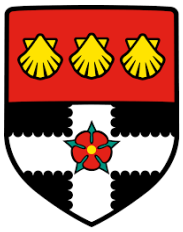

THE UNIVERSITY OF READING

The comparative effects of milk containing A1/A2 β-casein vs milk containing A2 β-casein on gut and cardiometabolic health in humans

By

Areej A. Almuraee

A thesis is part fulfillment of the requirements for the degree of

Doctor of Philosophy

School of Chemistry, Food and Pharmacy

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Declaration

I can confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Areej A. Almuraee

March 2019

Dedication

This thesis is dedicated to the memory of my beloved father

General Abdulhamid Almuraee

I miss him very much because he was my hero offering the support always to make everything possible and achievable.

Acknowledgements

The completion of my degree has been indeed a long journey. I would like to gratefully and sincerely thank my academic supervisors Professor Ian Givens and Dr Sandrine Claus for their constant advice and support throughout these years. I am also thankful to Professor Glenn Gibson and Professor Julie Lovegrove, for advice and support in the intervention study. I would also like to thank Dr Kim Jackson in her support in the intervention study, lab, statistical analysis and feedback. I would also like to thank Dr Giulia Mancano for her support in NMR analysis.

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Last, but not least, I would like to thank my God for support and succeed; my small family; my husband Saleh and my sons Turki and Faeasl, for their patience and for respecting and supporting my decision; and my big family brothers, sisters and specially my mother Salma for taking care of my kids for three years. I am also very grateful to my brother Hamdan for his decision to support me looking after my kids in the UK before they went back home.

Abstract

Consumption of A2 milk is often believed to have a wide range of health benefits, however clinical data regarding effects on gut inflammation, gastrointestinal symptoms and function, blood lipids, body composition, glucose metabolism and blood pressure is either conflicting or limited. There is also a lack of evidence on the prebiotic effect of bovine milk and on the effects on health of the bovine BCM-5 and 7 peptides released from A1 β-casein.

The work described in this thesis combined an *in vitro* investigation comparing the effect of digested bovine milk containing A1/A2 with that containing only A2 β casein variants and their peptides on gut microbiota fermentation properties as well as a human intervention study comparing milk containing both A1 and A2 β casein variants with milk containing only the A2 β casein variant on gut inflammation and risk markers of cardiometabolic disease. In the *in vitro* studies, the milks were digested and then the digested milk and BCM-5 and 7 peptides were fermented in anaerobic pH-controlled faecal batch cultures and bacterial concentration and diversity and short chain fatty acids were analysed. In the human study, regular consumers of milk who experienced gastrointestinal symptoms after drinking milk not due to lactose participated in a double blind randomised crossover study for a 10 week period started and separated with 2 weeks washout. Commitment to the dietary regimes was achieved by specific dietary advice.

In the *in vitro* fermentation study, BCM-7 was released only from milk containing both β casein variants A1/A2 during the enzymatic digestion and both digested milks altered bacterial diversity similar to FOS (prebiotic) and this was seen in the increase of total bacteria and bifidobacteria, while BCM peptides behaved similarly to negative control that did not contain any treatment. Both milks resulted in a greater increase than BCM peptides in SCFA propionate, butyrate and acetate. In the human study, milk with only A2 β casein significantly decreased C-reactive protein and increased *Actinobacteria* in faeces, stool frequency and haemoglobin relative to the A1/A2 milk. A2 milk had a tendency to lower bloating and abdominal cramps whilst A1/A2 milk significantly decreased serum high-density lipoprotein cholesterol (HDL-

C), glucose, diastolic blood pressure (DBP) and heart rate. However, there were no effects on local gut inflammation, other blood lipids and body composition.

This study demonstrates that milks behave similar to prebiotic *in vitro* and this could be influenced by the type of oligosaccharides that alter the microbiota composition. In addition, consumption of milk had no pro-inflammatory effects on the human gut, but A2 milk may improve or eliminate gastrointestinal symptoms associated with milk intolerance.

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Abbreviations

ACE	Angiotensin converting enzyme
Ang	Angiotensin
AOAC	Association of Official Analytical Chemists
ApoB100	Apolipoprotein B100
AQ	Acquisition
ASV	Amplicon sequence variant
AUC	Area under the curve
BCFA	Branched chain fatty acid
β CM	β-casomorphin
β CM-5	β-casomorphin-5
β CM-7	β-casomorphin-7
BM	Bovine milk
BMI	Body mass index
BMRB	Biological magnetic resonance data bank
BP	Blood pressure
CD	Crohn's disease
CFC	Corrected faecal calprotectin
CH_4	Methane
CNS	Central nervous system
CPP	Caseinophosphopeptide
CRP	C-reactive protein
CVD	Cardiovascular diseases
DBP	Diastolic blood pressure
DIGI	Dietary intervention & gastrointestinal function

DPP-4	Dipeptidyl peptidase-4
DPPH	Diphenyl-picrylhydrazyl
DRI	Dietary reference intake
EFSA	European Food Safety Authority
EPIC	European prospective investigation into cancer and nutrition
FC	Faecal calprotectin
FISH	Fluorescence in situ hybridisation
FOS	Fructo-oligosaccharide
GALT	Gut associated lymphoid tissue
GID	Gastrointestinal digestion
GIP	Glucose –dependent insulinotropic polypeptide
GLP-1	Glucagon like peptide-1
GOS	Galacto-oligosaccharide
H NMR	High resolution nucleated magnetic resonance
H_2	Hydrogen
HDL-C	High-density lipoprotein –cholesterol
HM	Human milk
HMO	Human milk oligosaccharide
HPLC	High pressure liquid chromatography
HR	High resolution
HR	Heart rate
HS-CRP	High sensitive C-reactive protein
IAUC	Incremental area under the curve
IBD	Inflammatory bowel disease
IHD	Ischaemic heart disease

IL	Interleukin
IL-10	Interleukin-10
IL-6	Interleukin-6
ISAPP	International Scientific Association of Probiotics and Prebiotics
LAP	Leucine aminopeptidase
LI	Lactose intolerance
LMT	Letter memory task
LPS	Lipopolysaccharide
LTT	Lactose tolerance test
MANT	Modified attention network task
MO	Milk oligosaccharide
MPO	Myeloperoxidase
MS	Mass spectrometry
OTU	Operational taxonomic unit
PANAS	Positive and negative affect schedule
PCA	Principal component analysis
PCoA	Principle coordinate analysis
PFA	Paraformaldehyde
PP	Pulse pressure
PPM	Parts per million
QIIME	Quantitative insight into microbial ecology
CCT	Controlled clinical trial
RD	Relaxation delay
SBP	Systolic blood pressure
SCFA	Short chain fatty acid

SCIT	Subtle cognitive impairment test
SEM	Standard error of the mean
SFA	Saturated fatty acid
SFDA	Saudi Food and Drug Authority
SGID	Simulated gastrointestinal digestion
SIBO	Small intestinal bacterial overgrowth
SNPS	Single nucleotide polymorphism
T1DM	Type 1 diabetes mellitus
TAG	Triacylglycerol
TC	Total cholesterol
TE	Total energy
TFA	Trans fatty acid
TNF	Tumour necrosis factor
TSP	Trimethylsilylpropanoic
UPLC	Ultra performance liquid chromatography
UV	Ultra violet
UV	Unit variance
VLDL	Very low-density lipoprotein
WHO	World Health Organisation
βC	β-casein

Chapter 1

Introduction

1.1 Milk composition

Milk is a natural source of nutrition since it contains proteins that play a crucial role in human growth, and provides essential amino acids that are used for maintenance of cell and tissues as well as growth. The composition of bovine milk varies among breeds and individual cattle and this is due to numbers of factors that significantly influence milk production and value (Park, 2013). It has been reported that the compositional content of total bovine milk includes per 100g, 3.6g protein, (of which 80% is casein, 20% is whey protein), 3.2g fat, 4.9g lactose, 0.12g calcium, 119-124mg potassium, 151-166mg chloride, sodium and biologically active compounds such as essential amino acids (leucine, isoleucine, valine, lysine, histidine, methionine and phenylalanine) (Looper, 2012, Pereira, 2014).

Woodford (2007) reported that 1 litre of bovine milk consists of the fractions shown in Figure 1. These compounds may have a beneficial impact on health and metabolism since milk has been associated with antihypertensive, antidiabetic, anticarcinogenic, anticholesterolaemic, antiobesity, prebiotic, probiotic, and immunomodulatory effects (Meisel, 1998, Park, 2013, Fox, 2003).

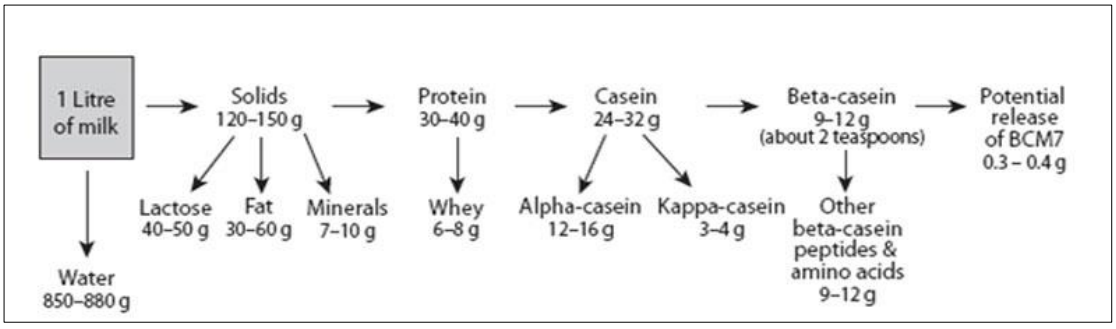

Figure 1.1. Contents of 1 litre of milk (Woodford, 2007).

According to the Canadian Dairy Information Centre in November 2015, the largest cow's milk producer worldwide is the United States accounting for 14.6% followed by India 8.7% in the last five years (Table 1). The United Kingdom is the 10th largest producer at 2.2%.

Table 1,1. Top 10 countries producing cow's milk in 2010-2014 (' 000 metric tonnes) ranked by production in 2015

Countries	2010	2011	2012	2013	2014
United States	87,474	88,978	90,962	91,277	93,461
India	54,903	57,387	59,805	61,258	64,750
China	35,756	36,560	37,436	35,310	37,246
Brazil	31,637	33,054	33,274	35,283	37,082
Germany	29,630	30,336	30,506	31,326	32,395
Russia	31,847	31,646	31,501	30,286	30,800
France	24,010	25,070	24,698	24,441	25,759
New Zealand	17,169	18,966	20,572	20,202	21,898
Turkey	12,419	13,802	15,978	16,655	16,867
United Kingdom	13,852	14,071	13,843	13,935	15,084

Although there are many breeds of milk-producing cattle globally, in the UK the most commonly used breed is the black and white Holstein-Friesian breed, followed by small numbers of other breeds including Jersey, Guernsey and Ayrshire (Park, 2013).

Milk proteins have been studied for around two hundred years (Fox, 2003). Bovine casein proteins consist of three major milk proteins including α- casein, κ- casein and β- casein. The casein protein composition largely determines the nutritional value of milk. These casein proteins are bound together in the casein micelle electrostatically with calcium phosphate (Figure2) (Dalgleish and Corredig, 2012).

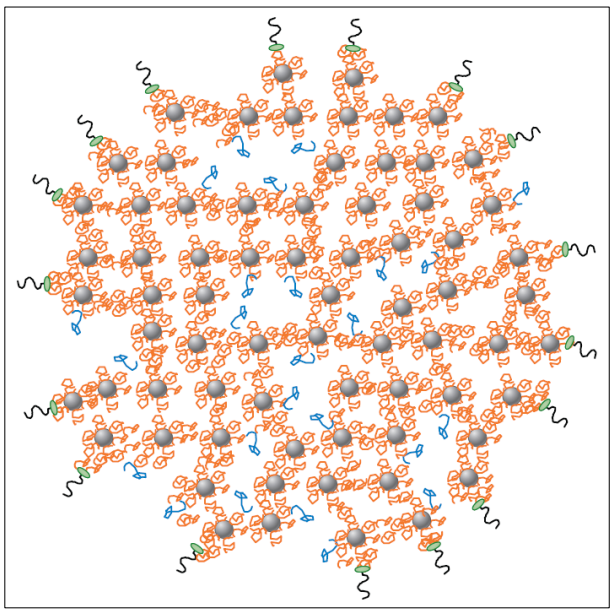

Figure 1.2. Representation of casein micelle structure. α- casein and β- casein (orange) interact with calcium phosphate (grey). Some other β- casein (blue) interacts with other casein by hydrophobic interaction in the same system. κ- casein is located on the surface (green) (Adapted from (Dalgleish and Corredig, 2012).

According to a review of milk proteins (Farrell Jr et al., 2004), α-casein is a multimer of αs1-CN (33.1% of casein proteins) with four αs2-CN molecules (8.2% of casein proteins). There are eight different variants of αs1-CN (A, B, C, D, E, F, G, and H) and four different variants of αs2-CN (A, B, C, and D). Both of these α-caseins play different roles in food production and health. The α-caseins have a critical role in the transport of calcium phosphate, and produce the antioxidant peptide 2, 2-diphenyl-1-picrylhydrazyl (DPPH) that helps reduce free-radical activity. κ- casein represents 9.1% of casein proteins whereas β- casein represents 32.3% (Farrell Jr et al., 2004, Pal et al., 2011, Kriščiunaite et al., 2012).

The protein in milk produced by British cows is comprised of about 20% whey proteins and 80% is casein, of which 36% is β casein. Within the β casein, there are several protein variants that are genetically different, including A1, A2, B and C. In the UK, the most dominant proteins variants in cow's milk are A1 and A2, the

proportion of A2 variant (58%) being higher than that of A1 (31%) in previous studies (Givens et al., 2013). The ratio of A1/A2 beta-casein depends on cow breed as reported by Kaminski et al. (2007) who observed that the A2 variant was the most frequent in milk from Guernsey and Jersey cattle, whereas a high frequency of A1 has been observed in Holstein-Friesian, Ayrshire, and Red cattle (Kamiński et al., 2007).

It is important to note that dairy cows can be either homozygous (A2/A2 or A1/A1) or heterozygous (A1/A2) genotypes. Most UK milk contains a mixture of A1/A2 protein variants, presumably as a result of most dairy cows being heterozygous genotypes. However, commercial A2 milk contains only the A2 variant produced from animals selectively bred to be A2/A2 homozygotes. A2 is recognized as being the original or ancestor β casein gene in dairy cattle (Givens et al., 2013). A1 developed following a genetic mutation in some European dairy herds several thousands of years ago. The A1 and A2 type proteins differ by only one amino acid, as discussed in the next section.

1.2 Bioactive peptides derived from beta-casein

Bioactive peptides can be described as food-derived bioactive components that play physiological roles in the body (Hajirostamloo, 2010). These peptides have been of interest since 1950, when Millander found that vitamin D-independent calcification was enhanced by casein-derived phosphorylated peptides in rachitic infants (Gobbetti et al., 2004). Such bioactive peptides can range in length from 2 to 50 amino acids and significantly influence the activity of other peptides and exert multiple functions in health, as can be seen in Figure 3 (Park, 2009).

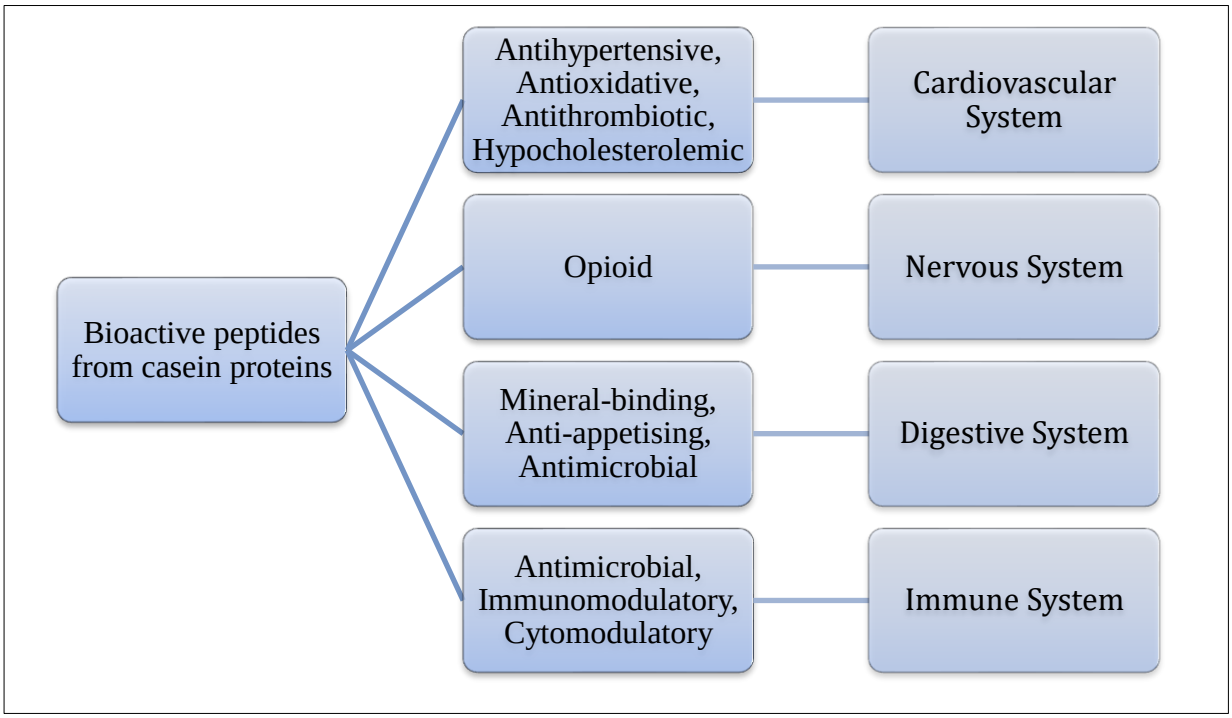

Figure 1.3. Physiological functions of casein derived bioactive peptides. (Adapted from Park, 2009)

Opioid peptides may form from various food proteins, including those found in milk, cereals, vegetables, meat, and eggs. However, milk proteins are considered the most abundant source of bioactive peptides. In human and bovine milk these include β-casomorphins (BCMs) notably BCM4, BCM5, BCM7 and BCM8. It is found that human BCMs have 30 times less affinity for opiate receptors than bovine BCMs (Shahidi and Zhong, 2008, De Noni et al., 2009).

These opioid peptides can be classified in two different categories. One opioid peptide, which is known as the 'endogenous opioid peptide' is characterised by a Tyr-Gly-Gly-Phe peptide in its terminal sequence. The other opioid peptide, known as the 'exogenous opioid peptide', is characterised by a Tyr residue at the N-terminal region (Teschemacher, 2003). These exogenous peptides can be released or identified by

enzymatic action during, for example, gastrointestinal digestion (GID) of their parent protein molecules (Meisel, 1998). The majority of milk proteins can release opioid receptor ligands that usually differ from each other. The α-caseins contain exorphin and casoxin D ligands, whereas β-casein contains β-casomorphins and β-casorphins ligands, and κ-casein contains casoxins opioid receptor ligands. Both the α- and β-caseins act as agonists, whereas casoxins act as antagonists (Meisel, 1998, Teschemacher et al., 1997).

Genetic variation plays a significant role in the primary sequence of all food proteins, including those in milk and will considerably influence the types of bioactive peptides that are released from milk proteins. Interestingly, β-casein A1 and B differ from β-casein A2 at amino acid position 67. The residue present in position 67 in β-casein A1 is histidine, whereas in β-casein A2 the amino acid in this position is proline. This amino acid difference has been reported to prevent the enzymatic hydrolysis of A2 beta-casein and prevent the release of the peptide β-casomorphin-7 (BCM-7) (De Noni et al., 2009, Ul Haq et al., 2015), which potentially has health implications that will be discussed later.

1.3 Release of proinflammatory peptide BCMs in milk/dairy foods during in vitro simulated gastrointestinal digestion

There are several reports that BCM peptides form during food processing, or after enzymatic digestion of milk. Peptides including BCM can be identified, separated and quantified using techniques such as High Pressure Liquid Chromatography (HPLC) and tandem mass spectrometry (MS/MS) (De Noni, 2008, De Noni and Cattaneo, 2010) and Ultra Performance Liquid Chromatography coupled to High Resolution

Mass Spectrometry (UPLC/HR-MS) (De Noni et al., 2015). The release of BCMs during simulated gastrointestinal digestion (SGID) has been investigated in different studies. The digestion protocol can vary for gastric pH values and gastric emptying rates, especially to reflect digestion in adults or children as will be described later. According to Jinsmaa et al. (1999), pepsin plays a significant role initially in the system of hydrolysis of β-casein, as shown in Figure 4 below (Jinsmaa and Yoshikawa, 1999).

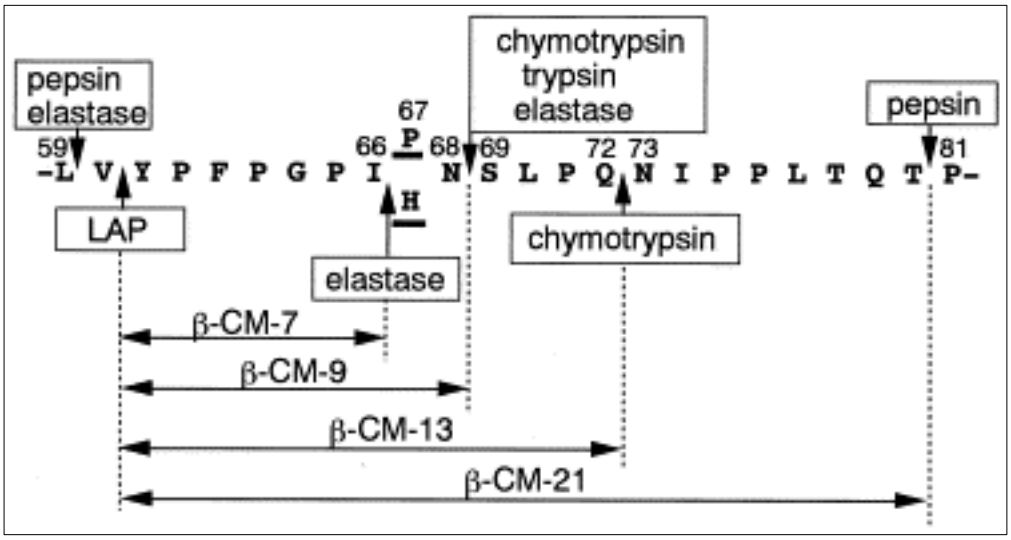

Figure 1.4. Cleavage of β-casein into BCM peptides by the action of various proteases, reproduced from Jinsma et al. (1999)

Pepsin cleaves the bond between Leu[58] and Val[59], whereas leucine aminopeptidase (LAP) removes Val from the amino terminus to form BCM.

BCM-3 can be detected in a pepsin hydrolysate using second-order derivative spectra and UV-spectra (Macaud et al., 1999), whereas BCM-7 could not. Using LC-MS, (Schmelzer et al., 2007) found that BCM-7 and BCM-3 from A1 and A2 variants also did not form after about 10-60 min of pepsin digestion at pH 2.0. However, BCM-7 was detected using HPLC/UV in milk from both homozygous A1 and A2 variants 24

hours after digestion with pepsin at pH 2.0. The yield of BCM-7 was four times higher in milk from homozygous A1 compared with homozygous A2 cows (11.9 mg/g and 2.87 mg/g respectively) (Cieslinska et al., 2007). However, these data could be biased because the incubation time in pepsin was long compared with what is generally used in other SGID studies, which ranges between 90-180min depending on the stage of the digestion. Moreover, using HPLC (High-Performance Liquid Chromatography) alone for identification and quantification of BCM-7 rather than a combination of HPLC, MS-MS, and ELISA recognised many different peptides with the same retention time due to similar hydrophobicity.

BCM-5 and BCM-7 formation was investigated using LC-MS/MS after SGID of milk from A1, A2, and B variants by pepsin digestion at three different pH values 2.0, 3.0, and 4.0, followed by hydrolysis with corolase (De Noni, 2008). No BCM-7 was released from these three variants upon initial peptic digestion. However, the largest amount of BCM-7 was detected from the B variant, followed by the A1 variant (5–176 mmol/mol, 0.2–0.5 mmol/ mol casein respectively). BCM-7 was not released at any stage of digestion from the A2 variant. Recently, BCM-5 and BCM-7 formation was also investigated during simulated gastrointestinal digestion of milk β-casein from A1A1, A1A2 and A2A2 variants from Indian crossbred cattle. The milk from these variants was hydrolysed with enzymes including pepsin, trypsin, chymotrypsin, and pancreatin. Detection of BCM-5 and BCM-7 was carried out by HPLC, MS-MS and competitive ELISA, which showed greater release of BCM-7 in milk from homozygous A1 compared with heterozygous A1A2. Consistent with previous studies, neither BCM-5 nor BCM-7 were detected in milk from the homozygous A2 variant (Ul Haq et al., 2015). A recent study (Asledottir et al., 2018) examined, ex

vivo, the release of BCM-7 from bovine milk containing only A1 or A2 β-casein (not a mixture of both) using human proteases notably pepsin and pancreatin and the digestion carried from 60-120 min depending on the stage of the digestion. They found that BCM-7 with the same amino acid sequence YPFPGPI was released after digestion of both variants. However, large differences were observed in the amount of BCM-7 between A1 variant and A2. A1 milk released higher amount of BCM-7 (1.85 - 3.28 /g β casein digested) compared with A2 milk (0.01 – 0.06 mg /g β casein digested). This study showed some limitation starting with the level of BCM-7 that was released from A1 milk. This level was lower compared to previous reported data from digested A1 milk. Another limitation could be degradation through the digestion because the early release of BCM-7 in duodenal phase of the digestion (Asledottir et al., 2018).

Most milk-based infant formulae are comprised of a combination of A1 and A2 β-casein variants. According to de Noni et al. (2008), BCM-5 was not detected after SGID in any milk-based infant formula samples, whereas BCM-7 was found with the highest values at pH 3.0 and the next highest at pH 4.0. De Noni et al. (2008) tested the release of BCM-7 from infant formula digested at different pH values which ranged from 2.0 to 4.0 and the reason for this was to take in to account the reported stomach pH value for infant (3-5). The lowest amounts of BCM-7 were found in milk-based infant formulae in which whey protein was the main protein ingredient (De Noni, 2008, De Noni and Cattaneo, 2010).

1.4 Biological studies of the impacts of peptides derived from the A1 β-casein variant versus the A2 β-casein variant

β-casomorphins in general have been studied over the last few years *in vitro* and *in vivo*. These food-derived peptides can target different tissues and organs including the GI, central nervous (CNS), and cardiovascular systems following absorption. For example, an animal study conducted in 2013 suggested that the administration of bovine BCM7 would alleviate high glucose-induced renal oxidative stress *in vivo* and *in vitro* (Zhang et al., 2013). More detailed examples are discussed below.

1.4.1 Effects of β-casein A1 on T1 Diabetes Mellitus (T1DM)

It is well known that T1DM is a multifactorial and autoimmune disease due to the destruction of pancreatic β-cells that play a role in the secretion of insulin by infiltration of T-lymphocytes and macrophages leading to insulin deficiency. Research found that in T1D subjects, A1 β-casein might affect the autoimmune reaction involved in the destruction of β–cells because research found increased antibody production in T1D against β-casein (ul Haq et al., 2014). An early animal study claimed that A1 β-casein has a diabetogenic characteristic for diabetic mice that are not obese compared with A2 β-casein. (Elliot et al., 1997). A recent, 30 week, animal study looked at whether A1 β-casein increases the incidence of T1D. Authors supplemented non- obese diabetic mice (from F0 generation to F4 generation) with A1/A1 or A2/A2 β-casein containing diets with free access to water. This study found similar diabetes incidence in generation F0-F2 in both mice groups receiving A1/A1 or A2/A2. However, the incidence of T1D was doubled in generation F3 mice that were fed the A1 β-casein diet. Also, the research group confirmed the presence of

subclinical insulitis that developed in 10-weeks old F4 female mice suggesting that T1D may be affected by epigenetic phenomenon due to the fact that diet and microbiota may affect the expression of insulin resistance and insulin signaling gene. The study concluded that A1 β-casein may affect glucose homeostasis and T1D progression that appear later in life (Chia et al., 2018).

Epidemiological studies have linked β-casein A1 with T1D in children. According to Elliott et al. (1999), there was no correlation (r = 0.402) between total milk protein consumption and T1DM incidence in data collected from 10 countries for children from 0 to 14 years old. However, there was a relationship (r = 0.726) between the incidence of T1DM and consumption of milk containing the β-casein A1 variant (Elliott et al., 1999). Similar results were also observed from epidemiological studies (based on supply of milk per capita) by McLachlan and Laugesen (2001). McLanchlan reported that β-casein A1 was strongly correlated with T1D incidence in 0-4 years olds which suggested that β-casein A1 shared one causative risk factor with ischaemic heart diseases as discussed later. In addition, Laugesen and colleagues (2003) confirmed the finding of Elliot et al. (1999) that A1 β-casein from milk and cream correlated significantly with T1D, but correlation was not significant with A2 β-casein (McLachlan, 2001, Laugesen and Elliott, 2003). Despite a number of reports demonstrating positive correlations between the risk of T1D and dairy consumption, the New Zealand Food Safety Authority (2004) and European Food Safety Authority (EFSA) (2009) concluded that the scientific evidence failed to find a cause-effect relationship between the consumption of milk and its bioactive peptide and T1D (Swinburn, 2004, De Noni et al., 2009)

1.4.2 Effects of β-casein A1 on cardiovascular disease

A strong correlation has been suggested between consumption of β-casein A1 and ischaemic heart diseases (IHD) using data pooled from several populations (McLachlan, 2001). McLachlan (2001) suggested a strong correlation between ischaemic heart disease mortality and the estimated intake of β-casein A1 (per capita) in 16 countries (Europe, USA, Israel and Japan). This study was based on correlation between the consumption of milk protein and mortality rate from IHD in men aged 30-69 y (McLachlan, 2001). (Laugesen and Elliott, 2003) later found similar results when they examined the association between the intake per capita of the β-casein A1in 1990 with the mortality rate in men and women in 1995. These authors concluded that there was a significant positive correlation between bovine β-casein A1 with IHD mortality from 20 countries and the correlation was stronger for male than female mortality. An animal intervention study was conducted by Tailford et al. (2003) to investigate the direct effect of β-casein A1 vs β-casein A2 consumption on atherosclerosis development using a rabbit model. Sixty rabbits had their right arterial endothelium injured using a balloon catheter placed into the carotid artery at baseline before dividing them randomly to 10 groups (n = 6/group). They were then fed a diet for six weeks containing 0, 5, 10 or 25 g/100g of casein isolate (either β-casein A1or A2) made up to 20 g/100g milk protein with whey. Group one had diet containing 20% whey protein, group two and three had diet containing 10% β-casein A1or A2 and 10% whey, the three groups had no added dietary cholesterol. The diet of the remaining seven groups were supplemented with 0.5 g/100g dietary cholesterol and 20 g/100g milk protein that only differed in the proportions of whey and casein. Atherosclerosis was assessed by taking blood samples at baseline and after 3 and 6 weeks whereas aortic fatty streak was assessed at week 6. The authors reported that in

the absence of cholesterol supplementation, β-casein A1 fed animals had significant higher serum cholesterol, LDL, HDL and triglyceride levels compared with whey alone fed animals and this higher level was also higher than β-casein A2 fed animals. In addition, the thickness of the fatty streak lesion in the aorta was higher significantly in β-casein A1 fed animals as well as in the animal groups with their diet supplemented with 0.5 g/100g cholesterol and 5, 10 and 20 g/100g β-casein A1. Therefore it was concluded that β-casein A1 had an atherogenic effect (Tailford et al., 2003). The outcome was due to the high proportion of the surface area of the aorta covered by fatty streaks and the thickness of these fatty streaks was also significantly higher in rabbits fed β-casein A1 compared with rabbits fed with β-casein A2. Usually, fatty streaks are an accumulation of foam cells that is known as the first sign of plaque development or atherosclerosis. In normal atherosclerosis the foam cells develop from macrophages that swallow oxidised LDL (oxLDL). However once the vessel wall is damaged, oxidative stress formation follows which in turn induces oxLDL. OxLDL results in low grade chronic inflammation in the vessel wall that leads to the expression of pro-inflammatory cytokines such as IL-1 and IL-6 and other immune molecules (Baker et al., 2018).

Another explanation for the effect was that β -casomorphin-7 released from β-casein A1 is involved in LDL oxidation during a peroxidase-dependent process (De Noni et al., 2009). LDL is a lipid carrier in plasma that is derived in the circulation from very low density lipoprotein VLDL. LDL consists of cholesterol ester, phospholipids, triglyceride, free cholesterol and apolipoproteinB100 (ApoB100) (Matsuura et al., 2008). It is involved in the transport of cholesterol to peripheral tissues and the regulation of cholesterol metabolism at these tissues. Oxidised LDL is also known to

be immunogenic and the release of antibodies against oxidised LDL is considered as a marker of CVD (Zebrack and Anderson, 2002).

However, the first human trial that examined dietary supplementation of β-casein A1 and A2 in a randomised crossover trial found no significant effect of consumption of β-casein A1 compared with β-casein A2 on plasma cholesterol concentrations of LDL cholesterol, HDL cholesterol and triacylglycerols (Venn et al., 2006). Similarly, a double-blind crossover human study also found no evidence of any disadvantage associated with consumption of A1 β-casein compared to A2 β-casein on cardiovascular health risk evaluated using total plasma cholesterol, LDL cholesterol, HDL cholesterol, triacylglycerols, plasma insulin, homocysteine, C-reactive protein, fibrinogen and resting blood pressure (Chin-Dusting et al., 2006). It has been suggested that the decrease of CVD risk biomarker levels measured in observational studies comparing milk consumption with CVD may be due to confounding factors driven by genetic differences, physical activity and uncontrolled changes introduced to the diets of the participants.

1.4.3 Effects of β-casein A1 on gastrointestinal health and inflammation

Two animal studies have investigated the effects of A1 versus A2 β-casein on the gastrointestinal tract directly. Barnett et al. (2014) showed that feeding rodents milk containing A1 β-casein resulted in significantly delayed gastrointestinal transit time compared with milk containing A2 β-casein. This delay was eliminated by administration of the opioid receptor blocker naloxone, suggesting that the gastrointestinal transit delay with A1 feeding is an opioid-mediated effect. They also demonstrated a significant 40% upregulation of dipeptidyl peptidase-4 (DPP-4) in the jejunum of A1- relative to A2-fed rodents, thereby increasing localised inflammation

effects in intestinal tract or systemically after BCM-7 absorption and distribution. This study suggested that upregulation was a response to BCM-7 released by A1 β-casein. This means that the effect of A1 β-casein on DPP-4 was independent of opioid receptors. However, this suggestion was not based on scientific evidence because the author stated that they had no information on the normal level of DPP-4 in the rat jejunum. It is well known that DPP-4 is involved in hormone regulation such as glucagon like peptide-1 (GLP-1) that plays a role in insulin secretion and glucose metabolism (Barnett et al., 2014). Based on these animal studies and previous mentioned quantification studies by (De Noni, 2008). It is suggested that little information is available in the literature concerning the minimum amount that reach the colon and can exert physiological effects in vivo or ex vivo. However De Noni reported that 0.05% of BCM-7 may be sufficient to cause intestinal contraction suggesting that lower amount of BCM-7 (200-4000 fold) may reach the infant gut after the digestion of 800ml of the infant formula.

Similarly, Haq et al. (2014) showed in mice fed a milk-free basal diet supplemented with β-casein isolated from milk A1/A1 or A1/A2 relative to β-casein isolated from milk A2/A2 that myeloperoxidase (MPO) levels were increased significantly by 204%, whereas A2 β-casein had no effect relative to control group fed only basal diet. Further, they showed significant increases in intestinal interleukin-4, immunoglobulin E and leukocyte infiltration, from murine small intestine (ileum), with A1 compared with A2 feeding. Consequently, the gut inflammatory response was induced by A1 β-casein variant through activating Th_2 pathways (Haq et al., 2014b). The same research group found similar results that indicate a significant increase in inflammatory

biomarkers (indicated above) in male Swiss-albino mice that received orally for 15 days (7.5 x $^{10-8}$ mol/day/animal) chemically synthesized BCM-5, BCM-7 (peptides) compared with control group who received a saline diet. This amount of peptide used was calculated based on two previous studies, one done by the same author (Haq et al., 2014b) where β-casein was fed according to the dose –translation formula (human/mouse) and De Noni (2008) work in the release of BCM-7 from A1 β-casein (Haq et al., 2014a, Barnett et al., 2014, Haq et al., 2014b).

A recent blinded randomized cross-over pilot human study (Ho et al., 2014) found that consumption of A1 β-casein milk led to significantly higher stool consistency values (Bristol Stool Scale) and a significant positive association between abdominal pain and stool consistency compared with A2 β-casein milk. Furthermore, some individuals may be susceptible to gut inflammation induced by A1 β-casein, as evidenced by higher faecal calprotectin values and associated intolerance measures.

