In Vitro Studies on Gum Acacia and its Potential as a Prebiotic in an Elderly Population

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Declaration

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

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Abstract

Gum acacia (GA) is a soluble dietary fibre derived from acacia trees. It is widely used in African countries and in the Middle East as a traditional medicine. Recently, the consumption of GA has been related to potential health benefits in terms of its potential prebiotics properties, this may be particular relevant in elderly people. Elderly are experiencing negative changes in their gut microbiota and their immune system. Therefore, in this study GA was assessed in *in vitro* models, and resulted in increased *Bifidobacterum spp.*, which can be important for a targeted population such as elderly. Further evaluation of GA looked at the potential to induce cytokines production with PBMC cells from elderly volunteers. Promising increases in IL-10 were observed.

The bacteria able to utilise GA, whilst possessing anti-microbial potential were further studied using enrichment culture techniques. Here *Lactobacillus spp.* were isolated and shows anti-pathogenic activity against known pathogens. The antimicrobial activity was related to the lowering pH regards to organic acid production. Finally, further investigation to evaluate the probiotic bacteria and the associated synbiotic was evaluated in the presence of *E. coli*. within *in vitro* mixed batch cultures. The synbiotic combination led to increases lactobacilli and inhibition in *C. histolyticum* group, this effect was more apparent than with the GA, or probiotic alone treatments.

Overall, the potential of GA as a prebiotic has been explored, furthermore, a possible synbiotic combination has been developed. These products could show great potential to an ageing population.

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1. Chapter (1) Introduction

1.1 General background on the gut microbiota

The human gastrointestinal (GI) tract harbours a complex and diverse bacterial community [2]. Some studies suggest that the colonisation of the gastrointestinal (GI) tract begins before birth and subsequently develops over the first 2 years to resemble that of an adult microbiota [3], [4]. At this point, there are many factors that could influence the diversity of bacterial composition, such as environmental factors, birthing regime and feeding. The bacterial inhabitants exist in different numbers through the GI tract, stomach, with the small intestine having less than the large intestine, this might be due to the gastric acidity and rapid transit time that influence the growth of bacteria. The large intestine, however, contains complex bacterial groups with up to 10^{11} or 10^{12} cells per gram [5]. It is dominated by four phyla: Firmicutes, Bacteroidetes followed by Proteobacteria and Actinobacteria, with minor contributors, including Verrucomicrobia and Fusobacteria [6]. The most copious human anaerobic bacterial genera are *Bacteroides, Eubacterium, Bifidobacterium, Clostridium, Peptococcus, Peptostreptosus* and *Ruminococcus* [2]. The gut microbiota can be affected by many factors, including, amount of fermentable substrates (diet), exogenous bacteria, ionic and pH conditions and transit time [7, 8]. Through fermentation of non-digestible carbohydrates the gut microbiota serves different functions to the host, these include:

i. Metabolic activity, providing energy to the host
ii. Trophic function, control of epithelial cell proliferation and differentiation; development of homeostasis of the immune system
iii. Protective properties, as the barrier function prevents invasion by pathogens [2].

1.2 Probiotics, prebiotics and synbioitcs

1.2.1 Probiotics

The term probiotic refers to "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [9]. A probiotic has been recommended to have the following attributes:

- Must be safe
- Be viable and stable under storage conditions
- Be viable to be prepared in wide manner
- Resist the host digestion and survive in the large intestine
- Should lead to beneficial effects to the host [10, 11].

Most probiotics used belong to the genera lactobacilli (*Lactobacillus acidophilus, L. casei, L. delbruckii*) or bifidobacteria (*Bifidobacterium adolescentis, B. bifidum, B. longum, B. infantis*). Gibson and Roberfroid (1995) reported that bifidobacteria and lactobacilli possess health promoting properties [5]. These probiotics could be used as single species or in mixed with other bacteria. There is evidence that probiotics play a crucial role in regulating human health, including reducing diarrhoea [12], alleviating constipation [13], [14] immunostimulation [15], [16] and reducing hypercholesterolemia [17].

1.2.1.1 Probiotics, mechanisms of action

Probiotics exert their effects in a number of ways. Some of these mechanisms are outlined below:

i. The production of antimicrobial substances [18] and end products such as SCFA, which can lower colonic pH, making an environment less favourable for pathogenic bacteria [19], [20], [21].
ii. Out-competing other bacteria for nutrients, possibly reducing substrate availability for non-desirable bacterial groups [22].

iii. Modulating the immune system, probiotics could promote gut barrier function and anti-inflammatory response through several mechanisms, including modulating dendritic cells [23].

1.2.2 Prebiotics

Prebiotics were redefined at the 6th International Scientific Association meeting of probiotics and prebiotics (ISAPP, 2008) as ”selectively fermented ingredients that result in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health” [24]. A prebiotic has been recommended to have the following attributes:

i. Be resistant to gastric acidity and hydrolysis by host enzymes and GI absorption
ii. Selectively stimulate the growth and /or activity of limited microorganisms in the gut
iii. Be fermented by intestinal microbiota [25].

There are several types of common prebiotics including inulin, fructo –oligosaccharides (FOS) and galacto-oligosaccharides (GOS) [26], while other oligosaccharides show potential prebiotic activity, such as soya-oligosaccharides [27], xylo-oligosaccharides [19], isomaltooligosaccharides [28]. Traditional dietary sources of inulin include soya beans, banana, Jerusalem artichoke, garlic, wheat, asparagus, rye, and chicory root. In order to obtain a recommended prebiotic dose of inulin, however, large quantities of these foods would be required (e.g. 10 bananas), this may not be so practical.

Recently, extracts of gum, such as Tragacanth gum, with arabinogalactan and fucoxylogalacturonans, have been considered as potential prebiotics [29]. Tragcanth gum is obtained from small shrubs of the *Astragalus* and grow widely in Pakistan to Greece, and in particular, in Iran and Turkey (Whistler, 1993). Moreover, partly hydrolysed guar gum has been demonstrated to have a positive effect on IBD patients in a human study [30]. From this

view gum acacia (GA), made up of arabinogalactan, could possess health promoting prebiotic potential. There is a great interest in developing new prebiotics with the aim of modulating the gut microbiota in the distal colon, as most commercial prebiotics are fermented in the proximal colon, in order to induce a positive health effect. As the protolytic fermentation end products might be responsible for the differ disorders occur in the left part within the colon [31]. Proteolytic fermentation within the colon, is also increased with aging [32, 33]. Moreover, the polyamines and ammonia production are increased, which have been linked to colon carcinogenesis [34].

1.2.3 Synbioitcs

Synbiotics as defined by Gibson and Roberfroid (1995) "are mixtures of pro- and pre-biotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract" [5]. The addition of prebiotics could improve growth of probiotics by offering a food source. Prebiotics could also act to increase both the numbers of probiotics and beneficial bacteria such as bifidobacteria and lactobacilli. The combination may be able to optimise the manipulation of the gut microbiota composition towards a healthy gut.

1.3 Diet and elderly persons

The World Health Organisation reported that the population of those aged 60 years or older is rapidly increasing [35] and over one-third of the UK population will be aged over 65 years by 2050. Ageing is defined as "the regression of physiological function accompanied by the development of age" [36]. Diet is a major influencing factor on the gut microbiota, and the high-fat, sugar-rich Western diet contributes to Bacteroidetes dominating the microbiome, whereas a high-fibre diet results in Firmicutes dominating the microbiome [37].

1.3.1 Changes in GI tract in elderly

Gastrointestinal function is essential for sustaining good nutrition. Therefore, age-related changes in GI tract are important considerations in elderly populations. The increased threshold for taste and smell, resulting in foods tasting bland and uninteresting. Furthermore, masticatory dysfunction caused by loss of teeth and muscle bulk [38] is combined with swallowing difficulties, resulting in an imbalanced diet. Subsequently low levels of nutrients and micronutrients could cause problems in elderly populations, such as constipation. Aging is subject to a number of physiological changes that impact on food digestion, absorption and immune function [39] these changes correlate with alterations to the colonic microbiota and increased occurrence of disease. There is a growing body of evidence indicating that *Bifidobacterium* spp. numbers are lower notably in aged populations (Claesson 2012). It is well established that reductions in this beneficial bacterial group can impair immune function [40]. Additionally, a decline in the genus *Bacteroides* has been observed in the elderly when compared to younger adults [41]. Faecal impaction and constipation, are symptoms associated with elderly gut function [42]. The dietary changes are able to effect diversity and numbers of the bacterial colon inhabitants, and subsequently influence metabolites and immune function [41].

1.3.2 Changes in immune function when age

Immunosenescence is defined by a decline in the immune response to exogenous infections agents as well as an increase in endogenous signals, observed in elderly population [43], [44]. During aging, a decline in immune function and response has also been observed [45, 46]. Additionally, increased levels of pro-inflammatory cytokines, including interlukin 6 (IL-6) , IL-1 β, and tumour necrosis factor α (TNF-α) occur [41, 47, 48]. The percentage of natural killer (NK) cells are increasing due to decline in T cells associated with aging [49]. In addition, during aging, the apoptotic cells decline, as a result of necrotic cells accumulate

due to impaired of clearance of autoantigen producing cells [48]. Moreover, naïve B cells, generated in bone marrow, also decrease with aging resulting in impaired defence against infections and intrinsic B cell dysfunction or declined CD4-T cell helper function [50].

1.3.3 Changes in gut microbiota when age

The diversity of species comprising the gut microbiota changes with age. Zwielehner et al. (2009) observed less *Bifidobacterium* spp. and *Clostridium* cluster IV in elderly subjects compared to those seen in middle aged subjects [51]. In another study, Marathe et al., (2012), reported that within an Indian cohort, the changes in the gut microbiota associated with age reflected a gradual decrease in Firmicutes and an increase in Bacteroidetes [52]. Claesson, 2012, found an association between diet, host health, environment, and gut microbiota; in particular, there was an association between a lower diversity in the diet and decreased gut diversity, subsequently there were increased inflammatory markers [53]. Additionally, individuals living in a community had more diverse microbiota than those in residential care. Ageing is associated with a decrease in probiotic bacteria such as bifidobacteria, which can act to inhibit pathogens such as *Escherichia coli (E. coli)* and *Salmonella* [54]. As such these changes in the intestinal bacteria may increase susceptibility to infections and diseases [38].

1.3.4 How could the microbiota impact on the immune system?

With ageing elderly experience a potentially negative shift in their gut microbiota, as well as significant increases in inflammation and dysfunction of the immune system, hence a relationship between the gut and immune system can be observed [51]. These changes are thought to result in increased risks for development of chronic disease such as diabetes, colorectal cancer (CRC) and inflammatory bowel disease (IBD). Furthermore, a decline in mucin production is observed within this population, such a change increases the potential for

pathogenic adhesion to the gut epithelial barrier [51]. Figure (1) shows the relationship between shifting in the gut microbiota in elderly and the impaired immune function.

1.3.5 Modulation of intestinal microbiota with prebiotics, probiotics and synbiotics, in elderly

The relationship between prebiotics and elderly gut microbiota has been widely investigated, Table 1shows *in vivo* studies on the modulation of gut microbiota. Walton et al (2012) found that GOS (4 g/d in juice) increased bifidobacteria in a crossover study when compared with placebo twice daily for 3 weeks [52]. In another human trial, Vulevic and others demonstrated that a prebiotic could modulate the elderly gut microbiota and modulate the immune responses [41]. These changes are relevant for an elderly population undergoing microbial changes and increased inflammatory status.

Moreover, a human study used prebiotic scFOS 8g/d for 4 weeks Bounhink et al (2007) led to significant increases in bifidobacteria in healthy elderly people [53]. Not all studies give a rise to positive reults as a study of Maukonen *et al* (2008) showed that after daily consuming GOS 10g/d for three weeks no changes in the elderly gut microbiota occured. The discrepancy results could be due to the short period of wash out during the study which make it difficult to return to baseline [54].

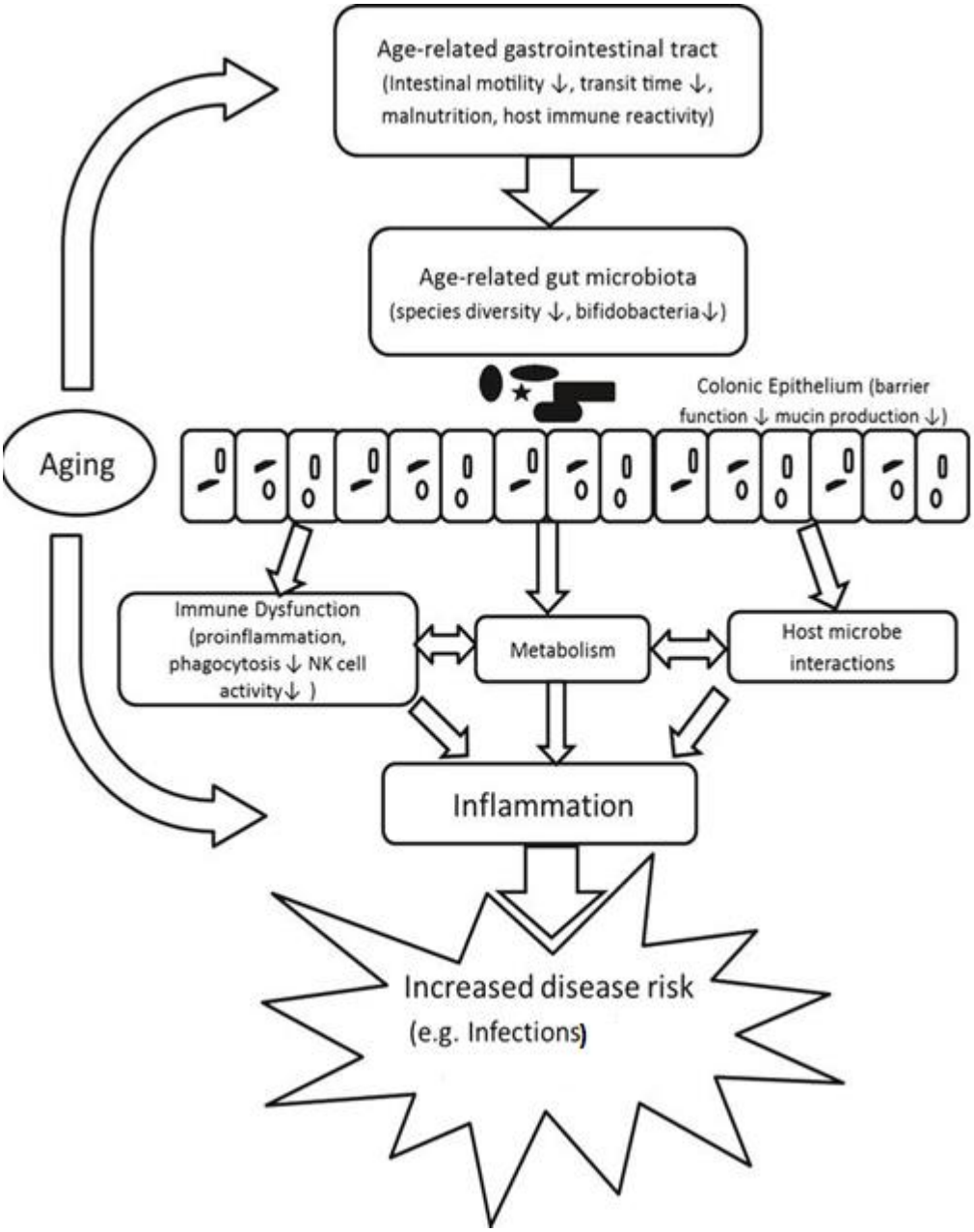

Figure 1: This figure indicates that elderly are at risk for inflammation and infections. With ageing there is an increase in inflammation and a decline in bifidonbacteria and diversity of bacterial numbers and mucin production. Figure adapted from [55].

