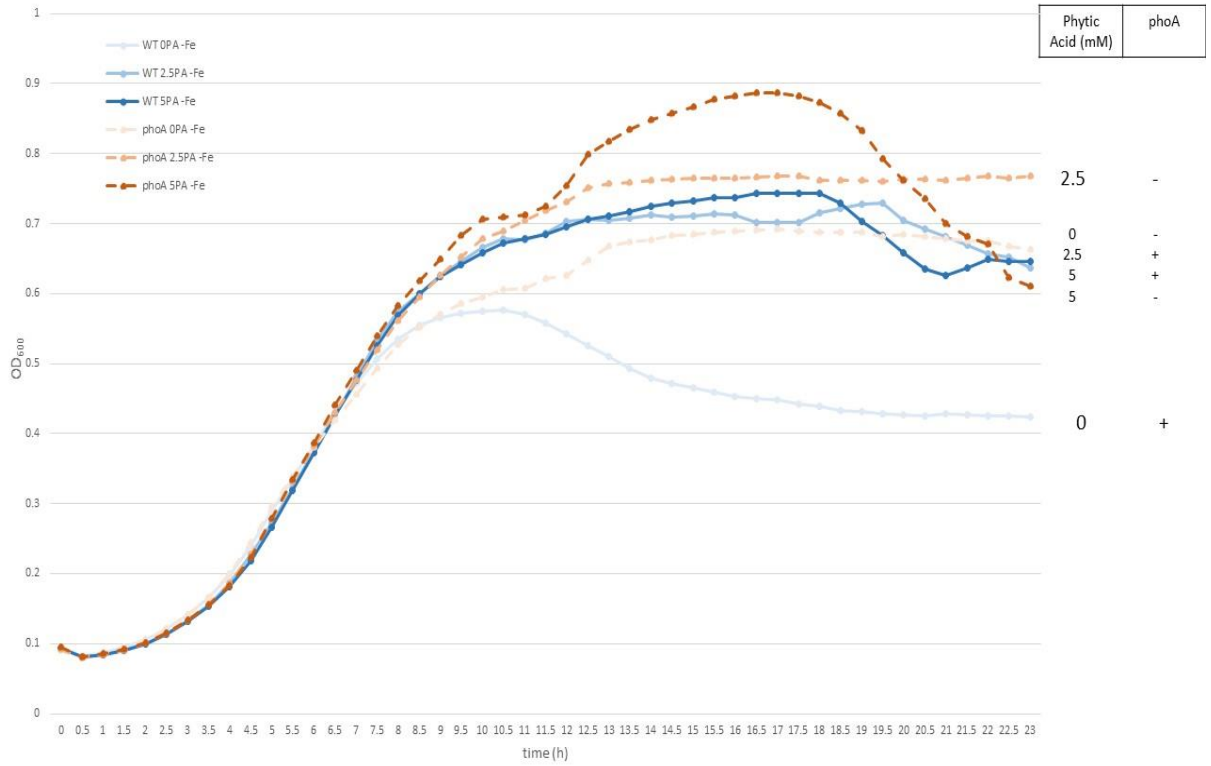
Suppl 3. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*agp* with/without phytate, in the absence of iron. Details are as for suppl 1 except that BW25113 Δ*agp* was employed.



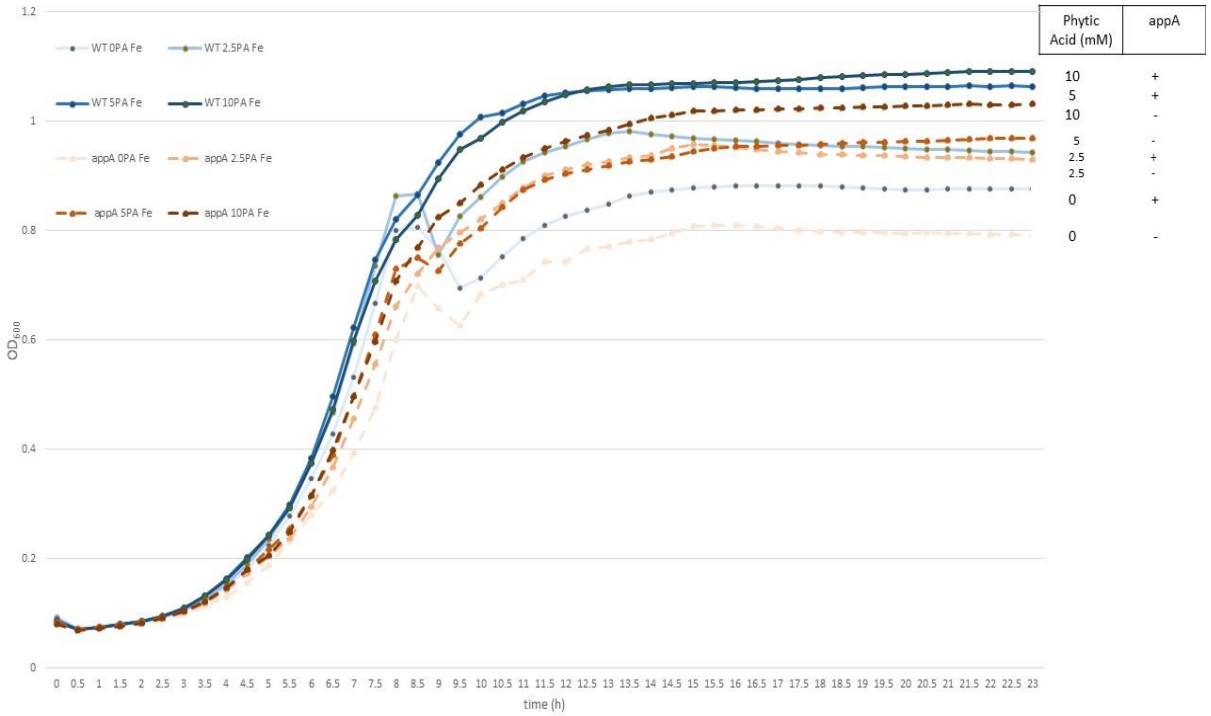
Suppl 4. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*agp* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.



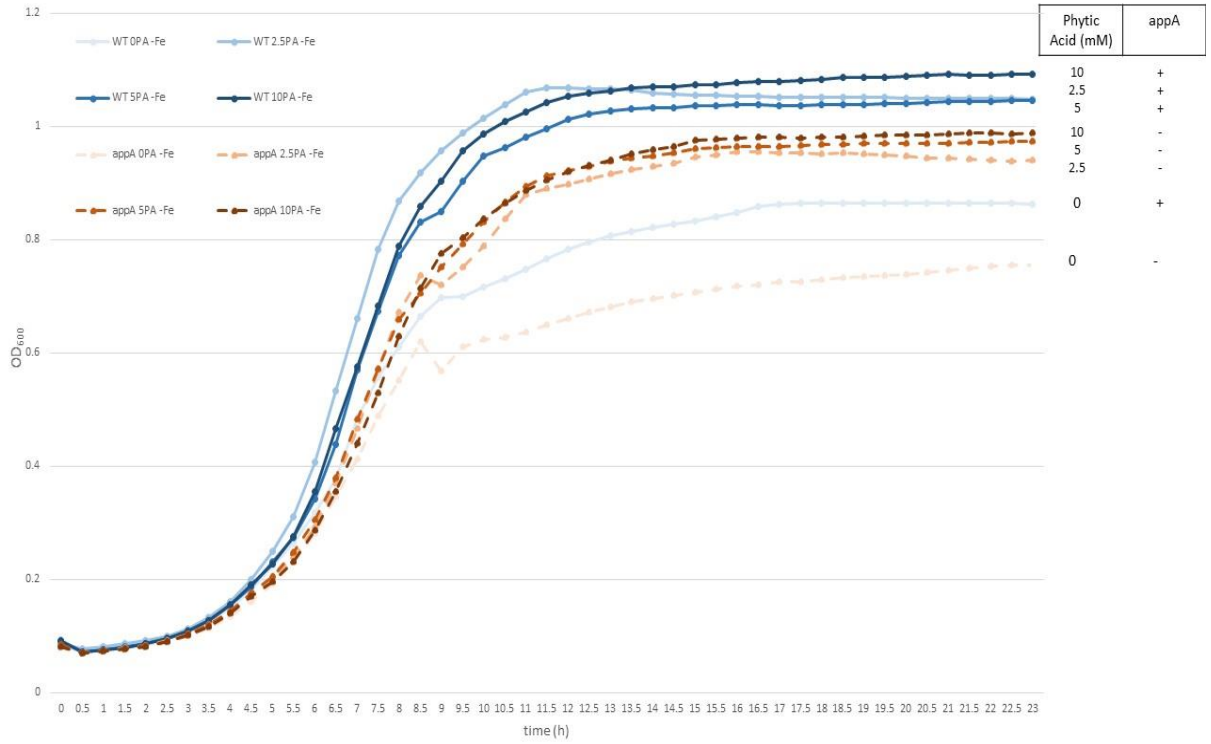
Sppl 5. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*phoA* with/without phytate, in the absence of iron. Details are as for suppl 1 except that BW25113 Δ*phoA* was employed.