Another recent study (Jianqin et al., 2016) concluded that subjects who consumed A1 β-casein milk had significantly high stool consistency values, a significant positive association between abdominal pain and stool consistency, delay in transit time, elevated inflammation-related biomarkers and immune response compared with subjects who consumed A2 β-casein milk. In addition, they also found that consumption of milk containing only A2 β-casein did not aggravate post-dairy digestive discomfort symptoms relative to baseline (i.e., after washout of dairy products) in lactose tolerant and intolerant subjects. However, consumption of milk containing both β-casein types was associated with worsening of post-dairy digestive discomfort symptoms relative to baseline in lactose tolerant and lactose intolerant

subjects. These findings suggest that gastrointestinal symptoms associated with milk in lactose intolerant subjects may in part be related to A1 β-casein rather than lactose itself (Jianqin et al., 2016). A recent randomised crossover double blind study with 600 male and female Chinese subjects aged 20-50 y with self-reported lactose intolerance compared the effect of consuming A1/A2 β- casein with A2/A2 β- casein on gastrointestinal function. Participants were asked to stop consumption of all dairy products 3 days before the start of the study and on the day 1 of the study participants provided a urine sample for urinary galactose (baseline) and then consumed 300ml of milk containing A1 and A2 β- casein or milk with only A2 β- casein after overnight fasting. One hour later they had breakfast and then completed a visual analogue scale for gastrointestinal symptoms. This was also done after 3h and 12h (by phone not for all participants) from milk intake as well as urine samples at 3h for urinary galactose. After this day, participants had a washout period of 7 days and then returned on day 8 for a study visit and consumption of the second milk in the same way. The study concluded that consuming 300 ml/d of conventional milk containing A1 β- casein decreased lactase activity and increased gastrointestinal symptoms than milk containing A2 β-casein (He et al., 2017).

It is clearly seen from the previous section the formation and release of BCM-7 from A1/A2 milk. BCM-7 and 5 are known as the most active BCMs which is belong to the fragment f60-66 and f60-64 of bovine B-casein, respectively, both of them has the same biological activity which is characterised by high content of proline, hydrophobic character and the presence of tyrosine on the N-terminus, required for interaction with μ-opioid receptors present in the gut. The intestine known as the first site where opioid peptides exert their physiological functions through opioid receptors. In vitro research indicated that the transport activity of BCM-7 was

increased across Caco-2 cell lines (intestinal epithelial cell) with glucose and calcium ions that involves in the pathogenesis of inflammation and food allergy in infants. In vivo animal trial (Haq et al., 2014) demonstrated the mechanism where BCM-7 and 5 may induce the inflammatory immune response throughout Th2 pathway by increasing level of inflammatory molecules including MPO, MCP-1,IL-4 and histamine in intestinal fluid and leucocyte infiltration in villi. This mechanism of action were confirmed the results of the second animal trial that tested the direct effect of isolated A1/A2 bate casein relative to A2 beta casein on murine gut inflammation. It was reported that BCM-7 in nano and picomolar concentrations resulted in formation of IL-8 from peripheral blood mononuclear cells and high production of IL-4.

1.5 Gut immune system

The gut immune system plays a critical role in the distinction between harmful and harmless substances that escape into the system. The intestinal epithelial membrane is a part from this system that provides a physical and chemical barrier protecting the human body from substances released by pathogens and environmental xenobiotics. Epithelial cells, through their tight junctions, also play a crucial role as a physical barrier to prevent pathogen invasion (Figure 6). Mucus-producing cells located in the epithelial layer are also involved in the secretion of antimicrobial peptides and lysozyme, which contribute to regulation of gut microbiota proliferation and decrease survival of pathogenic microbes (Parkin and Cohen, 2001). The largest adaptive immune tissue in the human body is the gut-associated lymphoid tissue (GALT), which is found along the basal surface of the mucosal membrane. It is involved in the regulation of the immune response by a number of physiological components not only immune cells that are located in Peyer’s patches, lymph nodes and lymph follicles,

but also single immune cells that are scattered throughout intestinal epithelium and lamina propria.

Cytokines are small protein molecules produced from a number of immune cells. Functionally, they can control cell proliferation and differentiation and regulate the behaviour of other target cells. Interleukins (IL), interferons and chemokines are examples of cytokines. Interleukins particularly affect leukocytes (Parkin and Cohen, 2001). Cytokines can be either anti-inflammatory or pro-inflammatory, depending on their role in infection or inflammation. Pro-inflammatory cytokines clearly promote inflammation such as IL-1, IL-6 and tumour necrosis factor (TNF). Anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 suppress the activity of pro-inflammatory cytokines but pro-inflammatory cytokines are not usually not produced in healthy persons and the balance between the effect of pro and anti-inflammatory cytokines determine the outcome of diseases. An example of this is IL-10, low IL-10 gene expression in mice results in the development of inflammatory bowel disease (IBD) (Dinarello, 2000).

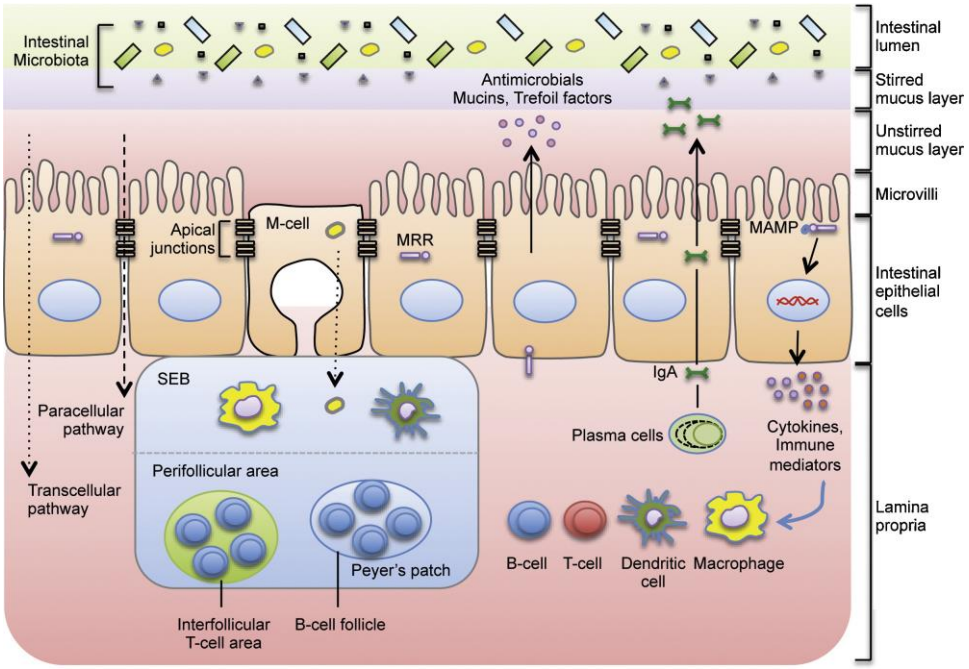

Figure 1.6. Intestinal homeostasis modulated by intestinal barrier. (Adapted from (Natividad and Verdu, 2013).

Distinguishing between local and systemic inflammation is very important to control disease. IL- 6 (pro-inflammatory cytokine) and IL-10 (anti- inflammatory cytokine) are cytokines produced at the local tissue level and released into the circulation during acute infection. In Crohn's disease (CD), active inflammation is associated with an acute phase reaction that leads to migration of leukocytes to the gut. Therefore, measuring proteins in faeces and serum is a reliable method to identify inflammation (Schoepfer et al., 2010). During the assessment of intestinal inflammation, the accuracy of diagnosis can be increased by measuring the combination of stool inflammation markers such as faecal calprotectin with serum CRP (Langhorst et al., 2008).

Calprotectin is known as local intestine inflammation marker. Calprotectin is a calcium binding protein predominantly found in neutrophils and to a lesser extent

from monocytes and reactive macrophages (Konikoff and Denson, 2006). Increased gut inflammation characterised by a high level of neutrophil infiltration that is released into the lumen and then passed in faeces where calprotectin can be measured (Banerjee et al., 2015). Calprotectin is found in plasma and stool, and the concentration in stool is higher (~6 times) than in normal plasma. High levels of calprotectin (> 50µg/g) have been detected in faecal samples from patients with bowel inflammation. The suggested cut off value for normal faecal calprotectin (FC) is 50µg/g. Calprotectin is stable in faeces in the presence of calcium and is resistance to proteolytic degradation. Samples can be kept at room temperature for up to 5 days. Spot sampling of stool for calprotectin analysis is as reliable as 24 h collections with an excellent correlation (r= 0.90). It has been reported that FC concentration increases with age in adults (Konikoff and Denson, 2006). Literature indicates that diet may play a role in increased FC concentration. It has been reported that breast fed infants had lower FC concentration than formula fed counterparts (Banerjee et al., 2015).

A strong positive correlation between FC and faecal excretion of neutrophils has been observed in patients with IBD. This means that an increased level of FC concentration in IBD patient results from increased level of neutrophils released into the gut lumen across inflamed mucosa. Therefore, faecal calprotectin is a marker of neutrophilic intestinal inflammation and a negative faecal calprotectin result should not be interpreted as reflecting a healthy intestine but of the absence of significant neutrophilic intestinal inflammation (Konikoff and Denson, 2006). One of the systemic inflammatory markers is high sensitive C- reactive protein (hs-CRP). This CRP is normally produced as a result of stimulation by pro-inflammatory cytokines such as IL-1 and IL-6. Studies have reported an association between CRP and IBD

(Schoepfer et al., 2010). In addition, hs-CRP is known to be the best validated and standardised marker for cardiovascular risk assessment (Zebrack and Anderson, 2002) with evidence of a strong relationship between CRP with the prevalence of CVD (Pihl et al., 2003).

1.6 Introduction to the gut microbiota

It is evident that the human colonic microbiota is a complex and large microbial community. According to Rowland et al. (2017), over 1000 bacterial species have been described in the human gut, but there are many more which are unknown. Approximately 200 bacterial species are found in a single individual's gut. The large intestine, part of the digestive system that plays an important role in physiological function introduced later in this section, consists of approximately 10^{13} bacterial cells. One of the metabolic functions of this large bacterial population is to complement mammalian enzymes that are unable to digest complex fibres. Another crucial role is the contribution of proteins that are not encoded in the human genome and provide essential vitamins (Rowland et al., 2017). Hence, the gut microbiota significantly contributes to an individual's energy and micronutrient intake.

It is worthy of note that the colonic microbiota composition and activity are affected by diet, which comprise mainly of carbohydrates and proteins, and this may result in modulation of body function and metabolism. Intestinal microbiota not only contributes to the protection of the mucosa from the invasion by dangerous pathogens, but also promote the immune system at regional and systemic level by producing anti-

infectious agents, compete for nutrients, improve intestinal barrier function, and produce organic compounds including lactate, short-chain fatty acids (SCFA), and some vitamins (Sekirov et al., 2010).

It is also important to mention that the colonization of bacteria starts after birth and is a gradual process. The mode of birth delivery is also known to influence the composition of the bacterial ecosystem. Facultative anaerobes from the surrounding environment colonise the sterile gut laying the ground for strict anaerobes to dominate later. Infants who were born through vaginal delivery have a gut microbiota characterised by high lactobacilli during the first few days due to the occurrence of lactobacilli in vaginal flora. On the contrary, infants born by C-section are more colonized by Clostridiales species (Thursby and Juge, 2017). Another example is that breast fed babies have significant amounts of bifidobacteria that colonize their gut in comparison with formula-fed infants, which is thought to be due to the characteristics of breast milk, in particular its high content in specific (prebiotic) oligosaccharides. The population of bifidobacteria then decreases gradually after weaning when the microbiota composition begin to resemble the adult type that is characterised by the predominance of *Firmicutes*, *Bacteriodetes*, *Actinobacteria (*bifidobacteria) and *Proteobacteria* (Isolauri et al., 2001). Although healthy individuals share a common bacterial profile, the composition of these microbiota varies from one individual to another due to differences in several aspects including health status, age, genotype, ethnicity, immunological condition and lifestyle factors such as nutrition and physical activity (Scott et al., 2013, Nicholson et al., 2012, Gibson et al., 2017). Throughout adulthood the gut microbiota are considered stable until older age where a reduction in microbiota diversity occurs.

1.6.1. The prebiotic concept and mechanism of action

In 1995 the prebiotic concept was defined by Gibson and Roberfroid as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon" (Gibson and Roberfroid, 1995). Today this concept has been extended to reflect the modern understanding of the host-gut microbiota interactions. In 2017, the International Scientific Association of Probiotics and Prebiotics (ISAPP, June, 2017) proposed a new definition as "a substrate that is selectively utilized by host microorganisms that confer a health benefit" Figure (7)(Gibson et al., 2017).

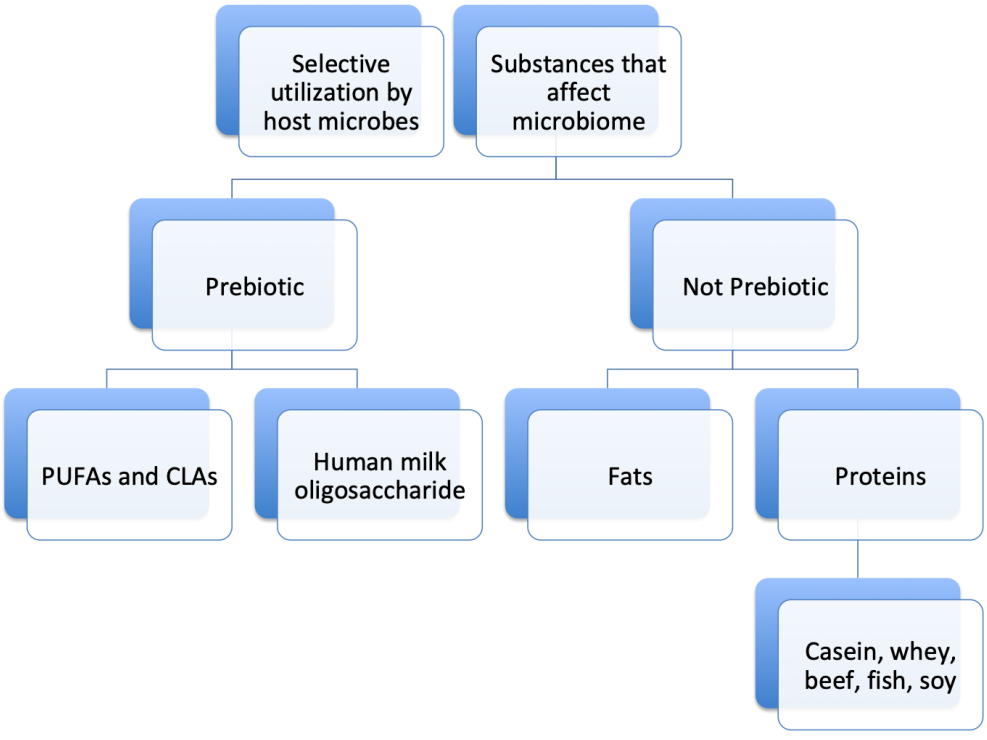

Figure 1.7. Suggested classification of foods into prebiotics and non prebiotics after the current definition released by Gibson et al. (2017)

Inulin, fructo-oligosaccharide (FOS), and galacto-oligosaccharide (GOS) are classified as prebiotics. The functional properties of those prebiotics can resist digestive juices at the upper digestive system and are fermented by selective beneficial bacteria present in the colon leading to growth stimulation. One of their benefits is to lower the level of blood lipids by inhibiting liver synthesis of very low density lipoprotein (VLDL) and triacylglycerols resulting in low blood cholesterol levels (Taylor and Williams, 1998). Furthermore, they exert protective effects against the development of colon cancer (Roberfroid et al., 2010) and enhance mineral bioavailability by creating an acidic environment that promotes absorption (Greger, 1999). Hence, prebiotics affect the relationship between beneficial gut bacteria and pathogens.

Inulin is characterised by a long chain and FOS a short chain and both of them are widely present in nature and can be in human diets especially in plant- based food such as onion, Jerusalem artichoke and asparagus. These prebiotics are resistance to human digestion because humans lack the enzymes that break down the linkages β (2-1) within inulin and FOS. Gut bacteria have the ability to produce enzymes that hydrolyse this bond. One of these gut bacterial types is bifidobacteria that have cell associated β-fructosidases. In vitro data by Gibson and Wang (1994) revealed the bifidogenic effects of FOS resulted from the high production of bifidobacteria. Hence, increasing the intake of prebiotics such as FOS and inulin may alter the balance of the gut microbiota towards bifidobacteria (Gibson and Wang, 1994). Also human studies carried out to explore the bifidogenic effects of FOS and inulin under a controlled diet reported a significant growth of bifidobacteria from 8.8 to 9.5 $\log^{10}$ g/ stool and 9.2 to 10.1 $\log_{10}$ g/ stool respectively and a decreased level of clostridia, bacteroides and

fusobacteria after the supplementation of the diet with 15 g FOS (15 days) and 15g inulin (15 days) (Gibson et al., 1995). A randomised control study supplemented the diet of 42 type 2 diabetes females with 10g/d inulin for 8 weeks compared with control group who received 10g/d maltodextrin, found that supplementation with inulin significantly lowered fasting blood sugar, total cholesterol, triacylglycerols, LDL-C, LDL-C/HDL-C ratio and TC/HDL-C ratio and increased HDL-C suggesting that inulin may control diabetes by improving glycemic and lipid parameters (Dehghan et al., 2013). Another human study in older people conducted with 19 participants investigated whether supplementation with 4g twice a day FOS for three weeks exerted bifidogenic effects. Faecal bacteria composition and immunity were measured before and after the intervention periods, It was found that bacterial count increased significantly (bifidobacteria) and decreased phagocytic activity in the FOS group compared with the control group who did not received any FOS , suggesting that FOS may decrease the inflammation process in older individuals (Guigoz et al., 2002).

Lactic acid producing bacteria such as *Lactobacillus* and *Bifidobacterium* have been categorised as beneficial bacteria that play an important role in health. *Lactobacillus* is a Gram-positive bacterium that is found in the intestine and gut mucosa. It is involved in the production of natural antibiotics, relieves intestinal inflammation due to food allergies, and can enhance immune system function and antitumour activity (Gibson and Roberfroid, 1999, Sekirov et al., 2010). *Bifidobacterium* is also a Gram-positive bacterium mainly found in the large intestine that participates in the production of lactic acid and acetate, and inhibits pathogen growth. *Bifidobacterium* is associated with a lower incidence of allergies, reduces the risk of chronic disorder and

can cure constipation and diarrhoea (Sekirov et al., 2010). The main factor behind this role of gut microbe is the availability of bioactive compounds. For example, saccharolytic bacteria species metabolise fibre and non-digestible carbohydrates that are found in the diet to produce energy and short chain fatty acids (SCFAs) including acetate, propionate and butyrate. It is estimated that 95% of these SCFAs are absorbed in the large intestine (Gibson et al., 2017, Rowland et al., 2017, Macfarlane and Cummings, 1991). The principle products of protein fermentation in the large bowel are SCFAs and branched chain fatty acids (BCFAs), which include isobutyrate formed from valine, and isovalerate formed from leucine and 2-methylbutyrate, which are formed from isoleucine. More than 95% of SCFAs produced by intestinal bacteria are metabolised by the host and the occurrence of BCFAs is an indicator of amino acid fermentation. The activity of proteolytic bacteria increases when there is limitation in the source of carbohydrate available in the gut i.e. in more distal areas (Macfarlane and Cummings, 1991).

1.6.2. Carbohydrates and proteins as prebiotics

Milk oligosaccharides fit the prebiotic definition since they are resistant to digestion and delivered to the colon where they contribute a source of carbon for gut microbiota (Gopal and Gill, 2000). Milk oligosaccharides are defined as carbohydrates that contain two to ten monosaccharide residues linked together via glycosidic bonds. There are two classes of oligosaccharides. The first one is neutral as it does not contain any charged carbohydrates. The second class is acidic and consists of one or more negatively charged residues such as sialic acid (*N*-acetylneuraminic acid). In human milk, lactose and oligosaccharides are classified as the major component available at a higher concentration than total protein (Table 2). In contrast, the composition of bovine milk oligosaccharides is relatively simple and the major

component is proteins. Based on advanced analytical techniques such as (HPLC), approximately 80 oligosaccharides have been isolated from human milk and 18 from bovine milk Hence, the structure and composition of oligosaccharides are different in human milk than bovine milk (Gopal and Gill, 2000).

Table 1.2. Composition of human milk and bovine milk (g/ 100ml or as stated) adapted from (Gopal and Gill, 2000, Martinez-Ferez et al., 2006).

	Protein	Lactose	Oligosaccharides	Fat
Human milk	1.0 [a]	6.8*	0.5-0.8*	3.0 [a]
Bovine milk	3.4[a]	4.6*	0.003-0.006*	3.7[a]

[a] Gobal and Gill (2000), * (Martinez-Ferez et al., 2006)

Evidence suggests that human milk oligosaccharides (HMO) work as growth promoters for beneficial bacteria. For instance, *Bifidobacterium* usually tend to be higher in infants who are breast-fed compared with infants who are formula fed. In contrast, breast fed infants have lower clostridia and more *Bifidobacterium* spp., associated with a less active proteolytic metabolism (Harmsen et al., 2000, Heavey et al., 2003). It has been shown that supplementation of bottle-fed infants with 6-galactosyl-lactose increased the population of bifidobacteria (Gopal and Gill, 2000, Deya et al., 1982). Increasing *Bifidobacterium* metabolic activity in the lumen results in decreasing the intestinal pH followed by inhibiting the growth of potentially pathogenic bacteria such as *Escherichia coli* (gram negative bacteria).

The amount of protein reaching the colon is depend on the protein consumed in the diet and its digestibility. Digestibility of animal protein (dairy and animal proteins) is

usually higher (90%) than the digestibility of plant proteins (70-90%). For instance, whey protein (fast protein) and casein (slow protein), are slightly more digestible than meat proteins (Windey et al., 2012). Fish proteins are digested slower than beef and chicken proteins. Soya proteins have a faster digestion rate than milk proteins due to nitrogen (N) absorption by the gut (Gilbert et al., 2011).

Upon ingestion, proteins are initially hydrolysed by the stomach acid and pepsin enzyme generating free amino acids that are cleaved from the amino terminus of aromatic amino acids followed by further enzymatic breakdown in the small intestine by pancreatic enzymes and epithelial brush border membrane peptidases. Proteins or peptides that have not been fully digested are delivered to the large intestine where they may be fermented by gut bacteria. Approximately 7-8 g of proteins will reach the colon following ingestion of 46-56g protein.

The main classes responsible for protein fermentation are proteolytic bacteria such as *Bacteroidales* and *Clostridiales*. These micro-organism become efficient at pH > 6 to generate end products of protein and peptide fermentation as CO_2, SCFA and *N*-containing products including ammonia, amines, and indoles (Rowland et al., 2017).
The fermentation of branched chain amino acids via gut bacteria generates BCFA. For instance, valine produces iso-butyrate whereas leucine and isoleucine produces iso-valerate and 2-methylbutyrate respectively. While the fermentation of aromatic amino acids such as tyrosine, phenylalanine and tryptophan generates phenolic and indolic compounds. For instance, tyrosine fermented by bacteria generates phenol and *p*-cresol. These latter products have been reported to be toxic compounds that should be detoxified by the liver and the colonic mucosa to release water soluble derivatives in

urine such as *p*-cresol sulphate and *p*-cresol glucuronide (Macfarlane and Cummings, 1991, Guarner and Malagelada, 2003, Rowland et al., 2017, Thursby and Juge, 2017, Windey et al., 2012). These protein fermentation products including BCFAs, phenols and indoles are also considered as markers of the degree of protein fermentation in the colon since these metabolites are not produced by human enzymes and are therefore specifically produced by colonic bacteria (Windey et al., 2012).

1.7. Lactose and milk sensitivity/ intolerance / maldigestion; metabolism, symptoms, diagnosis and complications

1.7.1. Lactose metabolism

Lactose is the major disaccharide found in dairy products and milk. It consists of one molecule of glucose and one of galactose linked together by a β1-4 glycosidic linkage (Figure 6). Lactose is produced by lactose synthase in the mammary glands during the end stage of pregnancy before secretion in milk during lactation. Cow's milk consists of about 5g lactose/100g of milk and hence 250 mL of cow's milk contains about 12g lactose (Campbell et al., 2005)

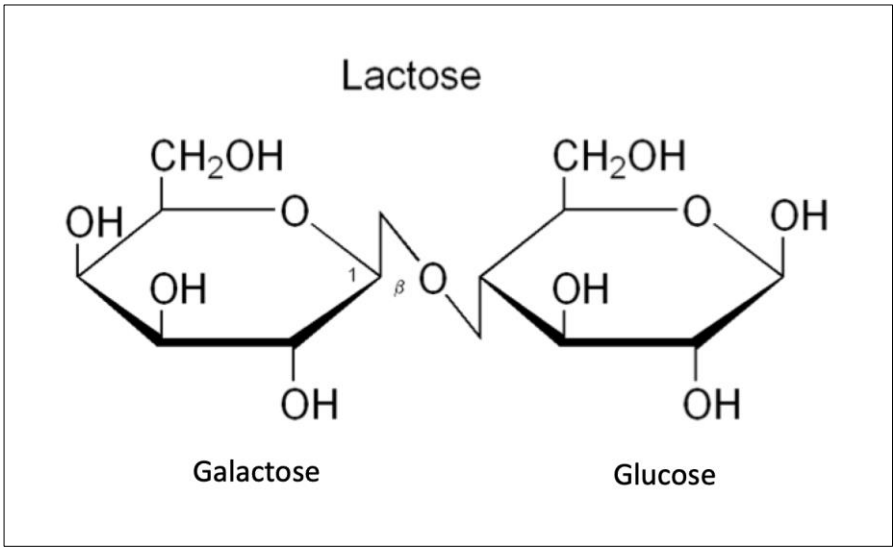

Figure 1.6. Lactose structure indicating galactose and glucose linked by β1-4 glucosidic linkage (Britz and Robinson, 2008).

In the small intestine, lactose cannot be absorbed and has to be broken down to glucose and galactose by the lactase enzyme. Lactase is a glycopeptide that consists of two active sites in the polypeptide chain. One hydrolyses lactose while the other hydrolyses the glycoside phlorizin to glucose and phlorizin (Vesa et al., 2000). The lactase enzyme is located on the apical surface of the brush border of epithelial cells in the small intestine. It is attached to the membrane by its C-terminal end, with the bulk of the molecules turned toward the lumen. It is encoded by a single gene (LCT) of around 50kb located on chromosome 2 in humans (Corgneau et al., 2017).

Once hydrolysis by lactase has occurred, the galactose is usually metabolized first in the liver to glucose to contribute to the internal glucose pool. Excess galactose is excreted in urine. In the small bowel lactose that has not been absorbed is delivered to the colon where the colonic microbiota ferments disaccharides that escape hydrolysis. In the colon lactose is broken down to glucose and galactose, which are then fermented by bacterial beta-galactosidase, expressed by bifidobacteria, lactobacilli and *E. coli*. Galactose then enters the mammalian Leloir pathway that contributes to the conversion of galactose to glucose. Subsequently, the fermentation end products of lactose consists of SCFA including acetate, propionate, butyrate and gases such as CO_2, H_2 and CH_4. Furthermore, a number of intermediate products are also produced and then further metabolized to ethanol and SCFAs such as succinate and lactate (Rowland et al., 2017). As discussed previously, the principle SCFA produced from the fermentation is acetate, which is absorbed and reaches the liver before entering the

general circulation and being distributed to peripheral tissues. Butyrate is largely consumed by colonocytes and only a small portion reaches the liver where it is further metabolized alongside propionate (Macfarlane and Cummings, 1991). Some of the gases are absorbed from the intestine to the blood to be further expelled by the lungs. The rest are used for cross-feeding of other bacterial species or passes as flatus (Bond and Levitt, 1976). These end products of lactose fermentation can result in common symptoms of lactose intolerance such as abdominal pain and bloating, as discussed later.

1.7.2. Definition and classes of lactose digestion / maldigestion

It is important to distinguish between different terminologies and types of conditions that are related to lactose sensitivity/intolerance/maldigestion. Lactose sensitivity is a term that describes abnormal symptoms after the consumption of lactose due to a deficiency of lactase enzyme that is responsible for the hydrolysis of lactose. The low activity of lactase enzyme in the small bowel mucosa is considered as lactase insufficiency. Lactase deficiency can either be primary such as genetic or secondary which is disease-related. The primary condition has two types. Firstly, congenital lactase deficiency is an autosomal recessive disorder that mainly affects babies at weaning age. This is often a severe lactase deficiency but is rare (Berg et al., 1969). Diekmann et al. (2015) suggested in their research that the main strategy to eliminate symptoms is to remove lactose containing food from the diet (Diekmann et al., 2015). Secondly, the other form of lactase insufficiency is lactase non-persistence, which is less severe and appears after weaning by gradual down-regulation of lactase expression, which results in a loss of lactose tolerance.

Secondary lactase deficiency is caused by diseases or injured intestinal mucosa, for instance in Crohn's disease, chronic intestinal inflammation and cancer chemotherapy. This can be transient since lactose tolerance is usually restored once the epithelium has recovered lactase activity (Corgneau et al., 2017).

Finally, lactose maldigestion or malabsorption refers to a condition where a high proportion of undigested lactose enters the colon. Lactose intolerance is a non-immunological reaction that is charactarised by systemic and gastrointestinal symptoms include abdominal pain, bloating, flatulence, diarrhoea, cramps, nausea and headache (Corgneau et al., 2017).

It is worth mentioning here that there is another condition or abnormality called small intestinal bacterial overgrowth (SIBO) where the individuals suffer from similar gastrointestinal symptoms. The clinical definition of SIBO is known as "a total growth of $>10^5$ colonic type bacteria forming units/ml intestinal fluid". In the gastrointestinal tract for normal individuals the concentration of bacteria increases in a caudal direction (meaning toward the tail) (Table 3). From the top, the stomach is almost sterile (no bacteria) and the upper bowel of the small bowel contains small numbers of bacteria mainly gram positive bacteria (aerobes). In the distal part of the small bowel the microbiota are more similar to the large bowel that are characterised with the predominance of gram negative aerobes and strict anaerobes. In SIBO, individuals the carbohydrate lactose is fermented in the upper gastrointestinal tract instead of the colon due to the high number of bacteria in this area that would normally contain less bacteria. This results in an increased production of fermented products such as hydrogen within 60 min (1hour) during a breath test. Breath test is a

non-invasive method to diagnose carbohydrate digestion. This method will be discussed in detail in the following section for lactose intolerance. However, just to mention here that SIBO can be diagnosed using hydrogen breath test with the most common use of either glucose (50-70g) dissolved in water or lactulose (10g). The interpretation of results indicate an early peak (30-60 min) of the hydrogen concentration due to jejunal and ileal bacterial overgrowth (Simrén and Stotzer, 2006a, Rezaie et al., 2017)

Table 1.3. The population of bacteria present in the gastrointestinal tract in normal individuals (bacteria/ml) (Adaptive from (Simrén and Stotzer, 2006a).

	Stomach	**Jejunum**	**Ileum**	**Caecum**
Aerobes	10^2-10^3	10^2-10^4	10^5-10^8	10^2-10^9
Anaerobes	0	0	10^3-10^7	10^9-10^{12}
Total count	10^2-10^3	10^2-10^4	10^5-10^8	10^{10}-10^{12}

1.7.3. Symptoms and diagnosis of lactose digestion / maldigestion

Lactose sensitivity can be found in both males and females and common symptoms usually appear in the form of bloating, abdominal pain, flatulence, gases and diarrhoea. These symptoms usually occur about 30 minutes to 2 hours after drinking milk or eating milk containing products. The severity of those symptoms vary from one person to another and tend to correlate with the type of diet, amount of lactose consumed, small intestine transit time and gastric emptying rate (Vesa et al., 2000, Corgneau et al., 2017, Stephenson and Latham, 1974). Several clinical tests can be used to measure human lactose digestion. All these techniques, whether direct or

indirect, differ in terms of accuracy, reliability, sensitivity and availability. One of those methods is the Lactose Tolerance Test (LTT). This test determines the increase in blood glucose concentration after administration of 50g lactose. Blood samples are usually taken at regular intervals every 15 or 30 min up to 2 hours post lactose load. An increased level of blood glucose higher than 1.7 mmol/L is considered normal whereas less than 1.1 mmol/L is considered hypolactasia (Arola, 1994). As it relies on circulating glucose measurements, this test is not effective in diabetic subjects (Lerch et al., 1991). It is therefore not recommended for clinical practice. A modified LTT can also be used by adding ethanol to the laboratory protocol. With this modification, ethanol stops the conversion of galactose to glucose, which results in galactose being excreted in the blood stream by the liver and then cleared in urine. Urinary or circulating galactose concentrations can then be used as an indicator for lactose digestion. Blood galactose levels can be measured at a single time point at 40 min after the ingestion of lactose. This test is considered positive if the blood galactose concentration is <0.3 mmol/L or urine galactose levels are < 2.0mmol/L 40 min post administration of lactose and ethanol (Arola, 1994). Although this test is suitable for diabetics, it is not recommended for pregnant women, children and infants due to the unfavourable effects of ethanol.

Another test to measure lactose digestion is known as the Stool Acidity Test. This test is usually used for infants and young children as it only involves a drink containing lactose. Stool samples are then collected to measure stool acidity. Therefore, this test is considered poorly reliable and not suitable for research purposes (Vesa et al., 2000). Intestinal biopsy is a direct diagnostic method to diagnose lactose malabsorption as it measures the enzymatic activity of the lactase enzyme in intestinal

biopsy samples. It is considered as the reference standard for the detection of lactase deficiency as it also allows to exclude other conditions that cause secondary lactose malabsorption such as coeliac diseases (Misselwitz et al., 2013). However it is rarely used due to its invasive nature and associated high cost.

Genetic testing is also a direct method to measure lactase deficiency. This test can detect adult- type hypolactasia where DNA samples are analysed in order to detect one of the two LCT-13910 C/T or LCT-22018 G/A gene variants (known as a single nucleotide polymorphism (SNPS). The lactase non-persistence is associated with the CC or GG genotype, whereas CT, TT, GA and AA are associated with lactase persistence (Misselwitz et al., 2013). Yet, this test is also rarely used due to the high cost involved.

1.7.4. Hydrogen and methane breath test

The introduction of the hydrogen breath test dates from the 1970's. Currently, breath hydrogen and methane tests are the most widely used techniques for the diagnosis of lactose malabsorbtion /intolerance in clinical settings because they are reliable, fast, cheap and non-invasive. The principle behind this test is that colonic bacteria ferment undigested lactose, which reaches the colon, to produce fermentation products such as SCFAs, lactate and gases including hydrogen (H_2) and methane (CH_4). These two gases are absorbed through the colon and exhaled in breath air. It is suggested that measuring the concentration of breath H_2 and CH_4 following an initial lactose challenge, is directly correlated to the level of lactose fermentation in the colon (Rezaie et al., 2017, Simrén and Stotzer, 2006a). Protocols vary in the administration of lactose or milk dose. For lactose breath tests, a high dose of lactose can be used (≥

50g), although 25g is the recommended dose because it is within the normal range of consumption, which is the equivalent of two cups of milk (500ml) (Rezaie et al., 2017). Breath samples are usually collected at intervals of 15 to 60 min for 2 to 6 hours and the change in hydrogen and methane from baseline determined to allow enough time for the substrate to reach the colon and to be metabolised by gut bacteria. Roa and colleagues (2007) recommended that 180 min is sufficient to detect colonic fermentation because it is reported that the gas peak is usually reached at 77 min for abnormal fructose breath test, which is transposable to lactose breath test (Rao et al., 2007). A recent guideline published by Rezaie et al. (2017) confirmed the criteria by running the test for at least 3h to ensure the presence of colonic fermentation (Rezaie et al., 2017) . In addition, it is suggested that the same cutoff value found in previous studies that confirm a rise of $\geq$ 1.78 mg/m^3 (20 p.p.m) in hydrogen and $\geq$8.48 mg/m^3 (12 ppm) in methane from baseline during the test is considered positive for lactose breath test (lactose malabsorption/intolerance) (KURT et al., 2003, Simrén and Stotzer, 2006a, Rezaie et al., 2017). Fermented unabsorbed substrate via colonic microbiota leads to the occurrence of GI symptoms such as gases, bloating and abdominal pain. Those symptoms generally appear after the consumption of 120-240 ml of milk (6-12 g lactose) (Rezaie et al., 2017, Corgneau et al., 2017).

It is worth noting that there are several risk factors that need to be addressed before performing breath tests, especially the day before the test, since these factors may influence the concentration of hydrogen and methane produced during the test. One of these factors is lifestyle, for example a smoking habit increases exhaled hydrogen concentration (Rosenthal and Solomons, 1983, Miller et al., 1989) and increases gastric transit time (Miller et al., 1989), while performing exercise lowers hydrogen

concentration (Simrén and Stotzer, 2006a). Another factor is diet since fermentation of complex carbohydrates such as oats, whole wheat, baked beans, corn and potatoes increase gas production which biases the measurement of lactose-induced gas production. However, rice seems to be acceptable the day before the test since it produces minimal amounts of exhaled hydrogen (Levitt et al., 1987). In addition to previous factors, antibiotic intake also alters the composition of exhaled hydrogen and methane since it reduces gut bacterial load (Rezaie et al., 2017). This is the reason why it is usually suggested that in the preparation of the test, volunteers are fasted (i.e. should not eat or drink for 8-12 hours anything but water), avoid alcohol as well as slowly digested food such as beans the day before the test (Simrén and Stotzer, 2006a, Rezaie et al., 2017).