Another approach to modulate the elderly gut microbiota composition is by probiotics, a range of studies have shown probiotics can modulate the microbiota in this population group. Most research investigating the gut microbiota has utilised traditional culture methods to assess the changes in the bacterial microbiota, in a study of Lahtnen et al (2009) *B. longum*

2C + *B. longum* 46 was used for 6 months by elderly people and resulted in a significant increase in bifidobacteria [56]. While other researchers have used molecular methods, a study on *B. lactis* Bb-12*;B. longum* 2C+*B.longum46* was administered at a dose of 10^9 CFU/daily, and resulted in enhancing *Bifidobacterium* [57], most of these studies used polymerase chain reaction (PCR) techniques to analyse the bacterial changes [58].

Additionally, synbiotics may be a good approach to modulate the gut microbiota composition an in elderly population. In *in vitro* studies, a batch culture of *Lactobacillus* combined with FOS and *Bifidobacterium longum* with isomaltoligosacchrides (IMO-BL) led to antimicrobial activity against enterohaemorrhagic *Escherichia coli* O157:H7 and enteropathogenic *E. coli* O86 [59]. Also in two human intervention studies it has been shown that synbiotics alter the gut microbiota which is relevant to elderly health improvement. The synbiotic *Bifidobacterium bifidum BB-02 and Bifidobacterium lactis BL-01* with inulin in elderly volunteers using PCR to determined the changes in the bacterial composition, additionally another study used *Lactobacillus acidophilus* with lactitol in a synbiotic combination in a double blind randomized trail in healthy elderly, the beneficial bacteria i.e bifidobacteria utilise the selected prebiotic and subsequently increases in the bifidobacteria and lactobacilli and reduction to the pathogens, these studies used culturing and molecular techniques to quantify the microbiota [42, 60-63].

Table 1: Studies *in vivo* on the modulation of probiotics, prebiotics and synbiotics on the elderly gut microbiota

Subjects	Substrates	Dose	Duration	Results	Reference
39 elderly people	GOS	4g twice daily	3 weeks	Significant increase in bifidobacteria (quantitative PCR)	[52]
44 healthy elderly people	B-GOS	5.5g/d (Bimuno)	10 weeks	Significant increase in beneficial bacteria (bifidobacteria, lactobacilli, and the *C. coccoides-E. rectale* group), and decrease in less beneficial bacteria (bacteroides, the *C. histolyticum* group, *E. coli*, and *Desulfovibrio* spp.) (FISH)	[41]
12 healthy elderly people	scFOS	8g/d	4 weeks	Significant increase in bifidobacteria (microbiological culture techniques)	[53]
41 elderly people	GOS	10g/d	3 weeks	No significant difference (DGGE)	[54]
66 elderly people	*B. longum* 2C +	Drink containing	6 months	Significant increase in bifidobacteria,	[56]

	B. longum 46	10^9 CFU/ml		especially, *B. catenulatum*, *B. Bifidum* and *B. breve*	
55 elderly nursing home people	*B. lactis* Bb-12; *B. longum* 2C + *B. longum* 46	10^9 CFU/d of *B. lactis* Bb-12; 10^9 CFU/d of both *B. longum* 2C and *B. longum* 46	6 months	*B. lactis* Bb-12 led to significant increase in *B. animalis. B. longum* 2C + *B. longum* 46 led to significant increase in *B. adolescentis* and *B. catenulatum* (PCR)	[57]
25 elderly constipated people	Inulin	20g/d from days 1 to 8, increased to 40g/d from days 9 to 11, kept at this dose from days 12 to 19	19 days	Significant increase in bifidobacteria for both doses. At 40 g/day, significant reduction in enterococci (analytical profile index system)	[42]
19 elderly nursing home people	scFOS	4g twice daily	3 weeks	Significant increase in bifidobacteria (microbiological culture techniques)	[64]
80 elderly people	*B. lactis* HN019	5×10^9 CFU/d, 1.0×10^9 CFU/d and 6.5×10^7 CFU/d	4 weeks	All the three doses caused a significant increase in bifidobacteria, lactobacilli and enterococci and a decrease in Enterobacteriaceae (microbiological culture techniques)	[62]

66 elderly nursing home people	*B. longum* 2C + *B. longum* 46	Drink containing 10^9 CFU/ml	6 months	Significant increase in bifidobacteria, especially, *B. catenulatum*, *B. bifidum* and *B. breve* (quantitative PCR and microbiological culture techniques)	[56]
10 elderly people	Probiotic-fermented milk containing *L. casei*	4 x 10^{10} cells/80 ml bottle, one bottle per day	2 months	Significant increase in *Bifidobacterium* and *Lactobacillus* and decrease in Enterobacteriaceae (PCR)	[58]
18 healthy elderly people	Synbiotic containing *B.bifidum, B. lactis* and the inulin-based prebiotic (Synergy)	3.5 x 10^{10} CFU of each probiotic plus 6 g/d Synergy	4 weeks	Microbiological culture techniques revealed a significant increase in bifidobacteria and lactobacilli. Real-time PCR revealed significant increase in numbers of copies of the 16S rRNA genes of *B. bifidum*, *B. lactis* and total bifidobacteria	
47 healthy elderly people	Synbiotic containing lactitol and *L. acidophilus*	5.0-5.5g synbiotic per day. 2 x 10^9 CFU/g	2 weeks	Real-time PCR revealed significant increase in *L. acidophilus* and bifidobacteria. Final bifidobacteria numbers similar to that of healthy adults	[61]

1.3.6 **Modulation of immune function using prebiotics, probiotics, synbiotics, in elderly**

Probiotic and prebiotic intakes have shown potential for immune stimulation in elderly populations. One interesting finding is that probiotic *Bifidobacterium longum* bv. infantis CCUG 52486 has been seen to modulate immune function in older populations [65]. Other probiotics are well documented as they have the ability to stimulate the immune system in elderly, such as *Bifidobacterium lactis* HN019 and *Lactobacillus rhamnosus* HN001 [66], [67], [68], [69], [57], [70], [71] Table 2 shows *in vivo* studies on the modulation of immune function of using pre, pro and synbiotics on elderly people.

Prebiotics also can regulate the pro and anti-inflammatory cytokines in elderly, scFOS is an example of a prebiotic that promote a positive effect on immune system by down-regulating pro-inflammatory cytokines [72],[63]. Vulevic et al (2008) reported that B-GOS treatment 5.5g/d for 10 weeks in a double-blind, placebo –controlled crossover study in elderly subjects B-GOS increased beneficial bacteria and IL-10, whilst reducing proinflammatory cytokines IL-6, IL-1 β, thus it was concluded that B-GOS can have a positive effect on elderly gut composition and immune responses [41]. Also in a human study on elderly (*Bacillus coagulans* GBI-30), led to an increase in *Faecalibacterium prausnitzii* which has a potential antiinflammatory properties [73].

More recent attention has focused on the provision of synbiotics on immune function in elderly populations, Przemska-Kosicka et al (2016) compared young and elderly seasonal influenza vaccination response by using a novel probiotic *Bifidobacterium longum* bv. infantis CCUG 52,486 combined with prebiotic glucooligosaccharides (*B. longum* + Gl-OS) there was a significant increase in the vaccine anti-body in both young and elderly and the changes in young group were greater, however, no differences were seen in the immune

markers, this could be due to the differences in baseline immunosenscence between randomised groups [74]. Similarly, in another study, a synbitoic (lactitol+ *L. acidophilus*) led to an increase in prostaglandin E2 (PGE_2) levels which are important in elderly immune function [61]. The *Lactobacillus* strains tended to promote T helper 1 cytokines, whereas bifidobacterial strains tended to produce a more anti-inflammatory profile [16].

1.4 Gum acacia (GA)

The natural plant exudate GA is obtained from the Acacia Senegal trees [75]. These trees are harvested predominantly in Sub Saharan Africa [76]. The production of gum, in a process known as gummosis, is a natural response of the tree to injury of bark. The gum exudes as nodules which are then removed by farmers as a raw product. Generally there are two varieties of the acacia tree that gum is harvested from, these being Acacia senegal and Acacia seyal [77]. Figure (2) shows the form of GA once it is picked from trees.

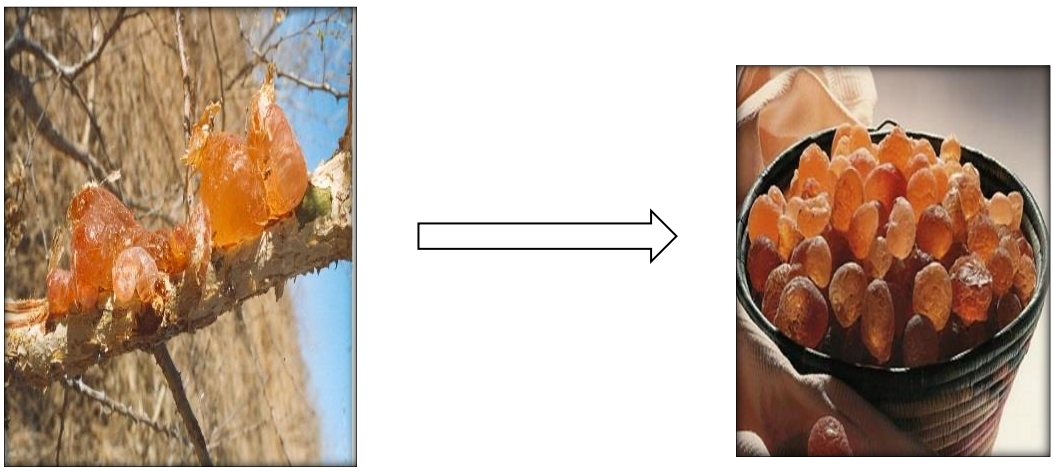

Figure 2 Raw GA pre and post-harvest.

1.4.1 GA in foods

The Federation of American Societies for Experimental Biology prepared a report for the United States Food and Drug Administration in March 1973, looking at the safety profile of gum acacia (GA) (Food and Agricultural Agency FDA). This committee looked at the safety of GA when used in foods. GA is "Generally Regarded as Safe" – GRAS and accepted as a food additive in the European Union (E414) (Directive 99/77/EC) and by Codex Alimentarius (INS414). GA is now also officially recognised as a dietary fibre in the EU directive 2008/100/EC [78].

Table 2: Studies *in vivo* on probiotic, prebiotic and synbiotic effect on immune function modulation in elderly populations.

Subjects	Substrates	Dose	Duration	Results	Reference
30 healthy elderly people	*B. lactis* HN019	5×10^{10} CFU/d; 5×10^{9} CFU/d	3 weeks	Both doses increased proportions of total, helper (CD4(+)), and activated (CD25(+)) T lymphocytes and NK cells	[66]
13 healthy elderly people	*L. rhamnosus* HN001	1.25×10^{8} CFU/ml, 200ml twice daily	3 weeks	Significant increase in phagocytosis, enhanced natural immunity	[68]
19 elderly nursing home people	FOS	8g/d	3 weeks	Reduced IL-6 mRNA expression	[64]

74 elderly people	oligosaccharides	1.3 g/250 ml/d	12 weeks	Reduced TNF-α mRNA and IL-6 mRNA	[72]
25 healthy elderly people	*B. lactis* HN019	$1.5x10^{11}$ CFU twice daily	6 weeks	Significant increases in interferon-alpha and phagocytosis, enhanced natural immunity	[71]
44 healthy elderly people	B-GOS	5.5g/d	10 weeks	Significant increase in phagocytosis, NK cell activity, IL-10 and decreases in IL-6, IL-1β, and TNF-α	[41]
27 healthy elderly people	*L. rhamnosus* HN001; *B. lactis* HN019	$5×10^9$ CFU/d of each probiotic	3 weeks	Both strains increased the proportion of CD56-positive lymphocytes, tumouricidal activity. Enhanced cellular immunity	[67]
55 heathy people (median 60,	*B. lactis* HN019	*B. lactis* in LFM (group A) or *B. lactis* in lactose-hydrolysed	3 weeks	Both groups significantly enhanced polymorphonuclear cell	[69]

range 41-81)		LFM (B)		phagocytosis and NK cell activity	
55 elderly people	*B. lactis* Bb-12; *B. longum* 2C + *B. longum* 46	10^9 CFU/d of *B. lactis* Bb-12; 10^9 CFU/d of both *B. longum* 2C and *B. longum* 46	6 months	Reduced inflammatory status	[57]
36 healthy elderly people	*Bacillus coagulans* BC30	(1×10^9) capsule per day	28 days	Significantly increased IL-10	[79]
47 healthy elderly people	Synbiotic containing lactitol and *L. acidophilus*	5.0-5.5g synbiotic per day. 2 x 10^9 CFU/g	2 weeks	Significantly increased PGE_2 levels	[61]
52 people (median age 63.5, range 44-80)	*L. rhamnosus* HN001	25 g/200 mL reconstituted low-fat milk powder, twice daily,10^9CFUs/g L	3 weeks	Increased phagocytic activity and NK cell activity	[70]

There are a number of benefits accrued to the GA, and it is widely known in food industry as thickening agent, emulisifer and as a stabilising agent in the pharmaceutical industry [80].

1.4.2 Bioactive compounds present in GA

According to Williams and Philips (2000), GA consists of polysacchrides with a high molecular weight (approximately 350-850kDa) containing galactose, rhamnose, glucoronic acid and arabinose residues, Table (3) indicates to the sugar content in GA but also minerals like calcium, potassium and magnesium. The total amount of protein is limited to less than 3% [75]. Figure (3) shows the GA structure.