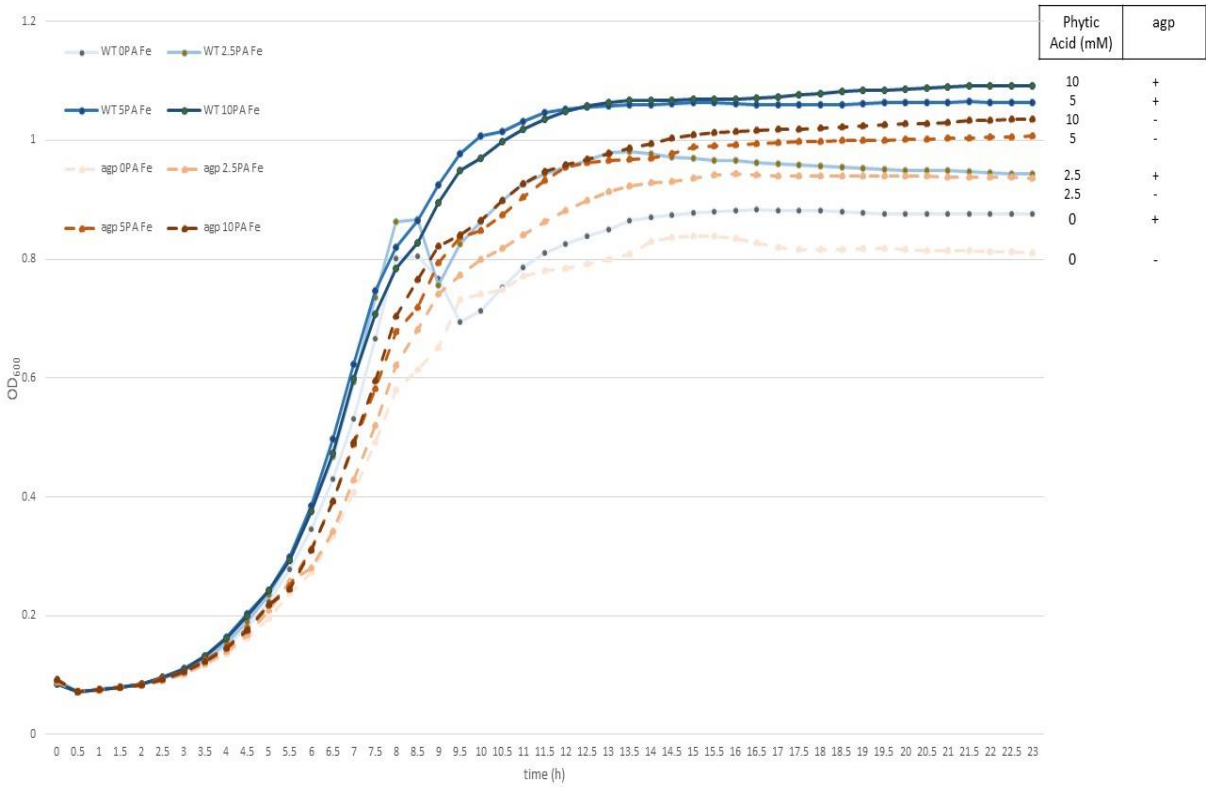
Suppl 6. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*phoA* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.



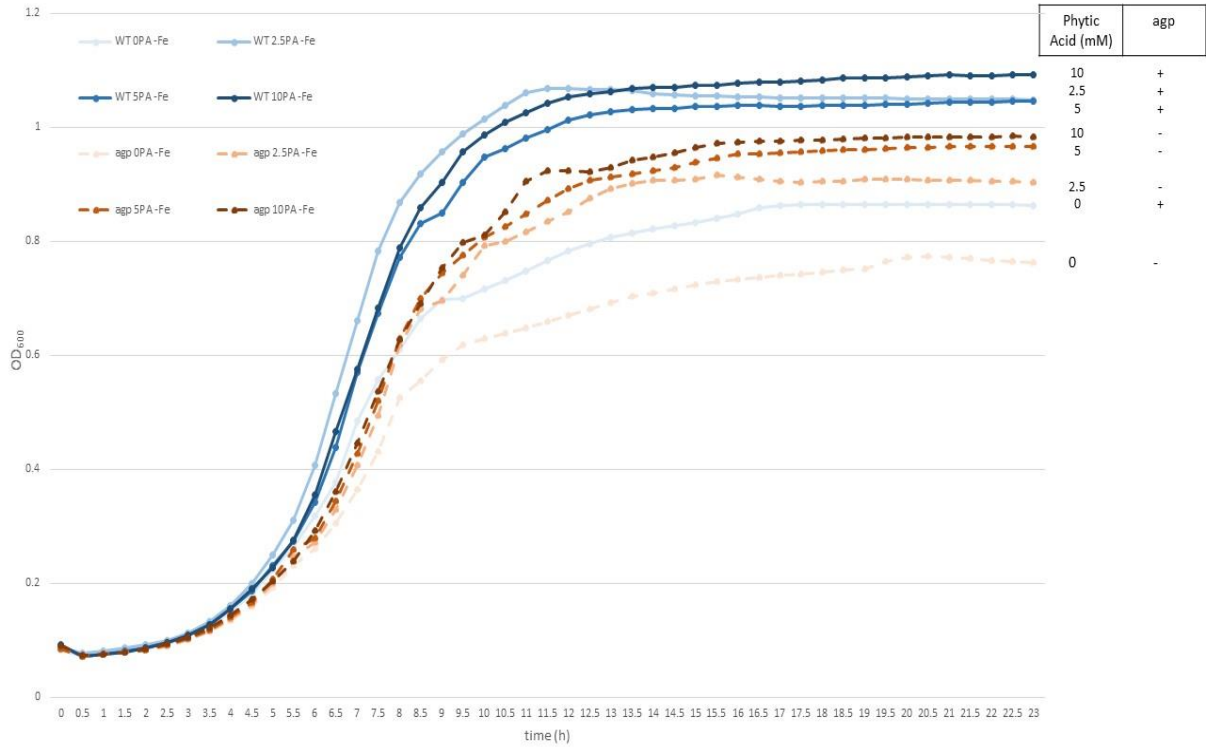
Suppl 7. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* with/without phytate, in the presence of iron. WT (solid lines, +), indicates the wild-type *E. coli*; and mutant (dashed lines, -) indicates the mutant. The bacteria were grown in low phosphate (3.5 mM) M9 minimal medium at pH 7 in the presence of 10 µM ferric citrate and phytic acid at 0, 2.5, 5 and 10 mM. Constant shaking under aerobic conditions in a Bioscreen C system. The results shown are average of triplicates. Precultures were grown overnight in M9 medium with 70 mM phosphate and 10 µM ferric citrate.