It is worth to note that although the hydrogen and methane breath test is widely used due to its simplicity, it has a number of disadvantages such as a lack of standardised protocol (i.e. various substrates are used at various doses and sampling times vary too), non-quantitative, it is time consuming and there is a lack of consensus regarding the symptoms (meaning when the symptoms should be recorded, during the test or after, and for how long, 3, 4 or 24h after the lactose challenge?).

1.8. Complications of reducing milk intake

The importance of bovine milk and associated dairy products in the human diet are well known as they have been studied at great length for their contribution to human nutrition. Haug et al. (2017), recently reviewed the nutritional package provided by milk and dairy products and concluded that it is difficult to compensate for a diet limited in dairy products. This is due to the fact that milk and dairy products are a rich

source of proteins that contain essential amino acids (particularly tryptophan) and branched chain amino acids (particularly leucine), calcium, potassium, phosphorus and iodine. Leucine is involved in the stimulation of insulin secretion and the ingestion of milk with a meal of high glycaemic load may stimulate the release of insulin and thus reduce the postprandial blood glucose concentration that results in a lower risk of diseases related to the insulin resistance syndrome. (Haug et al., 2007, Givens, 2018).

The dietary recommendations suggest consumption of 3 servings per day from dairy products such as 1 portion of cheese (35g), 1 yogurt (125g) and 1 glass of milk (200ml). This amount provides around 250mg of calcium and the UK reference nutrient intake (RNI) for calcium is 700 mg/day for adult males and females and 800-1000 mg/day for younger ages. RNI values are calculated for range of age/sex group at a level of intake considered to be enough to cover the requirements of 97.5% of the group.

In most guidelines, dairy products contribute to the dietary recommendation of calcium intake. It is suggested that during skeletal growth, in order to achieve peak bone mass, adequate dietary calcium and protein intakes are essential to prevent bone weakness later in life (i.e. elder stage) (Pereira, 2014, Rozenberg et al., 2016). This is due to the fact that calcium phosphate accounts for approximately 70% of bone weight (Givens, 2018). In addition, dairy proteins are important during weight loss and maintenance due to their satiety promoting effects (Bendtsen et al., 2013). Furthermore, low fat milk and dairy intake play an important role in lowering blood

pressure, which is supported by a recent meta-analysis of observational studies (Rideout et al., 2013).

There is scientific evidence to support the consumption of milk and dairy products because of its contribution to the nutritional recommendations and protective effects against chronic non-communicable diseases (Thorning et al., 2016, Givens, 2018).

1.9. Conclusions

The importance of bovine milk in the human diet has been studied at great length in terms of human nutrition and related health. A number of reviews have discussed the health benefits of milk and dairy products in providing a number of essential nutrients and their positive effects towards human health, mainly high quality proteins, essential amino acids, vitamins and minerals. Casein proteins are well documented milk proteins that play a physiological role in digestion, through the release of opioid peptide agonists, peptides with immunostimmulating and anti-hypertensive activities as well as contributing in calcium absorption. In this context, there is scientific evidence of the health effects of milk and dairy products that supports the consumption of milk and dairy products because it contributes to achieving public health recommendations and protects against common non-communicable diseases.

Therefore, there is a benefit to consume milk and to limit its withdrawal from the diet because of gastrointestinal symptoms such as gases, bloating and abdominal cramps that are not caused by lactose intolerance. It has been hypothesised that these gastrointestinal symptoms may result from the consumption of milk containing A1 β-casein variant rather than A2 β-casein variant because the A1 β-casein releases a pro-

inflammatory peptide, βCM-7. However, there is a lack of independent studies that have investigated the effects of A1 versus A2 milk and directly compared the substitution of conventional A1/A2 milk with A2/A2 milk on gastrointestinal function and inflammation, which is the topic addressed by this PhD thesis.

1.10. Objectives and hypotheses

- Identify βCM-5 and 7 peptides after simulated gastrointestinal digestion using liquid chromatography-mass spectrometry.
- Assess in vitro the impact of synthetic bovine βCM-5 and βCM-7, digested A1/A2 milk and digested A2 milk on gut microbial ecology.
- Investigate the effects of A2 milk on gastrointestinal inflammation, function and gut microbiota in healthy adults with mild to moderate non-lactose milk intolerance
- Explore the effects of A2 milk on cardiometabolic risk markers, cognitive function and mood in adults with mild to moderate non-lactose milk intolerance.

In order to achieve these objectives, two studies were designed as follows.

Study 1: Effects of β-casein-derived bioactive peptides on healthy children gut microbiota.

Objective: To compare *in vitro* the potential impact of digested milk containing A1/A2 β-casein variant, digested milk containing A2/A2 β-casein variant, synthesised

bovine βCM-7 and βCM-5 on the gut microbiota of healthy Saudi children using pH controlled, anaerobic, batch culture inoculated with three healthy faecal donors.

Hypothesis: β-casein-derived peptides will beneficially alter gut microbiota composition in batch culture and produce short chain fatty acids upon fermentation.

Study 2: Effects of A2 Milk on gastrointestinal function and gut microbiota in healthy adults with mild to moderate non-lactose milk intolerance

Objective: To determine whether substitution of A1/A2 milk with A2 milk influences inflammation, gut microbiota composition, gastrointestinal function and symptoms

Hypothesis: The replacement of A1/A2 milk with A2 milk in non-lactose milk intolerant individuals will lead to improvement of gastrointestinal symptoms and inflammation.

Secondary objective to study 2: Effect of A2 milk on cardiometabolic risk markers, cognitive function and mood in adults with mild to moderate non-lactose milk intolerance.

Objective: To investigate the chronic effects of A2 vs. A1/A2 milk consumption on serum lipids, glucose, body composition, blood pressure and psychological behavior and mood.

Hypothesis: The consumption of A2 milk will improve the serum lipid profile and body composition compared with conventional A1/A2 milk.

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Chapter 2

Effects of β-casein-derived bioactive peptides on gut microbiota in healthy Saudi children

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2.1. Abstract

Background: Bovine β-casein is produced predomenantly from genetic alleles, A1 and A2, which produce two protein variants which may lead to different digestive outcomes. Only the A1 protein releases the β-casomorphin 7 (βCM-7) and β-casomorphin 5 (βCM-5) peptides during digestion. βCM7 is one of the peptides that research suggests may be involved in the aetiology of a range of chronic diseases such as cardiovasculer diseases, type 1 diabetes and autism.

Objective: The aim of this study was to investigate the impact of β-caseins and their two main bioactive peptides with opioid activity, βCM5 and βCM7, on the colonic microbiota of children using *in vitro* batch cultures.

Design: A1/A2 and A2/A2 bovine milks were digested *in vitro* and their impact on the gut microbiota derived from 3 young healthy donors assessed using a validated *in vitro* pH controlled batch culture system over a period of 48 h. Changes in the gut microbial ecosystem were identified by fluorescence in situ hybridisation (FISH) and 16S rDNA, whilst the effects on microbial metabolism were investigated using metabolic profiling by high resolution ^{1}H NMR analysis. The primary focus was the effects of β-casein upon microbes that are already known to be either of benefit to health or that are pathogenic.

Results: βCM-7 behaved similarly to the negative control with no changes in the gut microbial population. A significantly higher growth in total bacteria in digested A1/A2, A2/A2 milk and FOS was seen compared with βCM-7 (P= 0.03, P= 0.01, P= 0.02 respectively). This increase could be derived from an increased production of bifidobacteria in A1/A2 digested milk (with a log1.82 ± 1.09) and A2/A2 digested milk (with a log 1.90 ± 1.07) similar to FOS. Significant differences were shown between donors 1 and 3. At the phylum level the relative abundance was differed significantly higher at T24 than T0. Proteobacteria and Actinobacteria were increased significantly over time in both digested milks. *Bacteroidetes* remained unaffected by both milks and by βCM-5 and βCM-7. A slight higher growth of bacteria but not significant in βCM-5 than βCM-7 and negative control. The relative concentrations of propionate, butyrate and acetate were higher in A1/A2 digested milk and A2/A2 digested milk was similar to FOS at 24h. Acetate has been indicated to have protective, trophic and metabolic host benefits.

Conclusion: Digested milks were fermented *in vitro* by gut bacteria in a way similar to FOS. βCM7 inhibited the growth of bacteria relative to βCM5.

2.2. Introduction

The human colon contains a large number of microorganism that have a major influence on host metabolism depending on their composition and activity (Gibson and Roberfroid, 1999, Rowland et al., 2017). Although human intestinal metagenomic studies illustrates that the major phyla represented in the human gut are Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria, (Jandhyala et al., 2015) the specific composition of gut microbes is highly individual at the species level due to various factors including diet (Qin et al., 2010, Scott et al., 2013). In young individuals by the age of 3 years old the gut microbiota starts to resemble the adult gut bacteria (Jandhyala et al., 2015). Dietary micronutrients have a driving effect on the composition and metabolism of colonic microbiota (Gibson et al., 2017). In fact, dietary micronutrients are substrates for metabolism by the intestinal microbial ecosystem, particularly influencing the growth and metabolic activities of dynamic bacterial populations thriving in the human colon (Gibson and Roberfroid, 1999, Nicholson et al., 2012). The metabolites released by microbial populations in the intestine as they process foods influence their own growth and function.

Casein proteins represent about 80% of total protein in cows' milk, about 36% of which is β-casein. β-casein can be derived from different allele variants with different proportions including A1, A2, B and C. In the UK, the A2 variant (58%) is the highest proportion in milk followed by the A1 variant (31%) (Givens et al., 2013). Dairy cows can be either homozygous (A2/A2 or A1/A1) or heterozygous (A1/A2)

genotypes. Most of the UK milk contains a mixture of A1/A2 protein variants as a result of most dairy cows being heterozygous genotypes. However, commercial A2 milk contains only the A2 variant produced from animals selectively bred to be A2/A2 homozygotes (Woodford, 2007).

β-casein (βC) protein contains 209 amino acids and the main difference between A1 and A2 β-caseins is in position 67 where histidine in A1 is replaced by proline in A2. The result of this is that during enzymatic digestion, the A1 β-casein releases the β -casomorophin-7 (βCM-7) peptide into the gut but this is not possible with the A2 variant (Jinsmaa and Yoshikawa, 1999). In addition, the literature provides evidence that βCM-7 may be further broken down to βCM-5 by brush border enzymes Dipeptidyl Peptidase-4 (DPP-4) (Barnett et al., 2014). In addition, peptides may be further broken down by microbial enzymes that released during the fermentation in the colon (Jandhyala et al., 2015).

The βCM-7 peptide has opioid characteristics which have been suggested to play a role in gut health including effects on the gut microbiota (Tuohy et al., 2015). *In vitro* and animal studies have also demonstrated that βCM-7 could cross the blood: brain barrier but its central effects are unknown (Sun et al., 1999, Sienkiewicz-Szłapka et al., 2009, Picariello et al., 2013, Haq et al., 2014b, Haq et al., 2014a). However, The European Food Safety Authority (EFSA) concluded that there was insufficient data to determine a causal relationship between ingestion of βCM-7 and these diseases (De Noni et al., 2009).

Milk oligosaccharides (MO) are defined as carbohydrates that contain two to ten monosaccharide residues linked together via glycosidic bonds. In human milk, lactose and oligosaccharides are classified as the second and third major component in milk compared with protein. The composition of milk oligosaccharides varies between human milk and cows milk with the latter containing considerably less. Approximately 80 oligosaccharides have been isolated from human milk and 18 from bovine milk (Gopal and Gill, 2000). This may explain why human milk oligosaccharides (HMOs) are studied as prebiotics which are defined as a substrate that is selectively utilised by host microorganisms that confer a health benefit (Gibson et al., 2017). They may modulate gut microbiota due to their structure and functional properties that prevent the attachment of pathogens to the intestinal mucosa, stimulate the immune system, provide bifidogenic effect and sialic acid that known as essential nutrient for infant (Chen and Gänzle, 2017).

Although human milk oligosaccharides are well studied *in vitro* in relation to alteration of gut microbiota composition, to date there is a lack of *in vitro* studies that have investigated the effect of bovine milk and casein bioactive peptides (βCM-5 and 7). pH controlled batch culture systems were used to provide a controlled environment in which to study the composition and metabolic activity of the gut microbiota in relation to diet. Children are generally high consumers of milk that provides them with energy and a number of important nutrients and micronutrients. The present study investigated the impact of β-caseins and their two main bioactive peptides with opioid activity, βCM5 and βCM7, on the colonic microbiota of children using *in vitro* batch cultures, inoculated with faecal samples from healthy donors. Bacterial populations were identified by fluorescence in situ hybridisation (FISH) of

targeted bacterial groups, DNA was also extracted for bacterial 16S rDNA sequencing for deep coverage of the microbial population, whilst the effects on microbial metabolism were investigated using metabolic profiling by high resolution ^{1}H NMR analysis.

2.3. Materials and Methods

2.3.1. Milk collection and preparation

Pasteurised semi-skimmed milk containing the protein variants A1/A2 (regular milk) and milk containing only the A2 variant were purchased from the local Waitrose supermarket in October 2015. 2L of each kind of milk were taken directly to the laboratory and frozen at -80 °C, then freeze-dried the following day for future analysis. Samples remained in the freeze-drier for one week to dry.

2.3.2. Determination of β-casomorphins 5 and 7 in milk digests by LC/MS-MS

Separation and identification of peptides were involved a modification of the method of (De Noni, 2008). Samples collected during digestion were filtered and centrifuged at 3000 g for 10 min and then bovine β-casomorphin 7 and 5 was identified using an Agilent 1100 HPLC-MS (Agilent Technologies UK Ltd., Wokingham, UK) apparatus and a micrOTOF-QII QTOF (Bruker). After passing through the detector and splitting with a micro-splitter valve for a flow rate of 30µl/min, the HPLC stream was electrosprayed into the MS. Peptides were eluted, solvent A was water + 0.1% trifluoroacetic acid. Solvent B was acetonitrile + 0.1% trifluoroacetic acid. The elution gradient, in terms of the proportion of solvent B was: 0–5 min, 5%; 5–55 min,

55%; 55–60 min, 95%; 60– 61 min, 5%, 61–70 min, 5%. A flow rate of 0.2 ml/min and detection at 210 nm were used with a ACE 5 C-18 300Å 5µM 150 mm x 2.1 mm with 30 nm pore size and 5 µm particle size held at 40 °C.

2.3.3. *In vitro* simulated human digestion (from mouth to small intestine) of milk

Digestion of freeze dried milk was carried out according to (Mills et al., 2008). In brief, A1/A2 or A2 milk (60 g) were mixed with 150 ml of distilled water and initially hydrolysed with gastric pepsin (2.7 g) at pH 2.0. The pH of the mixture was adjusted with 6 M HCL and then incubated at 37 °C for 2 h in a shaker (This condition is to mimic the gastric stage). Aliquots were removed from the reaction at the 0-h baseline and at 2 h for MS analysis. The *in vitro* gastric digestion was followed by small intestine digestion. The reaction was then stopped by adjusting the pH to 7.0 with 6 M NaOH. Next, the digests were hydrolysed further with pancreatin (560 mg) and bile acid (3.5 g) for 3 h. Aliquots were also removed from the reaction just before incubation at 37 °C and after incubation for MS analysis.

Samples then were then transferred to cellulose dialysis membrane (1 KDa molecular weight cutoff) (Cheshire Biotech, Cheshire, UK) and dialysed against NaCl (0.01 M, 5 °C) to remove low molecular mass digestion products. After 15 h the dialysis fluid was replaced with fresh fluid and dialysis continued for an additional 2 h. Samples were subsequently frozen and then freeze-dried the following day for 5 days. All samples were then ready to be used for *in vitro* fermentation experiments. All chemicals used for fermentation were purchased from Sigma-Aldrich, UK. Samples

collected during digestion were subjected to LC/MS for identification of βCM-5 and βCM-7.

2.3.4. Collection and preparation of faecal samples

Faecal samples were obtained from three healthy boys aged from 5-8 years old who had the same ethnicity, who had not consumed antibiotics within the last 6 months and were not taking pre- or probiotics. Stool samples were prepared on the day of the experiment and diluted (1:100, w/v) in an anaerobic phosphate buffer (0.1 M; pH 7.2). Faecal samples were then homogenised in a blender for 2 minutes (Stomacher 400, Seward, Worthing, UK).

2.3.5. *In vitro* fermentation

A sterile glass fermentation batch culture system was filled with 135 ml of sterile prepared basal nutrient medium containing peptone water 2 g/L (Oxoid, Basingstoke, UK), yeast extract 2 g/L (Oxoid), NaCl 0.1 g/L, K_2HPO_4 0.04 g/L KH_2PO_4 0.04 g/L, $MgSO_4$•7H2O 0.01 g/L, $CaCl_2$•6H2O 0.01 g/L, $NaHCO_3$ 2 g/L, Tween 80 2 mL (BDH, Poole, UK), hemin 0.05 g/L, vitamin K1 10 mL, cysteine HCl 0.5 g/L, bile salts 0.5 g/L, pH 7.0, resazurin 4 mg/L, and distilled water. Fermentation vessels were purged with oxygen-free nitrogen (15 mL/min), and the pH was adjusted to 6.8 before inoculation using an automated pH controller (Fermac 260, Electrolab, Tewkesbury, UK). The two pre-digested milk samples (1.5 g each), positive control (FOS), pure synthesised bovine βCM-5 (1.5 mg), or βCM-7 (2 mg) (Bachem, UK) were added to the vessels, including another vessel as a negative control, just prior to

the addition of faecal slurry. The temperature was adjusted to 37 °C and each vessel was inoculated with 15 ml of faecal slurry (1:10, w/v). Batch cultures were run for 48 h and 5 ml aliquots taken from each vessel at 0, 4, 8, 24, 30, or 48 h for fluorescence in situ hybridisation (FISH), ^{1}H NMR analysis and DNA extraction.

2.3.6. *In vitro* enumeration of bacterial populations by fluorescence in situ hybridization (FISH)

Batch culture samples were collected at different time points as described above and centrifuged at 13,000 x g for 5 min to concentrate the bacterial cells. Sample pellets were then re-suspended in 375 µl PBS and 1125 µl 4% paraformaldehyde (PFA). Samples then were incubated for 4–8 h at 4 °C. These samples then were washed with PBS to remove PFA and re-suspended in a mixture containing 300µl PBS and 300µl 99% ethanol. Samples were then stored at -20 °C prior to FISH analysis by flow cytometry (Grimaldi. 2017). The probes used in this study (Sigma Aldrich Ltd, Poole, Dorset, UK) are reported in Table 1.

Table 2.1. Oligonucleotide probes used in this study for FISH analysis of bacterial populations

Probe name	Sequence	Hybridisation / °C		W T °C[1]	Target group	Reference
Non EUB	ACTCCTACGGGAGGCAGC	Lysozyme	35	37	Control probe	(Wallner et al., 1993)
Eub338I+	GCT GCC TCC CGT AGG AGT	Lysozyme	35	37	Most bacteria	(Amann et al., 1990)
Eub338 I I+	GCA GCC ACC CGT AGG TGT	Lysozyme	35	37	Planctomycetales	(Daims et al., 1999)
EUB338 I I I+	GCT GCC ACC CGT AGG TGT	Lysozyme	35	37	Verrucomicriales	(Daims et al., 1999)
BIF164	CAT CCG GCA TTA CCA CCC	Lysozyme	35	37	*Bifidobacterium* species	(Langendijk et al., 1995)
LAB158	GGTATTAGCAYCTGTTTCCA	Lysozyme	35	37	Most *Lactobacillus* and species	(Harmsen et al., 1999)
BAC303	CCA ATG TGG GGG ACC TT	Lysozyme	35	37	Most *Bacteroide*	(Manz et al., 1996)
ATO291	GGT CGG TCT CTC AAC CC	Lysozyme	35	37	*Cryptobacterium* and *Collinsella*	(Harmsen et al., 1999)
EREC482	GCT TCT TAG TCA RGT ACCG	Lysozyme	35	37	Most of *Clostridium*	(Franks et al., 1998)
RREC584	TCA GAC TTG CCG YAC CGC	Lysozyme	35	37	*Eubacterium*	(Franks et al., 1998)
FPRU655	CGCCTACCTCTGCACTAC	Lysozyme	35	37	*Colstridium* Cluster	(Walker et al., 2005)
DSV687	TAC GGA TTT CAC TCC T	Lysozyme	35	37	Most *Desulfovibrionales*	(Ramsing et al., 1996)
CHIS150	TTATGCGGTATTAATCTYCCTT T	Lysozyme	35	37	Most member of *Colstridium* cluster I II	(Franks et al., 1998)

[1] WT, Washing Temperature.

2.3.7. DNA extraction and sequencing

DNA samples (available for 0 and 24 h time points) were extracted using DNA extraction kit (MO Bio Power Soil DNA Isolation Kit, Qiagen, MO Bio Laboratories, INC). The DNA was extracted following the manufacturer's instructions. The purity of nucleic acid was tested using Nano Drop TM 8000 Spectrophotometer (Thermo Fisher Scientific). The quality of the DNA was checked by performing Gel Electrophoresis that submitted to a Genesy G-box chem GRX5. Samples then sent to Wellcome Trust Center for Genomic Analysis at the University of Oxford. Illumina sequencing results were further processed through the Quantitative Insight Into Microbial Ecology (QIIME) software package with the help of bioinformatics centre in University of Reading, following similar work carried out by (Koutsos et al., 2017) and (Callahan et al., 2017). The DADA2 method was used for ASV inference, alpha and beta diversity were tested and relative abundances were calculated.

2.3.8. Metabolic profiling from fermentation by 1H NMR

Batch culture samples were collected at different time points as described above and then centrifuged at 13,000 x g for 10 min. Supernatants were transferred to new Eppendorf tubes for storage at -80°C. On the day of the analysis, samples were defrosted on ice and diluted with TSP as solvent. 500µl transferred into 5mm NMR tubes for analysis. All NMR spectra were acquired on a Bruker Avance DRX 700 MHz NMR Spectrometer (Bruker Biopsin, Rheinstetten, Germany). Each spectrum was phased manually, corrected for baseline and calibrated to the chemical shift of TSP. Metabolites were matched using data from literature (Saric et al., 2007) and from public databases including Biological Magnetic Resonance Data Bank (BMRB).

2.3.9. Statistical Analysis

Statistical analysis was performed using the software R package. One-Way ANOVA was used to determine differences between treatments during fermentation (negative control, FOS, A1/A2 digested milk, A2/A2 digested milk, synthesized bovine βCM-5 and synthesized bovine βCM-7) at the same time point 0h and 24h. This was followed by independent sample t-tests to compare each microbial response to different treatments after fermentation. For genomic data analysis, the raw data were analysed by Dr Bajuna Salehe (bioinformatician in University of Reading, School of Biological Sciences). The QIIME version 2.6 was used. Alpha diversity (within community diversity) was calculated using an ASV approach (Callahan et al., 2017). Statistical differences between samples (beta diversity) were analysed with the pairwise PERMANOVA test following the QIIME compare_categories.py script and using unwieghted phylogenetic UniFrac distance matrices. Principal Coordinate Analysis (PCoA) plots were generated using beta diversity plots workflow. Metabolic profiling data were analysed using both MestReNova software (version 10.0 MestreLab Research) and MATLAB software (version R2015a). All processed spectra were digitalized and imported into Matlab. The residual water signal was removed and spectra were normalized to the probabilistic quotient. All statistical models were performed using unit variance scaling. Principal component analysis (PCA) for all spectra was performed to identify any outliers and patterns associated with time and treatments. A partial least square discriminant analysis was also performed using one predictive component in order to optimise statistical separation between samples. Metabolites were matched using data from the literature (Saric et al., 2007) and from standard databases (BMRB, http://www.bmrb.wisc.edu, HMDB, http://www.hmdb.ca, Chenomx soft ware).

2.4. Results

2.4.1. Release of βCM-5 and βCM-7 during stimulated gastro-intestinal digestion of different variants of β-casein

LC/MS-MS was used to investigate the release of βCM-5 and βCM-7 during intestinal digestion of different β-casein variants. Standards of βCM-5 and βCM-7 demonstrated retention times of 21.82 and 27.90 min respectively at 500 fm/µl (femtomol per microlitre) on LC/MS-MS as seen in Figure 2.1A. In both milks no detection of βCM5 or βCM7 was observed when digested with pepsin alone at baseline and after incubation at 37°C for two hours. However, βCM7 but not βCM5 was seen from A1/A2 regular milk after addition of pancreatin and bile acids (Figure 2.1B). This recovery continued after incubation for 3 hours at 37°C and after dialysis for 19h. Similar spectra were also obtained for digests of homozygote milk containing A2/A2 variant under the same conditions but as expected, neither βCM7 nor βCM5 were detected during all parts of the digestion system (Figure 2.1C).

2.4.2. Changes in faecal bacterial populations measured by FISH

Florecent in situ hyperdisation flow fish (FISH) was used to assess bacterial population by target microorganisms after incubation with digested milk samples and synthesized βCM-5 and 7. Bacterial numbers from each treatment were compared with negative control and FOS (positive control) to see if particular treatment stimulates the growth of bacteria similar to other treatment. Significant different were observed (Figure 2.2 A and B) between treatments in total bacteria ($P<0.05$).

Significant higher growth in total bacteria in digested A1/A2 milk compared with βCM-7 ($P= 0.03$) was seen and in digested A2/A2 milk compared with βCM-7 ($P=$

0.01) and in FOS compared with βCM-7 (P= 0.02). This increase in total bacteria could be related to the increased production of bifidobacteria in A1/A2 digested milk (with a log1.82 ± 1.09) and A2/A2 digested milk (with a log 1.90 ± 1.07) compared with FOS. βCM-7 inhibited the growth of bifidobacteria significantly (Figure 2.2 B) compared to FOS (P= 0.03). A slightly increased but not significant of *Atopobium* and *Colinsella* growth detected by (Ato291) in βCM-5 containing vessels compared with negative control (P= 0.05) and decreased in βCM-7 containing vessels compared with FOS (P= 0.06).

No significant changes were observed between treatments in *Lactobacillus* groups, most Bacteroidaceae, most of the *Clostridium* group and sulphate-reducing bacteria (P>0.05). However, all type of milks and synthesized peptides were noted to stimulate the growth but not significantly (P>0.05).

2.4.3. Faecal bacterial alpha diversity, beta diversity and relative abundance at the phylum level.

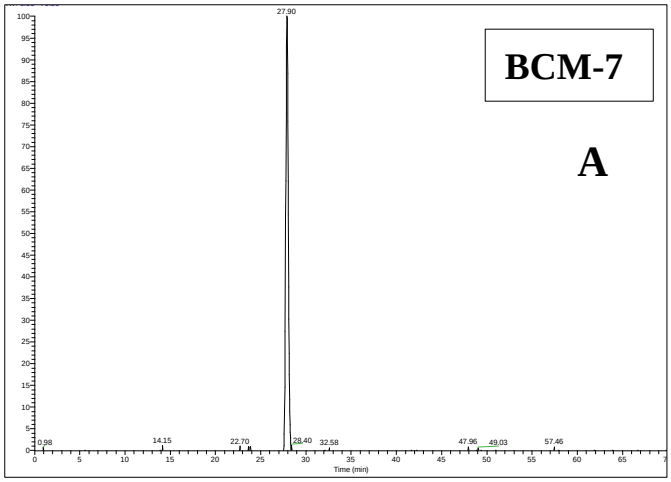
A total of 287,845 reads and 1,520 of features were generated. The gut microbial diversity within a community was evaluated with alpha diversity. Observations showed no significant differences in the richness of species at T0, T24 and all treatments (P = 0.16, P = 0.21 respectively). However, highly significant differences were shown in the richness of species between donor 1 and donor 3 (P = 0.003) (Figure 2.3). Bacterial diversity between samples for all the datasets was examined using beta diversity, data are shown in (Figure 2.4). The principal coordinate analysis (PCoA) showed a clustering observed based on faecal donor and time point. This (PCoA) was based on an unweighted phylogenetic Unifrac distance matrix. The cluster was significantly separated according to donor (PERMANOVA test, P = 0.001) and time (PERMANOVA test, P = 0.001). For all the data sets togetherm there

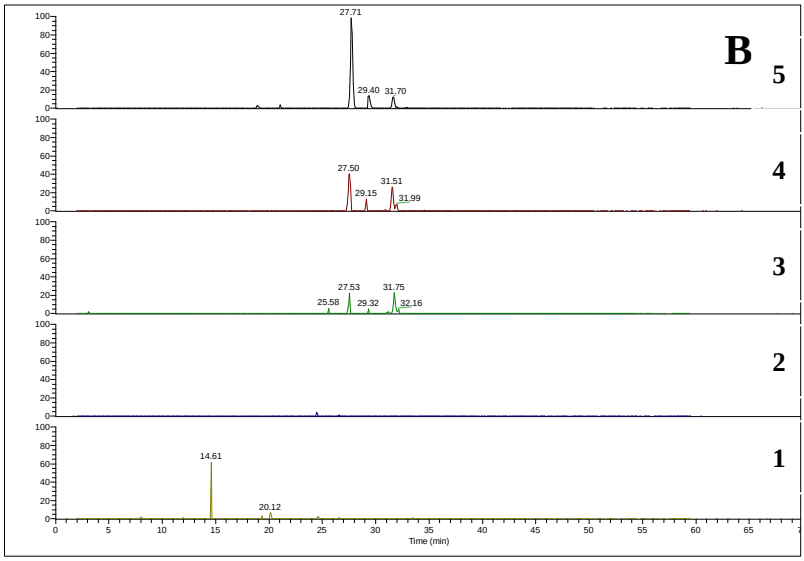
was no significant effect of treatments on beta diversity (PERMANOVA test, $P > 0.05$). The relative abundance at the phylum level is shown in (Figure 2.5). At time 0 the bacterial communities in all cultures were dominated by *Firmicutes* bacteria (48- 73%) followed by *Bacteroidetes* (19- 43%) then *Proteobacteria* (3- 8) and *Actinobacteria* (1- 4). A small percentage (0.1- 0.3%) was observed for *Synergistetes, Verrucomicrobia, Fusobacteria* and an unassigned phylum. On the relative abundance of phylum level, treatments did not have any significant effect. However, at time 24 h the relative abundance differed significantly more compared to time 0 h. Focusing on changes over time for each treatment separately, *Proteobacteria* and *Actinobacteria* were significantly increased over time in all digested milks and FOS. However, *Firmicutes* abundance was decreased significantly and *Bacteroidetes* remain unaffected over time. Donor 3 had less *Proteobacteria* abundance compared with donor 1 and 2 and high *Bacteroidetes* abundance compared with donor 1.

2.4.5. Metabolic profiling from fermentation by ^{1}H NMR

Metabolic profile of fermentation supernatants from digested A1/A2 semi-skimmed milk, digested A2/A2 semi-skimmed milk, synthesised bovine βCM-5 and synthesised bovine βCM-7 were assessed at T0 and T24 post inoculation by High Resolution 700 MHz NMR Spectrometer. Principle component analysis (PCA) that was normalized under total area with unit variance scaling (Figure 2.6A) was performed, the first two component explain together 43% of the variation between all treatments in two time points which are T0 and T24 and a clear separation can be observed visually between the time points. Metabolites were identified by OPLS-DA that includes all time points to identify global effect of the substrates (Figure 2.6B). This model showed that the concentration of propionate, butyrate and acetate were the

corresponding metabolites that separate the observation in 24h. Supplementation with A1/A2, A2/A2 milk and FOS were found to modulate the metabolic profile of healthy children (Q^2= 0.38) compared with βCM-7 and negative control (Figure 2.6C).





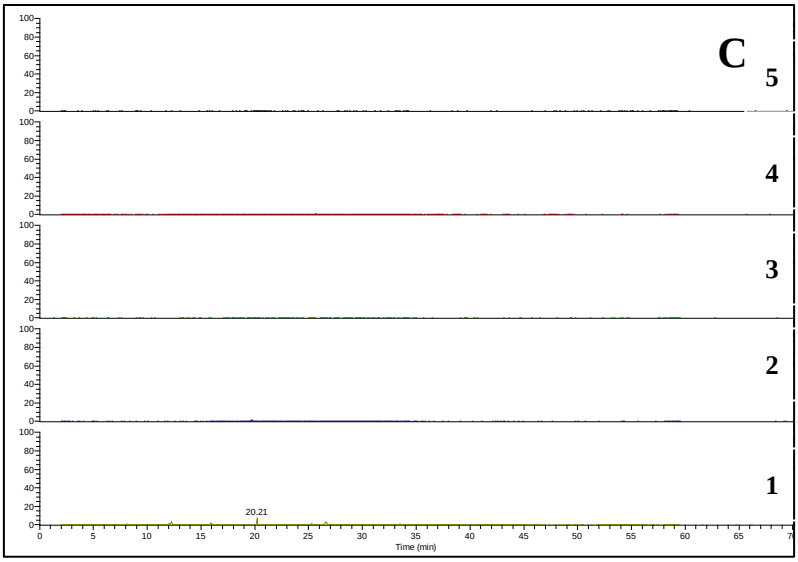
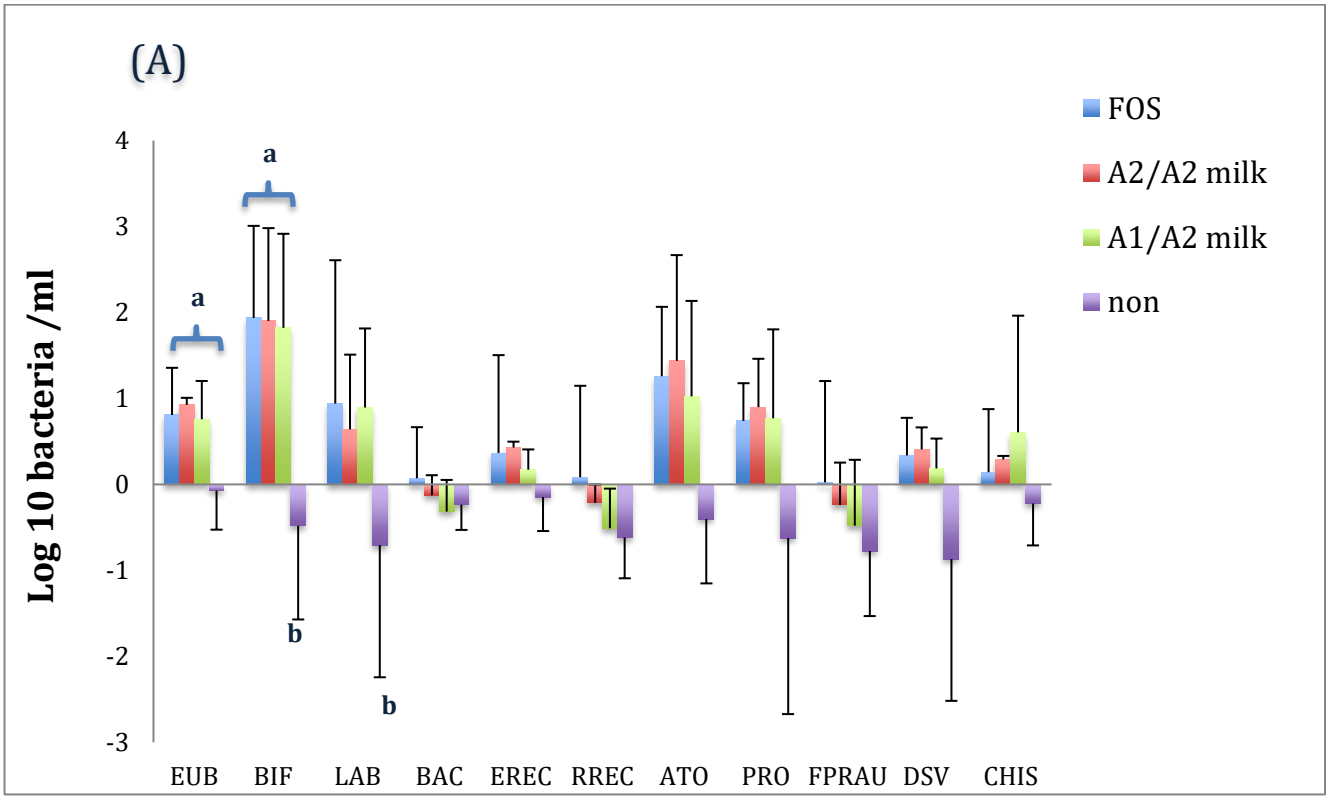


Figure 2.1. Chromatogram of (a) standard βCM7, chromatogram of (b) regular milk containing A1/A2 variant and (c) A2/A2 variant respectively representing only βCM-7 following digestion at different time points with pepsin, 0h (1) and 2h (2), pancreatin and bile acid at 0h (3) and 3h (4) and after dialesis (5)



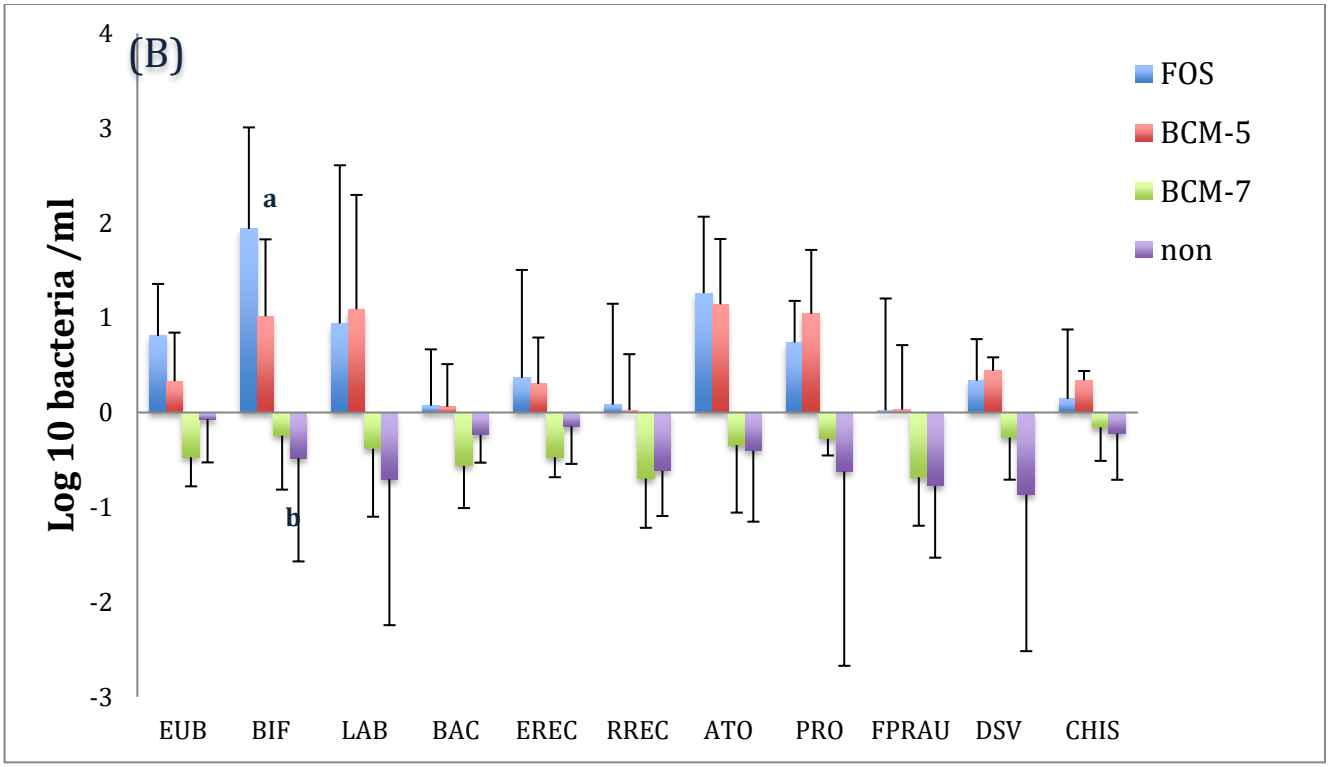


Figure 2.2. Bacterial group detected after analysis by fluorescent in situ hybridization (FISH) in a batch culture fermentation (A) comparing digested A1/A2 semi-skimmed milk, digested A2/A2 semi-skimmed milk with FOS as positive control and negative control (non) (B) containing synthesised bovine βCM-5 and synthesised bovine βCM-7 with FOS as positive control and negative control (non). Result reported as the mean of three independent fermentations with faecal samples from three different donors used as inoculate (n=3) in log10 CFU/ml ± standard deviation (SD). Significant differences are reported using a t-test ($P<0.05$).