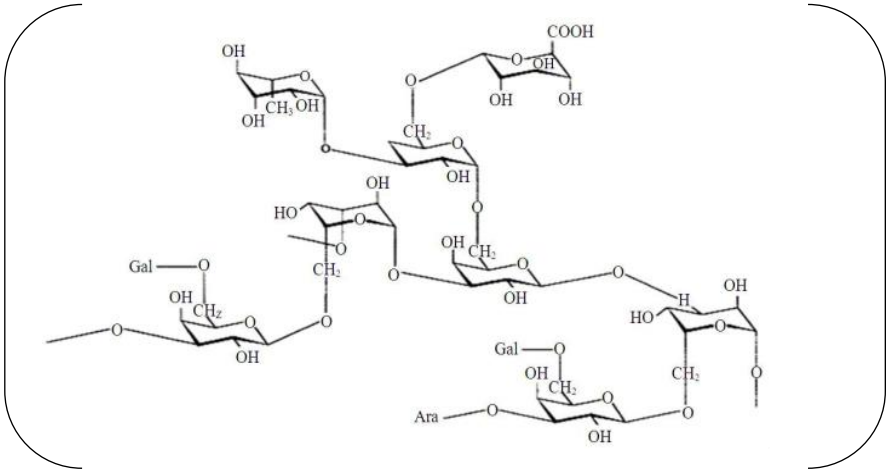

Figure 3: Structure of GA adapted from Dauqan and Abdullah (2013) [1]

Table 3: Characteristics of GA (Bhatti et al, 1970).

Component	% galactose	% arabinose	% rhamnose	% glucuronic acid	4-O-methyl glucuronic	% nitrogen	Specific rotation/degr	Average molecular
Result	45.0	27.1	12.9	14.8	1.6	0.316	−30	380.000

1.4.3 Health benefits associated with GA

GA is a major area of interest within the field of improving health, Traditionally, GA have been attributed to anti-obesity, anti-chronic renal failure, anti-diarrhoea properties [81] [82]. Additionally, a considerable amount of literature has been published on the effect of GA on the lipid metabolism and glucose level in human and murine studies [78] [81]. GA is resistant to digestion in the upper gastrointestinal tract and passes through to the colon where it can interact with gut microbiota [83, 84].[85-87].

1.4.4 Modulation of the intestinal microbiota by GA *in vitro*

Table (4) shows the effect of GA on the gut microbiota composition *in vitro*. Several studies have used traditional culture to determined changes in specific bacterial groups, Michels et al. (1998) demonstrated in an *in vitro* 24 h batch culture that GA (Fibergum) fermentation led to increases in lactobacilli and decreases in clostridia this study was enumerated using plate cultivation methodologies [88].

Previous studies have used molecular methods, Kishimoto et al (2006) reported a fermentation study of 2.5% GA enrichment culture to extract the predominant bacteria using porcine inoculum, resulting in *Prevotella ruminicola* like bacterium being predominant species, these might be responsible for propionate production observed. Temperature gel

electrophoresis (TGGE) was used to determine the gut composition, further studies are needed to acertain the microorganisms responsible for utilising GA anaerobically within the human colon [89].

Furthermore, *in vitro* studies indicate that GA could have a potential effect on the distal colon, Quantitative polymerase chain reaction (qPCR) and Denaturing gradient gel electrophoresis (DGGE) has been used to determined the changing numbers of bacterial groups, Terpend et al (2013) carried out a continuous culture experiment using arabinogalactan (AG), derived from GA, as a substrate, compared with fructo-oligosaccharides FOS. The apparatus consisted of five reactors simulating different parts of the human gastrointestinal tract:stomach, small intestine, and ascending, transverse and descending colon. After three weeks with a daily dose of 5 g the results showed that AG was mainly fermented in the distal colon while FOS was mainly fermented in the proximal colon. AG fermentation led to an increase in the phylum bacteroides and the genera bifidobacteria, along with an increase in *F. prausnitzii* which have been seen to possess anti-inflammation effects, these results were combined with an increase in butyrate and propionate in the distal colonic region and an increase in lactate and a decrease in ammonia in the descending colon. It was concluded that long term administration of AG derived from GA has potential benefits through increasing the saccharolytic metabolism distally in the colon and might play a key role in maintaining the gut by producing SCFA [90].

A study of Marzorati et al (2015) highlights that blending a blend of GA and FOS can help to extend the time of selective fermentation to latter colon regions, 16S rRNA-targeted Illumina sequencing, DGGE and qPCR has been used to determined the diversity of gut microbiota and changing in specific numbers of bacteria [91]. However, investigation of GA

alone is required to understand the fermentation characteristics and whether AG alone may function as a prebiotic that is distally fermented.

SCFAs are an end-product of dietary fibre fermentation in the human large intestine. *In vitro* batch studies allow SCFA production to be monitored in the absence of absorption [92]. In *in vitro* batch cultures inoculated with faeces from healthy adults GA led to a gradual increase in the amount of SCFA, such an effect was thought to be due to the complexity of the AG structure [93]. Faecal SCFA reflect the amount of SCFA left after microbial production with subsequent utilisation and coloncyte absorption [94]. Through the colonic epithelial absorption SCFA stimulate sodium dependent fluid absorption that is associated with improvements in diarrhoea [95].

Bourquin et al showed that in human subjects, ingestion of different dietary fibre rich substrates produced different quantities of SCFAs in the faeces [96]. The fibre rich substrates that they tested, in three human volunteers, were two varieties of oat hull fibre, gum arabic, carboxymethylcellulose (CMC), soy fibre, psyllium, and six blends containing oat fibre, gum arabic, and CMC in various proportions. Production of SCFA was directly proportional to the content of GA in the substrates and overall, proportions of the SCFAs were acetate, propionate, and butyrate, produced in the molar proportion of 64:24:12 [97]. Wyatt et al (1986) observed using human faecal inoculum in basal medium with 2% of GA resulted in isolation of *Bifidobacterium longum* and *Bacteroides ovatus*, *Bacteroides oris*, *Bacteroids buccae* and prevotella ruminicola-like bacterium [98].

Table 4: *In vitro* studies on the modulation of gut microbiota by GA

Subjects	Substrates	Dose	Duration	Results	Reference
Healthy adult	GA	10 g/day	18 days	Increase in bacteroides, bifidobacteria, lactobacilli	[98]
19 healthy adults	GA	10, 15 g/d	10 days	increase in bifidobacteria, lactobacilli	[84]
Healthy elderly	41%GA , 18% inulin).	serving as 3.3g	Gut model SHIME	Increase bif and lab significantly	[91]
Healthy elderly	Arabinogalactan		Gut model SHIME	distal fermentation and bifidogenic effect; increased in lactobacilli.	[90]

1.4.5 Modulation of the intestinal microbiota by GA *in vivo*

Table (5) shows the effect of GA on the gut microbiota composition *in vivo*. Although *in vitro* studies have found GA to stimulate the growth of bacterioides and bifidobacteria, *in vivo* human studies have found them to increase bifidobacteria and lactobacilli predominantly, using PCR [83].

A human study of Cherbuit et al (2003) used a traditional culture techniques to study a specefic numbers of bacterial groups in healthy adults following GA intervention [84] found

that *Bifidobacterium* and *Lactobacillus* selectively fermented GA, with no effect on bacteroides. A part double blind human trail, the healthy volunteers ingested sucrose 10 g or GA (10g) and after that the dose was increased to 15 g; stools were taken by day 5-10 of each period for bacterial enumeration, and in the same study after 2 months another human double blind randomised cross-over study was conducted to assess the bowel performance after GA consumption using (10-70 g/day) for 18 days and comparing to SUC and FOS and the results show that volunteers experienced excessive flatulence, this indicates that at higher doses may not alter the gut microbiota efficiently. Human studies have indicated that GA can be tolerated at higher doses than FOS Cherbuit *et al* (2003) [84] Babaker *et al* (2012) [99].

Research in human studies has indicated that a blend of fibres can modulate the gut microbiota and may provide additional health benefits. Goetze et al., (2008) compared FOS with a blend of GA (50% FOS and 50% GA) and observed similarity between the substrates in bifidobacterial numbers concluding that GA additional to FOS could be important to different health aspects [100]. Additionally, Glover *et al* (2009) investigated the effect of GA on Type 2 diabetes patients with normal renal function and observed improved function in the treatment group, however, in this study no analysis for intestinal bacteria composition and no measures for SCFA were made [78]. Min *et al* (2012) suggested that GA has therapeutic effects on IBS patients, thus suggesting that GA could give rise to positive effects within the gut [101]. GA have mainly been observed to support the growth of bifidobacteria and lactic acid bacteria with no changes in bacteroides and clostridia, indicating selectivity, which could result in positive health benefits [83, 84]. Calame *et al* (2008) explored the different doses on healthy adults in a double blind, double controlled trail where six parallel test groups and subjects consumed 5, 10, 20 or 40 g daily of substrates, (n=51). and found with higher doses of GA numbers of bifidobacteria were decreased while within the lower doses it increased,

suggesting that when higher doses are administered the specificity of the gum may be compromised due to cross-feeding. These two studies have not investigated this effect on older populations.

Cherbuit *et al* (2003) claim that structure of GA mainly consists of polysaccharides, these can be used by bifidobacteria amongst others, which secrete enzymes to breakdown the sugars and glycosidec bonds to resulting in acetate production [84]. However, enzymatic analysis is needed to confirm these claims. In the case where they are broken down, especially their polysaccharide component, they yield sugars and uronic acids. These products are also inert and therefore do not pose any toxic threat to the body. GA is a water –soluble dietary fibre, it is a polysaccharide based on branched chains of (1-3) linked β-D galactopyranosyle units. Side chains, 2 to 5 units in length, are attached by (1-6) units to the main chain. Both main and side chains contain α-L-arabinofuranosyl, α-L-rhamnopyranosyl, β-D-glucuronopyranosyl and 4-O-methyl-β-D-glucuronopyranosyl units [102]. In addition, the enzyme thought to involved in gum acacia breakdown is alpha-arabinofuranosidase, also the enzyme betagalactosidase also increased within GA fermentation which indicates that bifidobacteria should be able to utlise the sugars in GA [90].

1.4.6 Mechanisms of action of GA and prebiotic function

GA like many polysaccharides can be broken down by intestinal microorganisms but is not digestible within the human upper gastrointestinal tract due to the lack of appropriate enzymes. GA therefore has the potential to support the growth of probiotics within the large intestine [103]. Selective abilities of GA may be due to the main polysaccharide in GA being arabinogalactan. Bifidobacteria and lactobacilli secrete enzymes such as and β-galactosidase and α- arabinofuranosidase that are thought to be involved in the break down of

arabinogalactan breakdown [90]. Overall this mechanism could lead to the prebiotic potential of GA.

1.4.7 Mechanisms of action of GA and immune function

Much of the previous research on GA has focused on assessing the anti-inflammatory properties,table (6) presents some studies on GA effect on reduces inflammation and figure (4) shows possible mechanisms of GA in reducing inflammation. *In vitro* study have observed GA can improve renal function by reducing the inflammation [104].

Another study, Matsumoto *et al* (2006) examined the potential role of butyrate in regulating pro-inflammation in renal epithelial cells. Following 8 weeks of supplementation GA 25g/d in healthy human subjects, butyrate was increased (n=7, p=0.03) and *in vitro* work in the same study it was observed that butyrate could regulate the production of Transforming growth factor beta (TGF-β) [105], TGF-β is involved in pathphysiology and increases in circulating

of TGF-β in blood vessels occurs in hypertention patients [78]. Butyrate production is very important as it can help to stimulate mucins which is important for mucous layers maintainance and epithelial protection [106], furthermore, butyrate is a key colonocyte energy source and involved in regulation and growth differentiation of cells [107], [108]. Therefore, a modulation of gut microbiota after GA fermentaion and increases in SCFA production could associated with regulating in cytokine production and subsequnelty the immune system enhancement.

Table 5: *In vivo* studies on the modulation of gum acacia in human gut microbiota

Subjects	Substrates	Dose	Duration	Results	Reference
Healthy adult	GA	10 g/day	18 days	Increase in bif and bac	[98]
19 Healthy adults	GA	10,15g/d	10 days	Increase in bif, lab	[84]
54 Healthy adults	GA	5,10,20,40 g/d	4 weeks	Increase in bif, lab	[83]

Table 6: Inflammation reduction by GA

Subjects	Substrates	Dose	Duration	Results	Reference
Rats	GA	Rats (0.75 %, w/w in feed	4 weeks	Increase in IL-10	[104]
Dentric cells	GA (khartom company)	n/a	n/a	IL-10 enhanced , CD4+ enhanced.	[109]
10 healthy volunteers	GA	25g	8 weeks	Increase butyrate	[110]

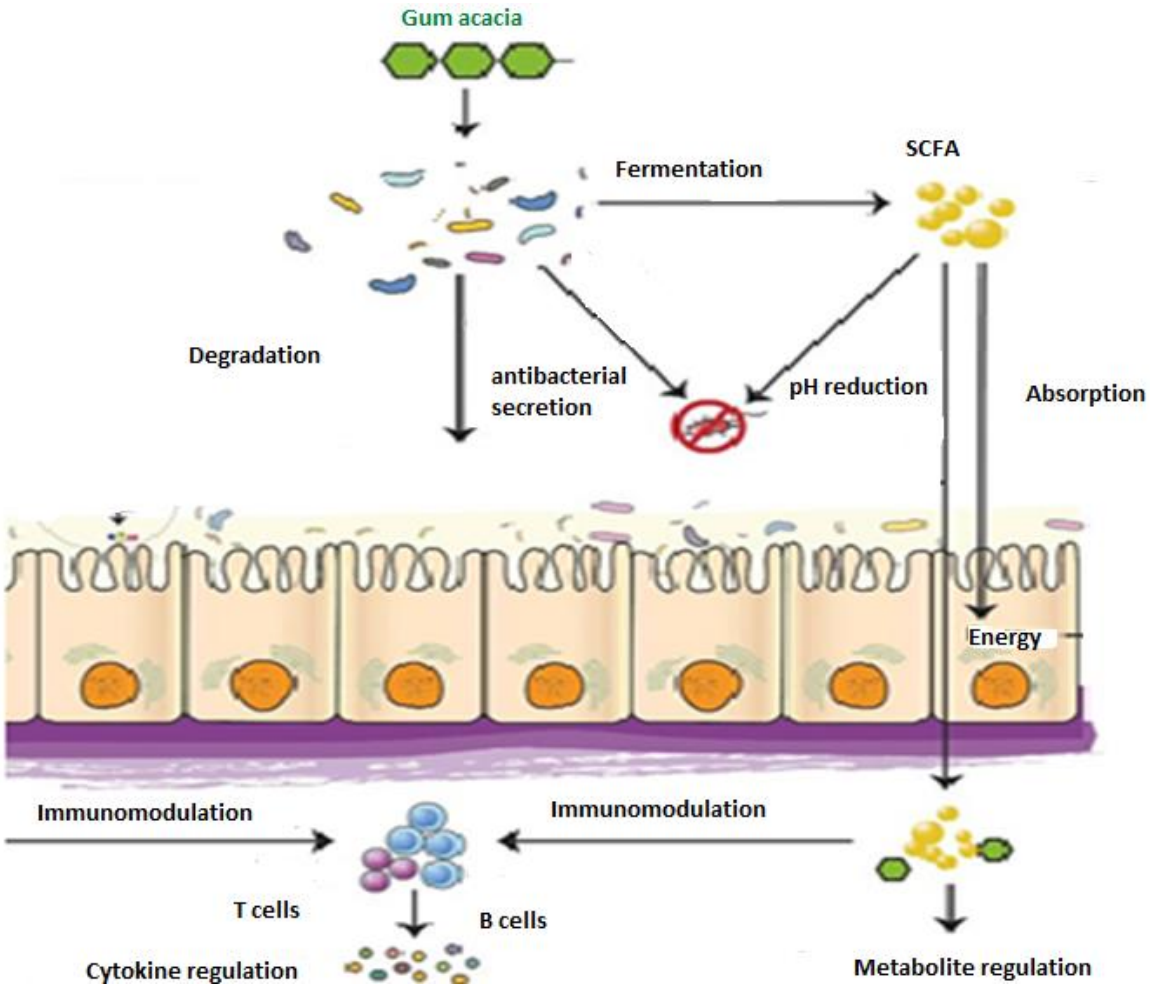

Figure 4: Pathway for gum acacia as a potential prebiotic that can modulate the immune function.