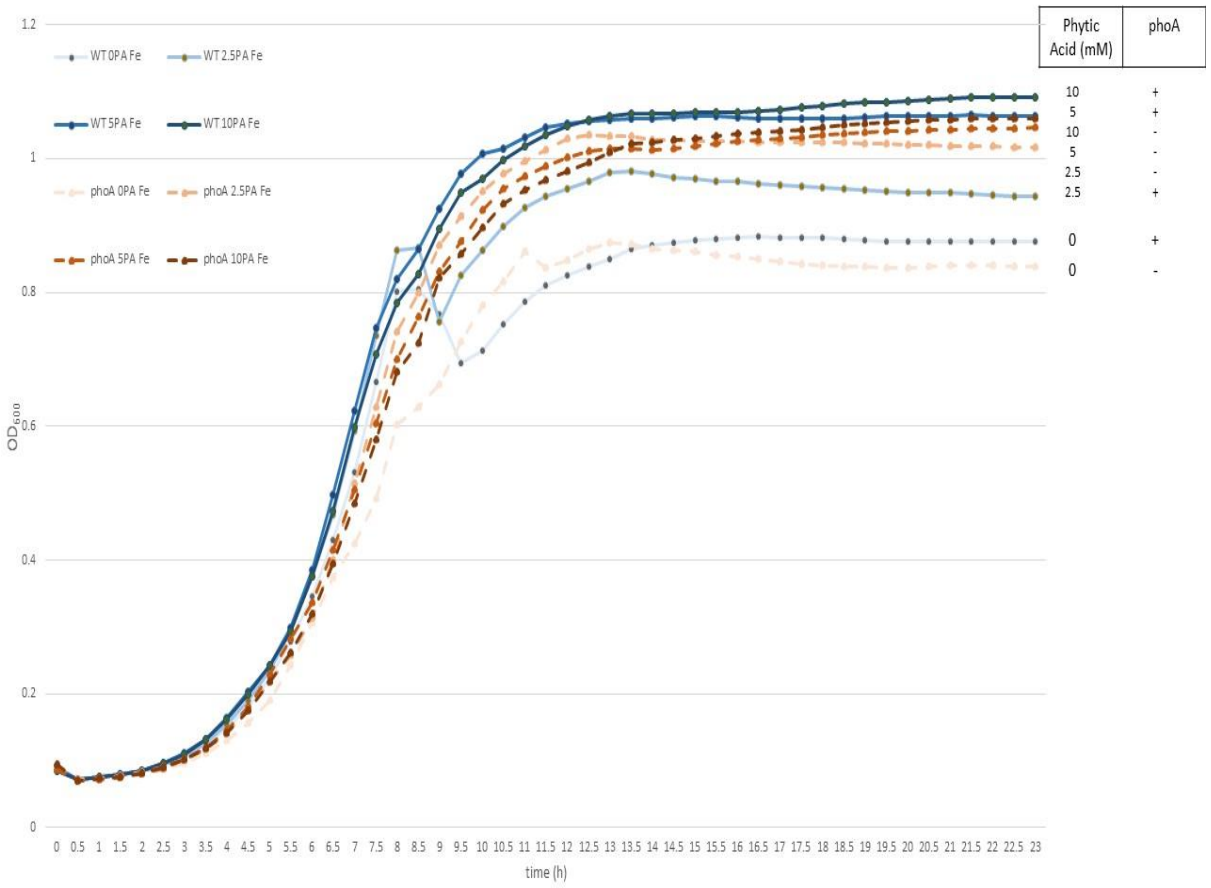
Suppl 8. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.



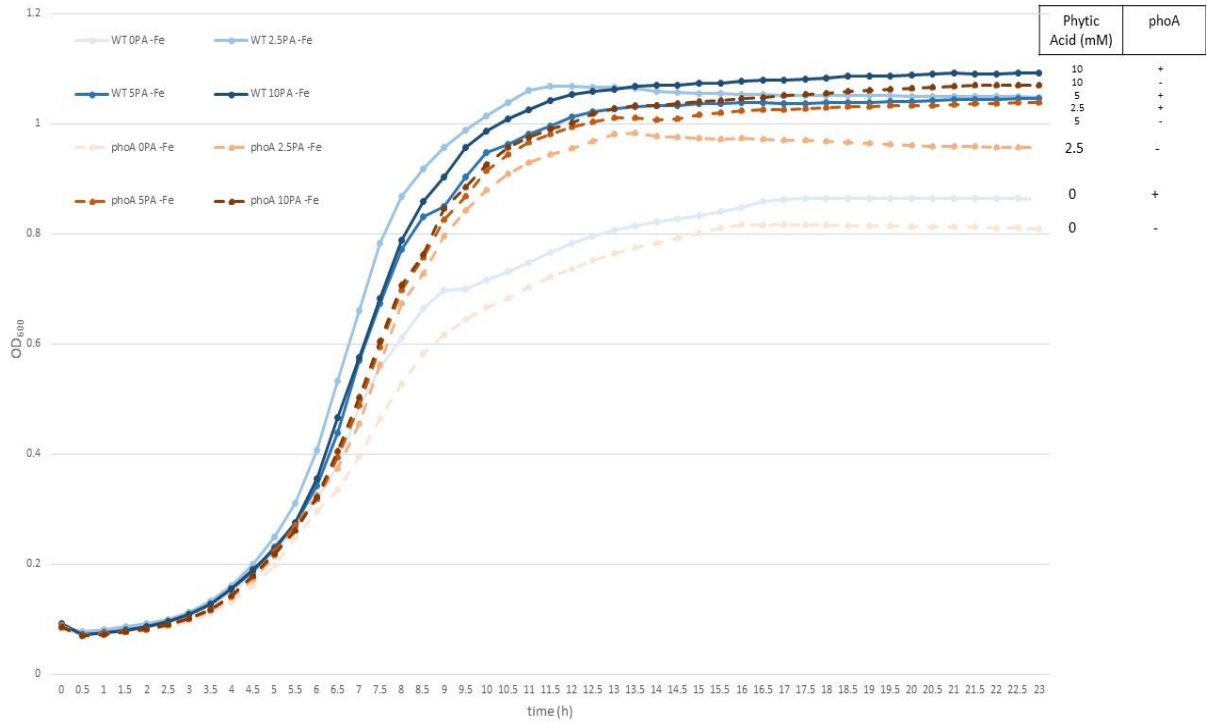
Suppl 9. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*agp* with/without phytate, in the absence of iron. Details are as for suppl 7 except that BW25113 Δ*agp* was employed.



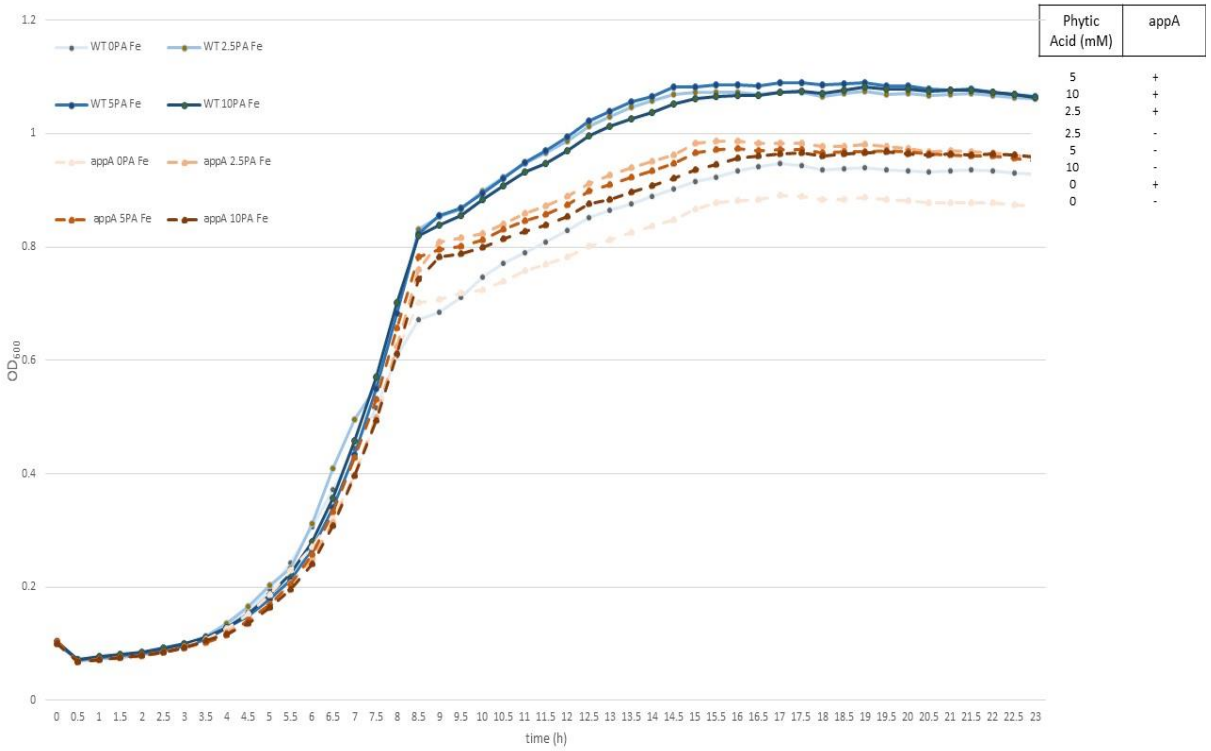
Suppl 10. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*agp* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.