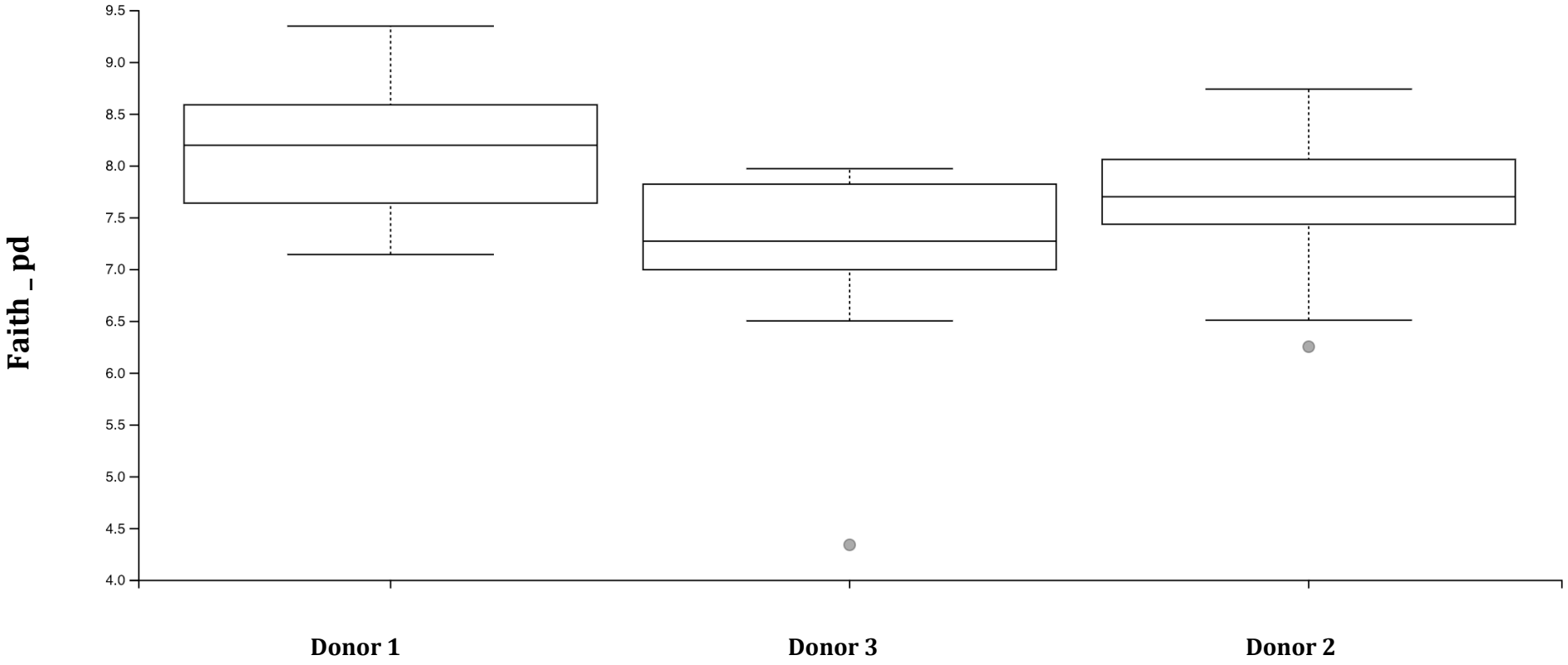


Figure 2.3. Alpha diversity based on ASV approach in 48-h *in vitro* batch culture fermentations inoculated with children's faeces (n = 3 healthy donors) administrated with FOS (positive control), A1/A2 milk, A2/A2 milk, synthesised βCM-5, synthesised βCM-7 and negative control. P value based on Kruskal-wallis (pairwise comparison). Significant differences observed when ($p < 0.05$).

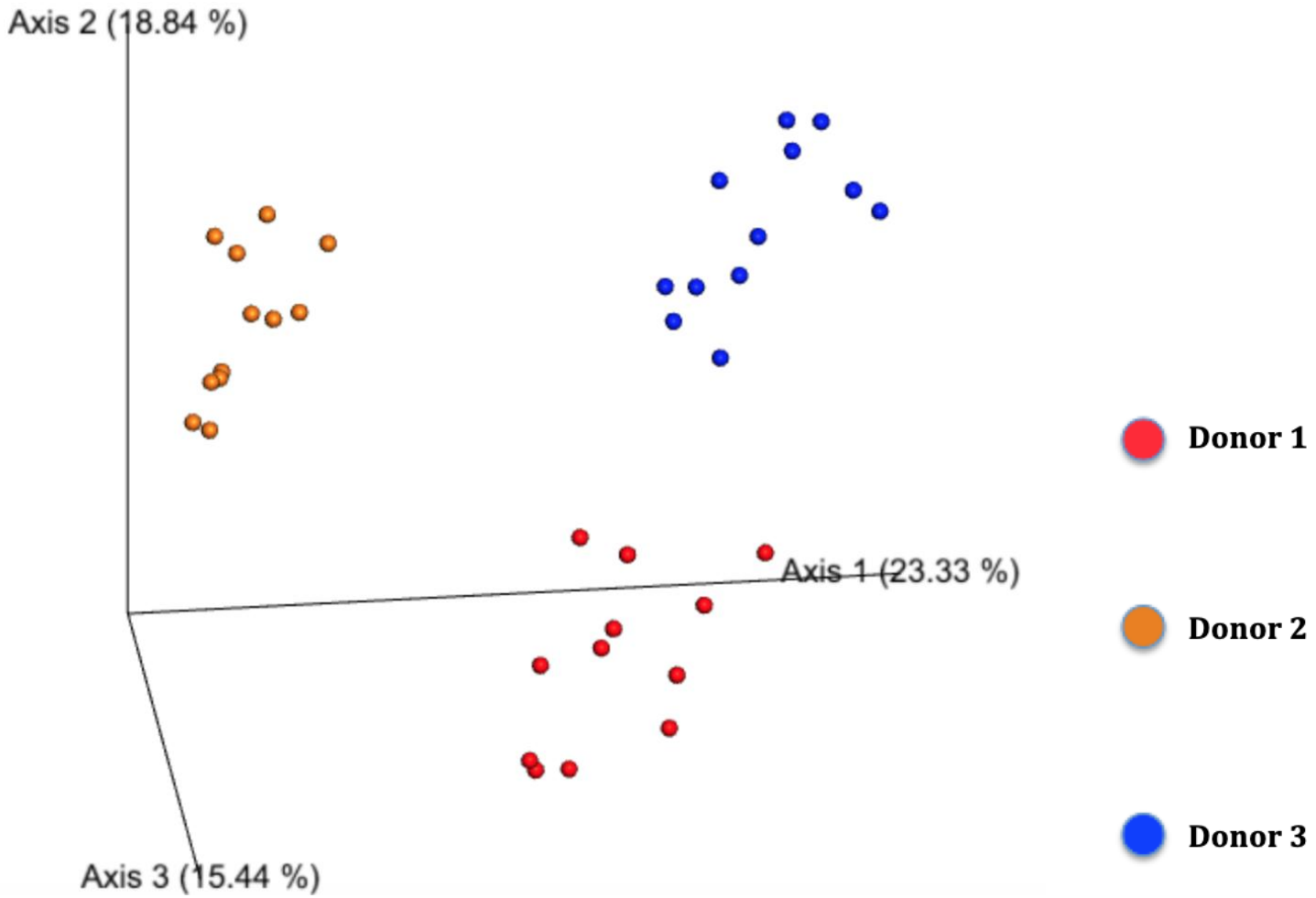


Figure 2.4. Principle coordinate analysis (PCoA) plot based on an unweighted phylogenetic Unifrac distance matrix calculated showing a clustering between donors. This was based on 24-h *in vitro* batch culture fermentations inoculated with children's faeces (n = 3 healthy donors) administrated with FOS (positive control), A1/A2 milk, A2/A2 milk, synthesised BCM-5, synthesised BCM-7 and negative control. Each color represents a different donor.

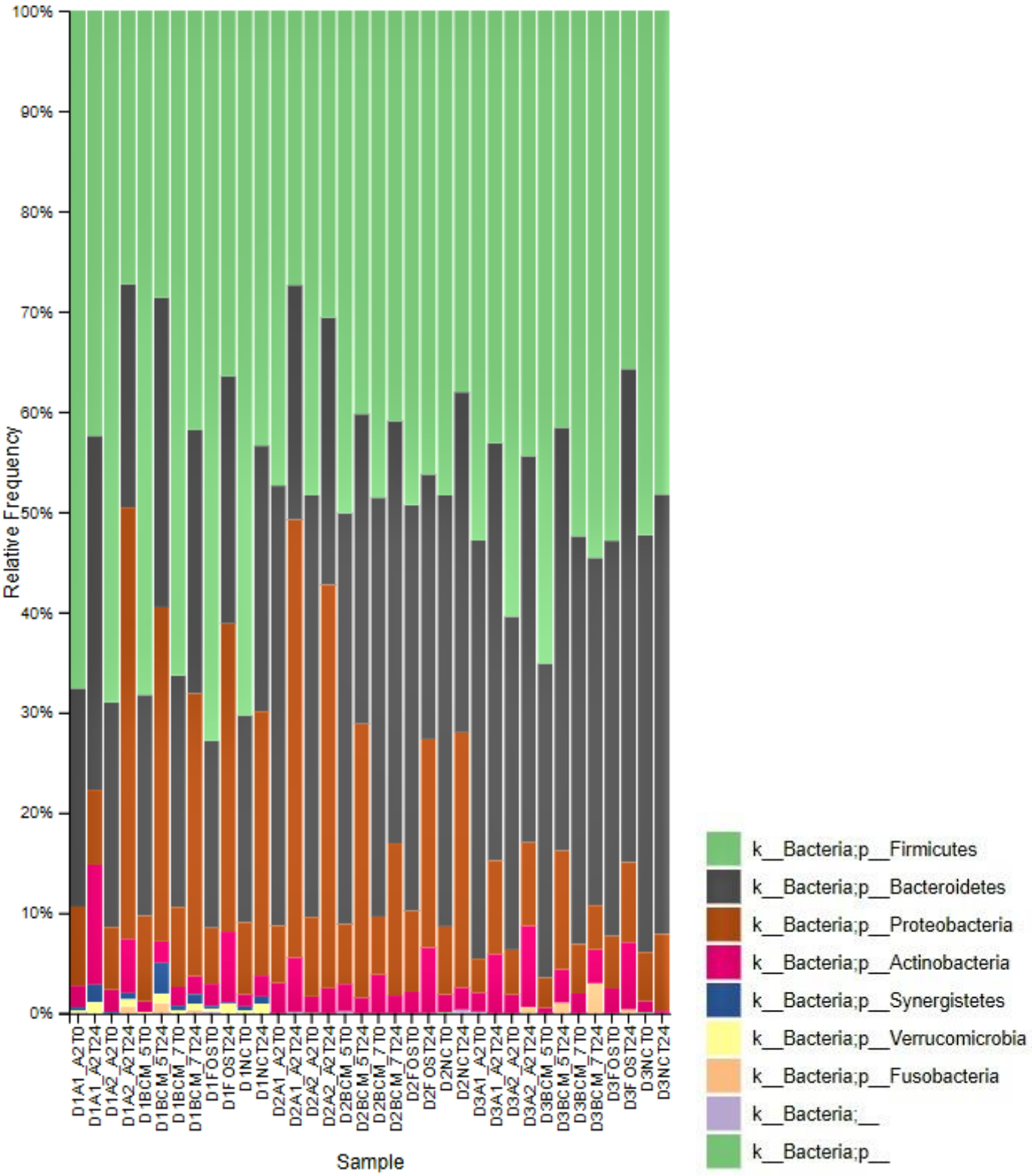
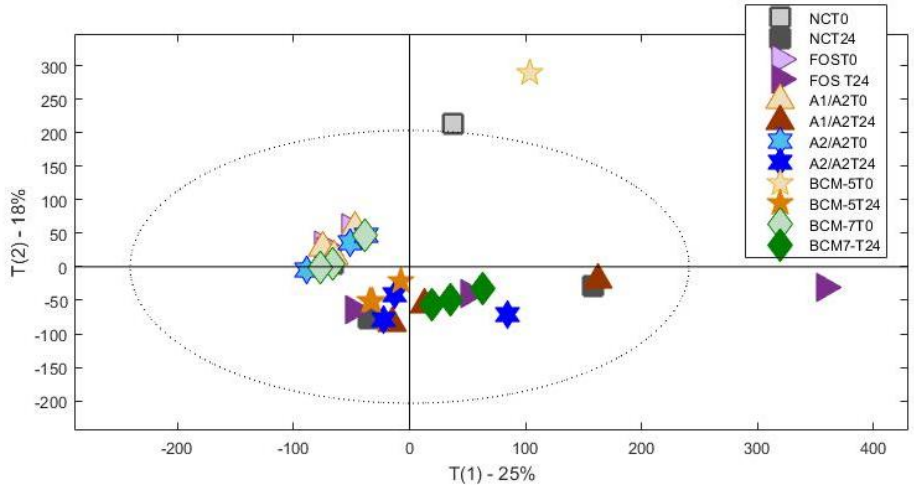


Figure 2.5. Relative abundance of dominant bacterial phyla (%) throughout 24-h *in vitro* batch culture fermentations inoculated with children feces (n = 3 healthy donors) administrated with FOS (positive control), A1/A2 milk, A2/A2 milk, synthesised βCM-5, synthesised βCM-7 and negative control.

A



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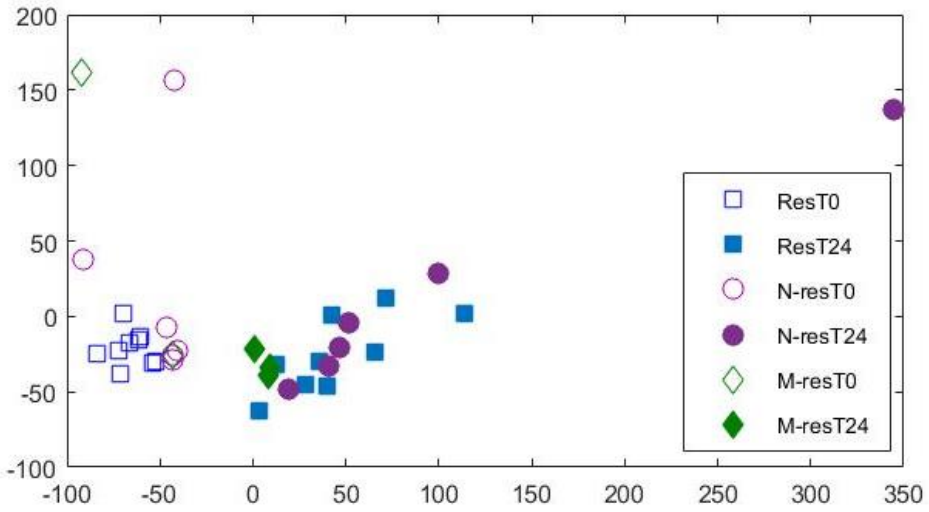

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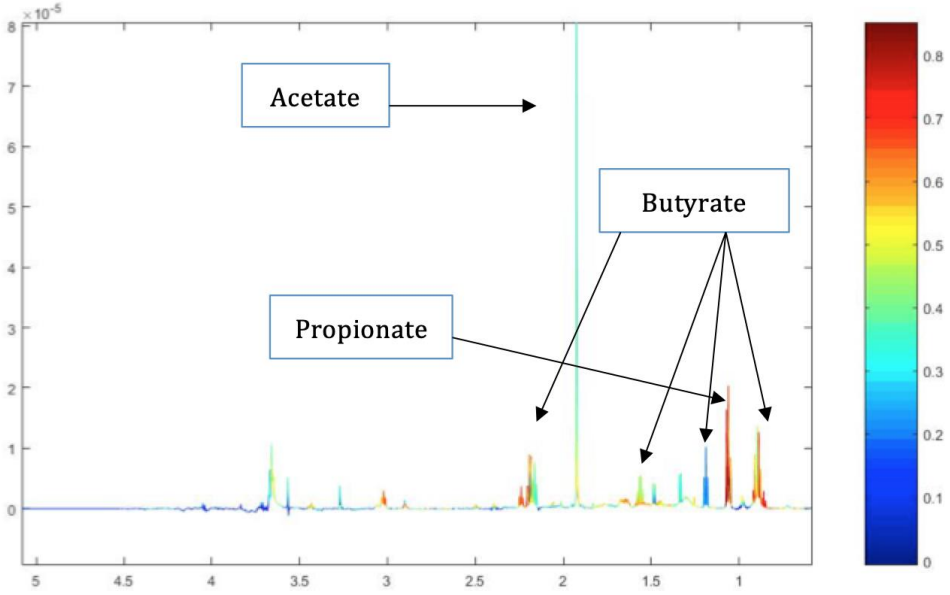

Figure 2.6. (A) is PCA and (B) OPLS scores plot for supernatant from all samples of digested milk samples and synthesized bovine BCMs compared with FOS as positive control and NCT as negative control. (C) is loading plot represent the OPLS model. Results derived from the 700 MHz ^{1}H NMR spectra of fermentation supernatants. Color and shape coded in PCA1 for time points 0 and 24 and treatments all together.

2.5. Discussion

The primary objective of this *in vitro* study was to investigate the potential impact of digested A1/A2 milk, A2/A2 milk and β-casein-derived peptides (βCM-5 and βCM-7) on the gut microbiota composition, diversity and microbial metabolism. Considering the fact that bioactive peptides including βCM-7 and 5 have been investigated since 1979 (Brantl et al., 1979) with both known to act as opioid peptides with morphine like activities that have been associated with diseases such as CVD, T1D and neurological disorders. This is due to the emerging evidence that A1 β-casein variant and βCM-7 have μ opioid agonist activity, both are pro-inflammatory, induce T cell-mediated immune response, induce jejunal mucin secretion, increase myeloperoxidase activity in intestinal tissue, increase IL-4, IgE, IgG, IgG1 and IgG2a concentration in intestinal fluid. Therefore, it is important to measure the release of βCM-7 and 5 first in order to examine their role in the gut. This current study showed the release of βCM-7 from A1/A2 regular milk but not A2/A2 milk throughout the digestion with enzymes. In addition, neither βCM7 nor βCM5 was detected during all parts of the digestion system from A2/A2 milk. These results are in agreement with previous results (De Noni, 2008, De Noni and Cattaneo, 2010, Ul Haq et al., 2015). According to De Noni (2008), βCM7 was released from heterozygote milk containing A1/A2 variants in negligible amounts (0.2–0.5 mmol BCM7/mol βCN). This could be due to the low amount of A1 variant in the milk. This suggestion is based on the fact that gene expression strongly plays a role in the amount and characterisation of casein family in milk (Bobe et al., 1999, Heck et al., 2009, Gustavsson et al., 2014). However, as expected, βCM 5 was not released from heterozygote milk containing A1/A2 at any stage of SGID. This finding is in agreement with other data from the literature that used the same enzymes or another combination including corolase (De

Noni and Cattaneo, 2010). However, (Cieslinska et al., 2007) showed that digestion of A2 milk with pepsin released βCM-7 with the amount four times less than βCM-7 that was released from A1 milk. This finding is discordant from other data from previous studies and the amount of βCM-7 seems to be unreliable because the digestion with pepsin was performed for 24 hours (not normally used hours in in vitro digestion) at pH 2 and identification and quantification of βCM-7 was performed using HPLC/UV, which resulted in many co-eluting peptides. It is thought that the formation of βCM-5 from βCM-7 is unexpected since βCM-7 is rich in proline which is about 43% of this peptide. As proline rich peptides are resistant to proteolytic hydrolysis so formation of βCM-5 is unlikely (Ul Haq et al., 2015).

It is well known that gut microbiota respond differently to different sources of substrate. For example, fructo-oligosaccharides (FOS) is known as a prebiotic which has been extensively studied in *in vitro* and *in vivo* studies.It has a bifidogenic properties that led industries to supplement infant formula with FOS. However, there is a lack of evidence on the role of bovine milk and β-casein-derived peptides on gut health. This current study investigated the fermentation profile of bovine milk and peptides using the same batch culture system inoculated with children's faecal microbiota. Bacterial groups were detected using flow FISH and results were confirmed with DNA sequencing. The present *in vitro* study showed that both milks effectively modified human faecal gut microbiota composition similarly to FOS during the fermentation period. Both milks significantly increased total bacteria and bifidobacteria. The growth pattern in both milks for *Lactobacillus*, *Atopobium*, *Colinsella* and *Clostridial clusterIX* were similar to FOS. BCM-7 significantly inhibited the growth of total bacteria and *Bifidobacterium spp*., with this group of bacteria behaving similarly to the negative control. βCM-7 had a tendency to decrease

Atopobium growth compared with a positive control. However βCM-5 had a tendency to increase *Atopobium* growth compared with negative control. In addition βCM-5 was observed to stimulate the growth of some bacteria. Sequencing results indicated significant separation between individual donors and a clear cluster was seen in time 0h and 24h for individual donors. Milks and synthesised peptides did not have a major impact on gut microbiota composition but did induce changes in relative abundances of certain bacteria at time 24. Significant differences were observed in relative abundance of *Proteobacteria, Actinobacteria, and Firmicutes* by supplementation of vessels with digested A1/A2 milk and A2/A2 milk.

Following A1/A2 and A2/A2 digested milks, the microbiota of healthy children produced greater amount of propionic acid, butyric acid compared with βCM-5 and 7. In addition acetate was also produced and this may be due to the significant production of bifidobacteria which has been shown to produce acetate (Macfarlane and Macfarlane, 2003). These results were consistent with a review by Isabelle and collegues (2010) which confirmed the high production of SCFAs by gut microbiota from the fermentation of formula- fed, which has composition similar to cows milk, in contrast with breast–fed infant (Le Huërou-Luron et al., 2010). Bovine milk is known to be the basis for most infant formula (Li et al., 2012).

It is important to highlight that after the consumption of milk, a high amount of carbohydrates, mainly lactose, may escape small intestine digestion and absorption then enters the colon where fermentation occurs. Parrett and Edwards (1997) confirmed that the end products of fermentation of breast fed and standard cow's milk based formula-fed (simple sugar and oligosaccharide) tend to be similar when measured *in vitro* cultures of faeces from healthy infants who were breast-fed or

formula-fed from 2-10 weeks of age. Although acetate is the predominant SCFAs in faeces from the fermentation of both milk sources, propionate and butyrate had a higher molar ratio in faeces from formula fed compared with breast-fed infants. Another study done at the University of Reading confirmed the same result after supplementation with HMOs in 24-h pH controlled batch culture inoculated with infant faecal slurries (Shen et al., 2011b).

In conclusion, this study showed that A1/A2 milk released βCM-7 during enzymatic digestion *in vitro* but A2/A2 milk did not. Both A1/A2 milk and A2/A2 milk beneficially modulated the gut microbiota composition and with a metabolic output *in vitro* similar to FOS. βCM-5 slightly increased the number of gut microbiota. Both types of milk may have positive consequences for human health by increasing bifidobacteria and SCFA.

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Chapter 3

Effects of A2 Milk on gastrointestinal function and gut microbiota in healthy adults with mild to moderate non-lactose milk intolerance

To be submitted to the American Journal of Clinical Nutrition (AJCN)

First author's main contribution: Areej Almuraee designed the study with the supervisors, conducted the *in vivo* study, all the lab analysis, statistical analysis and drafted manuscript. This trial was registered at clinicaltrials.gov as NCT03060395

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3.1 Abstract

Background:

The contribution of bovine milk containing a mixture of A1/A2 β-casein variant to the development of gastrointestinal dysfunction and inflammation has been debated recently. Despite animal studies demonstrating a detrimental relationship between β-casein A1 and gut health, only a very limited number of randomised clinical trials investigating the direct effect of milk on gut health have been reported.

Objective:

To determine whether substitution of A1/A2 milk with A2 milk influences inflammation, gut microbiota composition, gastrointestinal function and symptoms in healthy adults with mild to moderate non-lactose milk intolerance

Design:

A double blind randomised crossover design compared the daily consumption of semi-skimmed A2 milk with semi-skimmed A1/A2 milk for 14 days. 36 participants (men and women) aged between 18-56y were randomly assigned to their first intervention, either to consume A2 milk or A1/A2 milk. Participants refrained from consuming all dairy products for two weeks before each intervention and following each intervention for 2 weeks and a 2 weeks washout period separated interventions. Faecal calprotectin (FC), high sensitive C-reactive protein (hs-CRP), interlukin-6 (IL-6), interlukin-10 (IL-10), bacterial composition, gastrointestinal symptoms and faecal metabolome were measured at each study visit. Breath hydrogen (H_2) and methane (CH_4) concentration were measured after each intervention arm.

Results:

No significant effects were seen after consuming both milks on FC concentrations (P = 0.632) and a slight decrease in hs-CRP, which was significantly (P = 0.043) greater during A2 milk group (1.06 ± 0.21 - 0.68 ± 0.13 mg/l) relative to A1/A2 milk group (1.10 ± 0.14 - 0.98 ± 0.13 mg/l). Stimulated ex-vivo cytokine concentrations had no effects on IL-6 concentrations (P = 0.424) after consuming both milk but A2 milk had a tendency to lower the anti-inflammatory cytokine IL-10 (P = 0.076). No significant differences between the two milks in the incremental area under the curve (IAUC) response for breath hydrogen (H_2) and methane (CH_4) concentration. 24-h symptoms recorded after the 250 ml milk challenge showed a tendency for A2 milk to lower bloating and abdominal cramps (P = 0.069, 0.085) respectively. A2 milk increased

actinobacteria and stool frequency significantly ($P = 0.027$). Both milk types had no effect on faeces metabolome.

Conclusion:

This study showed a differential impact of milk containing only A2 β-casein variant on systemic inflammation, gastrointestinal function and microbiota composition, but no effect on a local gut inflammation marker in healthy adults with mild to moderate non-lactose milk intolerance. This trial was registered at clinicaltrials.gov as NCT03060395.

Keywords: milk, A2 β-caseins variant, BCM-7, inflammation, gastrointestinal function, microbiota, breath test.

3.2 Introduction

Casein proteins represent about 80% of total protein in cows' milk, about 36% of which is β-casein. β-casein can be the result of different allele variants in the animal leading to different proportions of A1, A2, B and C β-caseins (Fox, 2003). In UK milk, the A2 variant has been shown to be present in the highest proportion (58%) followed by the A1 variant (31%)(Givens et al., 2013). Dairy cows can be either homozygous (A2/A2 or A1/A1) or heterozygous (A1/A2) genotypes. Most UK milk contains a mixture of A1/A2 protein variants. However, commercial A2 milk contains only the A2 variant produced from animals selectively bred to be A2/A2 homozygotes (Woodford, 2007). The main difference between A1 and A2 β-casein variants is in position 67 where histidine in A1 is replaced by proline in A2. As a result of the A1 mutation, the enzymatic digestion of the A1 β-casein releases the β-casomorophin-7 (βCM-7) peptide but this is not possible with the A2 variant (Jinsmaa and Yoshikawa, 1999). The βCM-7 peptide has opioid characteristics which have been suggested to play a role in inflammation system and gut health including effects on the gut microbiota (Tuohy et al., 2015). A possible mechanism of action of βCM-7

may be through its ability to bind to μ-opioid receptors in the gut and stimulate myosin production (De Noni et al., 2009).

Animal studies investigating the effects of A1 versus A2 β-casein on the GI tract are limited (Barnett et al., 2014, Haq et al., 2014b) but suggest that A1 β-casein has a pro-inflammatory activity locally and systemically. In humans, three studies (Ho et al., 2014, Jianqin et al., 2016, He et al., 2017) conducted in subjects from two different populations (Australia and China) reported an association between gastrointestinal dysfunction and A1 β-casein milk with the milk containing A1 β-casein leading to significantly increased stool consistency values, significant positive association between abdominal pain and stool consistency, delay in transit time, elevated inflammation-related biomarkers and immune response. The H_2 and CH_4 breath test is a non-invasive and widely used method to assess lactose digestion. This method can be used to select non- lactose intolerant participants based on H_2 and CH_4 concentration resulting from the fermentation of lactose. In addition, FC and cytokines produced from monocytes are known as markers to reflect local gut inflammation whereas CRP is considered to be a marker for systemic inflammation. Activation of monocytes produces pro-inflammatory cytokines such as IL-6 as well as anti-inflammatory cytokines such as IL-10. Calprotectin is a calcium binding protein that is predominantly found in neutrophils, monocytes and macrophages (Konikoff and Denson, 2006). Normally, the concentration in faeces is higher (~6 times) than in normal plasma and it is stable in faeces and resistance to proteolytic degradation. In adults, FC concentrations higher than 50μg/g are indicative of gut inflammation (Konikoff and Denson, 2006).

The research studies noted above have a number of limitations including enrolling non-milk consumers, using the urinary galactose method for assessing lactose

intolerance and use of subjective methods such as self-diagnosed of milk intolerance to screen out participants and using milk containing A1/A1 β-casein (not available in the supermarket) which limit the ability to arrive a firm conclusions. Therefore, the present study was designed to determine whether substitution of A1/A2 milk with A2 milk influences inflammation, gut microbiota composition, gastrointestinal function and symptoms using local and systemic inflammatory markers, hydrogen and methane breath tests, and microbial effects.

3.3 Materials and Methods

3.3.1. Subjects

Thirty-eight healthy adults (7 male, 31 female), aged between 19 to 65 y, who were regular milk consumers were recruited from the local Reading area. Subjects were considered suitable if they met the inclusion criteria:

- Normal liver and kidney function
- Not anaemic, haemoglobin: ≥ 130 g/L (for women ≥ 115 g/L)
- Fasting total cholesterol <7 mmol/l and TAG < 4 mmol/l,
- Not diabetic
- Not hypertensive 120/80 mmHg (blood pressure <160/90 mmHg can be accepted).
- Not underweight or morbidly obese (BMI >35 kg/m^2)
- Self-reported intolerance to commercial milk.
- Not lactose intolerance or have a known dairy allergy.
- Not pregnant or planning to be a pregnant and lactating during the proposed study period

- Not taking any medication, supplements or medicines for cardiovascular or metabolic disease, or other dairy products including acidophilus milk
- Not taking antibiotics within the previous six months
- Not suffering from any gastrointestinal disorders or gastrointestinal disease.
- Not planning or on a regime to lose weight.
- Not enrolled in another interventional clinical research study while participating in this study.
- Fully aware of the nature, objective, benefit and the potential risks and side effects of the study.

Ethical approval for conducting the trial was given by The University of Reading Research Ethics Committee (Project No. 16/50: Comparison of the effect of milk containing A2/A2 β-casein variant vs milk containing both A1/A2 β-casein on inflammation and gastrointestinal tract of volunteers with mild to moderate non-lactose milk intolerance, details are in appendix (P 200) at the end of this thesis, and was registered at clinicaltrials.gov (NCT03060395). The study was conducted according to the guidelines laid down in the Declaration of Helsinki and its subsequent updates.

3.3.2. Recruitment

Potential subjects who responded to advertisements were contacted and sent a participant information sheet and medical and lifestyle questionnaire. Volunteers who were interested, completed the questionnaire either online or with guidance over the telephone and sent it back for evaluation. Potentially suitable subjects who met inclusion criteria were then invited for a screening visit at the Hugh Sinclair Unit of Human Nutrition in the Department of Food and Nutritional Sciences, University of Reading, UK. A flow chart of study recruitment for the study is shown in Figure 1.

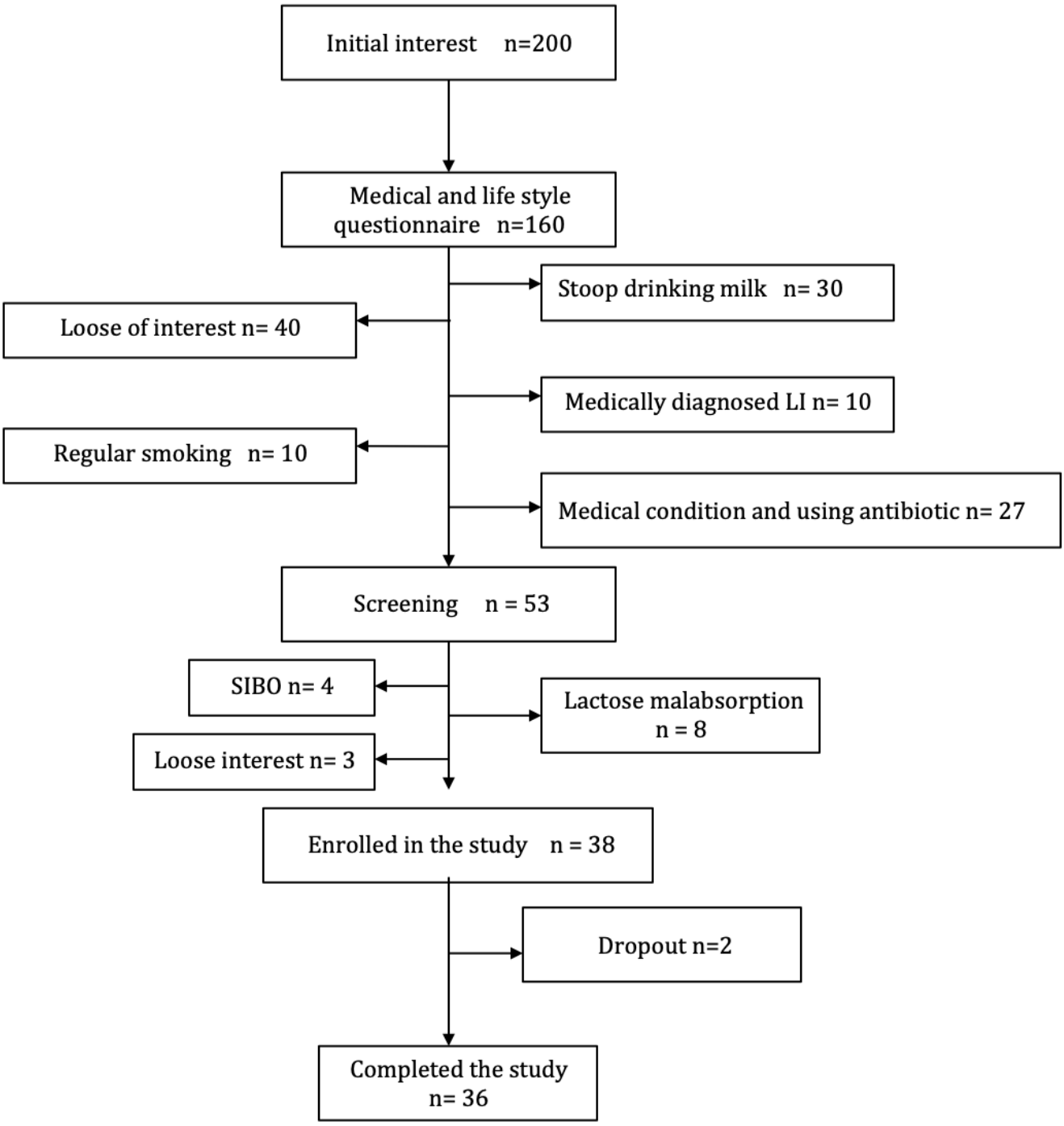

Figure 3.1. Flow of recruitment, screening and running the intervention study (DIGI). LI, Lactose Intolerance; SIBO, Small Intestinal Bacterial Overgrowth.