1.5 Methods of studying gut microbiota used in this study

1.5.1 *In vitro* models

In vitro models usually used to observe the effects of substrates such as probioics, prebioitcs, synbiotics. Such models do not alwayse provide an accurate model of what occurs *in vivo*, due the absence of mucosa colonocytes, absorption and immunological interactions. However, they can give a proper conditions to assess the metabolism and numbers of colonic gut microbiota.

1.5.1.1 Enrichment culture

An enrichment culture is a closed system used to isolate organisms that utilise a particular nutrient source from a complex microbial community. The enrichment sulture works on the basis that liquid growth medium replaced on a regular basis will provide optimal conditions for a key utilising organism, therefore enabling key substrate utilisers to be selected [111]. This allows microbes of interest to be isolated and further studied.

1.5.1.2 Batch culture

Batch cultures are a rapid method within a closed system in a pH controlled, stirred batch culture system. This system is maintained under anearobic conditions at 37Cº. the glass vessels contain a medium enable the growth of colonic bacteria, then the substrates is added before the addtion of faecal slurry 1%w/w total volume which is used presenting the gut microbiota. Batch cultures are a commonly used approach to determine the gut microbial activities, it where a substrate can be provided at the beginning of the fermentation. It can be used to assess how different substrates affect the colonic microbiota. This method used was

described by Olano-Martin [112]. Also it can be used to have more detail of what happens to the colonic gut microbiota.

1.5.1.3 Gut model

This model is a complex continuous system with a three vessels representing the proximal, transverse and distal colon, the three vessels linked to each other to sequentlly fed from a medium supply. The pH within the three vessels fed from 5.5 to 6.2 then 6.8 with an exit to a waste vessel [113]. The system is inoculated with faeces under anearobic conditions. The bacteria within the model are allowed to equilibrate before the addition of test substrates and a second equilibration stage will be reached before assessment of the changes. Continuous fermentation is an open system in which medium is continuously added to the bio-reactor and an equal volume of fermented medium is simultaneously removed [114]. The three stages gut model it can be a time consuming so often used after preliminary screening in batch culture system. It can give an overview of long term repeated exposure to a specific substrate may effect on colonic bacteria. This method can provide comprehensive metabolite data without the absorption that occurs *in vivo*. Subsequently SCFA production can be measured, thus the likelihood of a substrate to lead to SCFA generation can be determined. It has been validated by the colonic content of a sudden death victims thus it provide a decent method for testing substrates of interests [115].

1.6 Methods used in this study for detecting changes in colonic bacteria

1.6.1 Traditional methods: microbiological culture techniques

Over the last decade, methods used to quantify bacteria in the environment have changed dramatically. Traditional culture methods allow us to study the activity of bacteria to be studied under varying conditions, even when isolated from their natural ecosystem. However,

not all bacteria can be cultured. It is estimated that up to 70 % bacteria in the colon are unculturable [116]. Moreover, the cultural techniques does not give an indication of the changing in microbial anumbers.

1.6.2 Molecular techniques

1.6.2.1 *In situ* florescent hybridisation (FISH)

16S rRNA differs between microbial species, by targeted molecular probes. Synthetic oligoneucoltide probes can be hybridised to the bacterial group sequence of interest. A florescent label is used which allows for enumeration via florescence microscopy [117]. 16S rRNA is an excellent molecule to distinguish between different unknown bacterial species. It is an accurate technique that does not require cuturing of bacteria. Probes can be created and can be used for species level or genus specific. However, FISH technique has limit detection of 1×10^{6} , enabling bacteria to be accurately enumerated above this point [118]. 16S rRNA contains regions with different degree of sequence variation. there are conserved regions, where the DNA sequence is similar even between distantly related bacteria, and variable regions, where the DNA sequence is uniqe to a particular bacterial species or group of closley related species. Thus 16S Rrna is an excellent molecule for distingushing between different unknown bacterial species and evolutionary relationships.

1.6.2.2 Next generation sequencing (NGS)

Another approach to find out the gut function, to date, culture-independent molecular-based taxonomic assessment of microbiota has primarily relied on sequencing of PCR amplicons of targeted microbial genes at the deoxyribonucleic acid (DNA) level (DNA Amplicon-seq). Next generation sequencing (NGS) is a molecular method to sequence millions of fragments of bacterial genes. This method allows us to understand the diversity of

the environmental microbiome. Moreover, it can give information to the genus level. Previous data has used different methods to analyse the modulation in gut microbiota. However, searching more in depth about the species level to understand more about the interactions could aid in identifying the microorganisms that responsible for the fermentation of substrates. In order to achieve that NGS is provided as a new and comprehensive method, however, a limitation, might occur is that it is not fully quantitative as biased on primer selection [119] and it is also limited to discovering a new novel microbial phenotypes because the associated primers are designed based on known sequences [120].

Conclusion

From the literature, the increasing population of elderly provides the challenge of improving the general health and reducing intestinal inflammation. There exists the possibility to target the health of an ageing population through gut microbial modulation. Novel substrates that could impact on the microbiota are of great interest. One such substrate is GA, this has been considered a potential prebiotic and has been seen to possess anti-inflammatory properties in diabetes and renal failure patients.

1.7 Future Aspects

It must be stated that definitive proof of the modulation of gut microbiota by GA needs more research. Especially for elderly persons and improvements in systemic and mucosal immunity. Similarly, the science of *in vitro* fermentation offers the possibility of determining the more functional impacts of microbiota changes. The daily intake of GA as a supplement with different doses has shown a significant effect on human health, therefore, this study

indicates that there are other benefits of using GA worthy of investigation. It is essential to conduct further *in vitro* studies on the targeted population that have lower levels of beneficial bacteria such as the elderly. This group undergoes changes in the intestinal bacterial composition, due to various factors such as a slower GI transit time, which results in a subsequent increased inflammatory status and increased risk of pathogen infection. Furthermore, recent studies have indicated that prebiotics and probiotics play a role in improving the immune responses in elderly population, therefore, it is possible that GA can modulate the immune function via the microbiota in that target group. An *in vitro* approach could give a primary result that could base the knowledge to be the first step to continue the research *in vivo* studies. This study aims to assess systematically the fermentation characteristics and prebiotic potential of GA on elderly gut microbiota and their inflammation responses. Also to assess the bacterial profiles using modern molecular analysis, i.e. culture independent techniques such as bacterial analysis by fluorescent *in situ* hybridisation, 16S ribosomal ribonucleic acid (rRNA) gene sequencing, in addition to assess SCFA production using gas chromatography. Moreover, modulation of immune function will be assessed by using isolated peripheral blood mononeclear cells (PBMCs). The present research explores, for the first time, the effects of the immune modulation of the healthy elderly persons by using the fermentation metabolite of GA, and therefore, this could explain the therupatric properties of GA.

1.8 Aims and objectives

The overall aim in this study was to investigate the impact of GA fermentation on the gut microbiota and to assess if these changes if there offer potential to impact on health of elderly. Moreover, in order to produce a synbiotic of GA, putative probiotics were isolated from elderly faecal sample in extracted GA enrichment culture to generate a product more potent than GA alone. Therefore, in the present work *in vitro* approaches have been used to investigate changes in gut microbiota and immune markers on elderly and SCFA production.

The main objectives are:

1- Assess impact of GA *in vitro* fermentation on the microbiota and SCFA production on the gut microbiota.
2- Determine the likely impact of GA on inflammatory cytokines using *in vitro* models in elderly gut microbiota.
3- Isolate a putative probiotic using GA extract enrichment culture from elderly gut microbiota.
4- Assess the anti-pathogenic properties of isolated putative probiotic.
5- Investigate the anti-pathogenic properties of a synbiotic in elderly gut microbiota against *E. coli* in mixed culture.

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2. Chapter (2) *In vitro* fermentation of gum acacia - impact on the faecal microbiota

Abstract

Interest in the consumption of gum acacia (GA) has been associated with beneficial health effects, which may be mediated in part by prebiotic activity. Two doses of GA and fructooligosaccharide (FOS) (1% and 2%) were tested for their efficacy over 48 h in pH- and temperature-controlled anaerobic batch cultures inoculated with human faeces. Samples were taken after 0, 5, 10, 24, and 48 h of fermentation. The selective effects of GA (increases in *Bifidobacterium* sp. and *Lactobacillus* sp.) were similar to those of the known prebiotic FOS. The 1% dose of substrates showed more enhanced selectivity compared to the 2% dose. The fermentation of GA also led to SCFA production, specifically increased acetate after 10, 24, and 48 h of fermentation, propionate after 48 h, and butyrate after 24 and 48 h. Additionally, FOS led to significant increases in the main SCFAs. These results suggest that GA displays potential prebiotic properties.

2.1 Introduction

The colon has the most abundant and diverse population of bacteria in the human body and is inhabited by around 1000 different bacterial species, which can reach 10^{14} colony-forming units CFU [1]. The human microbiome is a complex and dynamic system that plays an important role in human health [2, 3]. By interacting with consumed material, colonic inhabitants ferment undigested food and secrete end products such as gases and SCFA [1]. Moreover, the composition of the bacterial population may shift, resulting in increases in bacteria associated with beneficial effects. Inulin and FOS are known prebiotics that are commercially used worldwide [4, 5]. A prebiotic is defined as 'a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health [6].

GA is derived from acacia trees of the *Leguminosae* family. It is an arabinogalactan protein complex with an approximate molecular weight of 350-850 K Da [7, 8]. It is a polysaccharide consisting of branched chains of (1-3) linked β-D galactopyranosyl units. Side chains, 2 to 5 units in length, are attached by (1-6) units to the main chain. Both the main and side chains contain α-L-arabinofuranosyl, α-L-rhamnopyranosyl, β-D-glucuronopyranosyl, and 4-O-methyl-β-D-glucuronopyranosyl units [9]. GA is widely used in the pharmaceutical and food industries as an additive , a stabilising, thickening, and an emulsifying agent [10] [11].

GA is not digestible in the small intestine and is fermented in the large intestine, and has been observed to lead to increases in *Bifidobacterium* spp. [12] [13] [14]. *Bifidobacterium* spp. have been shown to inhibit the growth of pathogenic bacteria, modulate the immune system, and produce SCFAs, which reduce the pH in the colon, imparting antimicrobial activity against pathogens [1]. A variety of GA doses ranging from 5 to 40 g/d have been reported to

be effective in increasing *Bifidobacterium* spp. and *Lactobacillus* spp. populations [12, 15]. These lines of evidence indicate that GA has bifidogenic potential in healthy humans. However optimal effective doses have not been defined. The results from most previous studies cannot be directly compared, as different methodologies, population groups, and types of GA have been studied. In addition, high daily doses of GA could result in the manifestation of adverse effects such as mild diarrhoea and bloating [16]. Therefore, following predigestion, we investigated the fermentation of two doses of GA over 48 h in pH-controlled batch culture systems and compared the results with those of FOS. *In vitro* batch culture systems are used to simulate the main physiological and microbiological processes in the distal colon and can be combined with metabolic and molecular analyses. This approach should provide more information on the prebiotic potential of GA.

2.2 Material and methods

Substrates

GA (KLTA-MF-Kerry Ingredients, U.K.) was in spray dried form as a water soluble, free-flowing powder (food-grade). Table 7 shows the composition of GA used in this study, dietary fibre were analysed by Campden BRI Laboratories (AOAC method 991·43). The FOS used was Orafti® P95 (Beneo, Belgium) extracted from chicory root.

Table 7 Composition and nutritional profile of GA used in the present study

Analysis	Results
Energy (kcal)	1205kJ/100g
Protein	2.1g/100g
Total carbohydrate (by difference)	82.6g/100g
Carbohydrate (avail)	56.5g/100g
Total Suger*	0.6g/100g
Fibre	26.1g/100g
Fat	0.1g/100g
Sodium	11.0mg/100g
Moisture	11.8g/100g
Ash	3.49g/100g

* Total sugars are the sum of glucose, sucrose and fructose expressed as monosaccharides

2.2.1 In vitro Upper Gut Digestion

Upper gut digestion was performed according to the protocol of Mills *et al.*(2008) [17]. Briefly, 60 g of GA powder was added to 150 ml of distilled water and the solution mixed with 20 mg α–amylase in 6.25 ml $CaCl_2$ (1 mM) and incubated on a shaker at 37 °C for 30 minutes. This simulated the initial oral digestion. Subsequently, 2.7 g of pepsin in 25ml of HCl (0.1M) was used to facilitate gastric breakdown of the sample. The pH was then reduced progressively to 2 by adding 6 M HCl, before incubating on a shaker at 37°C for 2 hours. A further 560mg of pancreatin and 3.5g of bile in 125 ml of $NaHCO_3$ solution was added to simulate the effect of the small intestine on the gum sample. The pH was increased to 7 by

adding NaOH (6M) and the resulting suspension incubated on a shaker at 37°C for 3 hours. Samples were transferred to cellulose dialysis membrane (1 KDa molecular weight), purchased from Cheshire biotech Cheshire, UK, and dialysed against 10Mm of NaCl solution at 5°C to remove low molecular mass digestion products. After 15 hours, the dialysis fluid was changed and dialysis continued for additional 2 h. The sample within the dialysis tubing was freeze dried (5 days) prior to use in batch culture systems.

2.2.2 Faecal sample preparation

Faecal samples were obtained from three healthy volunteers (31 35) years old, who had not been consuming antibiotics for at least 6 months before the study and had no history of gastrointestinal disease. Volunteers were not consumers of probiotic or prebiotic supplements. Samples were prepared on the day of the experiment and within 1 hour of production and were diluted to 1:10, w/v in anaerobic phosphate buffer (0.1 M; pH7.4). Samples were homogenised in a stomacher for 2 min, the resulting slurry was inoculated into batch culture fermenters.