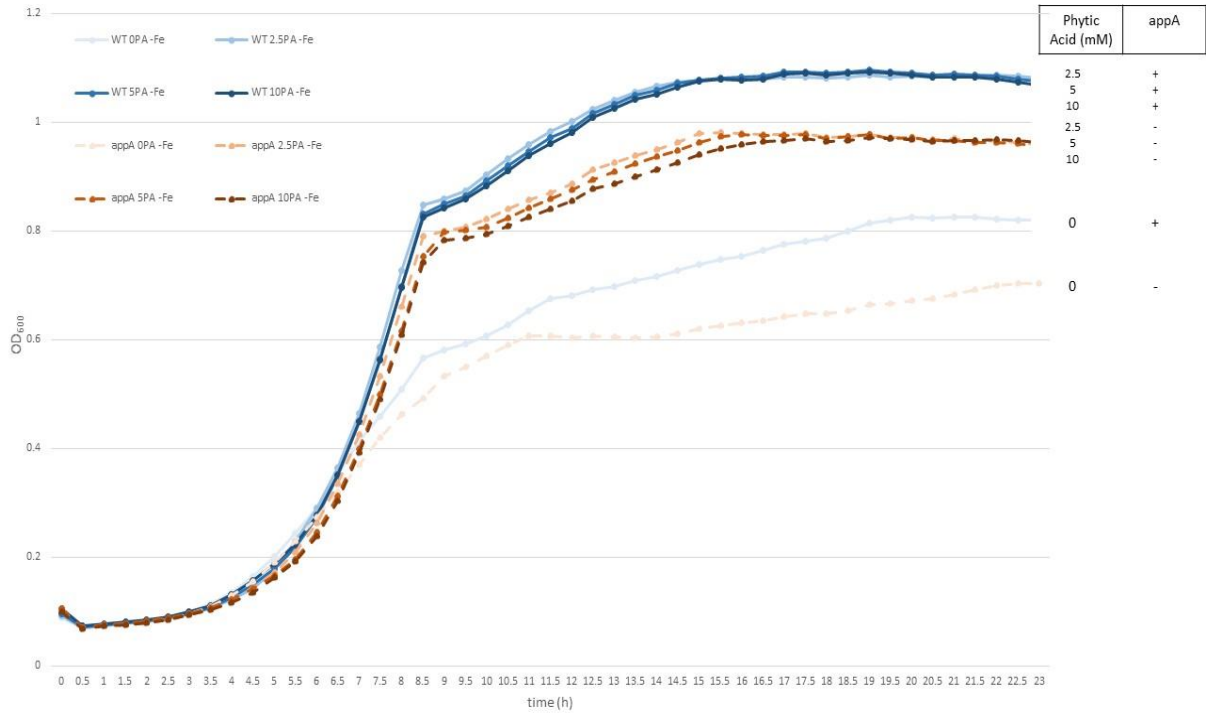
Sppl 11. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*phoA* with/without phytate, in the absence of iron. Details are as for suppl 7 except that BW25113 Δ*phoA* was employed.



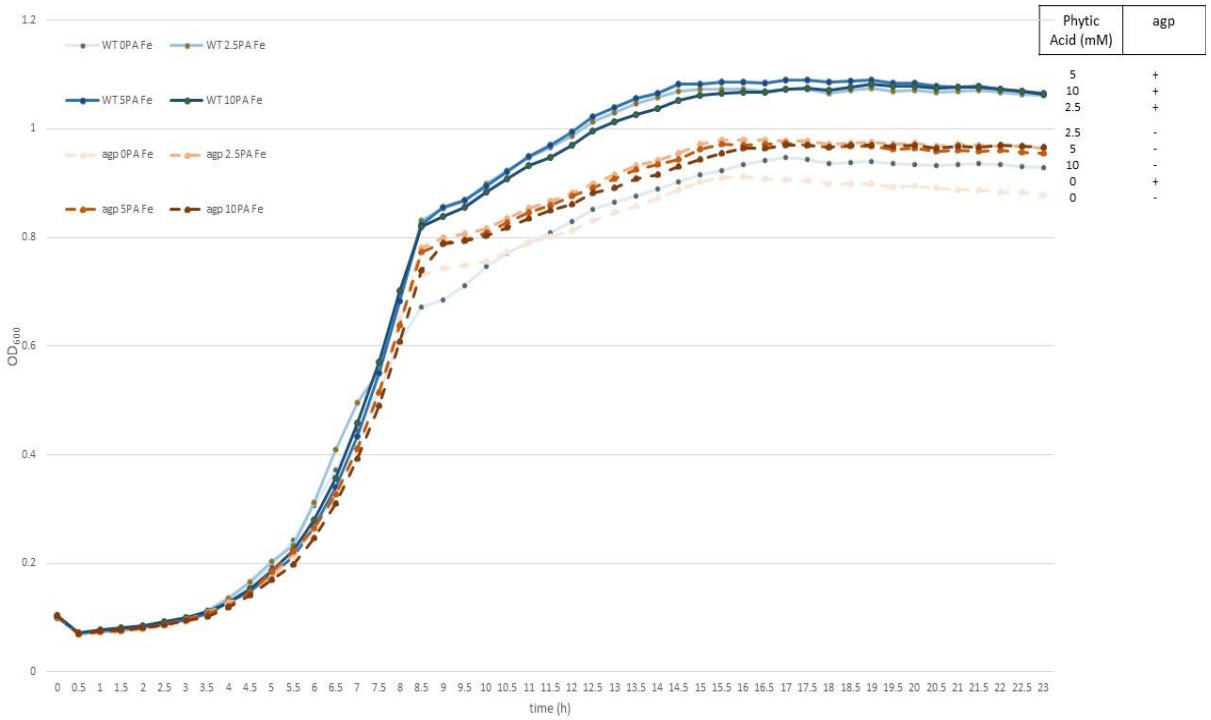
Suppl 12. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*phoA* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.



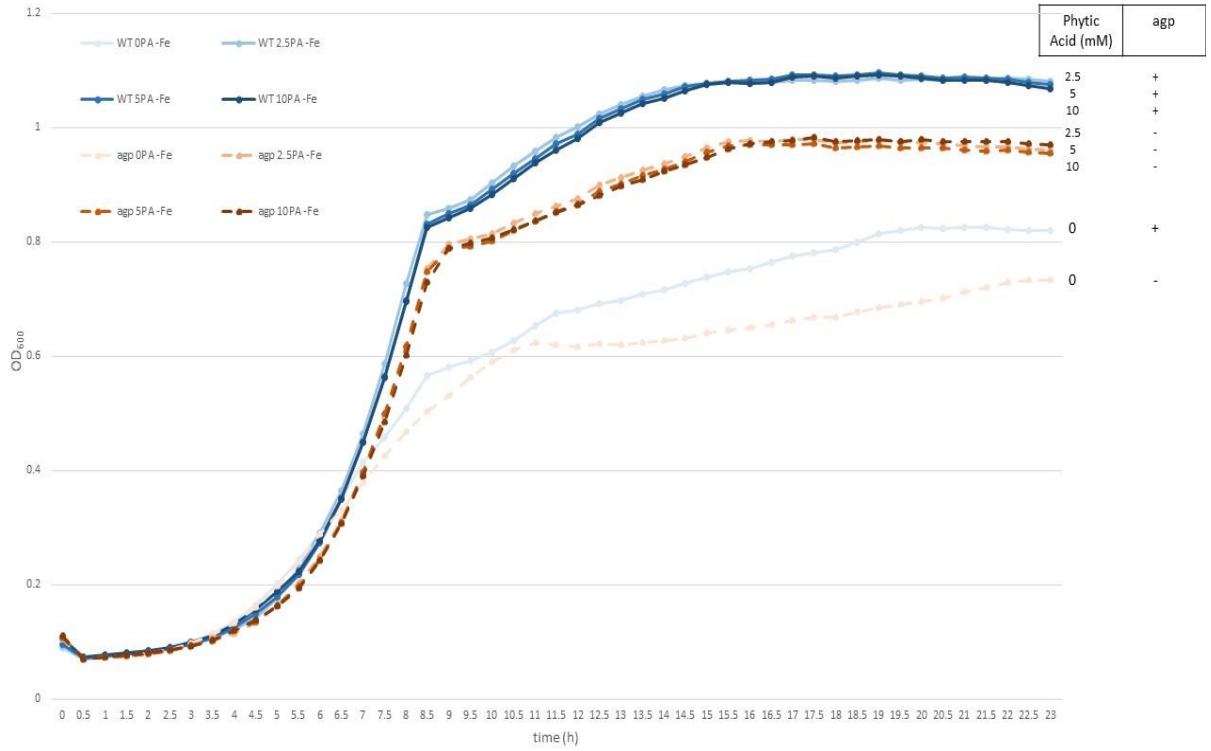
Suppl 13. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* with/without phytate, in the presence of iron. WT (solid lines, +), indicates the wild-type *E. coli*; and mutant (dashed lines, -) indicates the mutant. The bacteria were grown in low phosphate (3.5 mM) M9 minimal medium at pH 8.5 in the presence of 10 µM ferric citrate and phytic acid at 0, 2.5, 5 and 10 mM. Constant shaking under aerobic conditions in a Bioscreen C system. The results shown are average of triplicates. Precultures were grown overnight in M9 medium with 70 mM phosphate and 10 µM ferric citrate.