3.3.3. Screening

The screening visit was performed in the morning and participants were asked to be fasted, not eating or drinking anything but water, from 8 pm the night before. During this visit, the study was explained in great detail before subjects provided written informed consent. Measurements including height, weight and blood pressure were performed before a fasting blood sample was collected into a 5 ml serum separating tube and a 4 ml K2EDTA vacutainer. The serum separating tubes were used to analyse fasting blood glucose and lipids (TC, TAG, HDL-C) and markers of liver and kidney function enzymes (serum creatinine, total bilirubin, uric acid, alkaline transferase, alkaline phosphatase and γ-glutamyl transferease) using the ILAB 600 (Werfen UK Limited, Warrington, UK). The 4ml K2EDTA was sent to the Pathology Department of the Royal Berkshire Hospital (Reading, UK) to perform a full blood count.

A methane and hydrogen breath test was then performed using the GastroCH_4ECK gastrolyser (Bedfont, Scientific Ltd, UK) to screen out lactose intolerant subjects. In preparation for the screening visit and breath test, participants were instructed not to consume alcohol for 12 hours prior to the test, with only water to drink during this time and to avoid slowly digesting foods such as beans the day before the test. A baseline breath measurement was taken before the administration of substrate (25g of lactose dissolved in 250 ml of water). Following consumption, hydrogen and methane were measured in breath at 0, 15, 30, 60, 90, 120, 150 and 180 min (3 h). Compared with baseline, subjects with an increase in breath hydrogen concentration of ≥20 part per million (ppm) and breath methane concentration of ≥12ppm were classified as lactose intolerant. Subjects whose hydrogen concentration increased < 20 ppm and

breath methane concentration <12 ppm from the baseline were classified as lactose tolerant. Furthermore, in order to determine whether an individual could be defined as having mild to moderate symptoms of lactose intolerance resulting from consumption of the lactose drink, a rating scale dependent on symptoms (gases, bloating, cramp, headache, diarrhoea, nausea, constipation and rash) was distributed and completed during the screening visit. Subjects were then classified by the number and severity of symptoms experienced during the screening visit. Each symptom was rated according to the severity of symptoms graduated from not severe to very severe (see appendix [P 235]). Subjects were then asked to complete a symptoms questionnaire for 24 h following post lactose ingestion and send the questionnaire back by email or as a picture using their mobile phone. During the screening visit, mood and cognitive function was performed in an empty and quiet clinical room with a sign on the door to control any external noise to familiarise volunteers with the tasks that run on actual study visits. Subjects who fully met the inclusion criteria where then invited and scheduled to take part in the study.

3.3.4 Familiarisation visits

Before starting the study, subjects were invited to the Hugh Sinclair Unit of Human Nutrition and provided with a handbook giving details on how to prepare themselves before and during the study visits. This also described how to collect the 24 h urine sample prior to each visit and how to collect the stool sample on the day of the study visit.

3.3.4 Study design and actual study visits

The trial was a double blind randomised crossover dietary intervention study (Figure 2). Fourteen days prior to the start of the study, subjects were asked to exclude dairy products from their habitual diet (first washout) and advised to consume dairy alternatives. Instructions were given to control the overall dietary energy intake and match this intake with the intervention products incorporated into the diet during the first and second arms. After the washout period, baseline measures were taken at visit 1 with same measures at visits 2, 3, 4 and 5. Subjects were provided with intervention milk and a measuring cup and advised to follow the instructions written on the label of the milk container (See appendix P 238-239) for an example of the milk label). Milk was to be consumed twice a day and the amount increased gradually over the intervention period as follow; days 1 and 2: 100 mL, days 3 and 4: 150 mL, days 5 and 6: 200 mL, days 7 until 14: 250 ml. During each of the two intervention periods, volunteers completed a 3-day weighed food diary and kept a daily record noting the number of bowel movements and the average consistency of the stools using the Bristol stool chart (hard, solid, loose or watery), as well as the occurrence of abdominal discomfort, flatulence and bloating. Furthermore, the daily record form also enabled participants to report any adverse effects experienced during the study as well as any medication taken. During each study visit, anthropometric (height, weight, BMI), % body fat composition, clinical blood pressure (BP), heart rate (HR) and cognitive tasks were performed. A fasting blood, fresh stool and 24 h urine sample were collected, and questionnaires assessing mood and cognitive function were completed. In addition, after each 14 day intervention period, a 3 h methane and hydrogen breath test was performed after the ingestion of 250 ml of the assigned intervention milk, and symptoms (gases, bloating, cramp, headache, diarrhoea, nausea, constipation and rash) rated using a scale sheet. Furthermore, Bristol stool

chart and questionnaires to assess gastrointestinal symptoms (abdominal pain, intestinal bloating, flatulence) were completed during each intervention period.

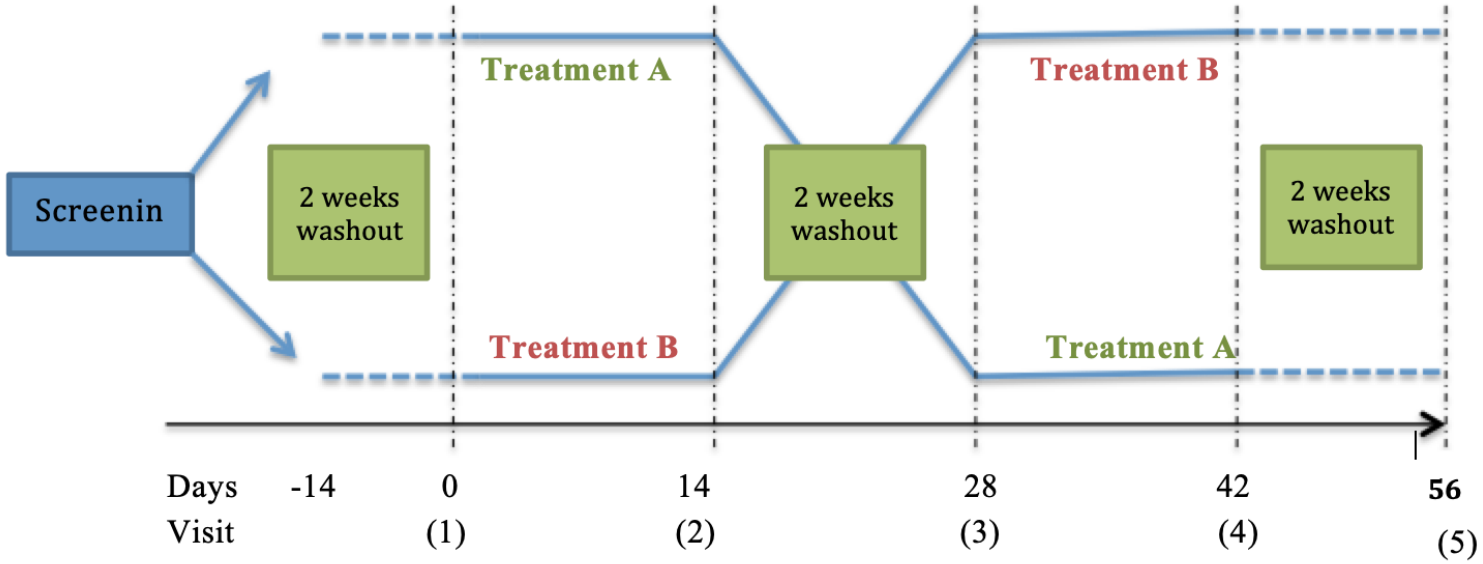

Figure 3.2. Overview of the intervention study design for the randomized double blind crossover study. Treatment A and treatment B refers to intervention milk either A1/A2 milk or A2/A2 milk. Amount of milk was consumed twice a day and milk amount was increased gradually (days 1 and 2: 100 mL, days 3 and 4: 150 mL, days 5 and 6: 200 mL, days 7 until 14: 250 mL) during the intervention period (14 days).

3.3.5. Diet diary analysis

Volunteers were asked to complete a 3-day diet diary to assess under-reporting during each of the intervention arms. This included all food and drink consumed during two-week days and on one weekend day. All food diaries were analysed with Dietplan6 software (Forestfield Software LTD) for macro and micronutrients based on the UK food database (McCance & Widdowson 7th edition).

3.3.6. Intervention products composition analysis

Samples of fresh milk used during each of the intervention periods were collected over several months between August 2017 and May 2018 and analysed for their nutritional composition. Milk components including proteins, fat, lactose and content of solids were measured using a milk product analyser Lactoscope Cn-2.3 (Delta Instruments, Netherlands) The micronutrient composition (calcium, iodine, phosphorus) was analyzed in 25 g of freeze dried intervention milk by Campden BRI laboratories, UK using their TES-AC-686 method with the extract then analysed by an inductively coupled plasma-mass spectrometer instrument. Quantitation of the β-casein protein variants analysis followed the protocol of Givens et al (2013). 1ml of semi skimmed milk from A1/A2 and A2/A2 variant were mixed with 1ml of an 8 M urea buffer. The mixtures then were left to stand for approximately 1 hour at room temperature, followed by centrifugation at 1200g for 5 min at 4°C. The upper fat layers were removed and then 0.5 ml from the middle of the lower aqueous layers that contains caseins was diluted to 2ml with distilled water (final solution). The final solutions were filtered through 0.45 μm Millipore syringe filter into an Eppendorf tube prior to injection of a 5 μl aliquot into the HPLC. Separation of the milk proteins by LC/MS was done according to a previous published method (Givens et al., 2013)

that used an Agilent 1100 binary gradient HPLC system (Agilent Technologies UK Ltd., Wokingham, UK) with a temperature-controlled column oven and cooled automatic liquid sampler. The column used was A C18 reversed phase column (150 mm long × 2.1 mm internal diameter) with 30 nm pore size and 5 μm particle size. Mobile phase used was consisting of A: a solution of 0.01% trifluoroacetic acid (TFA) in HPLC grade water (Rathburn Chemicals Ltd., Walk- erburn, UK) and mobile phase B, 0.01% TFA in far UV HPLC grade acetonitrile (Rathburn Chemicals Ltd., Walkerburn, UK) (Givens et al., 2013).

3.3.7. Faecal samples collections and processing

A fresh faecal sample was collected on the morning of each study visit into a pot kept under anaerobic conditions (Oxoid, AnaeroGe, 2.5L Sachet, 10269582, Fisher Scientific, UK) and kept for a maximum of 2 h until processing. To measure faecal calprotectin, 16S rDNA (gut microbiome) and metabolites, approximately 3 g of fresh sample was collected and stored at -80 ˚C. In order to measure the faecal water content, 2 g of fresh sample was weighted before and after being dried for 48 h at 70 ˚C.

3.3.8. Collection, processing and storage of blood samples

On each study, blood was collected using a safety blood collection set and holder (23G or 21G) from (Greiner bio-one Ltd, UK, 450086 or 450085) into a 5 ml serum separator clot activator tube (Greiner bio-one Ltd, UK, 456010), 2x 4 ml K2EDTA tubes (Greiner bio-one Ltd, UK, 454023). Immediately tubes were inverted 8-10 times after collection. One EDTA tube was sent to The Royal Berkshire Hospital for full blood count and the serum separator tube was left at room temperature for 20 min

before centrifugation. Then the serum tubes were centrifuged at 3000 rpm (1700 x g) for 15min at 4°C. The EDTA tube was left on ice until whole blood culture was performed as described below.

3.3.10. Faecal calpretectin analysis

Faecal calprotectin concentration was measured using the CALPRO ELISA kit based on polyclonal antibodies to human calprotectin. (Calpro AS, Oslo, Norway, CAL0100). Following the manufacturer's instructions, 100 mg of stool samples were homogenized with 4.9 ml of extraction buffer. This mixture was left on a shaker at 1000 rpm for 30 min, then left to stand on the bench for couple of minutes to settle. The supernatant that represented the extract (1:50) was further diluted (1:100) with the sample diluent. Fifty µl of provided standards and control as well as diluted samples were added to a 96 well plate (in duplicate) and incubated with a plate cover on a horizontal plate shaker at 650 rpm for 45 min at room temperature. After this incubation step, several washing steps were performed and then 50 µl of enzyme conjugate was added followed by incubation at room temperature for 45 min at 650 rpm with a plate cover. The plate was then washed before addition of 100 µl of the enzyme substrate solution and the plate was incubated at room temperature for 25 min protected from the light. The optical density (OD) was read at 405 nm using an ELISA reader that calculates FC concentration automatically. FC concentrations were also corrected for water content.

3.3.11. C- reactive protein analysis

CRP concentration was quantified by using autoanalyser ILAB 600 (Werfen (UK) Ltd, Warrington, UK) using kits supplied by Werfen. Each batch of samples was run

with the quality controls and all samples for each subject were analysed within a single batch.

3.3.12. Whole blood culture stimulation for cytokines analysis

This analysis followed the protocol published by Koutsos et al. (2014) . Blood samples collected into a K2EDTA tube were diluted 1:9 with RPMI1640 medium (Sigma, UK) containing 1% L-glutamine and 1% non- essential amino acid (MEM) (Sigma, UK). Diluted blood samples were cultured in 12 well plates with 0.5 µg/ml of LPS (bacterial lipopolysaccharide, *E.coli* 026:B6, Sigma, UK) and incubated at 37°C for 24 h followed by centrifugation for 5 min at 1000 rpm (700 x g). The supernatant was collected and then stored at -20°C until cytokine analysis. The full blood count including monocyte number was measured in the second EDTA blood sample by the Pathology Department at the Royal Berkshire Hospital, Reading, UK.

3.3.13. Measurement of cytokines concentration using Luminex method.

Cytokine concentrations were measured by a human high sensitive cytokine A premixed magnetic Luminex performance assay measuring IL-10, IL-6 and IL-4 concentrations (R&D System Europe Ltd) using Luminex 200 (Thermo Fisher Scientific). Whole blood culture supernatant samples were diluted 1:2 with the RD6-40 calibrator diluent provided by the kit prior to analysis according to the manufacturer's instructions. Cytokine concentrations were presented as $\mu g/10^3$ monocytes, as reported by Koutsos et al. (2014).

3.3.14. Breath hydrogen and methane test

Hydrogen and methane concentrations were evaluated as a measure of colonic lactose fermentation. Hydrogen and methane were measured after each intervention period at visit 2 and 4. Participants were asked to be prepared for the breath test as they were for the screening visit mentioned previously. In this test participants consumed 250 ml of the milk that they had consumed in the previous intervention arm.

3.3.15. Daily gastrointestinal function and symptoms recorded

Volunteers were asked to complete a 14-day diary to record gastrointestinal function and symptoms during each of the interventions arms. Stool consistency measured by using Bristol score that was provided with the diary. GI symptoms were scaled to 0, 1, 2 and 3 that reflect none, mild, moderate and severe then averaged for each participant.

3.3.16. Microbiota composition using 16S rDNA

Fecal samples for 16S rDNA profiling of the gut microbiome were sent to and analysed by clinical microbiomics (Denmark).

3.3.16.1 DNA extraction

DNA was extracted from faecal samples using NucleoSpin® 96 Soil (Macherey-Nagel). Bead beating was done on a Vortex-Genie 2 horizontally for 5 min at level 9. A minimum of one negative control was included per batch of samples from the DNA extraction and throughout the laboratory process (including sequencing). A ZymoBIOMICSTM Microbial Community Standard (Zymo Research) was also included in the analysis.

3.3.16.2 Normalization and sequencing

Products from the nested PCR were pooled based on band intensity and the resulting library cleaned with magnetic beads. The DNA concentration of pooled libraries was measured fluorometrically. Sequencing was done on an Illumina MiSeq desktop sequencer using the MiSeq Reagent Kit V3 (Illumina) for 2x 300 bp paired-end sequencing.

3.3.16.3 Bioinformatics analysis

The 64-bit version of USEARCH 10.0 (Edgar 2013), mothur 1.38 (Schloss et al. 2009), and in-house scripts were used for bioinformatics analysis of the sequence data. Following tag identification and trimming, sequences were trimmed at QS 10 and merged requiring a minimum overlap of 20 bp and a merged length of 400–500 bp. Sequences with ambiguous bases, without perfect match to the primers, homopolymer length greater than 10, or more than one expected error were discarded and primer sequences trimmed. Sequences were strictly dereplicated, discarding clusters smaller than 5. Sequences were clustered at 97 % sequence similarity with USEARCH's -cluster_otus command, using the most abundant strictly dereplicated reads as centroids and discarding suspected chimeras based on internal comparison. Taxonomic assignment of OTUs was done using SINTAX with a cutoff value of 0.8 (Edgar, 2016) against RDP training set v16 (Cole et al. 2014). All analyses, except for calculation of Shannon index, were done on rarefied data.

3.3.17. Metabolic profiling by ^{1}H nuclear magnetic resonance

3.3.17.1. Faecal Analysis

For assessment of faecal metabolites samples were prepared fresh for analysis on the same day and randomly run in batches to avoid batch differences. Faeces samples (200 mg) were mixed with 2 glass beads (3mm) (VWR international merck, 1.04015.0500) and 800 µl of NMR buffer (Sodium Phosphate buffer 0.2M, pH7.4 + TSP 1mM in 80% D_2O, 20% H_2O) (3-(trimethylsilyl)propionic acid-d_4, Sigma-Aldrich, 269913; Sodium Phosphate Monobasic Dihydrate, Sigma-Aldrich, 71505; Sodium Phosphate Dibasic Anhydrous, Sigma-Aldrich, S7907; Deuterium Oxide 99.9%, Sigma-Aldrich, 530867). This mixture was homogenized with the tissue-lyser for 5 min at 25 Hz (Qiagen). Then homogenized samples were centrifuged for 10 min at 4°C at 13000 x g. Supernatants (600 µl) were then transferred to 5mm NMR tubes for analysis by NMR spectroscopy.

3.3.17.2. Metabolic profiling by ^{1}H NMR nuclear magnetic resonance

NMR assessments on faeces samples were carried with the use of Bruker AV700 NMR Spectrometer equipped with a 5mm $^1H(^{13}C/^{15}N)$ inverse Cryoprobe®. All experiments were recorded at 300K by ^{1}H-1D NOESY-PRESAT (noesypr) using water signal suppression. For each spectrum, 8 dummy transients were followed by a total of 256 scans, with a relaxation delay (RD) of 2s and acquisition time (AQ) of 1.5s. Scans were accumulated in 64k data points with a spectral width of 9803.9 Hz. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening. Spectra were referenced to the single peak of trimethylsilylpropanoic acid (TSP). All spectra were manually phased and automatically baseline corrected applying a Whittaker smoother algorithm in MestreNova NMR version 10.0.1 (Mestrelab Research). The residual water signal was manually deleted.

3.3.18. Statistical analysis

The primary outcome measure was faecal calprotectin and a power calculation indicated that a total of 45 subjects were needed for this two-treatment crossover study. The probability is 80 % that the study will detect a treatment difference at a two-sided 0.05 significance level, if the true difference between treatments is 80.0 units. This is based on the assumption that the standard deviation of the difference in the response variables is 186, which was observed by Ho et al. (2014). It was decided to over recruit by 10% to allow for drop-outs. All statistical analyses were performed using IBM SPSS statistical software version 24. Normality tests were performed for all outcomes. Data that were not normally distributed, were transformed using Log10 and then checked for normality. Data that could not be normalized were processed with a nonparametric test (Mann-Whitney test). The effect of the treatments was evaluated by performing a linear mixed model analysis adjusted for baseline values, gender, age and BMI. $P \leq 0.05$ was considered significant. Data are presented in texts, tables and figures as mean ± SEM.

For metagenomic data, the alpha diversity of the samples was assessed by counting the number of OTUs (operational taxonomical units, similar to species richness) and calculating the Shannon index. The Shannon index takes not only the number of OTUs of a community into account, but also the relative abundance of the OTUs. The beta diversity is a measure of dissimilarity in the taxonomic composition between samples and this was measured by using generalized UniFrac with $\alpha = 0.5$ (Chen et al. 2012). The calculated generalized UniFrac distances were transferred to a 2D plot using principal coordinates analysis (PCoA). The change in relative sequence abundance at OTU, genus, and class level from start to end of treatment was calculated for all subjects and treatments. The effect of treatment was then tested with a linear mixed effect model (using treatment, group, and their interaction as explanatory variables and stratifying data by subject ID). Only taxa which showed a

change in abundance in ≥20 % of subjects were analyzed (290 OTUs, 72 genera, and 16 classes).

For metabolomic data, spectra were digitalised and imported into Matlab, where they were normalised under total area and unit variance (UV) scaled. Principal Component Analysis (PCA) was performed to detect metabolic group variations, detect possible outliers and clustering of groups under investigation. Data were further analysed using orthogonal projection to latent structure-discriminant analysis (O-PLS-DA) with 0 or 1 orthogonal components where ^{1}H-NMR spectroscopic profiles were used as a matrix of independent variables (X) and treatment, time and biochemical measurements as response vectors (Y). Changes from baseline based on the treatment were measured by subtracting the matrix of independent variables at baseline from the values measured after treatments. The evaluation of the quality of statistical models was based on the values of R^2Y (goodness of fit: percentage of Y explained by the model) and Q^2Y (the goodness of prediction: percentage of Y predicted after 7-fold cross validation). This evaluation was completed by visual observation of plots of the scores (T) compared with cross-validated scores (Tcv).

3.4. Results

3.4.1. Classification of volunteers after the screening visit according to their lactose tolerance.

After screening subjects using the lactose containing drink, they were classified into three groups comprising of lactose digesters (n=38), lactose malabsorption (n=8) and Small Intestinal Bacterial Overgrowth SIBO (n=4). The mean concentrations of both hydrogen and methane for the participants classified as lactose digesters are shown in

Figure 3.3A. This pattern of response after the lactose drink was considered negative because the increase of both hydrogen and methane from baseline was less than 20 ppm and 12 ppm respectively (Rezaie et al., 2017, Simrén and Stotzer, 2006b). The mean fasting breath hydrogen and methane concentrations were 5.7 and 9.4 ppm respectively. Both gases reached the first peak concentration at 60 min post-ingestion of the lactose challenge. Symptoms recorded 24 h after the lactose challenge are presented in Figure 3.4. The predominant symptoms were gases, bloating and abdominal cramps. Of these, 57% of participants experienced mild bloating, 32 % suffered from moderate gases and 11% experienced mild and moderate diarrhoea (Figure 3.4). No high percentage of symptoms such as headache, constipation and rash needed to be considered among participants. The data for lactose mal-digesters and SIBO participants who were excluded from the study are shown in Figure 3.3B and 3.3C. In lactose mal-digesters the mean concentrations of hydrogen started to increase at 120 min and continue increasing. In SIBO the mean concentrations of hydrogen started to increase earlier between 30 and 60 min and decreased again and then increased later. The SIBO group was excluded from the study because they fermented the lactose dose faster than the normal subjects and they experience GI symptoms during the screening visit.

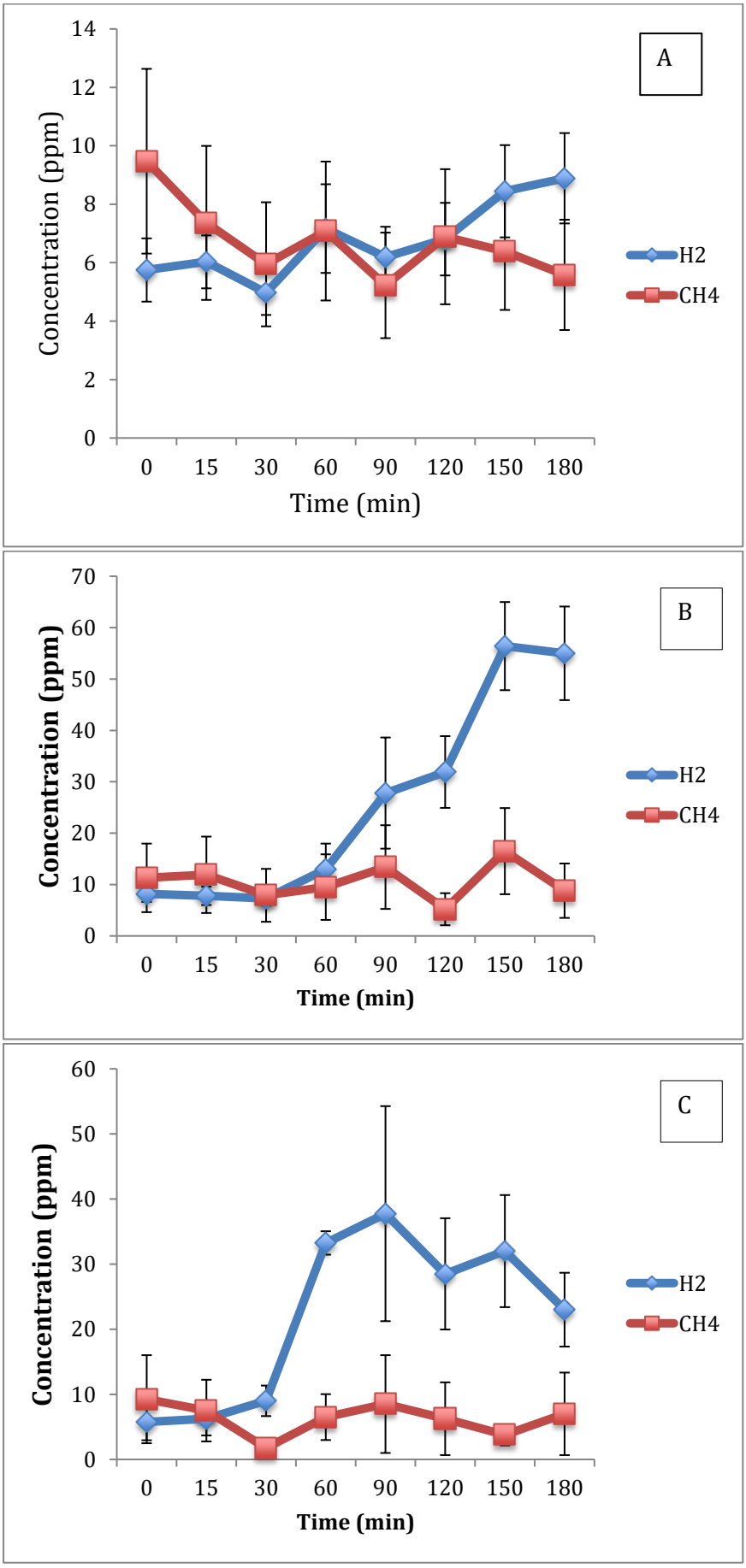

Figure 3.3. Breath hydrogen and methane response of (A) 38 participants after the ingestion of 25g lactose dissolved in 250ml water. (B) 8 mal-digester participants after the ingestion of 25g lactose dissolved in 250ml water. (C) 4 SIBO participants after the ingestion of 25g lactose dissolved in 250ml water Values are mean ± SEMs.

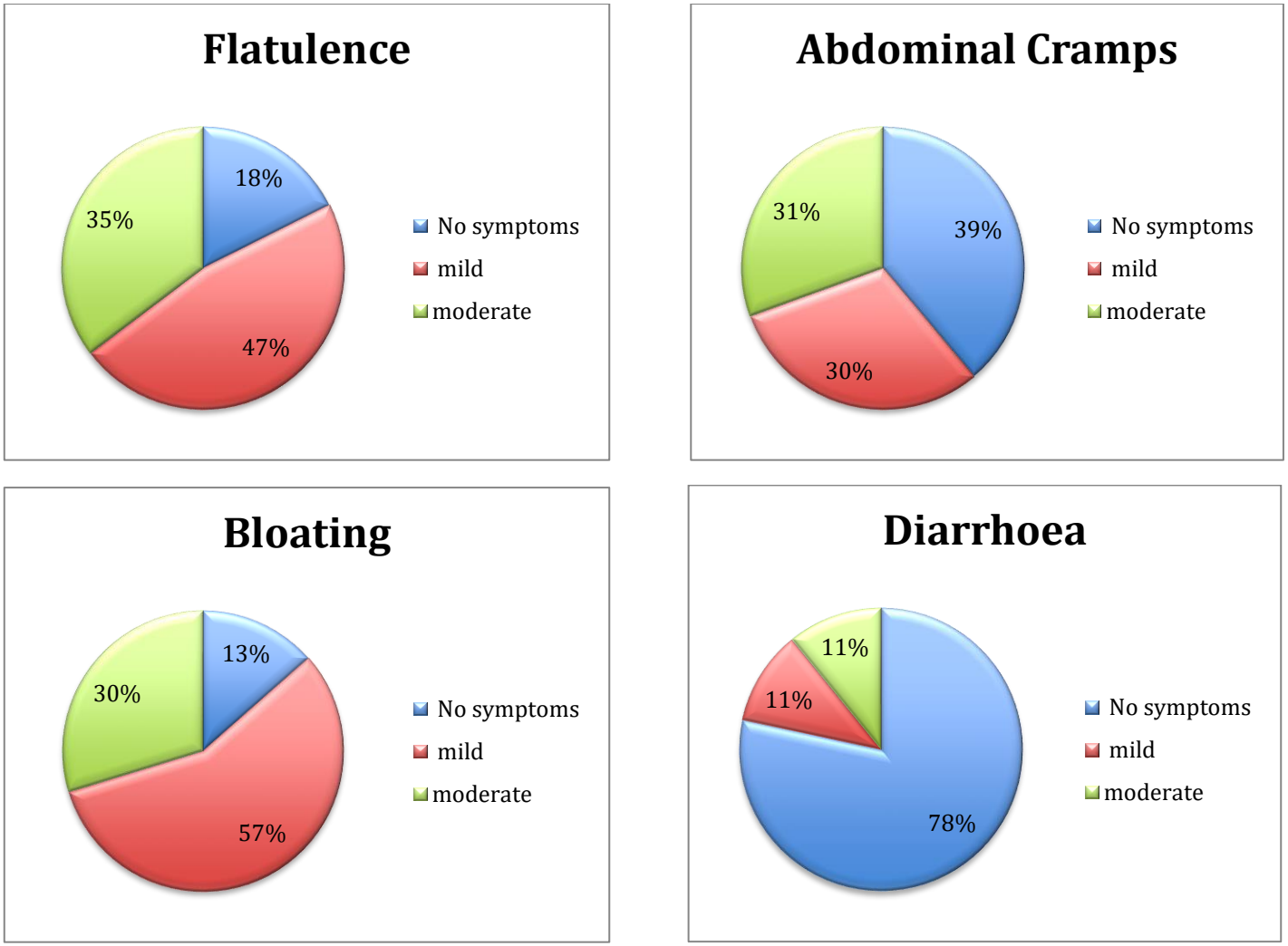

Figure 3.4. Symptoms recorded throughout 24h after the lactose challenge in the screening visit for lactose digesters subjects.

3.4.2 Compositional analysis of intervention products

The detailed compositional analysis of the intervention products is presented in Table 3.1. The total casein fraction of semi-skimmed A1/A2 milk (British conventional milk) contained 2/3 of A2 β-casein and 1/3 of A1 β-casein whereas the total casein fraction in A2 milk consists mostly of A2 β–casein. For micronutrient analysis, both A1/A2 and A2 milk powders contained similar amounts of calcium (mean 1033.0 vs 1066.0 mg/100 g), iodine (mean 4000 vs 4100 μg/kg) and phosphorus (mean 0.77 vs 0.78 g/100g, respectively). The normal range for iodine in milk powder is (1110.0 - 2050.0 μg/kg), calcium (1148.0 - 1341 mg/100g) and phosphorus (90 - 105 mg/100g).

3.4.3 Study population characteristics

Of the 38 volunteers who started the study, 36 completed all visits (seven males, 29 females). Two volunteers withdrew during the study (1 volunteer withdrew after visit 3 because of cold and flu and the other one withdrew after visit 1 because of relocation to another part of the country). Baseline characteristics of the completing participants (at day 0) are summarised in Table 3.2. The age of volunteers ranged from 18- 56 years (mean of 35 y) with a mean BMI of 24 kg/m^2. No significant differences between the two interventions were observed at baseline level in all baseline characteristic (P >0.05).

3.4.4 Dietary intakes

Dietary intakes during the intervention period are shown in Table 3.3. There were no significant differences during the interventions in terms of daily energy intake, carbohydrates, total fat, fibre, alcohol, and micronutrients including calcium, magnesium, phosphorus, iodine and vitamin D intakes (P >0.05). However, a significantly different intake of protein during the two intervention periods was found. The consumption of protein was significantly higher during A2 milk group compared with A1/A2 milk group (P=0.012). A tendency was observed for a difference in the consumption of total fat between milk interventions (P=0.057) (Table 3.3).

Table 3.1. Composition of intervention milks purchased from TESCO supermarket in Reading during August 2017-May 2018.

	Tesco commercial semi-skimmed milk					Tesco A2 semi-skimmed milk					
	Aug 2017	**Jan 2018**	**Mar 2018**	**Apr 2018**	**May 2018**	**Aug 2017**	**Jan 2018**	**Feb 2018**	**Mar 2018**	**Apr 2018**	**May 2018**
Fat g/100g	1.22±0.11	1.43±0.34	1.06±0.19	1.37±0.27	1.35±0.27	1.40±0.13	1.43±0.22	1.54±0.35	1.27±0.35	1.37±0.27	1.34±0.30
Protein g/100g	2.34±0.24	2.91±0.70	2.20±0.50	2.80±0.61	2.74±0.63	2.65±0.33	2.95±0.51	2.99±0.70	2.92±0.70	2.81±0.61	2.61±0.65
Lactose g/100g	4.28±0.73	4.30±0.80	3.34±0.80	4.18±0.99	4.20±1.00	4.15±0.83	4.26±0.81	4.17±0.90	4.17±0.99	4.18±0.95	4.05±1.05
Total solids g/100g	8.81±0.93	9.52±1.60	7.65±1.30	9.22±1.70	9.16±1.72	9.12±1.4	9.51±1.4	9.61±1.73	9.52±1.87	9.29±1.7	8.94±1.81
A1 β-casein[1] %	40	38	38	37	39	0	0	0	0	0	0
A2 β-casein[1] %	56	57	59	58	55	100	100	100	100	100	100
B[2] β-casein[1] %	4.58	6.39	3.34	5.62	6.38	0	0	0	0	0	0

Values are mean ± SEMs, n = independent samples each month (A1/A2 milk, A2/A2 milk) run in triplicate. Samples from the same brand and bought from the same supermarket (TESCO, Reading branch). [1] % of total casein protein. [2] B variant is now classified as an A1 type protein.

Table 3.2. Baseline characteristics of the participants

	Value
Total (male/female)	37 (7/30)
Age (y)	35.1 ± 1.8
BMI (kg/m^2)	24.0 ± 0.7
SBP (mm Hg)	115 ± 1.6
DBP (mm Hg)	70 ± 1.2
Total cholesterol (mmol/L)	4.76 ± 0.2
Triacylglycerol (mmol/L)	0.88 ± 0.1
Glucose (mmol/L)	5.15 ± 0.1

[1]BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. Values are Mean ± SEM

Table 3.3. Nutrient intake during two weeks consumption of either semi –skimmed A1/A2 milk or semi-skimmed A2/A2 milk [1,2].

	A1/A2 milk	A2 milk	*P* [4]
Energy (MJ/d)	8.3 ± 0.7	7.1 ± 0.7	0.143
Protein (%TE)[3]	16.8 ± 1.0	19.9 ± 1.0	0.012
Total fat (%TE)[3]	34.5 ± 1.6	32.0 ± 1.6	0.057
Carbohydrates (%TE)[3]	45.7 ± 1.9	47.6 ± 1.9	0.137
Dietary fibre (AOAC) g	20.7 ± 6.7	26.2 ± 6.8.2	0.496
Alcohol (%TE)[3]	2.1 ± 0.7	2.5 ± 0.9	0.810
Calcium (g/d)	1.0 ± 0.07	1.0 ± 0.07	0.479
Magnesium (g/d)	0.3 ± 0.02	0.3 ± 0.02	0.329
Phosphorus (g/d)	1.1 ± 0.1	1.0 ± 0.1	0.416
Iodine (µg/d)	156.6 ± 71.6	248.3 ± 72.2	0.252
Vitamin D (µg/d)	3.5 ± 0.6	3.5 ± 0.6	0.984

[1]Values are mean ± SEMs. n = 36. AOAC, Association of Official Analytical Chemists. [2]Differences between the two interventions were analysed with linear mixed model analysis, adjusted for baseline values, gender, age and BMI. [3] % of total energy intake. [4] P <0.05 considered significant from both test statistic.