2.2.3 Batch cultures

Three independent batch culture experiments were carried out using faeces from a different donor each time. Vessels were autoclaved and then aseptically filled with 135 ml of basal medium (peptone water (2 g/l), yeast extract (2 g/l), NaCl (0.1 g/l), K_2HPO_4 (0.04 g/l), KH_2PO_4 (0.04 g/l), $NaHCO_3$ (2 g/l), $MgSO_4 \bullet 7H_2O$ (0.01 g/l), $CaCl_2 \bullet 6H_2O$ (0.01 g/l), tween 80 (2 ml/l), hemin (50 mg/l), vitamin K1 (10 ml/l), L-cysteine (0.5 g/l), bile salts (0.5 g/l), 0.5 ml/l of 10% cysteine –HCl, resazurin (1 mg/l)). Vessels were left overnight with nitrogen pumping (15mL/min) through the vessel to provide an anaerobic environment. Before addition of faecal slurry, temperature of basal medium was set at 37 °C and pH was

maintained at 6.7-6.9 using a pH meters (Electrolab pH controller, Tewksbury, UK) by the addition of 0.5 M HCl or 0.5M NaOH. The vessels were stirred using magnetic stirrers. 1.5g, 3g (1% w/v) of FOS and 0.6 g and 0.3 g (1:10 w/v) of pre-digested GA (taking to account the intake daily 10g according to (Calame et al.,2008) of GA and reach the distal colon) were added to the vessel 1% and doubling the dose 2% just prior to the addition of 15 ml of faecal slurry (10%w/w). The vessels were left for 48h, with samples taken at 0, 5, 10, 24 and 48h. Samples were centrifuged in preparation for GC analysis, or prepared for microbial enumeration by FISH.

2.2.4 Florescence in situ hybridisation (FISH) analysis

To asses diffrences in bacterial population, samples hybridised as described by Daims et al., 1999 [18]. A sample of 375-µl obtained from each vessel was fixed for four hours 4˚C in 1125 µ L (4% w/v) paraformaldehyde. Fixed samples were then centrifuged at 11,337g (Eppendorf centrifuge minispin, Eppendorf, UK) at room temperature for 5 minutes. The supernatant removed and discarded. The pellet was resuspended in 1 ml of cold 1×PBS by aspirating carefully using a pipette. This step was conducted twice. The washed cells were suspended in 150 µL of cold 1×PBS, then 150 µL of ethanol (99%) was added and the samples were stored at -20˚C.

The oligonucleotide probes used were commercially synthesised and labeled with the fluorescent dye Cy3 (Sigma Aldrich Co. Ltd. UK). These were: Bif164 for *Bifidobacterium spp.* (BIF), Lab158 for *Lactobacillus/enterococcus* (LAB), Ato291 for *Atopobium cluster* (*Atopobium, Coriobacterium, Collinsella spp.)* (ATO), Chis 150 for *Clostridium histolyticum* group (CHIS) Erec 482 for *Eubacterium rectale – Clostridium coccoides* group (EREC), Bac 303 for *Bacteroides–Prevotella* group (BAC). EUB 338 mixture consisting of EUB338,

EUB338II and EUB338III for total bacteria (Total) see Table 8. For the hybridisation 20 µ L of diluted sample was pipetted onto a teflon poly-L-lysine-coated six-well slide (Tekdon Inc., Myakka City, FL). The samples were dried onto the slides at 46-50˚C for 15 minutes and after that dehydrated in an alcohol series 50, 80, and 96%. The ethanol was allowed to evaporate from the slides before hybridisation buffer was added. A probe/hybridization buffer mixture (5 µL of a 50 ng/µL stock of probe plus 50 µL of hybridization buffer). To permeabilise the cells for use with probes Bif164 and Lab158, samples were treated with 20 µ L of lysozyme at room temperature for 15 min before being washed briefly for 2−3 seconds in water and then dehydrated in the ethanol series. Then slides were placed in hybridisation oven for 4 hours (ISO 20 oven, Grant Boekel). For the washing step, slides were placed in 50 ml of washing buffer (0.9 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.005 M ethylenediaminetetraacetic acid (EDTA) solution (pH 8.0, Table 8, warmed at the appropriate temperature for each probe and 20 µL of 4;6-diamidino-2-phenylindole di hydrochloride (DAPI) was added to the washing buffer for 15 min. They were then briefly washed (2−3 s) in ice-cold water and dried under a stream of compressed air. Five microliters of ProLong Gold antifade reagent (Invitrogen) was added to each well and a coverslip applied. Slides were stored in the dark at 4 °C until cells were counted under a Nikon E400 Eclipse microscope. DAPI stained cells were examined under UV light, and a DM510 light filter was used to count specific bacteria hybridised with the probes. For each slide, 15 random different fields of view were counted.

Table 8 Hybridisation and washing conditions for oligonucleotide probes

Probe name	Sequence (5' to 3')	Hybridisation pre-treatment	Formamide (%) in hybridisation buffer	Hybridisation temperature (°C)	Washing temperature (°C)	Reference
Ato 291	GGTCGGTCTCTCAACCC	Lysozyme	0	50	50	[19]
Lab 158	GGTATTAGCAYCTGTTTCCA	Lysozyme	0	50	50	[20]
Bif 164	CATCCGGCATTACCACCC	Lysozyme	0	50	50	[21]
Erec 482	GCTTCTTAGTCARGTACCG	None	0	50	50	[22]
Chis 150	TTATGCGGTATTAATCTYCCTTT	None	0	50	50	[22]
Bac 303	CCAATGTGGGGGACCTT	None	0	46	48	[23]
EUB338*	GCTGCCTCCCGTAGGAGT	None	35	46	48	[18]
EUB338II*	GCAGCCACCCGTAGGTGT	None	35	46	48	[18]
EUB338III*	GCTGCCACCCGTAGGTGT	None	35	46	48	[18]
* These probes are used together in equimolar concentrations (all at 50 ng μl^{-1})						

2.2.5 Preparation sample for short chain fatty acids

Samples were extracted and derivatised as previously described [24]. Samples were defrosted and 1ml of each sample or standard solution was transferred into a labeled 100 mm × 16mm glass tube with the internal standard of 50µl of 2- ethyl butyric acid (0.1M). 0.5 ml concentrated HCl and 2 ml of diethyl ether was added to each glass tube and samples

vortexed for 1 min. samples were centrifuged at 2000 g for 10 min (SANYO MSE Mistral 3000i; Sanyo Gallenkap PLC, Middlesex, UK). The diethyl ether (the upper layer) was transferred in a new glass tube. A second extraction was conducted by adding 1 ml of diethyl either to the sample followed by vortex and centrifugation. 400 µl of pooled ether extract and 50 µl N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) was added in a GC screw-cap vial. Samples were heated at 80°C for 20 minutes and then left at room temperature for 48 hours to allow lactic acid in the samples to completely derivatise.

A 5890 SERIES II Gas Chromatograph (Hewlett Packard, UK) using an Rtx-1 10m×0.18mm column with a 0.20µm coating (Crossbond 100% dimethyl polysiloxane; Restek, Buckinghamshire, UK) was used for analysis of SCFA. Temperatures of injector and detector were 275°C, with the column programmed from 63°C for 3 minutes to 190°C at 10°C min-1 and held at 190°C for 3 minutes. Helium was the carrier gas (flow rate 1.2 ml min-1; head pressure 90 MPa). A split ratio of 100:1 was used. The standard solution contained (mM): sodium formate, 10; acetic acid, 30; propionic acid, 20; isobutyric acid, 5; n-butyric acid, 20; iso-valeric acid, 5; n-valeric acid, 5; sodium lactate, 10; sodium succinate, 20. The sample was injected onto the column, which was maintained at 140 ^{o}C for the first 5 minutes, temperature of the column was increased over 5 minutes to 240 ^{o}C. To maintain appropriate calibration after injection of every 20 samples an external standard solution, with known concentrations of SCFAs was injected. Peaks and response factors within samples were calibrated and calculated using ChemStation B.03.01 software (Agilent Technologies, Cheshire, UK).

2.2.6 Statistical analysis

GA and FOS both doses were tested in batch cultures inouculated with faecal samples collected from three individial donors in three separate experiments. The log $_{10}$ numbers of specific bacteria were expressed as mean values and standard diviation. Statistical tests were performed using SPSS, (SPSS Statistical Software, Inc., Chicago, IL, USA), version 18.0 A repeated measures one-way analysis of variance ANOVA to test the effect of time with the factor subjects, with five levels (0 h, 5 h, 10 h, 24 h and 48 h) and to assess the significant differences between the two subjects in the same time points. Significant differences between times point were represented by "*" $p < 0.05$, "**" $p < 0.01$ and "***" $p < 0.001$.

2.3 Results

2.3.1 Effects of different doses of GA and FOS on human faecal bacteria

To assess the impact of GA on the intestinal microbiota composition, pH-controlled, anaerobic, faecal batch cultures were conducted using FOS as a positive prebiotic control. Samples were taken after 0, 5, 10, 24, and 48 h of fermentation. Bacteria were enumerated by FISH.

The fermentation of GA at the 1% dose led to increased numbers of *Bifidobacterium* spp. after 5, 10, and 24 h of fermentation and of *Lactobacillus* spp. after 5 and 24 h compared with the levels at 0 h, as shown in Tables 9 and 10. However, a small but statistically significant drop in *Bifidobacterium* spp. compared with the negative control was seen after 24 h (7.53±0.10, 7.22±0.37 GA 1%) (7.46±0.21, 7.64±0.29 control). As shown in tables 9 and 10.

Fermentation of 2% GA a led to a significant increase in *Bifidobacterium* spp. after 5 and 10 h compared with the baseline levels (time 0 h). Total bacteria increased after the fermentation of 2% GA for 48 h compared with the baseline levels.

The 1% dose of FOS led to a significant increase in *Bifidobacterium* spp. after 5 h compared with baseline (7.39± 0.21, 7.83± 0.06). *Lactobacillus* spp. increased following fermentation of FOS at a dose of 1% at 5, 10, and 24 h. The 2% FOS dose significantly increased *Bifidobacterium* spp. numbers after 24 h compared with the negative control (8.12±0.16, 7.78±0.17) and after 5 h compared with baseline levels ($p = 0.03$), whereas an increase in *Lactobacillus* spp. was observed after 5 h (7.75±0.14, 7.29± 0.07) and 24 h (7.51± 0.10, 7.29± 04) ($p = 0.01$ and 0.02, respectively) compared with the negative control.

The number of bacteria in the *C. histolyticum* group decreased after fermentation of 1% FOS and 1% GA for 5 and 10 h, respectively, compared with the baseline levels. Additionally, GA and FOS enhanced the growth of *Atopobium* spp. after 5 h of fermentation compared with the baseline levels. The *C. coccoides-Eubacterium rectale* group did not change with any of the tested substrates. Additionally, *Atopobium* also increased after 5 h of fermentation of 2% FOS.

In the current study both substrates led to increases in *Bacteroides* spp.; these changes occurred after 5 h of fermentation of GA and FOS at the 1% dose ($p = 0.01$ and $p = 0.02$, respectively) and with the 2% dose of GA and FOS compared with the baseline levels ($p = 0.02$ and $p = 0.00$, respectively). Moreover, 1% FOS increased *Bacteroides* spp. after 24 h ($p = 0.01$). On the other hand, the prebiotic FOS at the 1% dose enhanced the growth of total bacteria, achieving statistical significance after 10 h and 24 h. Additionally, total bacterial growth was enhanced with 2% FOS after 5 h compared with the negative control.

Table 9 changes in the bacterial composition figures are presenting the mean bacterial populations in pH-controlled batch cultures at 0, 5, 10, 24, and 48h.Values are mean ± SD.*, significant differences from the 0 h value within the same treatment, p<0.05. small letters differences from the negative control. 1% faecal batch culture inoculated with vessel 1 negative control, vessel 2 FOS 1%, vessel 3 FOS 2%, vessel 4 GA 1%, vessel 5 GA 2%.

	Bif164		**Lab158**		**Ato291**		**Bac303**		**Erec482**		**Chis150**		**EubI-II-III**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
Control 0h	7.46	0.21	7.24	0.09	7.40	0.18	7.85	0.24	7.96	0.20	6.91	0.24	8.26	0.35
Control 5h	7.76	0.20	7.29	0.07	7.60	0.15	8.04	0.20	7.36	0.07	6.42	0.38	8.45	0.08
Control 10h	7.75	0.24	7.47	0.07	7.56	0.02	8.25	0.28	7.51	0.07	6.99	0.16	8.44	0.04
Control 24h	7.78	0.16	7.29	0.04	7.54	0.14	8.12	0.21	7.54	0.08	5.73	0.47	8.53	0.36
Control 48h	7.64	0.29	7.14	0.50	7.50	0.61	7.76	0.17	7.51	0.06	5.68	0.73	8.42	0.18
FOS 0h	7.39	0.21	7.31	0.08	7.52	0.09	7.80	0.11	7.72	0.2	6.62	0.53	8.21	0.14
FOS 5h	7.83 *****	0.06	7.78 ******	0.04	7.83 *****	0.10	8.16 *****	0.09	7.41	0.16	5.71 *****	0.55	8.78	0.11
FOS 10h	8.00	0.07	7.53 ******	0.09	7.77	0.30	8.30	0.10	7.83	0.29	6.23	1.09	8.72 *****	0.08
FOS 24h	8.17	0.23	7.75 *****	0.14	7.79	0.31	8.24 *****	0.01	7.50	0.12	6.27	0.88	8.83 *****	0.14
FOS 48h	7.82	0.15	7.44	0.15	7.58	0.18	7.93	0.18	7.28	0.19	5.65	0.69	8.57	0.17
GUM 0h	7.53	0.10	7.00	0.16	7.43	0.05	7.70	0.22	7.81	0.37	6.08	0.50	8.29	0.22
GUM 5h	7.92 *****	0.11	7.32 *****	0.05	7.80 ******	0.06	7.97 *****	0.19	7.42	0.11	6.64	0.42	8.54	0.18
GUM 10h	7.90 *****	0.14	7.46	0.11	7.60	0.26	8.01	0.35	7.44	0.166	5.87 *****	0.82	8.79	0.71
GUM 24h	7.93 *****	0.06	7.59 ******	0.22	7.66	0.22	8.08	0.52	7.65	0.17	5.87	0.82	8.53	0.25
GUM 48h	7.22 **a**	0.37	7.39	0.11	7.53	0.40	8.20	0.24	7.39	0.49	6.28	0.86	8.49	0.56

Table 10 changes in the bacterial composition figures are presenting the mean bacterial populations in pH-controlled batch cultures at 0, 5, 10, 24, and 48h.Values are mean ± SD.*, significant differences from the 0 h value within the same treatment, $p<0.05$. small letters differences from the negative control. 1% faecal batch culture inoculated with vessel 1 negative control, vessel 2 FOS 1%, vessel 3 FOS 2%, vessel 4 GA 1%, vessel 5 GA 2%.