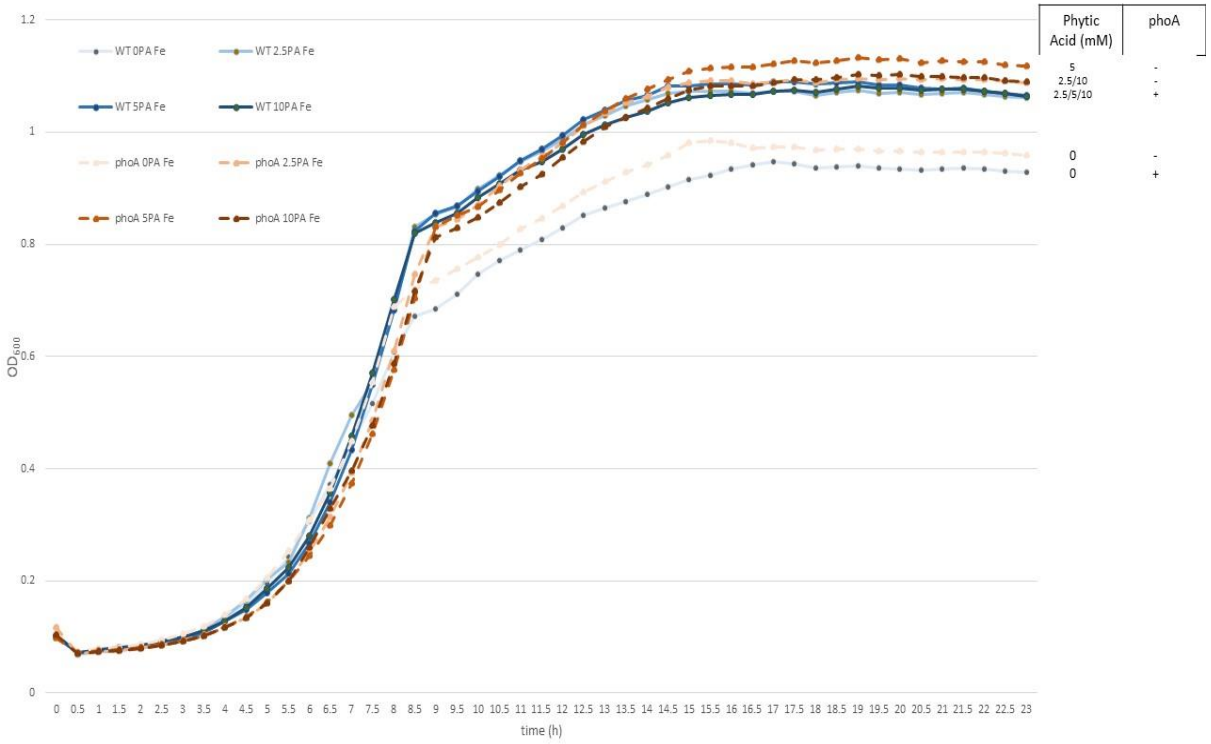
Suppl 14. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.



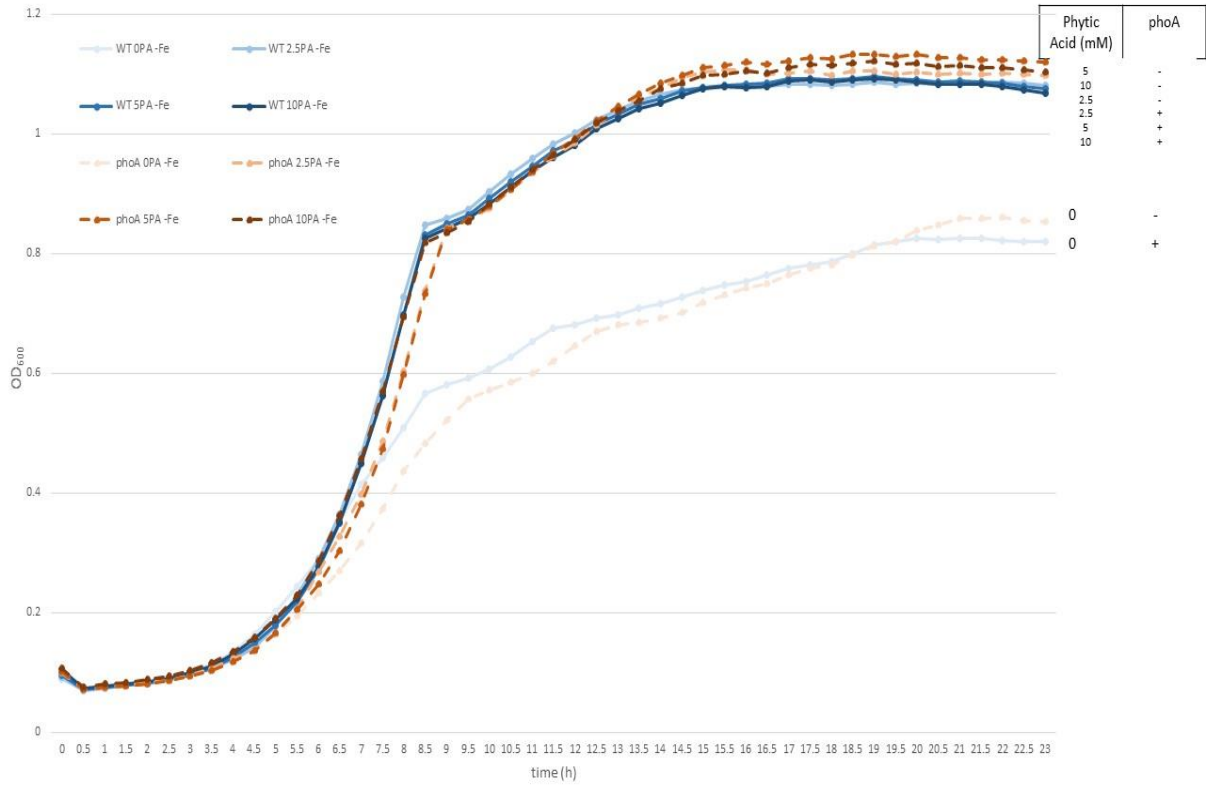
Suppl 15. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*agp* with/without phytate, in the absence of iron. Details are as for suppl 13 except that BW25113 Δ*agp* was employed.