3.4.5 Inflammation markers

Effects of the milk interventions on inflammatory markers are shown in Table 3.4. There were no significant effects of treatment on faecal calprotectin concentrations (*P* >0.05). However, a small decrease was observed in CRP during both interventions, which was significantly (P = 0.043) greater during A2 milk group (mean = 1.06 ± 0.21 to 0.68 ± 0.13 mg/l) compared with A1/A2 milk group (mean= 1.10 ± 0.14 to 0.98 ± 0.13 mg/l). Changes in CRP following the milk treatments are shown in Figure 3.5.

3.4.6 Ex vivo cytokines production

Plasma neutrophil, lymphocyte, monocyte numbers results are shown in Table 3.5. There were no significant differences in plasma concentrations of neutrophil, lymphocyte and monocyte numbers. Cytokines responses were corrected based on the monocyte count because cytokines are produced mainly from monocytes following LPS stimulation in whole blood. Ex-vivo cytokines concentration following LPS stimulation are shown in Table 3.5. Although, both treatments had no effects on IL-6 concentrations (*P* = 0.424), A2 milk had a tendency to lower the anti-inflammatory cytokine IL-10 compared with A1/A2 milk (P = 0.076; Table 3.5). IL-4 results were not included in this thesis because the concentrations for 80% of participants were below the level of detection (1.8 pg/ml). Only small proportion of data is available.

Table 3.4. Effect of daily consumption of semi-skimmed A1/A2 milk and semi-skimmed A2/A2 milk on markers of inflammation

	A1/A2 milk			A2 milk			
	Pre-treatment	**Post-treatment**	**Δ**	**Pre-treatment**	**Post-treatment**	**Δ**	***P*[1]**
FC mg/kg	16.49 ± 1.71	14.09 ± 1.79	-2.45 ± 0.80	18.62 ± 2.35	15.56 ± 1.89	- 2.71 ± 0.46	0.362
CFC[2] mg/kg	56.84 ± 7.11	49.62 ± 9.91	- 7.22 ± 2.80	55.58 ± 8.41	52.80 ± 7.84	-2.78 ± 0.57	0.505

Values are mean ± SEMs. n = 35. FC, Faecal Calprotectin; CFC, Corrected Faecal Calprotectin;. [1]Comparesonce between the two interventions were analysed with a linear mixed model analysis, adjusted for baseline values, gender, age, period and BMI. [2] CFC is the corrected means expressed on faecal dry weight. .

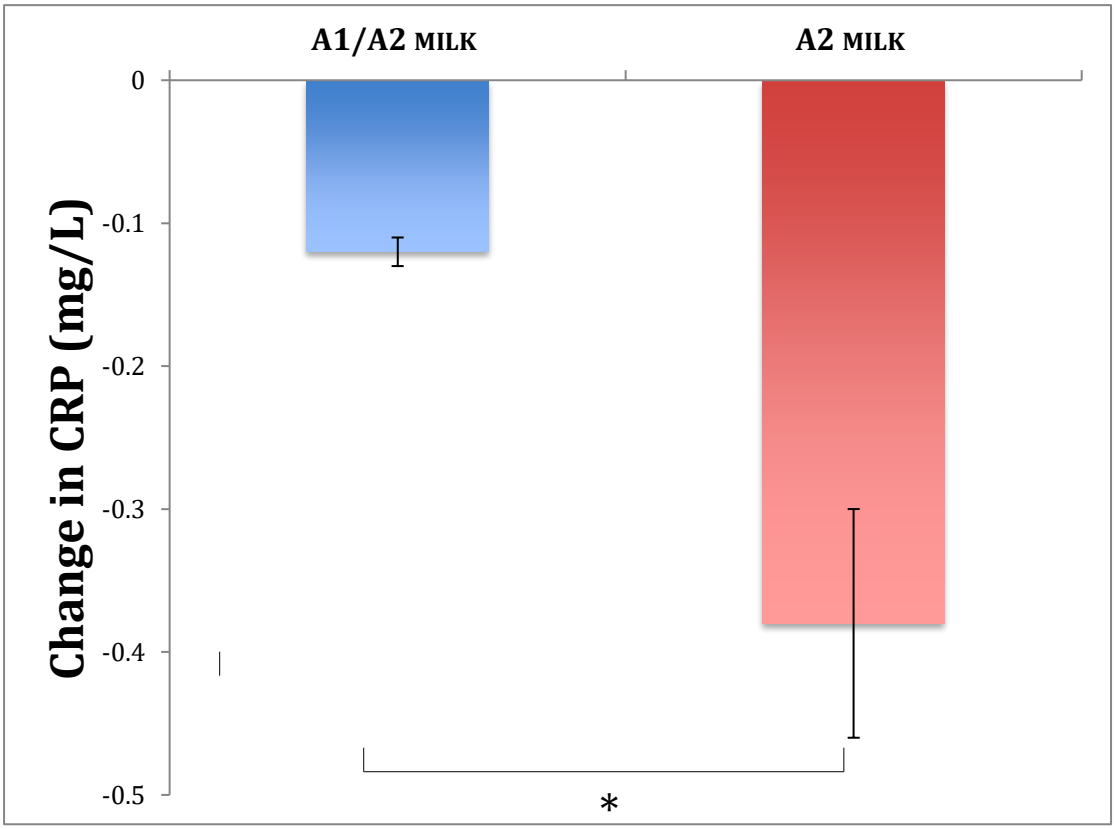

Figure 3.5. Mean change from baseline in hs-CRP concentration for A1/A2 milk and A2 milk. CRP, C-Reactive Protein. All data are expressed as mean ± SEMs. n = 35

Table 3.5. Plasma neutrophils, lymphocytes, monocytes and ex vivo cytokines production using whole blood culture

	A1/A2 milk			A2 milk			
	Pre-treatment	**Post-treatment**	**Δ**	**Pre-treatment**	**Post-treatment**	**Δ**	***P*[1]**
Neutrophils[2]	3.22 ± 0.25	2.95 ± 0.18	-0.27 ± 0.7	3.19 ± 0.19	3.29 ± 0.28	0.10 ± 0.09	0.332
Lymphocytes[2]	1.73 ± 0.07	1.74 ± 0.07	0.01 ± 0.00	1.67 ± 0.07	1.75 ± 0.07	0.08 ± 0.00	0.391
Monocytes[2]	0.41 ± 0.02	0.41 ± 0.02	0.00 ± 0.00	0.41 ± 0.02	0.40 ± 0.02	-0.01 ± 0.00	0.712
IL-10[3]	0.38 ± 0.04	0.39 ± 0.03	0.01 ± 0.01	0.43 ± 0.05	0.39 ± 0.04	-0.04 ± 0.01	0.076
IL-6[3]	53.98 ± 3.85	54.60 ± 3.39	0.62 ± 0.19	58.41 ± 2.64	57.32 ± 3.61	-1.09 ± 0.97	0.424

Values are mean ± SEMs. n = 35. IL-10, Interleukin-10; IL-6, Interleukin-6. [1]Comparesonce between the two interventions were analysed with a linear mixed model analysis, adjusted for baseline values, gender, age, period and BMI.[2] Monocytes count x 10^9/L. [3] µg x10^3.

3.4.7. Hydrogen and methane production after the milk interventions

Hydrogen and methane breath test was also done after each milk intervention period to identify the effect of milk on hydrogen and methane production. Data on the production of hydrogen and methane in response to the milk interventions are presented in Table 3.6 and Figure 3.6. There were no significant differences between the two treatments in terms of baseline measure, AUC and IAUC response for breath hydrogen and methane production ($P > 0.05$). Although, breath hydrogen and methane AUC were lower in response to A2 milk compared with A1/A2 milk, this difference was not significant.

3.4.8. Symptoms recorded during 24 h following consumption of milk interventions

Symptoms recorded 24h after each milk challenge (250ml) are presented in Figure 3.7. These figures compare the predominant symptoms experienced by participants after the consumption of 250 ml of each type of milk. There were no significant differences in the symptoms scores between the treatments. However, there was a tendency for A2 milk to lower bloating and abdominal cramps ($P = 0.069, 0.085$) respectively (Table 3.7). Of these, 45% of participants experienced moderate flatulence after A1/A2 milk compared with A2 milk (17%). However, 48% of participants experienced mild flatulence after the consumption of A2 milk. Similar results was recorded for moderate bloating after the consumption of A1/A2 milk and A2 milk (25% and 22%) respectively. However, about half of the participants experienced no symptoms of bloating after consuming A2 milk. Mild abdominal cramps were experienced by 41% of participants after consumption of 250 ml of

A1/A2 milk and 78% of participants had no symptoms of abdominal cramps after A2 milk. Less than 10% of volunteers experienced diarrhoea and constipation after the consumption of each intervention treatment.

Table 3.6. Effect of semi-skimmed A1/A2 milk and semi-skimmed A2/A2 milk consumption on H_2 and CH_4 concentration in breath

	A1/A2 milk	A2 milk	*P*[1]	A1/A2 milk	A2 milk	*P*[1]
	H_2	H_2		CH_4	CH_4	
Baseline ppm	6 (2,16)	5 (2, 18)	0.925	1 (0, 17)	0 (0, 1)	0.484
AUC[2]	682 (198, 2070)	622 (243, 1972)	0.882	90 (60, 1586)	90 (52,172)	0.602
IAUC [2]	-315 (-1413, 11)	-382 (-952, -93)	0.894	15 (-352, 78)	22 (-138, 78)	0.597

Values are median and quartile range. n = 31. AUC, Area under the curve; IAUC, Incremental area under the curve.
[1]Differences between the two interventions were analyzed with Mann-Whitney test. $P<0.05$ considered significant. [2] ppm x 180 min

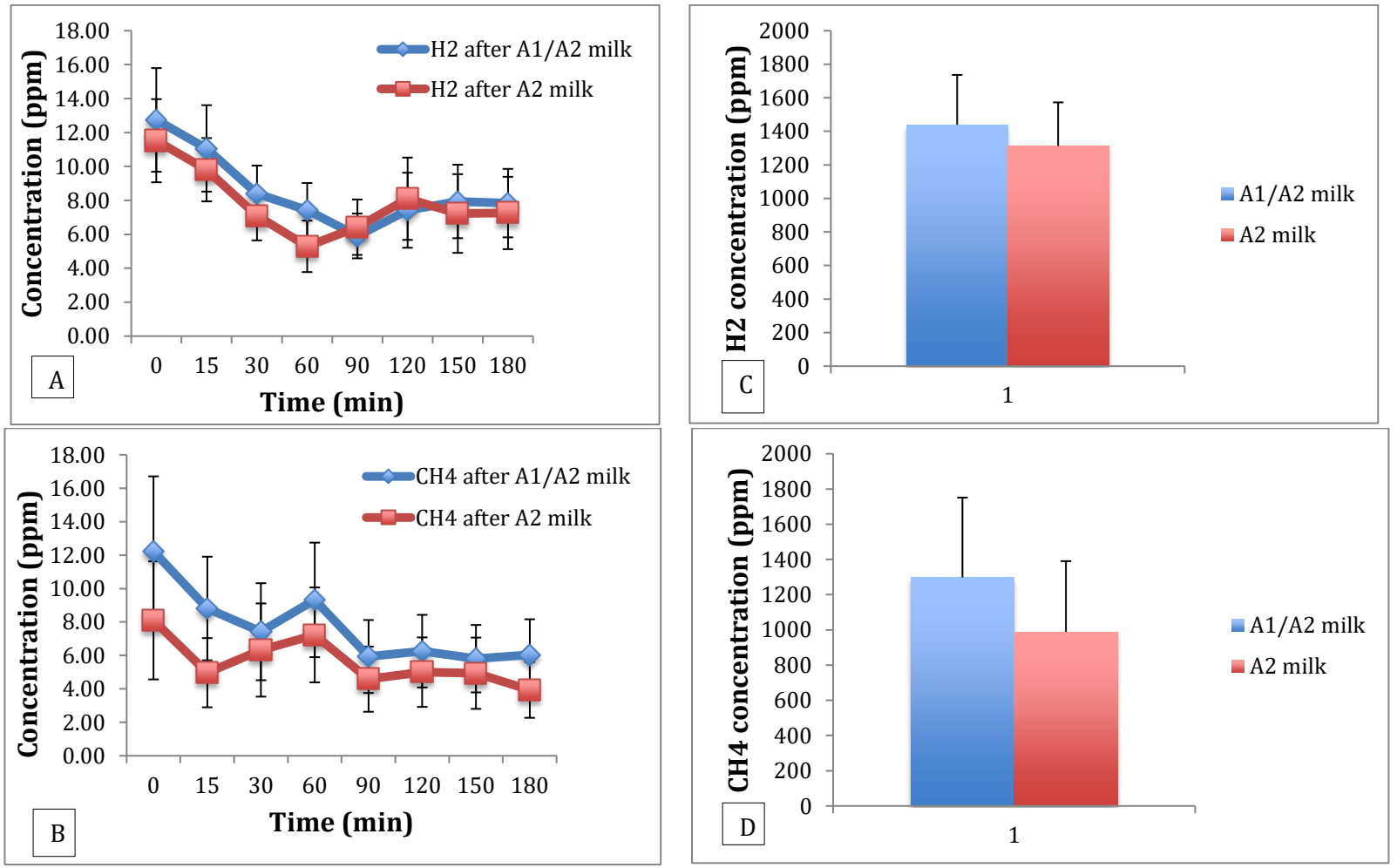

Figure 3.6. Breath A) hydrogen and B) methane production for 180 min after consumption of 250 ml of each milk intervention and AUC for the C) hydrogen and D) methane time response curves. All data are expressed as mean ± SEMs. n= 30.

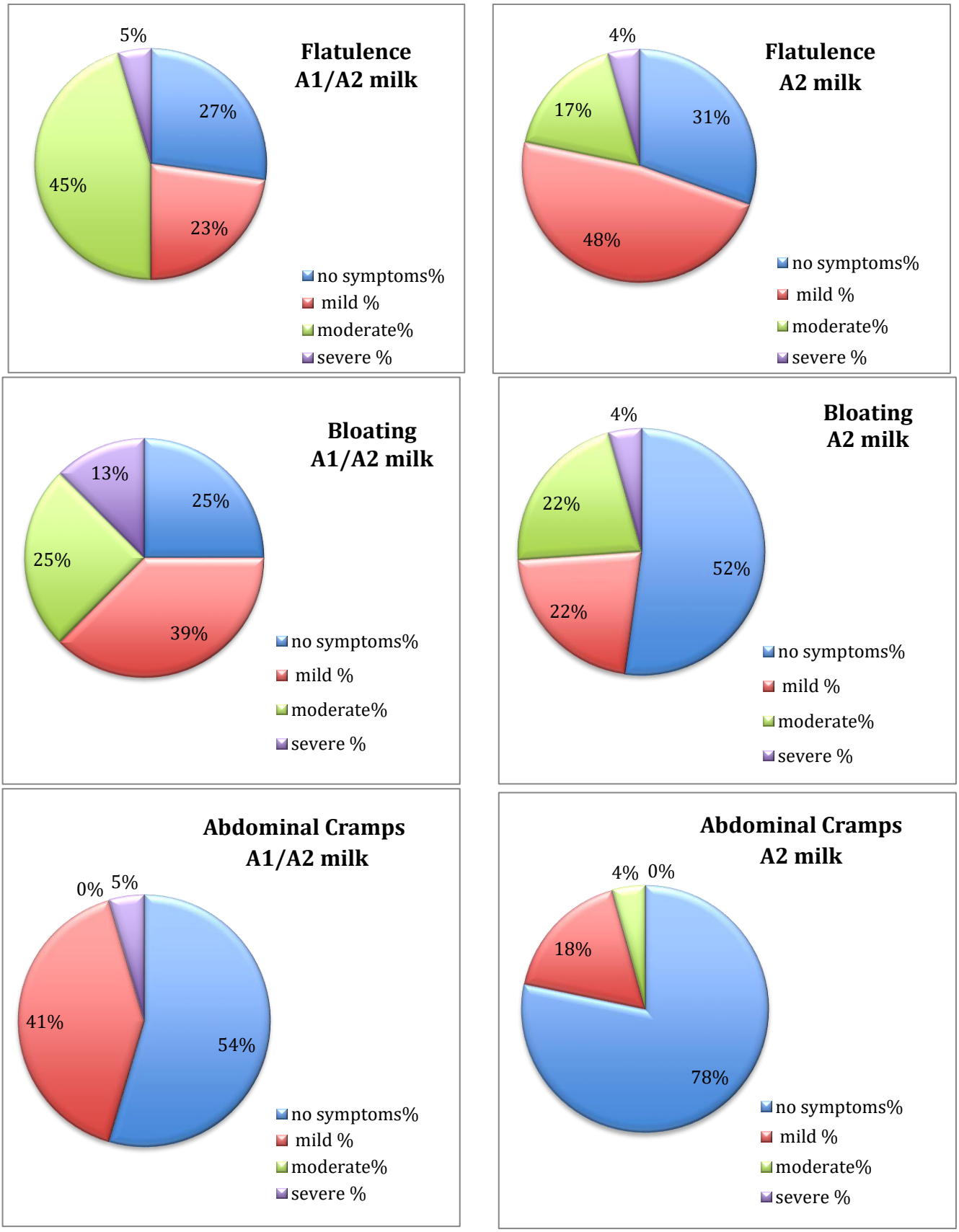

Figure 3.7. Symptoms recorded throughout 24h after the consumption of 250ml of each intervention milk. n=22. Values are percentage of symptoms experienced by participants

Table 3.7. Effect of consumption of 250 ml semi-skimmed A1/A2 milk and semi-skimmed A2/A2 milk on GI symptoms during 24h.

	A1/A2 milk	A2 milk	*P*[1]
Flatulence	1.5 (0.25, 2.0)	1.0 (0.0, 1.0)	0.103
Bloating	1.0 (0.25, 2.0)	0.0 (0.0, 1.5)	0.069
Abdominal cramps	0.0 (0.0, 1.0)	0.0 (0.0, 0.0)	0.085

Values are median and quartile range. n = 22

[1]Differences between the two interventions were analyzed with Mann-Whitney test $P<0.05$ considered significant.

3.4.9. Daily gastrointestinal function and symptoms recorded during intervention period (14 days)

Gastrointestinal function and symptoms were measured and recorded every day during each milk intervention period for 14 days. Both Bristol score and GI symptoms were calculated for the complete intervention period (14 days) and then according to days 0-7 and 8-14. To determine if participants experienced immediate symptoms when they drank the assigned milk or whether the symptoms improved when the dose of milk consumed increased during days 8-14. Data were compared between the intervention groups. The GI function and symptoms data are shown in Table 3.8. There was no significant difference between treatments for gastrointestinal symptoms (abdominal pain, bloating and flatulence) or bowel movements recorded for the complete intervention period (14 days) or according to days 0-7 or 8-14. The average severity of symptoms was below mild. Stool frequency was significantly increased with A2 milk (P = 0.027) compared with A1/A2 milk Figure 3.8. During the first week of the intervention when the dose of milk increased gradually, participants recorded stool texture as resembling a snake, smooth and soft. However, this stool became a little bit harder (lumpy sausages) during the second week of each intervention. Whereas, after 14 days of the intervention on average, participants had a stool like sausage with cracks on the surface.

Table 3.8. Effect of semi-skimmed A1/A2 milk and semi-skimmed A2/A2 milk consumption on gastrointestinal symptoms recorded over 14 days[1].

	A1/A2 milk	A2 milk	*P*
Overall symptoms (1-14 days)			
Bristol stool score	3.4 (2.7, 4.0)	3.3 (2.7, 4.2)	0.773
Abdominal pain[2]	0.2 (0, 0.8)	0.2 (0, 0.7)	0.636
Stomach bloating[2]	0.4 (0, 1.1)	0.4 (0.1, 1.0)	0.726
Flatulence[2]	0.6 (0.2, 1.0)	0.6 (0.4, 1.0)	0.836
First week symptoms (1-6 days)			
Bristol stool Score	3.7 (2.7, 4.2)	3.5 (2.8, 4.1)	0.939
Abdominal pain[2]	0.2 (0, 0.7)	0.2 (0, 0.6)	0.938
Stomach bloating[2]	0.5 (0, 1.3)	0.4 (0, 0.6)	0.578
Flatulence[2]	0.7 (0, 1.2)	0.7 (0.3, 1.0)	0.441
Second week symptoms (7-14 days)			
Bristol stool Score	3.3 (2.7, 4.1)	3.3 (2.6, 4.2)	0.846
Abdominal pain[2]	0.3 (0, 0.8)	0.3 (0, 0.9)	0.859
Stomach bloating[2]	0.6 (0, 1.3)	0.6 (0.2, 1.0)	0.977
Flatulence[2]	0.8 (0.3, 1.3)	0.6 (0.3, 1.0)	0.582

[1]All values are medians with quartile ranges. n=35. Between group treatments effects were evaluated using Mann-Whitney test. $P \leq 0.05$ considered significant. [2]Symptoms range 0 = no symptoms; 1 = mild; 2 = moderate; 3 = severe.

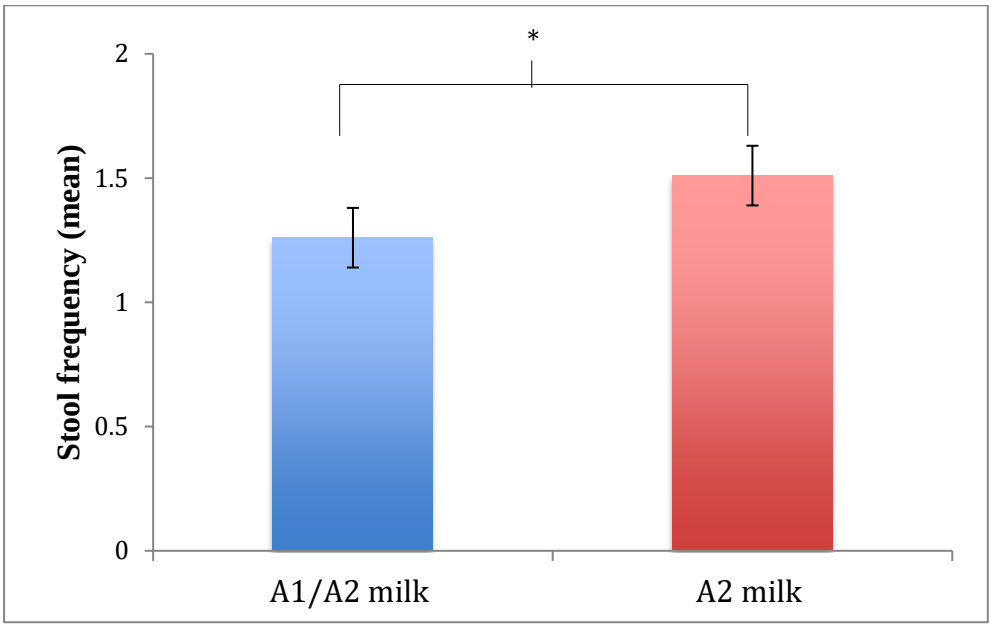

Figure 3.8. The average stool frequency. Values are mean ± SEMs. n = 35. Comparison between the two interventions were analysed with a linear mixed model analysis, adjusted for baseline values, gender, age, period and BMI frequency

3.4.10 Effect of milk intervention on the microbiome using 16S rDNA sequencing:

3.4.10.1 Alpha and beta diversity

Alpha diversity results are shown in Figure 3.9 A&B. A larger decrease (on average –14.41 compared to –0.177) was seen in the number of OTUs for A2 milk than for A1/A2 milk (LMEM, $p_{treatment}$ = 0.011, p_{group} = 0.297, $p_{interaction}$ = 0.454). No effect of treatment was seen on the Shannon index (LMEM, $p_{treatment}$ = 0.174, p_{group} = 0.433, $p_{interaction}$ = 0.623). Beta diversity data are indicated in Figure 3.10 A&B. As expected, looking at samples collected at start of treatment, no significant difference in microbiome community composition could be detected between treatments (permutational MANOVA, R^2 = 0.347 %, p = 1.00), but a significant difference could be detected for after-treatment samples (permutational MANOVA, R^2 = 1.071 %, p = 0.008). No effect of group (i.e. carry-over effect) could be detected when looking at start (permutational MANOVA, R^2 = 2.76 %, p = 1.00) or end of treatment samples (permutational MANOVA, R^2 = 3.47 %, p = 1.00).

3.4.10.2 Relative abundance

Changes in bacterial abundance within a treatment were also examined by comparing the abundance at the start and end of a treatment using a Wilcoxon test. This was done at OTU, genus, and class level. The class-level relative abundance in all samples are shown in Figure 3.11C. A2 milk decreased the genus *Barnesiella* significantly (median change −0.0993 percentage points, Wilcoxon test, *p*adjusted = 0.0290) and there was a tendency for the class Actinobacteria to increase also with A2 milk Figure 3.11A & B (median change 0.527 percentage points, Wilcoxon test, *p*adjusted = 0.0610).

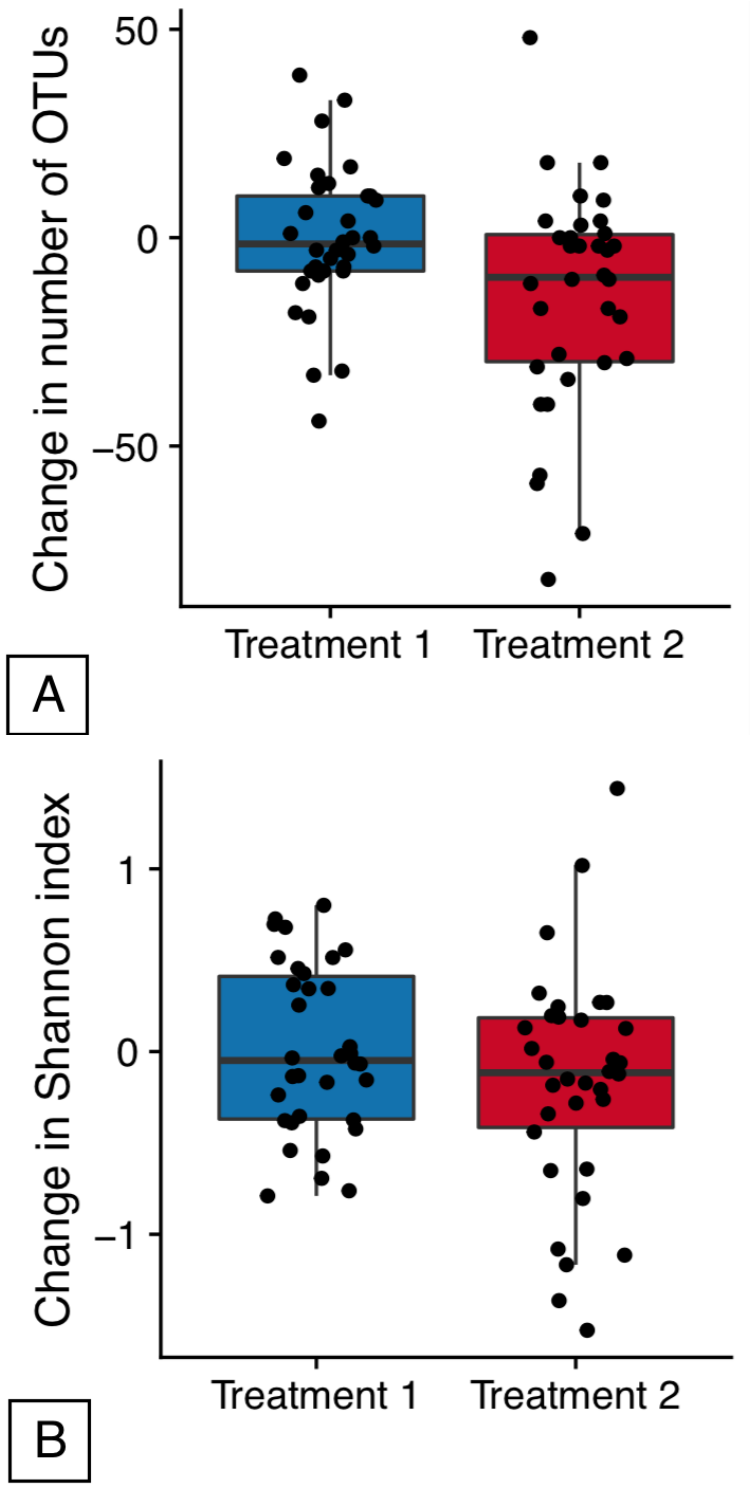

Figure 3.9. A; The change in number of OTUs and B; Shannon index as a function of treatment. Treatment 1, A1/A2 milk; Treatment 2 is A2 milk.

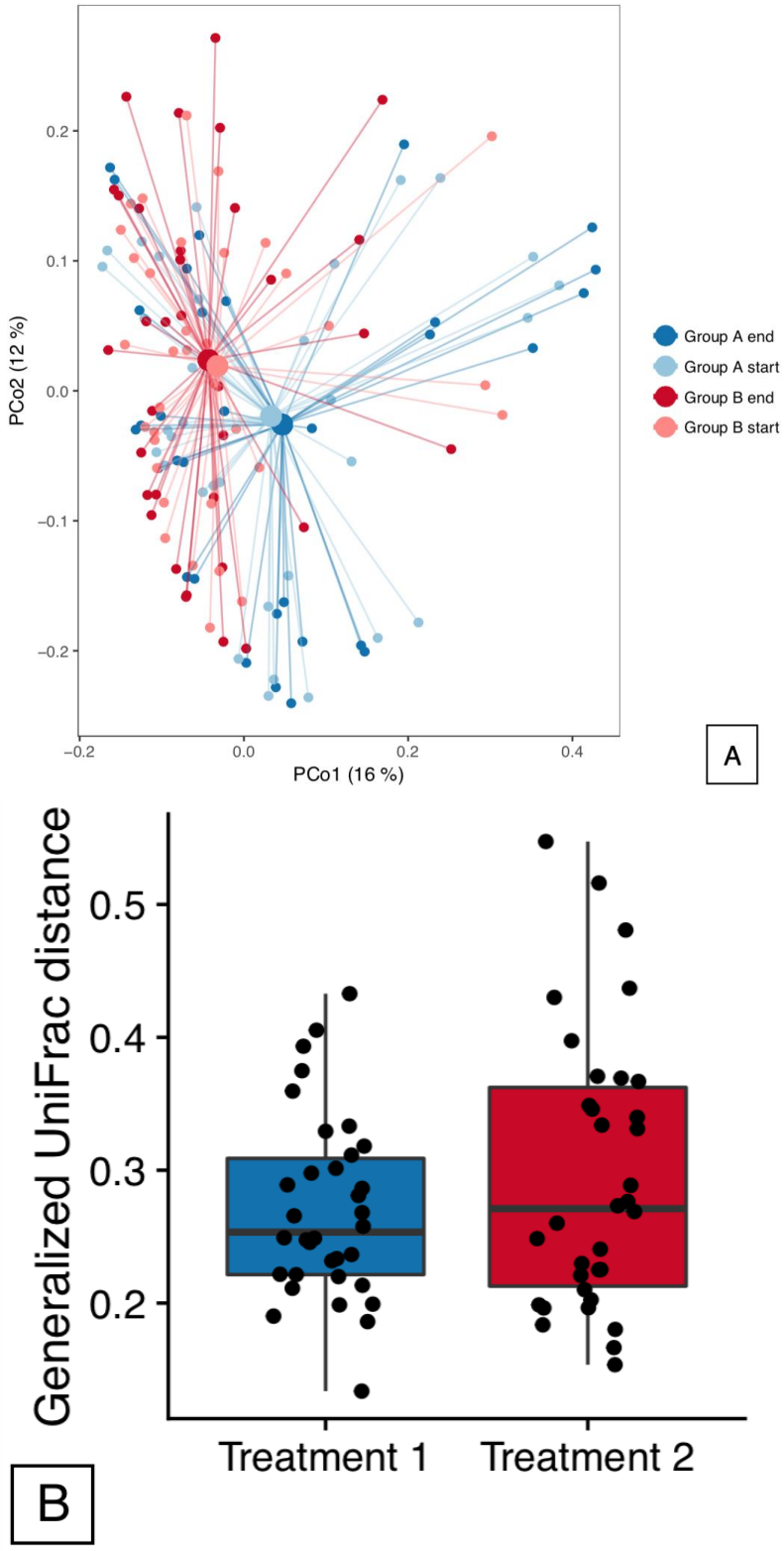

Figure 3.10. A; PCoA plot of generalized UniFrac distances. B; Change in microbiome community composition over the treatment period for each treatment. Treatment 1, A1/A2 milk; Treatment 2 is A2 milk.

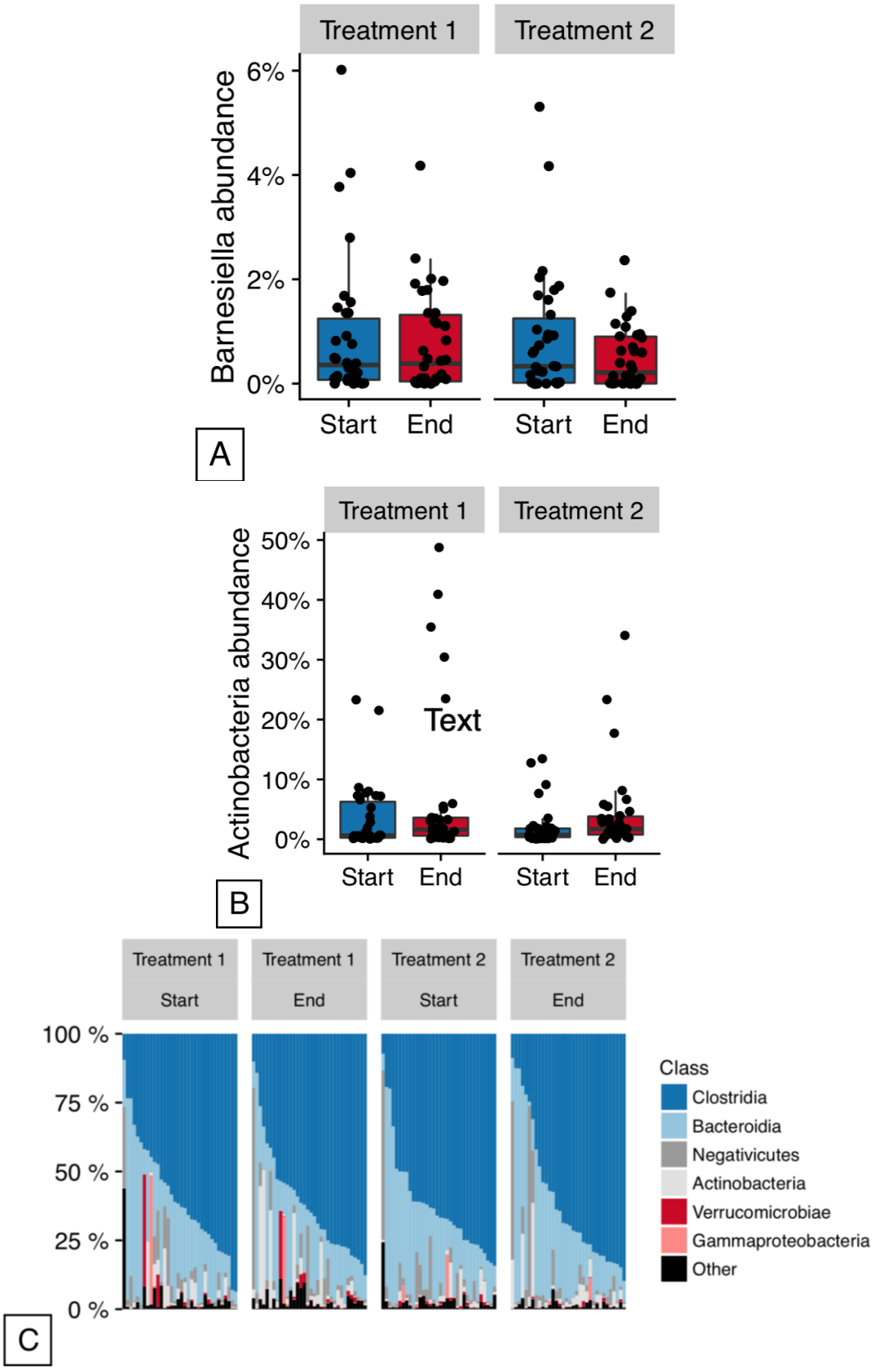

Figure 3.11. A; Abundance of the genus *Barnesiella.* B; Abundance of the class *Actinobacteria*. C; Class-level relative abundance. Samples are ordered by increasing Clostridia abundance. Treatment 1, A1/A2 milk; Treatment 2, A2 milk.

3.4.11. The effect of milk intervention on the metabolic profiling of faeces

A preliminary PCA unsupervised analysis was applied on the whole dataset but no clustering was detected between the 4 groups of samples. Then, a supervised OPLS-DA analysis computed using four distinct categories as response vectors, each built based on both treatment and time, confirmed no distinction between the groups. The dataset was split based on time points and comparison between treatments was investigated in each visit by PCA and OPLS-DA analyses. As shown in Figure 3.12 A & B no significant differences between A1/A2 milk and A2 milk at baseline level visits 1 and 3 respectively ($R^2Y = 0.025$, $Q^2Y = -1.223$; $R^2Y = 0.327$, $Q^2Y = -1.0886$ respectively). Similar comparison was made for after treatment visit and the model show no correlation. Baseline measurements were subtracted from the post-intervention measurements in order to obtain a new matrix of independent values which reflected the magnitude of the changes induced by the intervention. Both PCA and OPLS-DA analyses using treatment as response vector were applied but they both failed to reveal any distinction between the two treatments ($R^2Y = 0.424$, $Q^2Y = -1.282$) Figure 3.12 C & D. PCA and OPLS-DA analysis did not reveal any impact of both milks on the faecal metabolome.