	Bif164		**Lab158**		**Ato291**		**Bac303**		**Erec482**		**Chis150**		**EubI-II-III**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
Control 0h	7.46	0.21	7.24	0.09	7.40	0.18	7.85	0.24	7.96	0.20	6.91	0.24	8.26	0.35
Control 5h	7.76	0.20	7.29	0.07	7.60	0.15	8.04	0.20	7.36	0.07	6.42	0.38	8.45	0.08
Control 10h	7.75	0.24	7.47	0.07	7.56	0.02	8.25	0.28	7.51	0.07	6.99	0.16	8.44	0.04
Control 24h	7.78	0.16	7.29	0.04	7.54	0.14	8.12	0.21	7.54	0.08	5.73	0.47	8.53	0.36
Control 48h	7.64	0.29	7.14	0.50	7.50	0.61	7.76	0.17	7.51	0.06	5.68	0.73	8.42	0.18
FOS 0h	7.4	0.1	7.24	0.24	7.57	0.10	7.89	0.17	7.84	0.27	6.44	0.51	8.35	0.29
FOS 5h	7.93 *****	0.15	7.75 **a**	0.14	7.98 ******	0.12	8.38 *****	0.02	7.74	0.3	6.45	0.40	8.89 **a**	0.083
FOS 10h	8.02	0.11	7.58	0.14	7.89	0.18	7.8	0.32	7.88	0.19	6.25	0.94	8.76	0.26
FOS 24h	8.12 **a**	0.17	7.51 **a**	0.1	7.81	0.19	8.07	0.3	7.82	0.19	5.88	0.85	8.56	0.08
FOS 48h	7.88	0.11	7.43	0.15	7.67	0.10	7.95	0.34	7.62	0.18	5.87	0.15	8.69	0.29
GUM 0h	7.33	0.05	7.38	0.14	7.58	0.18	7.73	0.22	7.77	0.2	6.39	0.58	8.11	0.17
GUM 5h	7.78 ******	0.06	7.32	0.13	7.67	0.29	8.01 ******	0.23	7.6	0.16	6.31	0.67	8.44	0.16
GUM 10h	7.98 *****	0.13	7.6	0.31	7.70	0.25	8.21	0.54	7.58	0.2	6.09	0.75	8.74	0.47
GUM 24h	7.93	0.28	7.67	0.27	7.66	0.26	8.17	0.3	7.68	0.29	6.57	0.73	8.72	0.18
GUM 48h	7.69	0.149	7.34	0.12	7.78	0.27	8.2	0.25	7.46	0.3	6.04	0.91	8.66 *****	0.17

2.3.2 Impact of GA and FOS on SCFA production

Table 11 shows that within 1% GA, the concentration of acetate significantly increased after 24 h of fermentation ($p \leq 0.05$) and exhibited an increasing trend after 5, 10, and 48 h ($p = 0.06$, 0.06, and 0.08, respectively). Acetate levels were elevated at all time points after FOS fermentation compared with the levels at 0 h ($p = 0.00$, 0.00, 0.04, and 0.00, respectively), and 1% FOS led to an increase after 5 h compared with the negative control ($p = 0.01$). Butyrate production was significantly enhanced following the fermentation of FOS between 0 and 24 h ($p = 0.02$) and following GA 1% fermentation after 24 and 48 h ($p = 0.03$, 0.02); this was also the case at 24 h when compared with the negative control ($p = 0.04$). Compared with the levels at 0 h, propionate production increased following the fermentation of GA1% for 48 h ($p = 0.03$) and the fermentation of 1% FOS for 24 h ($p = 0.01$).

Additionally, with 2% FOS, acetate increased after 24 and 48 h ($p = 0.00$ and 0.01, respectively), and with 2% GA, acetate increased after 48 h compared with the baseline levels ($p = 0.02$). Butyrate production increased following the fermentation of 2% GA for 10 and 48 h compared with the levels at 0 h ($p= 0.01$ and 0.03, respectively). In addition, propionate increased after 10 h of fermentation of 2% FOS compared with the negative control ($p = 0.04$) and the baseline values ($p = 0.01$).

Table 11**:** changes in the SCFA concentration are presenting in table 3 in pH-controlled batch cultures at 0, 5, 10, 24, and 48h.Values are mean ± SD.*, significant differences from the 0 h value within the same treatment, $p<0.05$. small letters differences from the negative control. 1% faecal batch culture inoculated with vessel 1 negative control, vessel 2 FOS 1%, vessel 3 FOS 2%, vessel 4 GA 1%, vessel 5 GA 2%.

	ACETATE	**PROPIONATE**	**BUTYRATE**
Control			
0 h	1.68 ± 1.16	0.19± 0.12	0.15 ± 0.09
5 h	4.49 ± 0.99 a	0.82 ± 0.86	2.15 ± 3.19
10 h	6.47 ± 1.34	2.51 ± 1.19 a	1.47 ± 0.35
24 h	8.88 ± 1.71	4.50 ± 2.29	1.73± 0.85 a
48 h	10.57 ± 3.57	5.40 ± 3.11	3.10 ± 2.41
FOS 1%			
0 h	0.98 ± 0.04	0.10 ± 0.03	0.08 ± 0.02
5 h	8.84 ± 3.53 ** a	1.86 ± 0.31	0.66 ± 0.24
10 h	13.12 ± 2.02 **	8.45 ± 6.98	4.42 ± 2.02
24 h	11.89 ± 4.22 *	8.13 ± 2.10 *	8.26 ± 2.98 *
48 h	10.00 ± 1.11 **	4.32 ± 3.42	1.21 ± 1.58
FOS 2%			
0 h	1.35 ± 0.26	0.19 ± 0.10	0.13 ± 0.06
5 h	14.28 ± 4.93	4.24 ± 3.54	2.32 ± 2.10
10 h	15.08± 2.93	5.80 ± 1.04 * a	5.93± 4.34
24 h	13.00 ± 7.81 **	5.44 ± 2.56	6.21 ± 4.05
48 h	8.80 ± 0.32 *	2.31 ± 1.85	3.66± 1.52
GUM 1%			
0 h	2.01 ± 1.73	0.18± 0.10	0.16 ± 0.12
5 h	5.36 ± 0.37	4.79 ± 4.76	4.99 ± 7.61
10 h	12.72± 4.79	6.77 ± 4.78	6.96 ± 7.79
24 h	10.78 ± 8.27	3.28 ± 2.61	4.59 ± 1.49 * a
48 h	11.64 ± 4.10 *	4.79 ± 1.46 *	4.01 ± 0.88 *
GUM 2%			
0 h	2.37 ± 2.29	0.19±0.12	0.16 ± 0.12
5 h	5.24 ± 0.56	5.13 ± 5.93	0.73 ± 0.42
10 h	16.67 ± 7.59	12.03 ± 8.32	2.40 ± 0.51
24 h	11.41 ± 9.15	3.28 ± 2.51	3.09 ± 2.49 *
48 h	13.63 ± 3.17 *	6.15 ± 2.24	6.24± 2.22 *

2.4 Discussion

Prior studies have noted the importance of the effect of GA on improving human health [13]. GA is not digestible in the upper gastrointestinal tract, therefore it can reach the large intestine where it is fermented by intestinal bacteria [25],[26], [27]. Therefore this study aimed to determine the effects two doses of GA on human intestinal bacteria and to assess prebiotic potential as compared to prebiotic FOS. As such pH-controlled batch culture fermentation systems were used to evaluate the selectivity of GA when fermented with healthy human gut microbiota compared with FOS at two different doses.

Several studies have shown that GA can undergo a slow fermentation, specifically a more distal fermentation, whereas existing prebiotics typically undergo proximal fermentation [28], as proteolytic fermentation develops in the distal colon; therefore, this substrate may be able to be saccharolytically fermented in this part of colon. In this present study GA fermentation shows selectivity in bifidobacteria and *Lactobacillus* spp at time 10 h and continues to 24 h which could indicate slower fermentaion and is inline with others work [15]. However, it is worth noting that the bifidogenic effect of the 1% was not maintained at 48 hours in current study.

In the present study, GA significantly enhanced the growth of *Bifidobacterium* spp.. *Bifidobacterium* is considered an important group related to human health, having a favourable impact in the large intestine [29],[30]. These results are consistent with those of Calame *et al* (2008) in which the consumption of 10 g of GA daily by healthy adults had a beneficial effect on the gut microbial composition, and increases in bifidobacteria [12]. Furthermore, the 1% dose also led to increases in lactobacilli. Lactobacilli has long been considered a positive microbial group; as such, stimulation of this genera offers potential

benefits to the host [31]. The 2% dose did not lead to the same lactobacilli impact. The use of a higher doasge could have impacted on selectivity through a cross feeding network [32].

Furthermore, after 10 h, the numbers of bacteria in the *C. histolyticum* group decreased following the fermentation of 1% GA, which also agreed with results of *in vivo* studies [33], [12], [15]. This group of bacteria has sometimes been associated with inflammation and large bowel disease [34], [35]. It is thought that increased numbers of beneficial bacteria could lower the pH within the colon, therefore making the environment unfavourable for pathogenic groups; the results of the present study indicate that the fermentation of GA selectively increased the number of beneficial bacteria and reduced the number of harmful bacteria. This result further suggests the lower dose to offer improved selective potential.

In the current study GA fermentation resulted in a similar bacterial profile to FOS. Several *in vivo* and *in vitro* studies have confirmed that FOS can regulate the gut through the selective stimulation of the gut microbiota [36], [37], [38], [39], therefore, GA could has potentially prebiotic properties. GA can be incorporated into baked goods, therefore could provide an alternative prebiotic source for inclusion in the diet.

Moreover, an increase in *Bacteroides* spp. was observed with in GA and FOS, this group is associated with a range of colonic activities [40], [41]. *Bacteroides* spp. constitute a large proportion of the microbial population in the healthy adult gastrointestinal tract [42]. In previous *in vivo* and *in vitro* studies on GA fermentation, increased propionate levels were associated with *Bacteroides* spp. and *Prevotella* spp. which has relevance to the improving in lipid metabolism [43],[44].

The results show that both doses of GA were selectivly fermented. The higher dose was arguably less selective as the impact on lactobacilli and *C. histolyticum* groups were no

longer apparent. In fact Calame et al (2008) noted that an increase in the concentration of substrates results in less selectivity [12]. This might be explained by competiton for substrate, at higher dose other bacterial strains have easier access to the substrate and subsequently, become less selevtively than the lower dose.

GA fermentation induced modulation of the colonic microbiota, with increased levels of acetate, propionate, and butyrate. The best-known metabolic pathway for acetate and propionate production by gut bacteria involves the metabolism of polysaccharides. Acetate is produced mainly through the fructose-6-phosphate phosphoketolase pathway by bifidobacteria, and the increased production of this acid could be related to increased numbers of this group [45]. Furthermore, according to Hosseini *et al* (2011), propionate can be produced from fermentable carbohydrates by two pathways [46]. The first involves decarboxylation of succinate by the action of *Bacteroides fragilis* and *Propionibacterium* spp., and the second is the acrylate pathway, in which pyruvate is first reduced to lactate by lactate dehydrogenase by the action of the Cluster IX Clostridia groups; an increase in these bacterial groups was observed during GA fermentation. Butyrate is often used as an energy source by epithelial cells, and acetate plays an important role in controlling inflammation and resisting invasion by pathogens [47]. Furthermore, acetate and propionate may have a direct role in central appetite regulation. The propionate mechanism involves stimulating the release of the anorectic gut hormones peptide YY (PYY) and glucagon-like peptide-1 (GLP-1). They, in turn, are involved in the short-term signalling of satiation and satiety to the appetite centres of the brain, increasing satiety and reducing food intake by the host [48],[49], [50],[51]. Acetate administration is associated with the activation of acetyl-CoA carboxylase and changes in the expression profile of regulatory neuropeptides favouring appetite suppression [52]. Thus these SCFA increases could offer benefits to the host.

The pH-controlled stirred batch culture systems enabled rapid analysis of the effects of GA on the faecal microbiota. In the absence of absorption, colonic secretions, and epithelial interactions, the system has limitations. However, processes such as SCFA production can still be monitored away from the impact of additionally dietary factors. Thus, batch culture systems provide an alternative way of assessing how bacteria ferment a substrate and the end products they produce [53].

The comparisons of the substrates in the pH-controlled batch cultures indicated that GA has selective abilities that are at least similar to those of the known prebiotic FOS, as indicated by the bacteriology results and increased concentrations of acetate, butyrate, and propionate. These results could be relevant to improving host health by increasing the levels of the bifidobacteria group, especially in individuals with lower numbers such as elderly population. Tuohy *et al* (2001) reported that prebiotics can alter the gut microbiota in those with initially low numbers of bifidobacteria [39]. This may be particularly relevant in elderly people. Elderly are experiencing negative changes in their gut microbiota.

2.5 Conclusion

The aim of the current study was to use *in vitro* batch cultures to assess the effects of GA on the microbiota compared to FOS. Here, we showed that GA modulated the gut microbiota similarly to FOS, furthermore, the 1% dose showed additional selective potential. As such GA holds the potential to be used as a novel prebiotic source.

2.6 References

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3. Chapter (3) Metabolite Production of gum acacia in a gut model system.

Abstract

Gum acacia (GA) is a soluble dietary fibre derived from acacia trees. It is widely used in African countries and in the Middle East as a traditional medicine. The consumption of GA has been related to potential health benefits. The aim of this study was to examine the influence of GA on the metabolic activity of the human gut microbiota. We used a validated *in vitro* three-stage continuous culture system (gut model) system. The fermentation of GA led to an increase in *Bifidobacterium* spp. in vessel 2 and vessel 3, whereas FOS fermentation led to a significant increase in *Bifidobacterium* spp. in vessel 2 and decrease in *Clostridium histolyticum* in vessel 1. The fermentation of GA induced a significant increase in acetate in vessel 3 and propionate in vessel 2 in steady state 2 (SS2). We evaluated the fermentation of GA and FOS in a gut model system and the ability of the fermentation supernatants to modulate the activation and cytokine profile of PBMC subsets *in vitro*. FOS and GA led to an increase in IL-10 in vessel 1. In conclusion, GA shows an important factor to elderly inflammation, which could improve immune function.

3.1 Introduction

The World Health Organization reported that the population of those aged 60 years or older is rapidly increasing [1]. In Saudi Arabia, health care expenditures are expected to increase between 2015 and 2050 due the increase in elderly individuals from 5.4% to 25% of the population, and over one-third of the UK population will be 65 years old or older by 2050. Ageing is associated with an increased incidence of infectious diseases, which may be, in part, due to a decline in immune function [2]. Ageing is defined as "the regression of physiological function accompanied by the development of age" [3]. The process of ageing has a large impact on the physiology of the intestinal gastrointestinal tract due to effects on the gut microbiota. The reduction of intestinal motility results in a slower transit time that could lead to constipation [4]. As a result, the gut bacteria are altered so that the fermentation in the gut shifts to proteolytic fermentation [5],[6].

Immunoseescence is defined by a decline in the immune response to exogenous infections agents as well as an increase in endogenous signals [7],[8]. An intrinsic change occurs in T cells, which contributes to the decline in T cell function. Decreased innate and adaptive immune activities associated with ageing lead to different diseases [9] and exogenous infectious agents result in increased IL-6 levels [10]. Gomize *et al* (2005) and Bruunsgaard et al (2001) demonestrated that the inflammatory response produces increased levels of pro-inflammatory mediators, including IL-6, IL-1 β, and TNF-α, in older individuals, and NK cells are impaired [9], [11].

Diet is a major influencing factor on the gut microbiota, and the high-fat, sugar-rich Western diet contributes to Bacteroidetes dominating the microbiome, whereas a high-fibre diet results in *Firmicutes* dominating the microbiome [12].