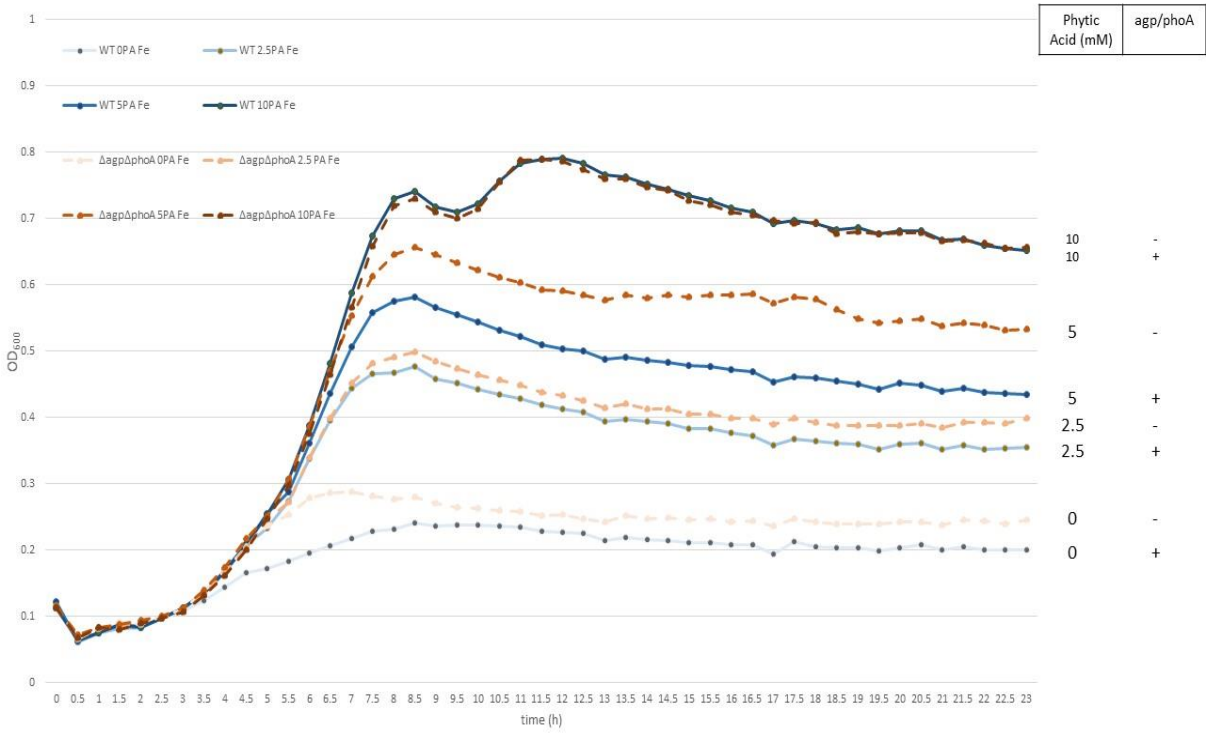
Suppl 16. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*agp* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.



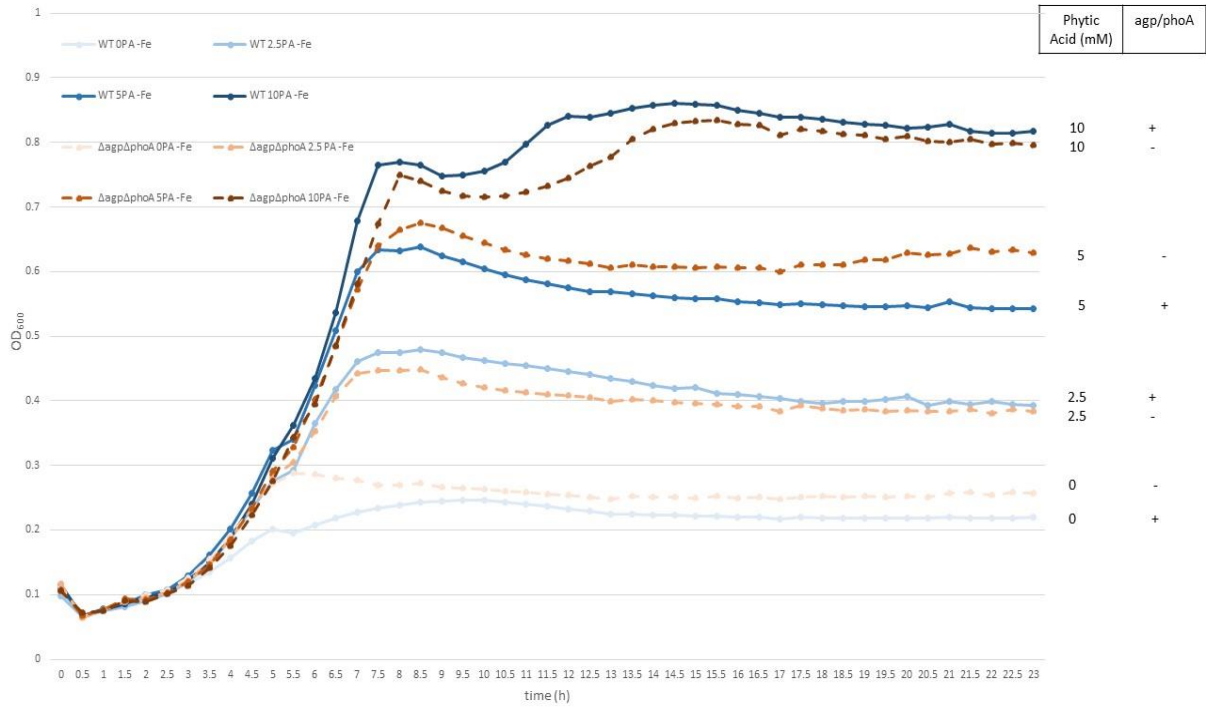
Sppl 17. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*phoA* with/without phytate, in the absence of iron. Details are as for suppl 13 except that BW25113 Δ*phoA* was employed.



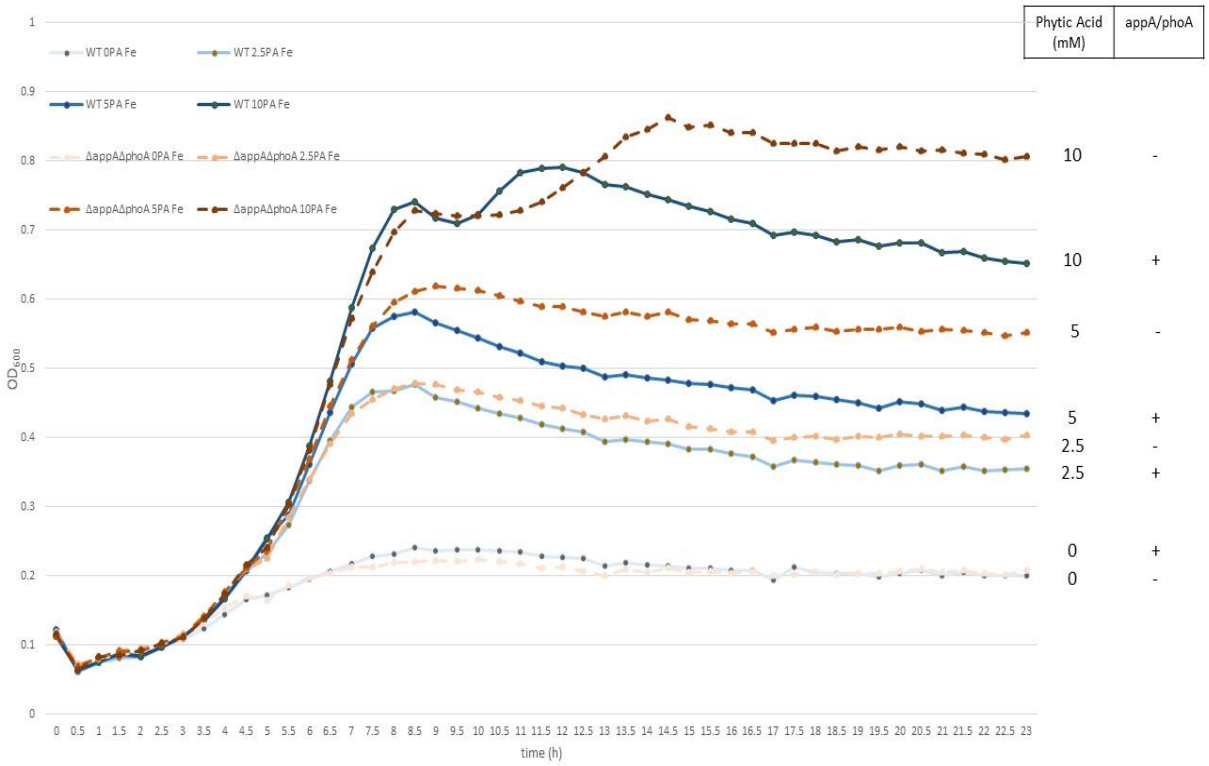
Suppl 18. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*phoA* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.