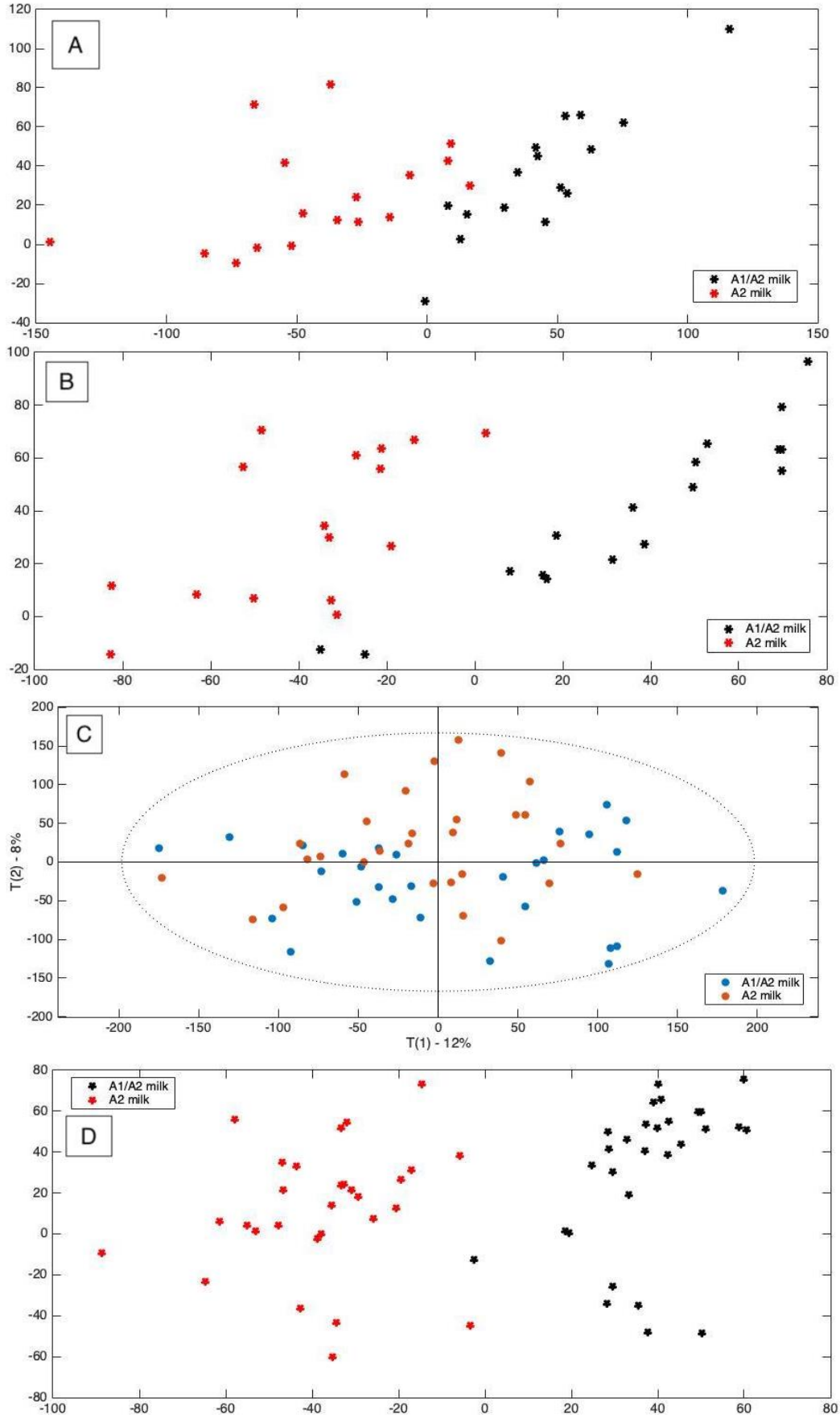

Figure 3.12. Supervised multivariate statistical analysis on faeces metabolic profile. A: Scores plot derived from a PLS model using *treatment* at visit 1as response vector; B: Scores plot derived from a PLS model using *treatment* at visit 3 as response vector. C: Principal component analysis (PCA) scores plot for the new matrix after calculating the change; D: Scores plot derived from a PLS model using *treatment* as response vector for the new calculated change.

Discussion

To our knowledge, the present study is the first independent study in UK adults with mild to moderate non-lactose milk intolerance to investigate the effect of replacing milk containing both A1/A2 β-casein variants with A2 milk that contains only A2 β-casein variant on gastrointestinal inflammation, function and symptoms. Our study showed differential beneficial effects of A2 milk that contains only A2 β- casein variants on systemic inflammatory markers (secondary outcome measure, hs-CRP), gastrointestinal symptoms and microbiota composition, with no significant impact of test milks on gut inflammation marker (FC as a primary outcome measure) and fermentation products including breath H_2 and CH_4 production. Compositionally, casein from semi-skimmed A1/A2 milk (British conventional milk) contained 2/3 of A2 β-casein and 1/3 of A1 β-casein whereas casein from A2 milk consists mostly of A2 β–casein. This finding is consistent with that of (Givens et al., 2013) who reported that the highest proportion in UK milk is A2 variant (58%) followed by the A1 variant (31%).

It is worth noting first that non-lactose milk intolerance is different to lactose intolerance/malabsorption. Lactose intolerance/ malabsorption is a condition characterised with the lack of lactase enzyme that is responsible for breaking down lactose which results in the appearance of symptoms including bloating, diarrhoea, flatulence and abdominal pain (Di Stefano and Corazza, 2009). Whereas, non-lactose milk intolerance is a condition that has not been well defined clinically but the current literature reports existence of subjects who are moderately milk intolerant and whose intolerance can neither be attributed to a defect in lactose digestion, nor to milk protein allergy (Stephenson and Latham, 1974, Johnson et al., 1993). Yet, they experience at least one or two of the following symptoms:

flatulence, bloating, abdominal cramp. These symptoms were highly variable from one individual to another and could range from mild to severe symptoms (Stephenson and Latham, 1974, Johnson et al., 1993).

Research has reported an association between A1 β-casein variant and gastrointestinal dysfunction and inflammation since A1 β-casein releases BCM-7 that has a pro-inflammatory activity locally and systemically (Barnett et al., 2014, Haq et al., 2014b, Ho et al., 2014, Jianqin et al., 2016, He et al., 2017). Inflammation is a consequences of allergy and autoimmune diseases and low grade inflammation is involved in the metabolic syndrome (Hakansson and Molin, 2011). FC is known as a local intestine inflammation marker that is predominantly found in neutrophils and to a lesser extent produced from monocytes and reactive macrophages (Konikoff and Denson, 2006). A strong positive correlation has been observed between FC and faecal excretion of neutrophils in patients with inflammatory bowel disease (IBD). This means that an increase level of FC concentration in IBD patient results from increased neutrophil release into the gut lumen across inflamed mucosa. In addition, Montalto and his group indicated that FC level remains normal in numbers of gastrointestinal disorders such as small intestinal bacterial overgrowth (Montalto et al., 2008), and celiac disease individuals (Montalto et al., 2007). Therefore using calprotectin as a biomarker to mmeasur gut inflammation was correctly selected. However, this kind of measure needs a large group of volunteers in order to identify significant level. In the present study, the lack of effect of both intervention milks on gut inflammation measured by FC in participants who experience mild to moderate non-lactose milk intolerance is similar to previous studies that failed to find any differential effect of milk containing only A1 β- casein variant compared with milk containing only A2 β- casein variant on FC in adult Australians (Ho et al., 2014). However, Ho et al. (2014)

reported that a sub-group showed high FC levels after 14 days of the consumption of A1 β-casein but not A2. This effect on FC may have resulted from the sub-group being self-reported lactose intolerants who were included in the study. Also this result was in agreement with (Rosti et al., 2011) who suggested that infant formula milk (cow proteins as a source) does not promote activation of an intestinal inflammatory reaction measured by FC compared to human milk, however activation of an inflammatory response may be triggered in sub-groups of infants with a family history of allergic disease. This means that the current study and other available evidence suggests that milk containing both A1/A2 β-casein variants has no inflammatory effects in the gut.

It is suggested that negative faecal calprotectin results should not be interpreted to reflect a healthy intestine but to reflect the absence of significant neutrophilic intestinal inflammation (Konikoff and Denson, 2006). Therefore, measuring another local inflammatory marker was important to further explore the effect of A1/A2 β-casein in the gut relative to A2. IL- 6 (a pro-inflammatory cytokine) and IL-10 (an anti-inflammatory cytokine) are two of the cytokines produced at local tissue and released in the circulation at acute infection. It is evident that IL-10 is clinically important in the prevention of IBD (Opal and Depalo, 2000). In the present study, one interesting finding was that we did not observe any significant changes in the concentrations of IL-6 and IL-10 after the consumption of both milks. Although these results have not previously been described in relation to the direct effect of milk with A1/A2 β-casein relative to A2 milk, these results could be related to milk proteins whey and casein since (Pal and Ellis, 2010) found no significant change in IL-6 in a chronic parallel study in overweight subjects who consumed either whey or casein protein and glucose as control for 12 weeks.

It is suggested that during the assessment of intestinal inflammation, the accuracy of diagnosis can be increased by combination of stool inflammation markers such as faecal calprotectin with serum CRP (Langhorst et al., 2008). CRP is one of the systemic inflammation markers that is produced normally as a result of stimulation by pro-inflammatory cytokines such as IL-1 and IL-6. Studies have reported an association between CRP and IBD (Schoepfer et al., 2010). In the current study, the most important finding was that both milks significantly decreased the acute phase protein hs-CRP the decrease of which was significantly greater in the group who had milk containing A2 β-casein variant compared with baseline. Although, this result differs from the finding of (Jianqin et al., 2016) who did not find any differential effect on CRP of milk containing A1/A2 β-casein relative to milk with A2 β-casein in Chinese adults who experienced milk intolerance, the current result could be due to the use of high sensitivity CRP that detects lower levels of the protein in human blood (0.5 to 10 mg/L) compared with the standard CRP (Gabay and Kushner, 1999).

A strong relationship between gut microbiota and inflammation has been reported in the literature since gut microbiota indirectly influence the modulation of metabolic endotoxemia, intestinal permeability and inflammation. Low-grade inflammation can be triggered by lipopolysaccharide (LPS) which is a component found in the cell walls of gram negative bacteria that are involved in the activation of macrophages, endothelial cells and neutrophils and these cells in turn release mediators such as proteins (CRP) and cytokines (IL-6) (Ashraf and Shah, 2014). Therefore, shifting gut microbiota to beneficial bacteria can inhibit inflammation and improve gut permeability (Hakansson and Molin, 2011). An example of such beneficial bacteria are bifidobacteria and lactobacilli, both of which are Gram positive bacteria that do not contain LPS in the cell membrane. Milk

oligosaccharides exert a bifidogenic effect *in vivo* and *in vitro* (Knol et al., 2005, Le Huërou-Luron et al., 2010, Shen et al., 2011a). Milk oligosaccharides fit the prebiotic definition since they are resistant to digestion and delivered to the colon where they contribute as a source of carbon for gut microbiota (Gopal and Gill, 2000). In the current study, the consumption of milk containing A2 β-casein variant resulted in a significant increase in the number of *Actinobacteria* abundance and a decrease in *Barnesiella* abundance. *Actinobacteria* is a phyla of *Bifidobacterium* (Hakansson and Molin, 2011) and *Bacteroidetes* is a phyla of *Barnesiella* (Kulagina et al., 2012).

This secondary outcome has not previously been investigated in relation to the direct effect of A2 milk on bacterial composition. *Bifidobacteri*aare considered to be non-gas producers and non-pathogenic bacteria that exhibit positive effects on human health. This includes production of acids such as acetate and lactate as metabolic end products that are involved in the inhibition of growth of gram positive and gram negative pathogenic bacteria and therefore inhibit inflammation. Moreover, *Bifidobacteria* can reduce blood ammonia levels and have the ability to not generate aliphatic amines, hydrogen sulphide and nitrites. They can also produce B vitamins, digestive enzymes such as casein phosphatase and lysozyme. In addition, *Bifidobacteria* can act as immunomodulators that stimulate the immune system against malignant cells, improve host resistance to pathogens and restore the normal intestinal microbiota in antibiotic therapy (Gibson and Roberfroid, 1995).

This modulation of microbiota composition suggests that semi-skimmed milk with A2 β-casein variant may have a prebiotic effect. Animal model studies have reported that administration of bifidobacteria to rodents results in improved barrier function, improved

immune response and decreased in inflammatory compound (intestinal LPS) (Wang et al., 2006).

It is well known that the fermentation of undigested lactose by gut microbiota in the large intestine produces H_2, CH_4, CO_2 and other metabolites (Enko et al., 2014). These gases can be execrated by the lungs and measured in the breath and also can cause abdominal and systemic symptoms (Simrén and Stotzer, 2006b). This study is the only study that used a Gastrolyser instrument to measure H_2 and CH_4 concentration after the consumption of each type of milk to assess any differences in these gaseous fermentation products. No significant differences were observed in the production of H_2 and CH_4 levels between the two milk types which suggests that both milks allow the microbiota to ferment lactose in the same way. However, little variation was observed between participants in the concentrations of H_2 and CH_4 produced and this may not be related to the treatment but due to the fact that numbers of individuals may produce H_2 more than CH_4. In addition, research has found that about 35% of healthy adults are CH_4 producers (Levitt et al., 2006).

It has been indicated that measurement of H_2 and CH_4 without a record of symptoms detects less than 50% of individuals who have lactose sensitivity. There are a number of studies which recommended evaluating the symptoms during the breath test (Stephenson and Latham, 1974, Hermans et al., 1997, Rezaie et al., 2017) because higher frequency and intensity of symptoms were associated with positive breath test result (Hermans et al., 1997). In this study, it is not clear whether symptoms experienced after the consumption of intervention milk was due to the exposure to different casein variants. To examine this possibility, symptoms that were recorded during 24h from the breath test after each milk showed no significant differences between milks and the predominant symptoms

experienced was flatulence, bloating and abdominal cramps. However, A2 milk had a tendency to lower bloating and abdominal cramps. This means that consumption of A2 milk improved gastrointestinal symptoms during 24h. Since this study compared the effect of A1/A2 milk with A2 milk on H_2 and CH_4 production and related symptoms over 24h, it is difficult to compare this finding with previous findings. However, this result is consistent with chronic studies (Ho et al., 2014, Jianqin et al., 2016) which showed that A2 milk improved gastrointestinal symptoms after 14 days of consumption.

Daily bowel habit and function during the intervention periods showed significant differences in the stool frequency between milk types. Higher stool frequency observed in A2 milk group relative to A1/A2 milk group. However, no significant difference was observed in the daily gastrointestinal symptoms (abdominal pain, stomach bloating and flatulence) for 14 days between the two milks. It is worthy of note that when the milk increased gradually during the second week of both milks the stool consistency changed slightly to become a little bit harder (lumpy sausages) but this was not significantly, unlike in the first week. This finding is contrary to the previous mentioned studies suggesting that A1 β-casein milk had significantly high stool consistency values and a significant positive association between abdominal pain and stool consistency as well as delay in transit time. A possible explanation for this might be due to the type of diet and amount of milk ingested by volunteers (milk was increased gradually from the start of the study until the end of the first week when participants started to consume twice a day 250ml of milk). Another possible explanation could be that in the previous studies they enrolled participants who were not regular milk drinkers and were lactose intolerant. Enrolling inappropriate subjects in human trials may affect the results and the interpretation of the results. The metabolism of milks in the gut generates SCFA and BCAA, which mainly derived from carbohydrate

and protein fermentation (Topping and Clifton, 2001). This study did not find any significant differences between interventions in bacterial metabolites including SCFA and BCAA. This finding is contrary to that of (Jianqin et al., 2016) who found that consumption of milk containing A1/A2 β-casein variants led to lower concentrations of SCFA than A2 milk. The current results may be explained by the fact that metabolites such as SCFA are rapidly absorbed (>95%) therefore their concentrations in the distal colon can be affected (Topping and Clifton, 2001).

The countification of BCM-7 from volunteers fluid such as urine and blood was not considered in this human study due to the lack of validated method to measure the release of BCM-7 in the circulation. Measuring this important outcome might identify and countify the amount of BCM-7 consumed during the intervention.

A strength of the study is the use of the Gastrolyser monitor to measure breath H_2 and CH_4 concentration in order to select the correct participants who experience milk intolerance but not lactose intolerance. When considering the availability of milk with β-casein variants, milk with A1/A2 β-casein variants is commercially available in all supermarkets used by the majority of individuals and can be used as representative of the main milk type consumed by the public. Therefore, the results of this study reflect the reality of symptoms when participants consume typically available milk. However, there are some potential limitations of this study. The sample size was somewhat under that indicated by the power calculation. We intended to recruit 45 volunteers in the study and we screened 53 subjects but because we needed a very specific group of participants we ended up with 38 participants including those who dropped out later. For the primary outcome measure (FC) research suggests more subjects reduce between subject variability. Another limitation may

include the unequal numbers of each gender that participated in the study. Therefore, recruiting more participants and more equal numbers of both genders in the study would increase the power of the primary outcome measure.

In conclusion, the findings of this study suggest that milk containing a mixture of A1/A2 β-casein variants does not affect gut inflammation and function. However, milk containing only A2 β-casein variants reduced systemic inflammation marker (hs-CRP), increased *Actinobacteria* and improved bowel habits relative to A1/A2 β-casein variants. These findings will contribute to the evidence related to A2 milk and gastrointestinal inflammation, function and symptoms for those groups of people who experience mild to moderate milk intolerance.

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Chapter 4

Effect of A2 milk on cardiometabolic risk markers, cognitive function and mood in adults with mild to moderate non-lactose milk intolerance.

This chapter contains secondary objective to the human study in chapter 3

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First author's main contribution: Areej Almuraee designed the study with the supervisors, conducted the *in vivo* study, all the lab analysis, statistical analysis and drafted manuscript

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4.1 Abstract

Background:

Evidence suggests that semi-skimmed milk containing A1/A2 β-caseins variants and its bioactive components are associated with beneficial effects on blood pressure, lipid metabolism and other markers of cardiometabolic health. However, very limited randomized clinical trials investigating A2 milk containing only A2 β-caseins variant and cardiometabolic health.

Objective:

This study investigated the effects of A2 milk consumption on serum lipids, glucose, body composition, blood pressure, plasma metabolome, psychological behaviour and mood in healthy men and women.

Design:

A double-blind, randomised, crossover, study was conducted to compare the daily consumption of semi-skimmed A2 milk with semi-skimmed A1/A2 milk for 14 days. 36 participants (men and women) aged between 18-56y were randomly assigned to the first intervention, either consume A2 milk or A1/A2 milk. Participants stopped all dairy products and followed each intervention for 2 weeks and a 2 weeks washout period separated interventions. Serum lipids, glucose, haemoglobin, clinic blood pressure, body composition, cognitive and mood were assessed. The plasma metabolome was assessed by ^{1}H-nuclear magnetic resonance (^{1}H-NMR). This trial was registered at clinicaltrials.gov as NCT03060395.

Results:

A1/A2 milk significantly decreased serum high-density lipoprotein cholesterol (HDL-C) (P = 0.018), glucose (P = 0.025), diastolic blood pressure (DBP) and heart rate (HR) (P = 0.006) compared with A2 milk. A2 milk significantly increased haemoglobin compared with A1/A2 milk. Both milks had no effect on serum total cholesterol (TC), low-density lipoprotein cholesterol LDL-C, Triacylglycerol (TAG), systolic blood pressure (SBP), body composition measures, plasma metabolome and cognitive function.

Conclusion:

Our study showed differential impact of milk β-caseins variant on HDL-C, glucose and blood pressure, but no effect on body composition and the rest of blood lipid.

Keywords: milk, A2 β-caseins variant, , BCM-7, cholesterol, blood pressure.

4.2 Introduction

Milk is known as the primary source of dairy products that are consumed by the majority of individuals. It provides a significant amount of energy, protein and micronutrients such as calcium, magnesium, phosphorus and vitamin B_{12} (Givens, 2018). Milk proteins are generally considered to be high quality proteins due to their bioavailability and pattern of essential amino acids. For instance, whey protein is rich in leucine, isoleucine and valine (branch chain amino acids). It is also a rich source of lysine whereas, casein protein is rich in histidine, methionine and phenylalanine (Pereira, 2014). Milk proteins are not only of high quality and biological value but bioactive peptides resulting from enzymatic hydrolysis during digestion have shown multiple health protective effects such as antibacterial, antioxidant, antihypertensive, antimicrobial, opioid and immunomodulatory effects as well as improving the absorption of other nutrients (Muro Urista et al., 2011, Pereira, 2014).

Casein proteins represent about 80% of total protein in cows' milk, about 36% of which is β-caseins. β-caseins can be derived from different allele variants with different proportions and include A1, A2, B and C protein variants (Fox, 2003). The A2 variant (58%) represents the highest proportion of β-caseins in UK milk followed by the A1 variant (31%) (Givens et al., 2013). Dairy cows can be either homozygous (A2/A2 or A1/A1) or heterozygous (A1/A2) genotypes and most of the UK milk contains a mixture of A1/A2 protein variants. However, commercial A2 milk contains only the A2 variant produced from animals selectively bred to be A2/A2 homozygotes (Woodford, 2007, Ul Haq et al., 2015).

Beta casein protein contains 209 amino acids and the main difference between A1 and A2 β-caseins is in position 67 where histidine in A1 is replaced by proline in A2. The result of this is that during enzymatic digestion, the A1 β-casein releases the β -casomorophin-7 (βCM-7) peptide in the gut but this is not possible with the A2 variant (Jinsmaa and Yoshikawa, 1999). The βCM-7 peptide has opioid characteristics which have been suggested to play a proinflammatory role in human health (Allison and Clarke, 2006) cardiovascular diseases (McLachlan, 2001, Chin-Dusting et al., 2006, Venn et al., 2006), type 1 diabetes (Elliott et al., 1999, Chia et al., 2018), sudden infant death and neurological disorders such as autism (Banerjee, 2018, Shashank et al., 2018). Torreilles and Guerin (1995) reported that βCM-7 has a pro-oxidant effect. This result came from ex-vivo stimulation of LDL oxidation by bovine casein hydrolysates with tyrosyl residues. This was the only study that reported the role of βCM-7 in LDL oxidation. However, an EFSA report, based on the current scientific literature, found no strong evidence for an association between the intake of β-casein A1 and increased risk of cardiovascular diseases (CVD) in humans (De Noni et al., 2009). In addition, current evidence that examined both milk proteins in relation to cardiometabolic health confirmed that whey and casein have beneficial impacts on plasma lipid profile, blood pressure (BP), inflammation and body composition (Arnberg et al., 2013, Visioli and Strata, 2014). This is supported by a recent review on the management of cardiometabolic health that shows how milk proteins including casein and whey protein have a beneficial impact on fasting plasma lipids, BP and inflammatory markers (Fekete et al., 2016).

To the best of our knowledge, only two clinical trials have compared direct effects of the A2 β-casein variant with A1 or the more common A1/A2 β-casein variants on markers of cardiometabolic disease risk (Chin-Dusting et al., 2006, Venn et al., 2006). Both studies

found no differential effect between A1 βcasein variant and A2 on cardiometabolic disease risk markers. However these studies has a number of limitations that might confound the result such as lack of control of dietary intake and the use of cocoa and citrus juice to make the shake and flavour the casein products. It is therefore clear that more studies with a wider range of outcome measures are needed to determine whether A2 milk can reduce cardiometabolic disease risk relative to A1 or A1/A2.

Accordingly, the aim of this chronic dietary intervention study was to test the hypothesis that the consumption of A2 milk will result in improvements in the serum lipid profile, blood pressure, body composition and cognitive function in adults aged 18-56y compared with conventional A1/A2 milk.

4.3. Materials and method

4.3.1. Participants

Thirty-eight healthy adults (7 male, 31 female), aged between 19 to 65 y, who were regular milk consumers were recruited from the local Reading area. Participants were considered suitable if they met the inclusion criteria including normal liver and kidney function, not anaemic, not diabetic, fasting total cholesterol <7 mmol/l and TAG < 4 mmol/l, not hypertensive (blood pressure <160/90 mmHg), not underweight or morbidly obese (BMI >35 kg/m^2), self-reported intolerance to commercial milk, not lactose intolerance or have a known dairy allergy, not pregnant or planning to be a pregnant and lactating during the proposed study period, not taking any medication, supplements or medicines for cardiovascular or metabolic diseases, or other dairy products including acidophilus milk, not taking antibiotics within the previous six months, not suffering from any

gastrointestinal disorders or gastrointestinal disease, not planning or on a regime to lose weight, not enrolled in another interventional clinical research study while participating in this study and fully aware of the nature, objective, benefit and the potential risks and side effects of the study.

An ethical approval for conducting the trial was similar to that given by The University of Reading Research Ethic committee that used in chapter 3. The study with the registration number (NCT03060395) was conducted according to the guidelines laid down in the Declaration of Helsinki and its subsequent updates.

4.3.2. Recruitment

Potential participants who responding to advertisements were contacted and sent a participant information sheet and medical and lifestyle questionnaire. Volunteers who were interested, completed the questionnaire either online or with guidance over the phone and sent it back for evaluation. Potentially suitable participants who met the criteria where then invited for a screening visit. A flow chart of study recruitment for the DIGI study is shown in **Chapter 3** (page 84).

4.3.3. Screening

Screening visits were performed in the morning and participants were asked to be fasted, not eating or drinking anything but water, from 8 pm the night before.

During this visit, the study was explained in greater detail before providing written informed consent. Measurements including height, weight and blood pressure were performed before a fasting blood sample was collected into a 5 ml serum separating tube and 4 ml K2EDTA vacutainer. Serum separating tubes were used to analyse fasting blood glucose and lipids TC, TAG, HDL-C and markers of liver and kidney function enzymes

(serum creatinine, total bilirubin, uric acid, alkaline transferase, alkaline phosphatase and γ-glutamyl transferease) using the ILAB 600 (Werfen UK Limited, Warrington, UK). The 4ml K2EDTA was sent to Pathology Department at the Royal Berkshire Hospital (Reading, UK) to perform a full blood count.

A methane and hydrogen breath test was then performed to screen out lactose intolerant participants (described in more detailed in Chapter 3). During the screening visit, mood and cognitive function was assessed in an empty and quiet clinical room with a sign on the door to control any external noise to familiarise volunteers with the tasks that would take place on actual study visits. Participants who fully met the inclusion criteria where then invited and scheduled to take part in the study.

4.3.4 Familiarisation visits

Before starting the study, participants were invited to visit for a discussion and were provided with a handbook giving details on how to prepare themselves before and during the study visits.

4.3.5. Study design and actual study visits

The present trial was a double blind randomised crossover dietary intervention study (see Figure 2 in Chapter 3). Fourteen days prior to the start of the study, participants were asked to exclude dairy products from their habitual diet (first washout) and advised to consume dairy alternatives. Instructions were given to control overall dietary energy intake and match this intake with the intervention products incorporated into the diet during the first and second arms. After the washout period, baseline measures were taken at visit 1 with the same measures at visits 2, 3, 4 and 5. Participants were provided with intervention milk and

a measuring cup and advised to follow the instructions written on the label of the milk container (See Appendix P 238-239) for an example of the milk label). Milk was consumed twice a day and the amount was increased gradually over the intervention period as follow; days 1 and 2: 100 mL, days 3 and 4: 150 mL, days 5 and 6: 200 mL, days 7 until 14: 250 ml. During each of the two intervention periods, volunteers completed a 3-day weighed food diary. During each study visit, anthropometric values (height, weight, BMI), % body fat composition, clinical BP, HR and cognitive tasks were performed. A fasting blood sample was taken and questionnaires assessing mood and cognitive function were completed.

4.3.6 Blood pressure and anthropometrics

Clinic BP, height, weight, body fat percentage, fat mass, fat free mass and body mass index (BMI) were measured at screening and at each study visit. BP was recorded as the average of three measurements using the Omron M2 automated blood pressure monitor. Pulse pressure was calculated by subtracting DBP from SBP. A pulse pressure of 40 is considered normal, but 40 to 60 is a relatively healthy range (Safar et al., 1987). Body mass index was calculated by dividing the body weight (kg) by the square of the body height (BMI =kg/m^2) and body fat composition was measured using Tanita body composition analyzer (Tanita BC-418 digital scale, Tanita UK, West Drayton, UK). This measurement was performed using standard settings for participants wearing light clothes.

4.3.7. Collection, processing and storage of blood samples

On each study, blood was collected using a safety blood collection set and holder (23G or 21G) from (Greiner bio-one Ltd, UK, 450086 or 450085) into a 5 ml serum separator clot activator tube (Greiner bio-one Ltd, UK, 456010), a 4 ml K2EDTA tubes (Greiner bio-one

Ltd, UK, 454023) and a 4 ml Lithium Heparin tube (for NMR) (Greiner bio-one Ltd, UK, 454084). Immediately tubes were inverted 8-10 times after collection. One EDTA was sent to the Royal Berkshire hospital for blood haemoglobin and the serum separator tube was left at room temperature for 20 min before centrifugation. Then both the serum and lithium heparin tubes were centrifuged at 3000 rpm (1700 x g) for 15min at 4°C.

4.3.8. Metabolic profiling by ^{1}H nuclear magnetic resonance

4.3.8.1 Plasma analysis

Plasma metabolites were quantified using ^{1}H NMR. Samples were prepared fresh for analysis on the same day and randomly run in batch to avoid batch differences. This work was done with the aid of ice to minimize any effect. Plasma samples (300µl) were mixed with 350 µl of D2O (Deuterium Oxide 99.9%, Sigma-Aldrich. 530867) and then centrifuged for 10 min at 4°C at 13000 x g Supernatants (600µl) were then transferred to 5 mm NMR tubes for analysis by NMR spectroscopy.

4.3.9 Biochemical parameters

TC, HDL-C, TAG and glucose were quantified in serum by using an ILAB 600 (Werfen (UK) Ltd, Warrington, UK) using kits supplied by Werfen. LDL cholesterol (LDL-C) was calculated using the Friedewald formula (Friedewald et al., 1972). Each batch of samples was ran with the quality controls and all samples for each subject were analysed within a single batch.

4.3.10 NMR acquisition and processing

NMR experiments on plasma samples were carried out in the Chemical Analysis Facility of the University of Reading with the use of Bruker AV700 NMR Spectrometer equipped with a 5mm $^{1}H(^{13}C/^{15}N)$ inverse Cryoprobe®. All experiments were recorded at 300K by ^{1}H-1D NOESY-PRESAT (noesypr) and Carr-Purcell-Meiboom-Gill^{1}H-1D CPMG-PRESAT (cpmgpr), both using water signal suppression. For each spectrum, 8 dummy transients were followed by a total of 256 scans, with a relaxation delay (RD) of 2s and acquisition time (AQ) of 1.5s. Scans were accumulated in 64k data points with a spectral width of 9803.9 Hz. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening. Spectra were referenced to the doublet peak of glucose at 5.223 ppm. All spectra were manually phased and automatically baseline corrected applying a Whittaker smoother algorithm in MNova NMR version 10.0.1 (Mestrelab Research). The residual water signal was manually deleted.

4.3.11 Mood and cognitive function

Mood and cognitive function was assessed using different executive tasks including The Positive and Negative Affect Schedule (PANAS), Modified Attention Network Task (MANT) and Letter Memory Task (LMT). E- prime V 2 (Psychology software tools, Inc) using on a laptop to record participant responses. Tasks were ran in an empty and quiet clinical room with a sign on the door to control any external noise. All participants completed these tasks during each study visit including the screening visit. In the screening

visit, participants were instructed on how to do these tests and practice was performed only for training, and not for including these data within the study.

4.3.11.1. Mood measure

Mood was assessed with the PANAS self-reported measure of Positive Affect (PA) and Negative Affect (NA). This is a reliable and valid 20-item tool consisting of 10 positive and 10 negative mood states. Using the method of Khalid et al. (2017), the participants were asked to rate the degree to which they were experiencing each mood state using a scale ranging from 1 to 5 with anchor points (not at all and extremely). The score that reflected PA and NA symptoms were summed separately to calculate the overall positive and overall negative affect (lower score indicates a lower level of positive or negative affect).

4.3.11.2. Cognitive function

Cognitive performance or executive function and selective attention were assessed using published methods (Whyte et al., 2018) namely the Modified Attention Network Task (MANT) that explores response interference. This method modified in (Whyte et al., 2016) in which five white arrow symbols are presented in a row (<" and ">) on a black background. The middle arrow was congruent or incongruent with the pair of arrows on both sides. Examples of congruent are (<<<<< or >>>>>) and incongruent are (<<><< or >><>>). Participants were instructed to press the arrow key on the keyboard based on the direction of the middle arrow. Response time and accuracy were measured separately for congruent and incongruent.

Monitoring and updating information in working memory was measured using Letter Memory Tasks (LTM) and the proportion of letters recalled correctly analysed. This involved a presentation of several letters from a list and the task simply was to recall and type the last 4 letters which appeared in the list. This tasks followed the method proposed by (Miyake et al., 2000). For example, letters were presented as "T, H, G, B, S, K, R," and for this tasks, participants should say "T… TH … THG … THGB … HGBS … GBSK … BSKR" then at the end of the trial recall "BSKR".

4.12. Statistical analysis

All statistical analyses were performed using IBM SPSS statistical software version 24. Normality tests were performed for all outcomes. Data that were not normally distributed, were transformed using Lg10 and then checked for normality. Data that could not be normalised were processed with a nonparametric test (Mann-Whitney test). Effects of treatments were evaluated by performing a linear mixed model analysis adjusted for baseline values, gender, age and BMI. $P \leq 0.05$ was considered significant. Data are presented in texts, tables and figures as mean ± SEM.

For mood and cognitive analysis, change from baseline was calculated and a two-way analysis of variance (ANOVA) was performed. The main effect of treatment and the interaction between time and treatment were considered significant at $P \leq 0.05$.

For metabolomics, spectra were digitalised and imported into Matlab, where they were normalised under total area and unit variance (UV) scaled. Principal Component Analysis (PCA) was performed to detect metabolic group variations, detect possible outliers and clustering of groups under investigation. Data were further analysed using orthogonal projection to latent structure-discriminant analysis (O-PLS-DA) with 0 or 1 orthogonal

components where 1H-NMR spectroscopic profiles were used as a matrix of independent variables (X) and treatment, time and biochemical measurements as response vectors (Y). Changes from baseline based on the treatment were measured by subtracting the matrix of independent variables at baseline from the values measured after treatments. The evaluation of the quality of statistical models was based on the values of R2Y (goodness of fit: percentage of Y explained by the model) and Q2Y (the goodness of prediction: percentage of Y predicted after 7-fold cross validation). This evaluation was completed by visual observation of plots of the scores (T) compared with cross-validated scores (Tcv). Significance of selected models was further validated by random permutation tests (500 permutations). Discriminant metabolites were identified based on visual examination of correlation coefficients colour-coded loadings plot, constructed from the model outputs by back-scaling transformation of the loadings. Colours represent the significance of correlation (r2) for each metabolite to class membership, where red is the maximum correlation value. Metabolites were assigned using Chenomx Software (Chenomx Inc.), public metabolic databases (HMDB, http://www.hmdb.ca, BMRB, http://www.bmrb.wisc.edu) and from the literature.

4.4. Results

4.4.1. Anthropometric measurements, blood pressure and haemoglobin

There were no significant treatment effects on BMI and body composition (fat %) as result of the milk interventions $P > 0.05$ (Table 4.1). Similarly, there were no treatment effects on SBP and pulse pressure. However, a treatment effect was observed for DBP and HR, with a

decrease (-1.0 ± 0.1 mm Hg, -2.0 ± 1.3 beats/min respectively) observed during A1/A2 milk whereas no change was observed during A2 milk (*P* = 0.006). Calculated pulse pressure was within the normal range (40-64 mm Hg) (Safar et al., 1987). An increase in plasma haemoglobin concentration was observed during A2 milk compared with A1/A2 milk intervention (*P* = 0.010). Data are summarised in Table 4.1.

4.4.2. Blood biochemistry

Blood biochemical results are presented in Table 4.1. There was a differential effect of treatment on serum HDL-C (*P* = 0.018) and glucose (*P* = 0.025) concentrations, with a decrease of both observed following A1/A2 milk compared with A2 milk. There were no significant differences observed between treatments on LDL-C, TAG, total cholesterol concentrations and total cholesterol: HDL-C ratio (*P* >0.05)(Table 4.1).

4.4.3. Plasma metabolome

Change from baseline in the plasma metabolome were analysed by ^{1}H-NMR, and metabolites with different concentration (relative to baseline) between groups were identified by OPLS-DA. OPLS-DA analysis did not reveal any linear correlation between the pre- and post-intervention metabolic fingerprints (A1/A2 milk: Q2Y= -0.1053; A2 milk: Q2Y= -0.4656). Baseline measurements were subtracted from the post-intervention measurements in order to obtain a new matrix of independent values, Z, which reflected the magnitude of the changes induced by the intervention. On matrix Z, PCA and OPLS-DA analyses using treatment as response vector were applied but they both failed to reveal any distinction between the two treatments (Q2Y= -0.0632). PCA and OPLS-DA analysis did not reveal any impact of both intervention milks on the plasma metabolome (Figure 4.1).