The diversity of species comprising the gut microbiota changes with age. In their study, Zwielehner *et al* (2009) observed less *Bifidobacterium* and *Clostridium cluster IV* in elderly subjects compared to those seen in middle age subjects [13]. Moreover, in another study, Marathe *et al* (2012), reported that within an Indian cohort, the changes in the gut microbiota associated with age reflected a gradual decrease in *Firmicutes* and an increase in *Bacteroidetes* [14]. Claesson *et al* (2012) found an association between diet, host health, environment, and gut microbiota; in particular, there was an association between a lower diversity in the diet and decreased gut diversity and, subsequently, health parameters and increased inflammatory markers [15]. Additionally, individuals living in a community had more diverse microbiota than those in residential care. Ageing is associated with a decrease in probiotic bacteria such as bifidobacteria, which inhibit pathogens such as *E. coli* and Salmonella [16]. These changes in the intestinal bacteria may increase susceptibility to infections and diseases due to the changes in diet and intestinal transit time [17].

Gum acacia (GA) is a non-starch polysaccharide, and previous *in vitro* studies have shown that GA has an immunomodulatory effect on intestinal dendritic cells (DCs) [18]. Furthermore, a study carried out in rats with adenine-induced chronic renal failure (CRF) showed that drinking a GA solution (15%) resulted in reduced inflammation [19]. The significant contribution of anti-inflammatory properties may be mediated by short chain fatty acids (SCFAs) produced by the fermentation of GA [20], [21]. Nasir *et al* (2012) reported that GA could have a systemic effect and not just affect the gut. However, no study has investigated this effect in the elderly population [22].

Several studies have shown that prebiotics can enhance the immune system in the elderly by increasing anti-inflammatory and decreasing pro-inflammatory cytokines. Vulvic *et al* (2008) observed that treatment of subjects over 65 years of age with bimuno-galacto-

oligosaccharide (B-GOS) had a positive effect on the gut microbiota while producing an anti-inflammatory effect [23]. A prebiotic is a selectively fermented ingredient that produces specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon the host's health [24]. Thus, the microbiota tends to be enriched in bifidobacteria and lactobacilli, as opposed to other pathogenic genera such as *Eubacterium, Faecalibacterium*, or *Clostridium*, after treatment with prebiotics [25].

The fermentation of GA produces several degradation products, including SCFA [26]. Accordingly, treatment with GA may enhance serum butyrate concentrations [27], [28]. Glover *et al* (2009) suggested that bacterial SCFAs increase SCFAs in the systemic circulation, which can impact immune function [27]. These studies indicate that systemic SCFAs resulting from the fermentation of GA by intestinal bacteria may reduce inflammation [28]. These previous studies used culture methods to assess changes in the gut microbiota of elderly individuals to investigate how the gut microbiota is modulated in the elderly after treatment with GA. This study aimed to determine the effect of GA on the elderly gut microbiota compared to FOS, a known prebiotic. Additionally, the effects of GA on cytokines induced in PBMCs obtained from elderly subjects were investigated.

3.2 Materials and methods

3.2.1 Materials

The following materials were used in this study: Lympholyte (Cedarlane Lonza), RPMI 1640 medium, L-glutamine (Bio-Whittaker), 24-well plates, 1% PBS, a centrifuge, an incubator, a counting chamber, a microscope, a balance, a gas chromatograph, and a fluorescent microscope. All nucleotide probes used for FISH were commercially synthesised and labelled with the fluorescent dye Cy3 at the 5′-end (Sigma Aldrich). All chemicals,

unless otherwise stated, were from Sigma Aldrich. Media and instruments were sterilised by autoclaving at 121°C for 15 min.

Substrates

The following substrates were used in this study: GA (Kerry Ingredients, UK) and FOS (Beneo, Belgium) as mentioned in chapter 2.

3.2.2 *In vitro* Upper Gut Digestion

To mimic human digestion, a previous method Mills et al (2008) was used in this study [29]. First, 60 g of GA powder was added to 150 mL of distilled water, and the solution was mixed with 20 mg of α-amylase in 6.25 mL of $CaCl_2$ (1 mM) and incubated on a shaker at 37°C for 30 min. This step stimulated the initial oral digestion. Subsequently, 2.7 g of pepsin in 25 mL of HCl (0.1 M) was used for stimulating the gastric digestion of the sample. The pH was then reduced progressively to 2 by adding 6 M HCl followed by incubation on a shaker at 37°C for 2 h. Next, 560 mg of pancreatin and 3.5 g of bile in 125 mL of $NaHCO_3$ solution was added to simulate the effect of the small intestine on the gum sample. The pH was increased to 7 by adding NaOH (6 M), and the resulting suspension was incubated on a shaker at 37°C for 3 h. The samples were transferred to a cellulose dialysis membrane (1 KDa molecular weight, purchased from Cheshire Biotech, Cheshire, UK) and dialysed against a 10 mM NaCl solution at 5°C to remove low-molecular-weight digestion products. After 15 h, the dialysis fluid was changed, and dialysis was continued for an additional 2 h. Afterwards, the samples were freeze-dried (5 d) prior to use in the gut model.

3.2.3 Medium for the three-stage continuous system (gut model)

The medium for the three-stage continuous system contained the following (g/L) : soluble potato starch, 5; peptone water, 5; yeast extract, 4.5; NaCl, 4.5; KCl, 4.5; porcine mucin type

III, 4; milk casein, 3; pectin, 2; xylan from oat spelts, 2; arabinogalactan, 2; $NaHCO_3$,1.5; $MgSO_4$, 1.25; guar gum, 1; cysteine HCl, 0.8; KH_2PO_4, 0.5; K_2HPO_4, 0.5; bile salts, 0.4; $CaCl_2 \cdot 6H_2O$, 0.15; $FeSO_4 \cdot 7H_2O$, 0.005; and hemin, 0.05 in addition to 1 mL Tween80, 10 μL vitamin K1, and 4 mL reasazurin. For V1, V2, and V3, 51.43, 66.67, and 82.50 mL, respectively, of culture medium was aliquoted into three Duran bottles and sterilised. Then, 5 L of basal medium was sterilised in a 5-L glass bottle and used as a medium reservoir.

3.2.4 Faecal sample preparation

Faecal samples were obtained from three healthy volunteers (65 years and older, two males, one female) who had not been prescribed antibiotics for at least 3 months before the study, had not consumed pre- or pro-biotic supplements, and had no history of a gastrointestinal or metabolic disease. The samples were collected onsite and used directly after collection. A 20% (w/v) dilution of the faecal sample was made using sterile, reduced, phosphate buffered saline (PBS; 0.1 mol/l, pH 7.0) and homogenised in a stomacher (Stomacher 400, Seward, West Sussex, United Kingdom) for 2 min at normal speed.

Three-stage continuous culture colonic model (gut model) system

Gut models, each comprising a cascade of three glass fermenters connected in series that simulate the different physical and nutritional characteristics of the proximal (V1), transverse (V2), and distal colon (V3), were implemented under conditions previously detailed by Macfarlane et al (1997) [30]. V1 simulated the acidic conditions, rapid turnover, and high substrate availability that prevail in the proximal colon, whereas V3 was held at a neutral pH with a slower turnover and limited substrate availability, simulating conditions similar to those in the distal colon. Each vessel was inoculated with 28.57, 33.33, or 37.50 mL (corresponding to V1, V2, and V3, respectively) of a 20% (w/v) faecal slurry from a healthy

elderly donor. The pH set points (±0.1) for V1, V2, and V3 were 5.5, 6.2, and 6.8, respectively. Each vessel was stirred magnetically and sparged continuously with oxygen-free nitrogen gas. The temperature (37°C) was maintained by a water jacket, and the culture pH was controlled (Electrolab, UK) by the addition of 0.5 M NaOH or HCl. Three independent models were inoculated with faeces from a different faecal donor for each acacia and FOS sample tested.

The system was operated in batch culture mode in the gut model medium for 24 h to enable the microbial populations to adjust to their new environment. After the stabilisation period, the medium pump was started. The flow rate of the basal medium was controlled by a pump set to 2.5 mL/h, resulting in a system retention time of approximately 48 h. The medium for the gut model was pumped into vessel one (V1) and sequentially fed into V2, then V3, and ultimately a waste unit. The three-stage continuous culture system was run for 35 d. Steady state 1 (SS1) was reached after eight full turnovers at 18, 19, and 20 d. SS1 was established by the stabilisation of SCFA concentrations, as assessed by gas chromatography (GC). Thereafter, the test product, pre-digested GA, or FOS was administered into V1 at a 1.6 g dose each day until the second steady state (SS2) was reached at 33, 34, and 35 d. Samples (1 mL) were collected from all vessels of the colonic system and centrifuged at 13,000 x g for 10 min to remove all particulate matter. Samples were stored at -20°C until analysed.

3.2.5 Sample preparation

Samples (375 µL) obtained from each vessel at each sampling time were fixed for 4 h at 4°C in 1.125mL (4% w/v) paraformaldehyde. The fixed samples were then centrifuged at 13,000 × g for 5 min at room temperature and washed twice in 1 mL of filter-sterilised PBS.

The washed cells were suspended in 150 µL of cold 1×PBS and 150 µL of ethanol (99%) at -20°C for FISH analysis for bacterial counts.

To measure the SCFA concentrations, we used the internal standard GC method. One millilitre of each sample was transferred to a labelled 100 mm × 16 mm glass tube. To prepare for the *in vitro* immunoassays, 1 mL of the gut model supernatant was sampled in triplicate, centrifuged for 10 min at 11337 x g, and then filtered through a 0.22-µm filter device (Millipore, Schwalbach, Germany). The cell-free supernatants were stored at -20°C.

3.2.6 *In situ* florescent hybridization (FISH) analysis

To assess the differences in bacterial number, FISH was used with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA. The probes were commercially synthesised and labelled with the fluorescent dye Cy3. The following probes were used: Bif164 for bifidobacteria (BIF), Lab158 for lactobacilli/enterococci (LAB), Chis 150 for the *Clostridium histolyticum* group (CHIS), Erec 482 for the *Eubacterium rectale–Clostridium coccoides* group (EREC), and Bac 156 for the *Bacteroides–Prevotella* group (BAC). The EUB 338 mixture consisting of EUB338, EUB338II, and EUB338III was used for total bacteria (Total). Samples were hybridised as described previously [31]. For the hybridisation, 20 µL of a diluted sample was pipetted onto Teflon- and poly-L-lysine-coated six-well slides (Tekdon Inc., Myakka City, FL). The samples were dried on the slides at 46-50°C for 15 min and then dehydrated in an ethanol series (50, 80, and 96%). The ethanol was allowed to evaporate from the slides before the probes were applied to the samples. To permeabilise the cells for use with the Bif164 and Lab158 probes, samples were treated with 20 µL of lysozyme at room temperature for 15 min before being washed briefly for 2−3 seconds in water and then dehydrated in the ethanol series. A hybridisation mixture of 5 µL of probe

plus 50 μL of a mixture of 0.9 M NaCl, 0.02 M Tris/HCl (pH 8.0), formamide (if required – Table 5), and 10% (w/v) sodium dodecyl sulphate (for a final concentration of 4.55 ng/mL probe) was applied to the surface of each well. Then, the slides were placed in a hybridisation oven for 4 h (ISO20 oven, Grant Boekel). For the washing step, the slides were placed in 50 mL of washing buffer (0.9 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.005 M ethylenediaminetetraacetic acid (EDTA) solution (pH 8.0, Table 2), H2O) warmed to the appropriate temperature for each probe, and 20 μL of 4,6-diamidino-2-phenylindole di hydrochloride (DAPI) was added to the washing buffer before the hybridisation was finished. Then, the slides were briefly washed (2−3 s) in ice-cold water and dried under a stream of compressed air. Five microliters of Pro Long Gold anti-fade re-agent (Invitrogen) was added to each well, and a coverslip was applied. The slides were stored in the dark at 4°C until the cells were counted under a NikonE400 Eclipse microscope. A DM510 light filter was used to count specific bacteria hybridised with the probes of interest. A minimum of 15 random fields of view were counted for each sample. The following formula was used to calculate numbers of bacteria: (0.8 × A1 × 6732.42 × 50 × Dilution factor), where A1 is the average count of 15 fields of view, 6732.42 is the area of the well divided by the area of the field of view, and multiplying by 50 takes the count back to millilitre of sample. The results are expressed as log10 (bacterial numbers per millilitre of batch culture fluid).

3.2.7 Preparation of samples for short chain fatty acid analysis

To determine the SCFAs, samples were extracted and derivatised as previously described [32]. Samples were defrosted, and 1 ml of each sample or standard solution was transferred to a labelled 100 mm × 16 mm glass tube with an internal standard of 50 μl of 2-ethyl butyric acid (0.1 M). Then, 0.5 ml of concentrated HCl and 2 ml of diethyl ether were added to each glass tube, and the samples were vortexed for 1 min. The samples were then centrifuged at

2000 x g for 10 min (SANYO MSE Mistral 3000i; Sanyo Gallenkap PLC, Middlesex, UK). The diethyl ether (the upper layer) was transferred to a new glass tube. A second extraction was conducted by adding 1 ml of diethyl ether followed by vortexing and centrifugation. Then, 400 µl of pooled ether extract and 50 µl N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) were added to a GC screw-cap vial. The samples were heated at 80°C for 20 min and then left at room temperature for 48 h to allow the lactic acid in the samples to completely derivatise.

A 5890 SERIES II gas chromatograph (Hewlett Packard, UK) with an Rtx-1 10 m × 0.18 mm column and a 0.20-µm coating (Crossbond 100% dimethyl polysiloxane; Restek, Buckinghamshire, UK) was used for the SCFA analysis. The temperature of both the injector and detector was 275°C, with the column programmed from 63°C for 3 min to 190°C at 10°C/min followed by holding at 190°C for 3 min. Helium was the carrier gas (flow rate, 1.2 mL/min; head pressure, 90 MPa). A split ratio of 100:1 was used. The standard solution contained the following (mM): sodium formate, 10; acetic acid, 30; propionic acid, 20; isobutyric acid, 5; n-butyric acid, 20; iso-valeric acid, 5; n-valeric acid, 5; sodium lactate, 10; and sodium succinate, 20. The sample was injected onto the column, which was maintained at 140°C for the first 5 min, after which the temperature of the column was increased over 5 min to 240°C. To maintain the appropriate calibration after the injection of each of the 20 samples, an external standard solution with known concentrations of SCFAs was injected. The peaks and response factor within the samples was calibrated and calculated using ChemStation B.03.01 software (Agilent Technologies, Cheshire, UK).