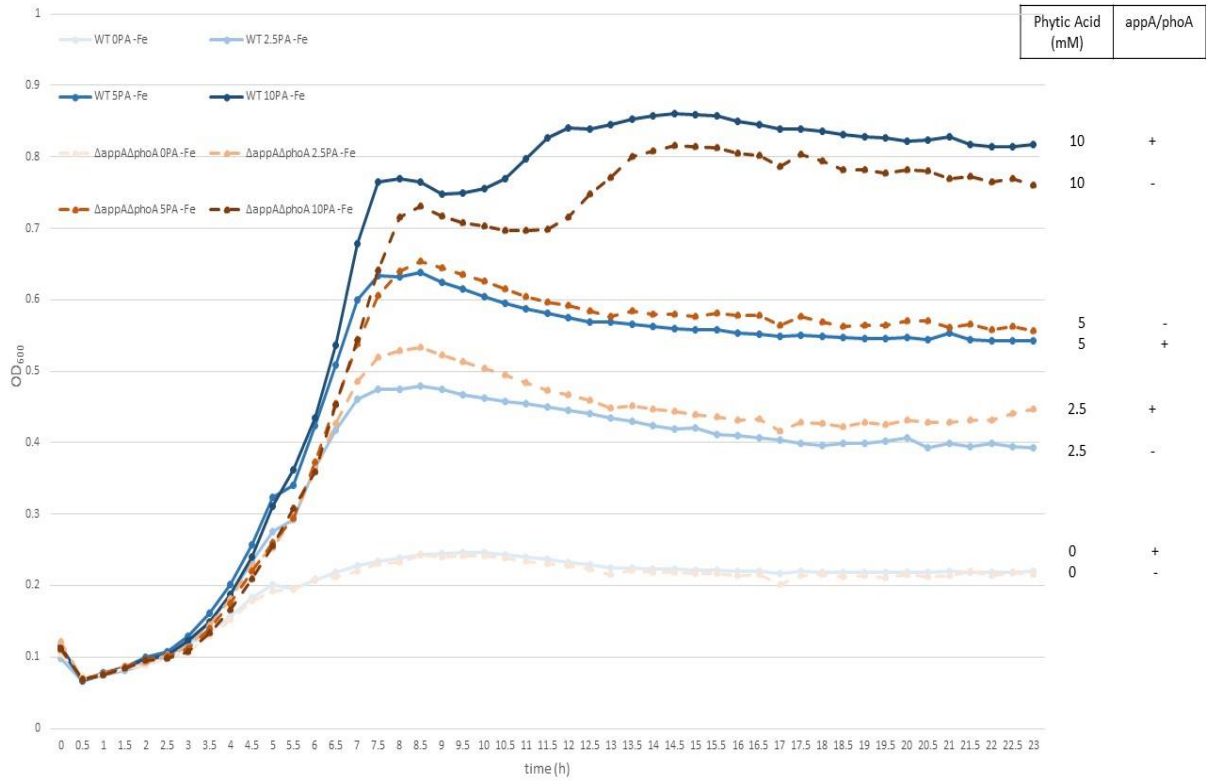
Suppl 19. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*agp ΔphoA* with/without phytate, in the presence of iron. WT (solid lines, +), indicates the wild-type *E. coli*; and *appA* (dashed lines, -) indicates the mutant. The bacteria were grown in low phosphate (3.5 mM) M9 minimal medium in the presence of 10 µM ferric citrate and phytic acid at 0, 2.5, 5 and 10 mM. Constant shaking under aerobic conditions in a Bioscreen C system. The results shown are average of triplicates. Precultures were grown overnight in M9 medium with 70 mM phosphate and 10 µM ferric citrate.



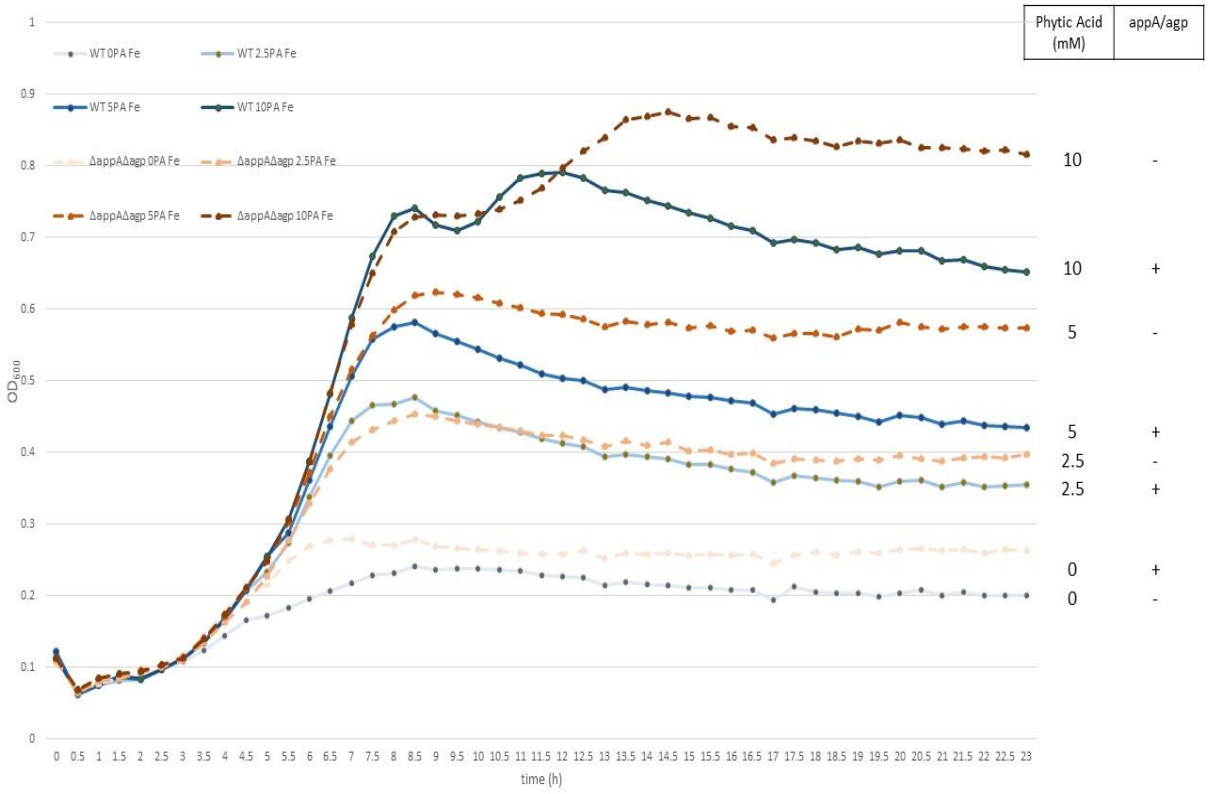
Suppl 20. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*agp ΔphoA* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.