4.4.4. Mood and cognitive function

There were no significant differences in the PANAS mood assessment results between intervention treatments (Table 4.2). For the attention network task, accuracy and response time measured separately for both congruent and incongruent, the results showed a significant treatment effect on congruence. In terms of accuracy, multiple post-hoc analyses indicated a tendency for a greater accuracy after A1/A2 milk than A2 milk (P = 0.066). Also, there was a significantly better performance on congruence in A1/A2 milk compared with baseline level (P = 0.008) and better performance on neutral also in A1/A2 milk P = 0.050. In terms of response time, there was also a significant effect for congruence $P <$ 0.05. Incongruent was much slower than congruent and neutral respectively (mean = 546.1 ± 14.4 min, 478.1 ± 13.3 min, 465.7 ± 12.9 min). There was no significant effect on the interaction between treatments with congruent $P > 0.05$. For the letter memory task, within subjects effects showed no differences between treatments on letter memory task (P = 0.146) and no differences in the interaction between the treatment, time and the length (P = 0.519). However, better performance was observed after A1/A2 milk (P = 0.034) than A2 milk compared with the baseline (Table 4.2).

Table 4.1 Effect of daily consumption of semi-skimmed A1/A2 milk and semi-skimmed A2/A2 milk on anthropometrics, hemoglobin, blood pressure and blood biochemistry

	Post- A1/A2 milk	Post- A2 milk	Δ	*P* [1]	95% Confidence Interval for Difference	
					Lower limit	Upper Limit
Anthropometrics						
BMI kg/m^2	24.20 ± 0.08	24.26 ± 0.07	-0.06 ± 0.08	0.484	-0.22	0.10
Fat %	27.34 ± 0.39	27.30 ± 0.38	-0.03 ± 0.32	0.906	-0.61	0.69
FM kg	18.10 ± 0.26	18.09 ± 0.25	0.00 ± 0.22	0.991	-0.43	0.44
FFM kg	47.31 ± 0.24	47.27 ± 0.24	-0.04 ± 0.23	0.850	-0.42	0.52
Blood haemoglobin (g/L)	133.17 ± 1.06	135.76 ± 1.02	-2.58 ± 0.96	0.010	-4.51	-0.65
Blood pressure						
SBP (mm Hg)	114.44 ± 1.32	115.55 ± 1.28	-1.10 ± 1.24	0.379	-3.59	1.38
DBP (mm Hg)	68.55 ± 0.98	70.29 ± 0.93	-1.74 ± 0.84	0.043	-3.42	-0.05
PP (mm Hg)	46.17 ± 1.24	45.37 ± 1.23	0.79 ± 0.98	0.423	-1.17	2.76
HR (beats/min)	69.29 ± 1.49	72.97 ± 1.39	-3.67 ± 1.30	0.006	-6.27	-1.08
Fasting Lipid Profile						
Total cholesterol,(mmol/L)	4.94 ± 0.08	5.00 ± 0.07	-0.06 ± 0.08	0.445	-0.22	0.10
TRIG, (mmol/L)	1.10 ± 0.10	1.05 ± 0.10	-0.04 ± 0.04	0.320	-0.04	0.13
LDL cholesterol (mmol/L)	2.92 ± 0.06	2.97 ± 0.06	-0.05 ± 0.06	0.387	-0.18	0.07
HDL cholesterol, (mmol/L)	1.51 ± 0.02	1.58 ± 0.02	-0.07 ± 0.03	0.018	-0.13	-0.01
Total cholesterol: HDL-C [2]	3.35 ± 0.05	3.27 ± 0.04	-0.07 ± 0.06	0.222	-0.04	0.19
Glucose, (mmol/L)	5.08 ± 0.05	5.21 ± 0.05	-0.13 ± 0.05	0.025	-0.24	-0.01

Values are mean ± SEMs. n = 37. BMI, Body Mass Index; FM, Fat Mass; FFM, Fat Free Mass; SBP, Systolic Blood Pressure; DBP, Diastolic blood pressure; PP, Pulse Pressure; HR, Heart Rate; TRIG, Triacylglycerol; HDL cholesterol, HDL-C. [1]Differences between the two interventions were analyzed with a mixed model analysis, adjusted for baseline values, gender, age and BMI. [2] ratio.

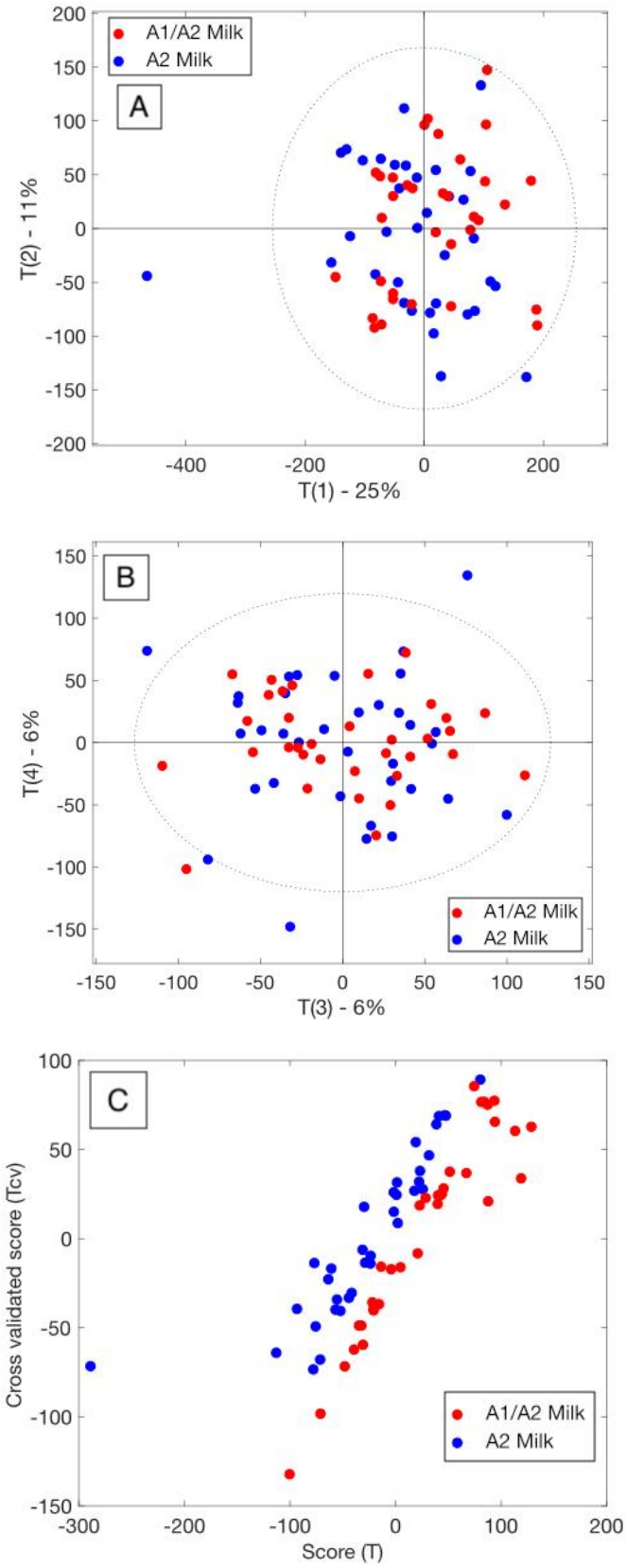

Figure 4.1. Unsupervised and supervised multivariate statistical analysis on plasma metabolic profile on Z matrix. A, B: Principal component analysis (PCA) scores plot; C: Scores plot derived from a PLS model using treatment as response vector. The calculated scores (X axis) are plotted against the cross-validated scores (Y axis); R2Y= 0.1581; Q2Y= -0.0632.

Table 4.2. Effect of daily consumption of semi-skimmed A1/A2 milk and semi-skimmed A2/A2 milk on mood and cognitive function [1]

	A1/A2 milk			A2 milk			
	Pre-treatment	**Post-treatment**	**Δ**	**Pre-treatment**	**Post-treatment**	**Δ**	*P*[1]
Mood assessment							
Positive affect	31.35 ± 1.41	30.62 ± 1.62	-0.73 ± 0.21	31.38 ± 1.33	30.05 ± 1.63	-1.33 ± 0.30	0.780
Negative affect	14.97 ± 1.21	14.73 ± 1.26	-0.24 ± 0.05	15.11 ± 1.00	14.81 ± 1.27	-0.3 ± 0.27	0.891
Cognitive function							
Letter memory	0.82 ± 0.178	0.79 ± 0.182	-0.03 ± 0.04	0.73 ± 0.17	0.77 ± 0.18	0.04 ± 0.01	0.102

[1] All data are expressed as mean ± SEMs. n = 36. [2]Differences between the two interventions were analyzed with repeated measure ANOVA analysis.

4.5. Discussion

The findings from this study suggest that A2 milk is similar to A1/A2 milk and had no differential effect on TC, LDL-C, TAG, systolic blood pressure, body composition, mood and cognitive function. However, A2 milk increased plasma haemoglobin level, fasted glucose, HDL-C and DBP.

It is well known that milk containing both A1/A2 β-caseins,that reflects most commercially available milk, releases βCM-7 in the gut during the digestion but this is not possible with the A2 milk containing only A2 β-caseins. The βCM-7 peptide is considered a proinflamatory peptide that may have an impact on CVD. Alison and Clark (2006) reported that A1 β-casein that releases BCM-7 during digestion may be proatherogenic since its involvement in the stimulation of the oxidation process of LDL (Allison and Clarke, 2006). LDL is a lipid carrier in plasma that is derived in the circulation from very low density lipoprotein (VLDL). LDL consists of cholesterol ester, phospholipids, triglyceride, free cholesterol and apolipoproteinB100 (ApoB100) (Matsuura et al., 2008). It is involved in the transport of cholesterol to peripheral tissues and the regulation of cholesterol metabolism at these tissues. Oxidised LDL is also known to be immunogenic and release of antibodies against oxidised LDL is considered as a marker of CVD (Zebrack and Anderson, 2002). On the other hand, milk and milk derived bioactive peptides may also act as antioxidants such as peptides derived from bovine casein and whey proteins (Thorning et al., 2016, Givens, 2018). In addition, EFSA (2009) concluded that the scientific evidence failed to find a cause-effect relationship

between food derived bioactive peptides such as βCM-7 and cardiometabolic risk markers including oxidised LDL (De Noni et al., 2009). Only limited evidence exists comparing the direct effect of A1/A2 milk and A2 milk on cardiomentabolic health in clincal trial. In the current study, there was no evidence of a statistically significant effect of A2 milk on TC, LDL-C, TAG, SBP and body composition compared with A1/A2 milk. These findings broadly support the work of (Venn et al., 2006) who compared the effect of β-casein A1 compared with A2 on risk markers for cardiovascular disease. Venn et al. (2006) found from a randomised clinical trial with 62 participants no differential effect of consumption of β-casein A1 compared with β-casein A2 on plasma total cholesterol, LDL-C and HDL-C concentrations. In agreement with Venn et al.(2006), (Chin-Dusting et al., 2006) concluded that daily supplementation with 25g of A1 β-casein for 12 weeks had no cardiovascular health disadvantage compared with A2 casein based on plasma lipid profiles.

HDL known as anti-atherogenic lipoprotein due to its involvement in the reverse transport of excess cholesterol from peripheral tissues back to the liver (Lund-Katz and Phillips, 2010). A high level of HDL-C is associated with a decreased risk of cardiovascular disease (Rothblat and Phillips, 2010) which is the leading cause of death. HDL-C level is commonly used as a marker of reduced CVD risk especially when used as the ratio of total cholesterol to HDL-C (Rico and Rico, 2018). Research has reported that consuming skimmed bovine milk containing enhanced concentrations of long chain n-3-fatty acids increased plasma HDL-C and reduced TAG (Visioli et al., 2000). One interesting finding from the current study is that A1/A2 milk decreased the concentrations of serum HDL-C, DBP and HR. These

results differ from Chin-Dusting (2006) who failed to find any differential effect of A1/A2 milk and A2 milk on HDL-C and DBP after the supplementation with 25g casein powder containing B-casein A1 or A2 consumed as a shake made with water or fruit juice. A possible explanation for this might be related to the intervention diet that plays a beneficial role in modulating gut microbiota composition as seen from our *in vitro* work in Chapter 2 increasing bifidobacterial species (Gibson et al., 2017). Bifidobacteria exert beneficial properties that lower the level of blood lipids by inhibiting liver synthesis of very low density lipoprotein (VLDL) and triacylglycerols resulting in low cholesterol levels (Taylor and Williams, 1998). Another possible explanation may be related to lifestyle factors such as dietary habit. Restriction of the diet to exclude dairy products during the current study may change the habitual diet that may lead participants to consume fibre, fruit and vegetables and this in turn improves cardiovascular health. Another possible explanation for this could be driven from a recent review that reported milk proteins including casein and whey proteins have beneficial impact on fasted lipids, BP and inflammatory markers, based on a high dose (28g) of milk proteins (Fekete et al., 2016). In addition, a randomised controlled trial conducted for 12 weeks in 193 overweight adolescents aged 12-15 years consumed 1 litre of water (as control), skimmed milk, whey or casein. The protein isolate content in milk-based test drink was 35g protein per litre. A significant decrease was observed in brachial and central DBP in the casein group compared with the control group who consumed water, but no treatment effects on blood lipid concentrations were seen (Arnberg et al., 2013). The mechanism behind the role of casein proteins in reducing BP results, at least in part, from the inhibition of ACE enzyme that regulates BP. This enzyme converts angiotensin I to angiotensin II

which is a vasoconstricting effect which increases BP. ACE inhibitory peptides have been found *in vitro* in the amino acid chain from whey and casein proteins (Giromini et al., 2017). *In vitro* studies suggest that the mechanism behind the improvement of blood lipid profile may be related to milk proteins down-regulating genes that are involved in the biochemical pathways of transport and synthesis of intestinal fatty acid and cholesterol. Also, leucine appears to play a role in the decreased synthesis of hepatic cholesterol and therefore decreased total plasma cholesterol and LDL-cholesterol (Arnberg et al., 2013). In addition, calcium, vitamin D and dairy proteins are leading factors in the mechanism of weight management by increasing satiety and lowering appetite, inhibiting gastric secretion by cholecystokinin and some branched chain amino acid, increased secretion of glucagon-like peptide 1 (GLP-1) and (GIP) glucose–dependent insulinotropic polypeptide (Visioli and Strata, 2014). Dove et al. (2009) reported that consuming 600ml of skimmed milk with the breakfast had a stronger satiating effect compared with fruit juice and results in a lower consumption (after 4h) of food offered at lunch time. In a meta-analysis of 29 randomised clinical trial, Chen et al. (2012) reported that dairy intake dose not increase body weight gain or body fat and consumption of dairy could result in weight or fat loose in chronic studies with energy restriction.

Evidence from the European Prospective Investigation into Cancer and Nutrition (EPIC) based on a large case-cohort n = 340,234 from eight nations in Europe reported an inverse association between the intakes of cheese and fermented dairy products and incidence of type 2 diabetes. They revealed that consuming 55g/d of cheese and yogurt was associated with a lower type 2 diabetes incidence by 12%

(Sluijs et al., 2012). Interestingly, in the present study blood glucose concentration reduced in A1/A2 milk but not in A2 milk. This result has not previously been reported in clinical trials that compared A1 with A2 milk in humans but is worthy of further investigation. The protective effect of dairy products on the incidence of type 2 diabetes results in part from dairy proteins, especially whey proteins, that stimulate insulin secretion and lower glycaemic excursions in healthy and diabetic participants. Gunnerud et al. (2012) found that whey protein provides a high insulin response compared with casein or milk that contains both casein and whey proteins. The effect of whey protein is primarily through the stimulation of GLP-1 and GIP, both identified as strong insulinotropic agents. The branched chain amino acids in whey protein contribute in the release of GLP-1 from intestinal cells (Gunnerud et al., 2012). There is however contrasting evidence from a very recent animal study looking at whether A1/A1 β-casein increases the incidence of Type 1-diabetes (T1D) in non-obese mice (Chia et al., 2018). The study supplemented non-obese diabetic mice from F0 generation to F4 generation with A1/A1 or A2/A2 β-casein containing diets with free access to water. The results showed a similar diabetes incidence in generation F0-F2 in mice receiving A1/A1 or A2/A2 β-casein. However the incidence of T1D doubled in generation F3 in mice fed A1 β-casein diet. Also, the research group confirmed the presence of sub-clinical insulitis that developed in 10-weeks old F4 female mice. The study concluded that A1 β-casein may negatively affect glucose homeostasis and T1D progression that appears later in life (Chia et al., 2018).

Another interesting finding of the present study is that A2 milk increased plasma haemoglobin. Comparison of this finding with available evidence from research

on casein derived bioactive peptides revealed that some of the peptides act as biocarriers such as caseinophosphopeptides (CPPs;casein-derived phosphorylated peptide). These CPPs appear in the stomach and duodenum following the consumption of milk and after *in vitro* and/or *in vivo* digestion of $\alpha_{s1,}$ α_{s2} and β-casein and have the ability to reach the distal ileum. Phosphopeptides are resistance to further hydrolysis due to the high concentration of negative charges that allow macroelements like Ca, Mg, and Fe to bind to them. This is one of the important roles that milk plays, that of preventing Fe deficiency since CPPs can bind to Fe resulting in the inhibition of the formation of ferric hydroxides (high-molecular weight) which are poorly absorbed. (Silva and Malcata, 2005). One *in vitro* study reported that binding Fe to one of the CPPs called β-casein f1-25 improved the ability to cure anaemia and restore Fe storage tissues (Aît-Oukhatar et al., 1999).

Cognitive decline and dementia are increasing major health concerns. Cognitive decline can be mild and simply associated with normal ageing or a severe decline that is associated with old age. There is a growing body of evidence that nutrition plays an important role in the prevention of age related cognitive decline. A positive relationship has been shown between a number of nutrients such as antioxidants, folate, omega-3 and 6 fatty acids and cognitive performance (Crichton et al., 2010). The results of this study indicates no effects of both milk types on mood, but A1/A2 milk was associated with improved cognitive function. This differs from observations of (Jianqin et al., 2016) who suggested that A1/A2 milk slows cognitive processing speed and decreases processing accuracy compared with the baseline value. However, results from published studies on dairy intake and cognitive function were contradictory. One systematic review

reported a direct association between dairy intake and better cognitive function (Crichton et al., 2010). According to Crichton et al. (2010), consuming 200ml/d milk, as the lowest effective amount, was associated with better memory and slower cognitive decline (Crichton et al., 2010). These studies indicate that milk and dairy products may have neuroprotective properties. It is important to mention that using appropriate methods that are suitable for specific age groups is very important when studying cognitive function because using methods such as Subtle Cognitive Impairment Test (SCIT) for older age subjects similar to that used by Jianqin et al. (2016) may be not suitable for adults with an average age of 46 years. SCIT methods are usually used in studies targeting older people such as in the study of Friedman et al. (2012) who used the test with participants aged 62-98 years.

In terms of limitations, the diet of volunteers was not restricted in polyphenols and fibre-rich products. Differences between the A1/A2 and A2 milk groups confirmed the effect of the milk as a whole rather than one specific protein or macronutrients and this could be regarded as a potential limitation, if the study was only interested in the effect of β-caseins. In addition, having a variety of alternatives to dairy products such as soya milk-alternative to consume during washout period, to provide nutrients matching those from the treatment milk consumed during the intervention period, may have a protective impact on the blood lipid profile by significantly reducing serum total cholesterol, LDL-C and TAG and increasing HDL-C that might be result from the naturally occurring isoflavones depending on the process used to obtain the final product (Bricarello et al., 2004, Zhan and Ho, 2005). Therefore, further control studies are required to confirm or otherwise the

health benefit of A2 milk vs. A1/A2 milk in individuals who are at risk of CVD, metabolic syndrome and chronic inflammation such as inflammatory bowel disease (IBD).

In conclusion, this controlled clinical trial (CCT) found that consumption of A2 milk did not affect total cholesterol, TAG, LDL-C, SBP, BP, plasma betabolome and cognitive function but increased haemoglobin and glucose. Furthermore, A1/A2 semi-skimmed milk reduced DBP and HDL-C compared with semi-skimmed A2 milk. Overall, compared with milk containing only A2 β-caseins variants, this chronic study showed milk containing A1/A2 β-caseins variants to have beneficial effects on blood pressure, lipid profiles and cognitive function. These findings will contribute to the evidence that support the beneficial consumption of semi-skimmed milk with A1/A2 β-caseins variants on blood pressure and CVD risk overall. More studies are also needed directly comparing milk and dairy foods containing A1 vs. A2 β-caseins on cardiometabolic risk markers, particularly in subjects at greater risk.

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Chapter 5

General discussion and future research

5.1. General discussion

The importance of bovine milk in the human diet has been studied at great length in terms of human nutrition. A number of reviews have discussed the health benefits of milk and dairy products in providing a great numbers of essential nutrients and their positive effects towards human health, mainly high quality proteins, essential amino acids, vitamins and minerals (Pereira, 2014, ul Haq et al., 2014, Thorning et al., 2016, Givens, 2018). Lactose is a disaccharide that is found in milk and dairy products in varying amounts. It can be hydrolysed by the lactase enzyme during the digestion. A recent review indicates that approximately 70% of the world population develops a reduction in lactase enzyme activity during their lifetime that results in lactose intolerance and gastrointestinal symptoms such as diarrhoea, flatulence and abdominal pain shortly after the intake of milk (Ugidos-Rodríguez et al., 2018). However, Thorning and colleagues (2016) reported that adults who are lactose intolerant generally have the ability to tolerate and digest lactose in a glass of milk which is recommended for the general population and public health (Thorning et al., 2016) due to the health benefits. Casein proteins are one of the milk proteins that play a physiological role including opioid-like features, immunostimmulating and anti-hypertensive activities as well as the contribution in calcium absorption (Silva and Malcata, 2005). A recent review on the available scientific evidence of the health effects of milk and dairy products supports consumption of milk and dairy products because they are contribute to the achievement of key dietary recommendations and protect against common non-communicable diseases (Thorning et al., 2016, Givens,

2018). Therefore, milk and dairy products should be consumed and not eliminated from the diet because of the appearance of symptoms such as gases, bloating and abdominal cramps that are not related to lactose intolerance. That why recent limited evidence (He et al., 2017) that these gastrointestinal symptoms could be resulted by the consumption of milk containing A1 β-casein variant rather than A2 β-casein variant is potentially important. During enzymatic digestion as seen in **Chapter 2**, the A1 β-casein releases the β-casomorophin-7 (βCM-7) peptide in the gut but this is not possible with the A2 variant. The βCM-7 peptide has opioid characteristics which have been suggested to act as a pro-inflammatory compound that play a detrimental role in gut and cardiomentabolic health (Barnett et al., 2014, Haq et al., 2014b, Haq et al., 2014a).

Diet considered as one of the major factors linked to the risk of developing inflammatory-related conditions such as cardiovascular disease, obesity, diabetes, metabolic syndrome and immune disorder (Round and Mazmanian, 2009). However, one of the potential modulators of these conditions is the gut microbiota and its fermentation products such as short chain fatty acids (SCFA) that are linked to several beneficial health effects. Milk contains non-digestible carbohydrate, such as oligosaccharides (Gopal and Gill, 2000), which may reach the large intestine and be in contact with the gut microbiota. Thus, milk oligosaccharides (MO) may modify bacterial composition in the gut which in turn uses these compounds to produce compounds that exert biological activities (Heavey et al., 2003). It has been shown that SCFAs affect neutrophils and lymphocytes *in vitro* by down regulating pro-inflammatory mediators, an example of this is butyrate that is involved in down regulating the production of pro-inflammatory cytokines and up-regulating secretion

of anti-inflammatory cytokines such as (IL-10). CRP is one of the systemic inflammation markers that is produced normally as a result of stimulation by pro-inflammatory cytokines such as IL-1 and IL-6. Low-grade inflammation can be triggered by lipopolysaccharide (LPS) which is a component found in the cell wall of gram negative bacteria that is involved in the activation of macrophages, endothelial cells and neutrophils and these cells in turn release mediators such as proteins (CRP) and cytokines (IL-6) (Ashraf and Shah, 2014). Therefore, shifting gut microbiota to a beneficial bacterial population can inhibit inflammation and improve gut permeability (Hakansson and Molin, 2011). Examples of beneficial bacteria are Bifidobacteria and Lactobacilli, both of which are Gram positive bacteria that do not contain LPS in the cell membrane.

Following the literature review in **Chapter 1** it was identified that a number of research studies have been conducted comparing the consumption of human milk oligosaccharides (HMO) with infant formula fed products to explore the effect on gut health. *In vitro* research has shown that SCFAs mainly acetate, propionate, and butyrate are produced after the administration of HM but not bovine milk (BM) in colonic models (Shen et al., 2011b). Therefore, to address these limited data, an *in vitro* study was performed to investigate the impact of β-caseins and their bioactive peptides on the colonic microbiota of three different children aged between 5 – 8 years old from the same ethnicity using *in vitro* batch cultures (**Chapter 2)**. The results showed that milk containing A1/A2 β-casein variants and A2 β-casein variants behaved similarly to a recognised prebiotic FOS in terms of modulation of microbiota composition by increasing beneficial bifidobacteria, while the βCM-7 peptide gave results similar to negative control which characterised with no growth of bacteria.

This effect of milk was translated by the production of SCFA mainly propionate, butyrate and acetate. It is worth noting that the reason for using the microbiota of children was because we intended to conduct a clinical trial in Saudi Arabian children in their home country but because of several circumstances that related to the Saudi Food and Drug Authority (SFDA) not permitting the import of A2 milk from Australia to Saudi Arabia the focus of the work had to be changed to adults in the UK.

The research focus then changed to explore further if milks has differential effect on gut health especially for those individuals who experience GI symptoms after drinking commercial milk. As discussed in **Chapter 1,** epidemiological studies have reported an association between the intake of β-casein A1 variant and type 1-diabetes (T1D) (Elliott et al., 1999), (McLachlan, 2001, Laugesen and Elliott, 2003), ischaemic heart disease (IHD). However, data from epidemiological studies lacked accuracy and may not have accounted for all confounding factors such as smoking (Thiese, 2014). In relation to gut health, two *in vivo* animal studies investigated the direct effects of A1 versus A2 β-casein on the GI tract, one conducted by (Barnett et al., 2014) suggested that A1 β-casein has a direct effect on the gastrointestinal function by the opioid pathway. This indicated that A1 β-casein has a pro-inflammatory activity locally and systemically. The second study conducted by Haq et al. (2014) concluded that the gut inflammatory response was induced by the A1 β-casein variant (isolated from cow milk) through activating Th_2 pathways (Haq et al., 2014a). It is clearly seen from those animal studies that the diets used to explore the effect used proteins in the form of isolates and not in the whole food/ matrix and individuals usually consume milk, cheese etc as a whole foods and not as specific nutrients. In humans, detailed literature in **Chapter 1** indicated very limited number of randomised controlled trials

conducted on whole semi-skimmed containing A1/A2 β-casein variant compared to semi-skimmed containing only the A2 β-casein variant to explore if commercial milk that contains A1/A2 β-casein variants has a pro-inflammatory property (Ho et al., 2014, Jianqin et al., 2016, He et al., 2017). Moreover, some of these trials suffer from methodological issues such as the use of inappropriate screening methods and having limited power. In addition, the longer term effects of milk consumption on the gut microbiota composition have not been explored. To address this evidence gap, a double blind randomised crossover design trial was conducted to compare the effect of daily consumption of semi-skimmed A2 milk with semi-skimmed A1/A2 milk in 36 healthy adults with mild to moderate non-lactose milk intolerance for 14 days **(Chapter 3)**. The use of the hydrogen and methane breath test to screen participants for lactose intolerance increased strength of the study. We have shown that daily consumption of both milk types did not trigger local gut inflammation measured by faecal calprotectin and cytokines although both milk types lowered systemic inflammation and this was greater in A2 milk group. Furthermore, our results indicated that milk containing A2 β-casein variant had the potential to significantly increase Actinobacteria (a phylum that includes bifidobacteria) populations and increase stool frequency. In addition, the acute effect of consuming 250 ml milk challenge showed that A2 milk has the ability to lower bloating and abdominal cramps compared with the milk containing A1/A2 β-casein variant.

According to World Health Organisation (WHO), in many countries particularly those with a high standard of living, the prevalence of lifestyle diseases such as CVD and type 2 diabetes is growing and becoming a key cause of mortality. CVD includes a group of diseases such as CHD, hypertension, heart attack, atherosclerosis and stroke.

There are numbers of risk factors which have been identified and some of them can develop over many years starting from childhood. A number of these risk factors can be modified such as high LDL-cholesterol, low HDL cholesterol, high BP, inflammation, smoking, central obesity and physical inactivity. Diet is an important means by which risk factors associated with the development of CVD can be moderated. For example, positive associations have been shown between CVD and the high intake of saturated fatty acids (SFA) and trans fatty acids (TFA) that may be delivered from milk and dairy products in the UK diet (contributing 27% to the adult diet). Therefore, reduction of SFA and TFA in the diet will be one of the protective strategies from CVD (Lovegrove and Givens, 2016). The current study has shown that milk containing both A1/A2 β-casein variants in a food matrix had no association with cardiometabolic risk factors as has been suggested by the previously mentioned epidemiological studies and one animal study. As mentioned in **Chapter 1,** using a rabbit model suggested that β-casein A1 (in form of casein isolate) fed animals led to a significant increase in serum cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride levels compared with β-casein A2 fed animals and this was due to the oxidation of LDL by β-casomorphin-7 released from β-casein A1 (Tailford et al., 2003). These data were in contrast with later human trials (Chin-Dusting et al., 2006, Venn et al., 2006) that examined the same hypothesis and concluded that no deferential effect exists between milk containing A1/A2 β-casein and milk containing only A2 β-casein. Moreover, some of these trials suffered from methodological issues such as a lack of control of dietary intake and the use of cocoa and citrus juice to make the shake to flavour the casein powder. Therefore, in order to gain a better understanding of the beneficial effect of both milk types on the cardiometabolic health markers, one secondary outcome of the randomised clinical trial was evaluated and

presented in **Chapter 4**. This human trial investigated the chronic effect of different milk (as food matrix) containing different β-casein variants on CVD risk markers in healthy adult subjects. The results showed that milk containing A1/A2 β-casein variant has the potential to lower serum high-density lipoprotein cholesterol (HDL-C), fasting glucose, diastolic blood pressure (DBP) and heart rate (HR) compared with milk containing only A2 β-casein, while A2 milk improved haemoglobin compared with A1/A2 milk. Both milks had no effect on fasted serum total cholesterol (TC), low-density lipoprotein cholesterol LDL-C, triacylglycerols (TAG), systolic blood pressure (SBP), body composition measures, plasma metabolome and cognitive function. These findings are in accordance with the evidence that looked at the milk as a whole not as a single nutrients as described in **Chapter 4**. However, it remains unanswered whether A1/A2 milk has a differential effect than A2 milk on cardiovascular risk markers in healthy individuals. Indeed, the outcome of the current study may have been influenced small sample size or the limited duration of the intervention period. However, comparing our findings with what is scientifically known about the beneficial impact of milk on blood lipid, PB and body composition (Fekete et al., 2016, Lovegrove and Givens, 2016, Givens, 2018) it seems clear that milk had no detrimental effect on LDL oxidation thus no disadvantage of consuming milk containing A1/A2 β-casein variants.

Factors that may influence evaluation of the impact of milk consumption on health include health status of subjects and the study power, primarily related to the number of subjects. Usually, human intervention studies that are conducted in a group of people with for example obesity, metabolic syndrome, family history of inflammatory disorders or allergy are more likely to report beneficial effects on inflammatory

markers, whilst intervention conducted in healthy individuals mostly report no effects. In addition, power is important in the interpretation of data. In our study, prior to the intervention study power was calculated based on a previous study. However our study fell short of its recruitment target due to difficulties in recruitment and was completed with 36 participants instead of 45 as indicated by the power calculation which was based on the primary outcome FC (local inflammation marker).

In conclusion, it has been shown that consuming milk containing A2 β-casein variants in the habitual diet of healthy adults who experience gastrointestinal symptoms after drinking A2 milk may improve gut health and reduce systemic inflammation and gastrointestinal symptoms and also through the modulation of gut microbiota composition by increasing beneficial bacteria such as bifidobacteria. Furthermore the beneficial activity was shown *in vitro* by the production of SCFA. In addition, daily consumption of normal milk that contains a mixture of A1/A2 β-casein variants may improve blood pressure and fasted glucose levels. Although this PhD research aimed to study the effects of milk with A1/A2 β-casein variants and milk with only A2 β-casein variants on the gut and cardiovascular system *in vitro* and *in vivo*, several research questions remain unanswered. However it is hoped that these findings will provide evidence for future research and for public health recommendations in order to obtain the valuable nutrients present in milk.

Finally, it is clearly seen from the literature discussed before and from this research that food derived bioactive proteins and peptides have been confirmed to affect numbers of biological process in the body. In addition, some of these bioactive peptides have more than one activity such as BCM-7. BCM-7 exhibits opioid and

ACE inhibitory and immunomodulatory effects. BCM-7 was found in considerable amounts in the small intestine after the in vitro digestion of normal bovine milk that contained a mixture of A1/A2 β-casein variants but not from A2 milk. Once these peptides are released from the parent protein they have the ability to act directly in the gut lumen or exert an effect after binding to specific receptor binding sites on brush border membranes. However, evidence suggests that the affinity of bioactive peptides released from dietary proteins to cellular receptors seems to be weak compared with synthesised (drug) and endogenous peptides. Furthermore, β-casein derived peptides have been shown to have concentration-dependent effects on proliferation of human lymphocytes, promote antibody formation and enhance phagocytic activity. In addition, the activity of bioactive peptides depends on the matrix it is in and other factors such as ingredient composition and pH that may impact on the degree of activity. For example, purified milk bioactive peptides when tested individually normally show activity, whereas when mixed together did not exert any activity on the mechanism of preventing overstimulation of the immune system (Calder and Yaqoob, 2013). Furthermore, bioactive peptides such as kappacin (antibacterial peptides) can also be lost or reduced at neutral pH. Last but not least, most of the research findings on the effect of bioactive peptides is derived from *in vitro* studies with limited data from human trials. Evidence suggests that milk is already naturally balanced since it contains numbers of bioactive peptides and proteins to prevent stimulation of the immune system. This current research supports the claim that milk as a food matrix does not exert any local gut inflammation and systemic inflammation in healthy adults. An overall summary of the key findings of this research is presented in Table 5.1.

5.2. Future research

The studies in the present thesis have addressed a number of important research questions, while highlighting some opportunities for future research. The randomised clinical trials in this thesis demonstrated beneficial effects of consuming milk with A1/A2 β-casein variants and A2 β-casein variants delivered via the parent food matrix in a healthy adult population. However, effects in healthy children and in children with disorders such as autism is of further interest since the A2 milk used in this study modulated microbiota composition. It is also of interest to determine whether the bioactive peptide BCM-7, which is released from milk with A1/A2 β-casein variants, appears in blood and is excreted in urine. As seen in **Chapter 2**, the potential effect of digested milk on modulation of microbiota composition was achieved from healthy children donors. It would be interesting to examine the same model in adult donors with some modifications in order to minimise confounding factors that may affect gut microbiota composition such as dietary habit, smoking, exercise and stress.

Table 5.1. Summary of the key findings of the current study

	A1/A2 milk	**A2 milk**
Milk Composition analysis		
Type of β-casein variants	A1 & A2	Only A2
***In vitro* digestion**		
Release of BCM-7	Yes	No
Release of BCM-5	No	No
***In vitro* fermentation**		

Modulation of gut microbiota	Yes	Yes
Total bacteria	↑	↑
Bifidobacteria	↑	↑
SCFA production	Yes	Yes
Impact on gut health and inflammation		
Local inflammation (FC)	NDE	NDE
Pro-inflammation (Il-6)	NDE	NDE
Anti-inflammation (IL-10)	NDE	NDE
Systemic inflammation (hs-CRP)	↓	↓ more
DNA sequencing		↑ Actinobacteria (Bifido)
Metabolome	NDE	NDE
Fermentation products (H_2 and CH_4)	NDE	NDE
SCFA production	NDE	NDE
GI symptoms (acute)		↓ abdominal pain ↓ bloating
GI symptoms (chronic)	NDE	NDE
Stool frequency		↑ number of stools
Impact on cardiometabolic health		
Total cholesterol	NDE	NDE
Triacylglycerol	NDE	NDE
Very low-density lipoprotein	NDE	NDE

High-density lipoprotein –cholesterol	↓	
Glucose		↑
Haemoglobin		↑
Body mass index	NDE	NDE
Body fat %	NDE	NDE
Systolic blood pressure	NDE	NDE
Diastolic blood pressure	↓	
Pulse pressure	NDE	NDE
Heart rate	↓	
Plasma metabolome	NDE	NDE
Cognitive function	Better performance	

SCFA, short chain fatty acid; FC, faecal calprotectin; IL-6, interleukin-6; IL-10, interleukin-10; Hs-CRP, high sensitive C-reactive protein; NDE, no differential effect; ↓, s□□□□□□□□□□□□□□□□□□□□□□d; ↑, s□□□□□□□□□□□□ increased relative to second intervention.

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Appendix