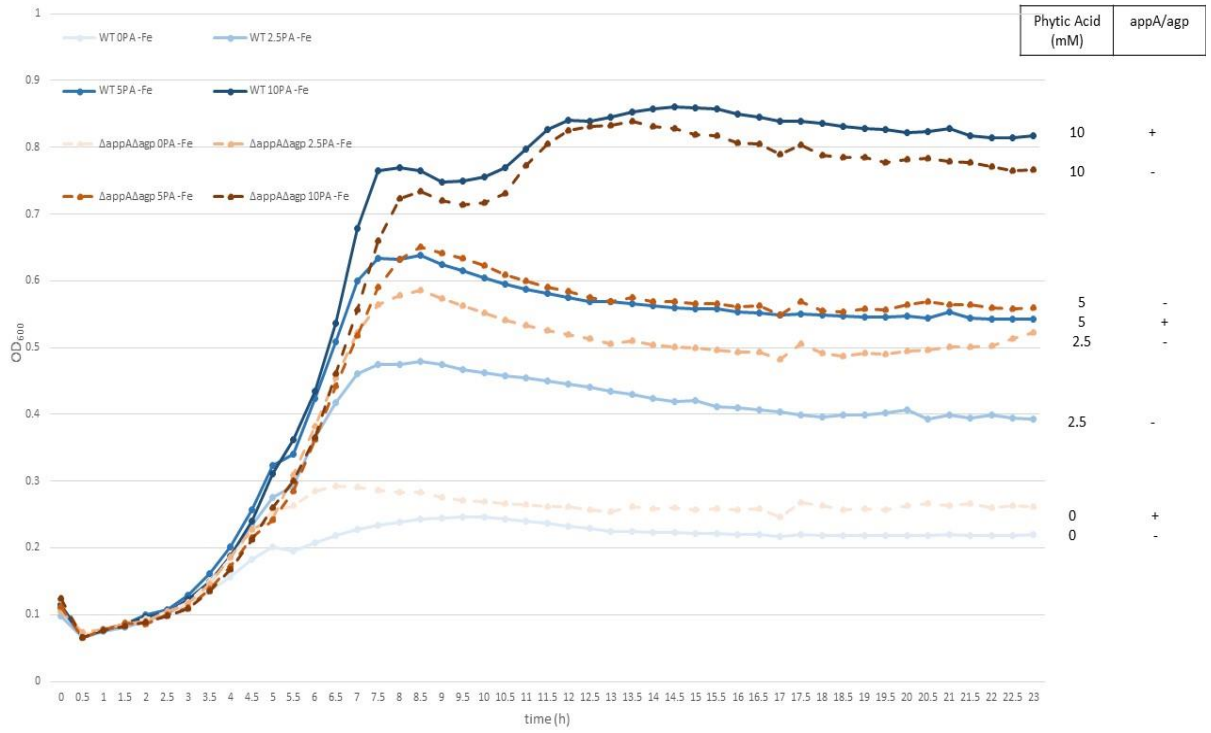
Suppl 21. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* Δ*phoA* with/without phytate, in the absence of iron. Details are as for suppl 19 except that BW25113 Δ*appA* Δ*phoA* was employed.



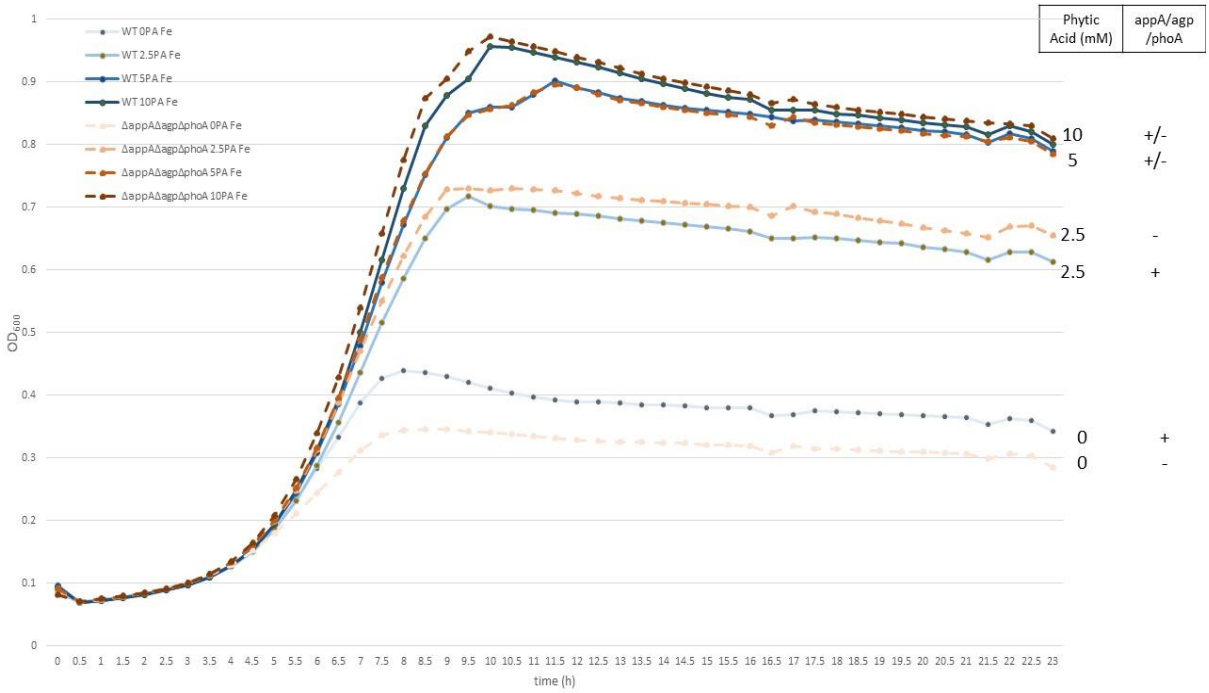
Suppl 22. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* *ΔphoA* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.



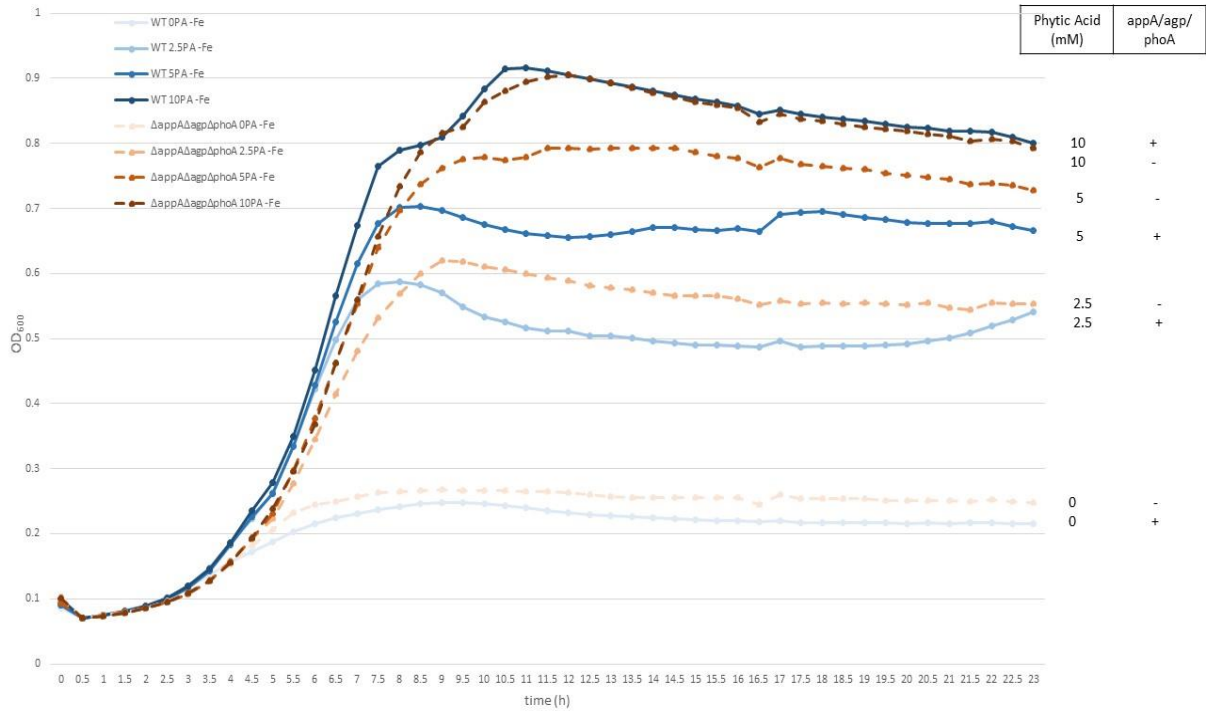
Suppl 23. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* Δ*agp* with/without phytate, in the absence of iron. Details are as for suppl 19 except that BW25113 Δ*appA* Δ*agp* was employed.



Suppl 24. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA* *Δagp* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.



Suppl 25. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA Δagp ΔphoA* with/without phytate, in the presence of iron. WT (solid lines, +), indicates the wild-type *E. coli*; and mutant (dashed lines, -) indicates the mutant. The bacteria were grown in low phosphate (3.5 mM) M9 minimal medium in the presence of 10 µM ferric citrate and phytic acid at 0, 2.5, 5 and 10 mM. Constant shaking under aerobic conditions in a Bioscreen C system. The results shown are average of triplicates. Precultures were grown overnight in M9 medium with 70 mM phosphate and 10 µM ferric citrate.



Suppl 26. Phosphate-limited growth of wild-type *E. coli* BW25113 and *E. coli* BW25113 Δ*appA Δagp ΔphoA* with/without phytate, in the absence of iron. Details are as above except for the absence of iron.