3.2.8 Preparation of peripheral blood mononuclear cells

Fasted blood samples were taken from six healthy donors aged 65 years and older in sodium heparin vacutainer tubes (Greiner Bio-One Limited, Gloucestershire, UK). The study was conducted according to guidelines laid down in the Declaration of Helsinki 1975 as revised in 1983. All procedures involving human subjects were approved by the Ethics Committee of the Reading University. Written informed consent was obtained from all subjects. Blood was layered over an equal volume of Lympholyte (Cedarlane Laboratories Limited, Burlington, Ontario, Canada) and centrifuged at 930 x g (Beckman Coulter Allegra TM X-I2 R centrifuge) for 15 min at room temperature. Cells were harvested from the interface, washed once, resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (containing glutamine, Roswell Park Memorial Institute, Autogen Bioclear Ltd., Wiltshire, UK). In addition, the above steps were then repeated to achieve a lower degree of erythrocyte contamination. The pellet was finally resuspended in RPMI medium, and the cell number was adjusted to the required concentration using a cell counter (Z1 Coulter ® particle counter, Beckman Coulter) and trypan blue.

3.2.9 Viability assays

PBMCs were tested to determine the proper supernatant concentration. Trypan blue was used to count the cells. PBMCs were adjusted to 2×10^6 cells/mL and then incubated in 24-well plates in RPMI 1640 medium (containing glutamine, Roswell Park Memorial Institute, Autogen Bioclear Ltd., Wiltshire, UK). These steps were repeated to obtain low contamination levels of erythrocytes. Gut model medium and samples from established SS1 and after treatment established SS2 were used, and supernatants were incubated with PBMCs for 24 h at 37°C in an air-CO_2 (19:1) atmosphere incubator. The concentrations of the supernatant were 1%, 2%, 3%, and 4% (w/v) in 2 mL (final working volume). After

incubation, cells were counted using the trypan blue test, and 1% was determined to be the appropriate concentration, with over 50% of PBMCs viable for the different treatment supernatant.

3.2.10 Cytokine stimulation and detection

PBMCs were adjusted to a cell concentration of 2×10^6 cells/mL and incubated for 24 h at 37°C in an air-CO2 (19:1) atmosphere incubator in the presence of 1mg/mL lipopolysaccharide (LPS; L4516, Sigma) and 1% (w/v) of the supernatants from each steady state vessel and gut model medium. At the end of the incubation, cell culture supernatants were collected and stored at -20°C until used for the cytokine production analysis. Non-stimulated cultures were used as a blank.

3.2.11 Cytometric bead array immunoassay

A BD Cytometric Bead Array (CBA) was used to quantitatively measure interleukin-8 (IL-8), interleukin-6 (IL-6), interleukin-10 (IL-10), tumour necrosis factor α (TNF) protein levels in a single sample. Samples or standards were incubated with specific fluorescent beads and biotin conjugates for each analyte for 3 h at room temperature in the dark. Samples were washed and centrifuged at 200 × g for 5 min and then measured using a BD™ Cytometric Bead Array (CBA) Human Soluble Protein Master Buffer Kit (BD Biosciences, Oxford, UK) and the corresponding BD™ Cytometric Bead Array (CBA) Human Flex Set (BD Biosciences, Oxford, UK) with a BD Accuri™ C6 flow cytometer according to the manufacturer's instructions. BDTM CBA analysis software FCAP Array v3.0.1 (BD Biosciences, Oxford, UK) was used to perform the data analysis.

3.3Statistical analysis

All statistical tests were performed using the SPSS software package. Repeated measures one-way ANOVA was used to compare the SS1 and SS2 data for bacterial counts and organic acid concentrations and the cytokine data. Statistical significance was accepted at $p < 0.05$ for all analyses.

3.4 Results

3.4.1 Effect of GA and FOS on human faecal bacteria

The average bacterial counts are displayed in Figures (5) and (6) and expressed as log10 ± standard deviation, as enumerated by FISH. Following the administration of GA, a significant increase in *Bifidobacterium* was observed in vessel 2 and vessel 3 ($p = 0.003$, $p = 0.009$ respectively). However, following the fermentation of FOS, *Bifidobacterium* increased significantly in vessel 2 ($p = 0.02$) and a trend to increase in vessel 3 ($p = 0.08$); furthermore, there was a significant decrease in *Clostridium histolyticum* in vessel 1. The total bacteria level also increased with FOS treatment in vessel 1, although it was not significant, as the p value shows ($p = 0.08$).

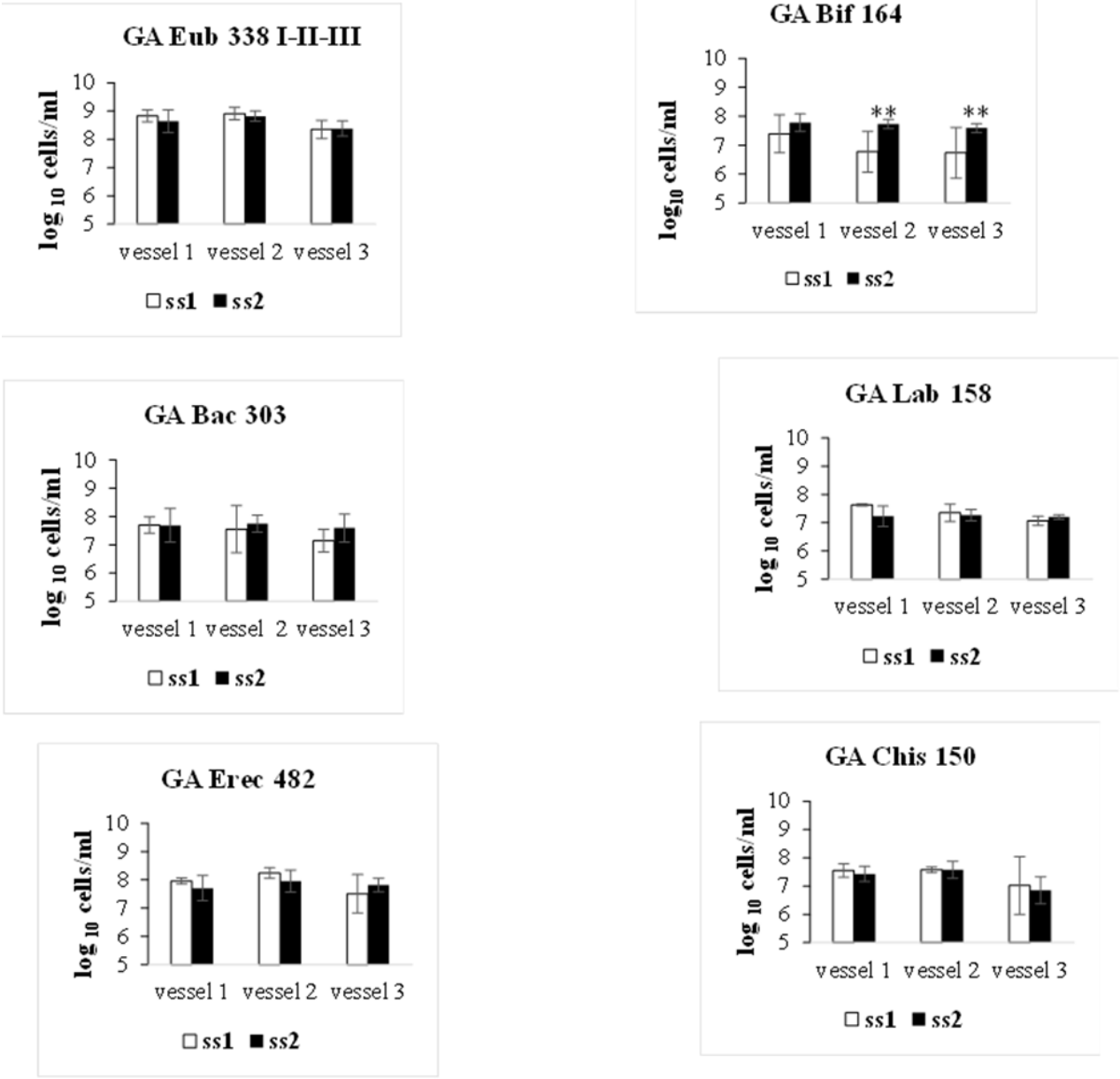

Figure 5: changes comparing SS1 (no treatment) and SS2 (treatment with GA). Gut model system. Values are the bacterial counts Log10 number of cells / ml using FISH analysis using three healthy volunteers in total six gut models.

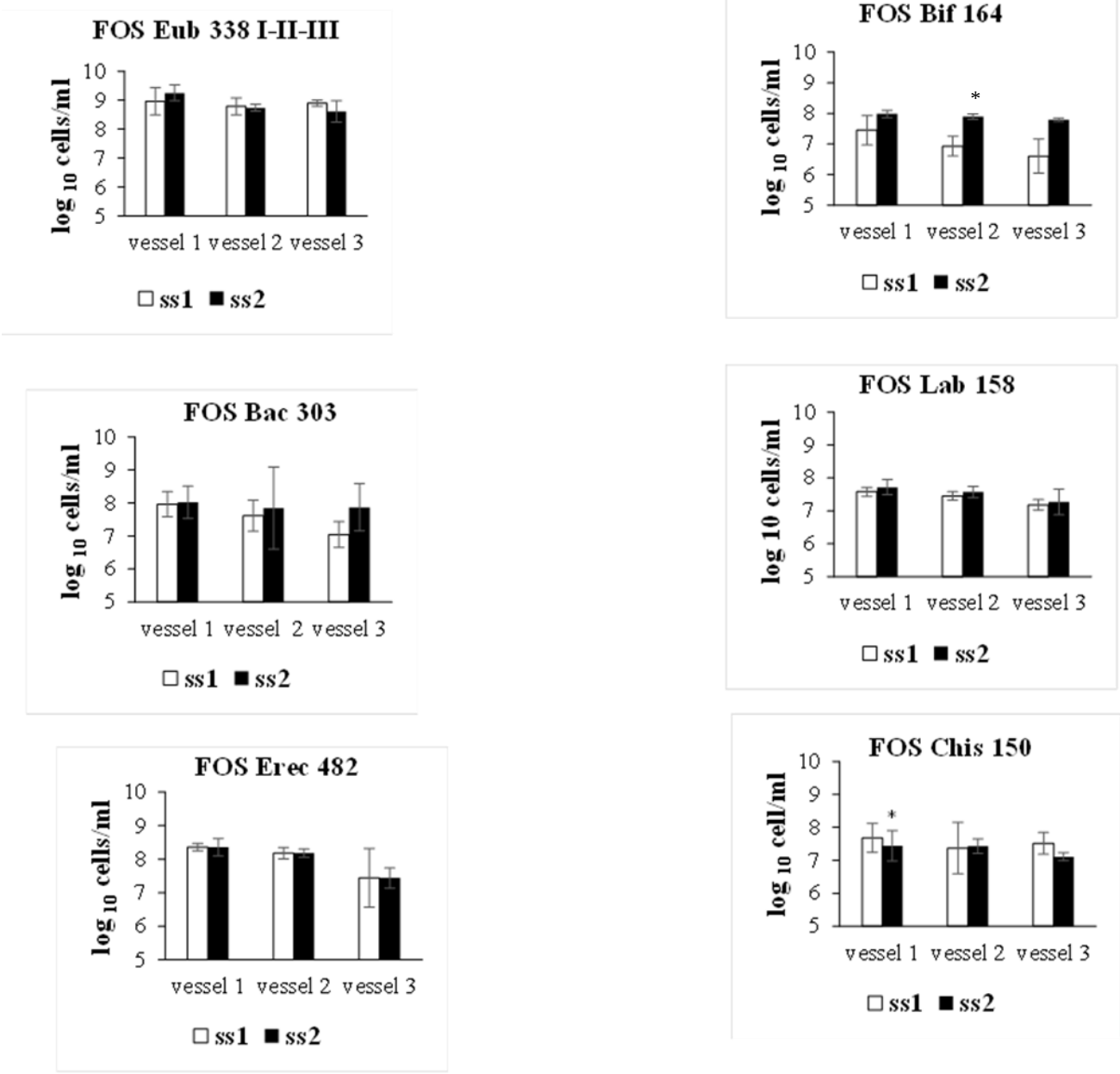

Figure 6 changes comparing SS1 (no treatment) and SS2 (treatment with FOS). Gut model system. Values are the bacterial counts Log10 number of cells / ml using FISH analysis using three healthy volunteers in total six gut models.

3.4.2 SCFA production

The relative concentrations of organic acids, as determined by GC, are reported in **Tables** 12 and 13 and are expressed as mM ± standard deviation. The fermentation of GA caused a significant increase in acetate in vessel 3 and propionate in vessel 2 at SS2. In addition, an increasing trend for propionate and butyrate was observed in vessel 1 and vessel 3 ($p = 0.092$, $p = 0.1$, $p = 0.074$, and $p = 0.11$ respectively). Additionally, GA fermentation resulted in a significant 21-mM increase in propionate ($p < 0.021$) in vessel 2.

FOS fermentation caused an increasing trend for acetate and butyrate in vessel 2 ($p = 0.16$ and $p = 0.08$, respectively).

Table 12: SFCA concentration. Changes comparing SS1 no treatment and SS2 treatment with FOS or GA. Gut model culture. vessel (ss1) no treatment, SS2 GA or FOS values are based on means and standard deviation of three gut models

FOS	**Acetate**		**Propionate**		**Butyrate**	
	SS1	SS2	SS1	SS2	SS1	SS2
Vessel 1	58.74±21.33	51.81±23.76	29.90±12.22	35.03±21.25	34.44±17.64	33.88±29.63
Vessel 2	52.76±14.98	65.10±23.36	34.36±19.77	40.06±19.35	30.92±23.00	44.49±28.08
Vessel 3	55.52±13.83	61.83±26.03	35.82±21.76	37.50±13.83	33.56±28.02	41.57±33.90

Table 13: SCFA concentration. Changes comparing SS1 no treatment and SS2 treatment with FOS or GA. Gut model culture. vessel (ss1) no treatment, SS2 GA or FOS values are based on means and standard deviation of three gut models

GUM	Acetate		Propionate		Butyrate	
	SS1	SS2	SS1	SS2	SS1	SS2
Vessel 1	53.66±0.54	46.11±23.02	28.89±15.17	54.64±4.89	18.27±8.59	24.00±3.19
Vessel 2	68.49±0.46	70.13±15.49	44.47±2.51	65.76±3.27 *	25.70±10.35	33.48±7.13
Vessel 3	71.12±5.22	84.35±10.59 *	37.50±15.07	70.85±2.72	31.25±8.78	41.05±8.52

3.4.3 Cytokine production

Figure 7 shows the effects of the fermentation of GA and FOS on the cytokine responses of PBMCs in the *in vitro* model of the large intestine.

The fermentation of GA resulted in increased in IL-10 activity in LPS-stimulated PBMCs (vessel 1, $p \leq 0.05$); furthermore, an increasing trend for the activity of this cytokine was observed in the supernatants of vessels 2 and 3 ($p = 0.19$, $p = 0.06$). The TNF-α activities of the PBMCs showed a decreasing trend in the presence of fermentation supernatants from vessels 2 following GA fermentation ($p = 0.18$). FOS fermentation also resulted in increased IL-10 activities in LPS-stimulated PBMCs in vessel 1with p value ($p \leq 0.00$).

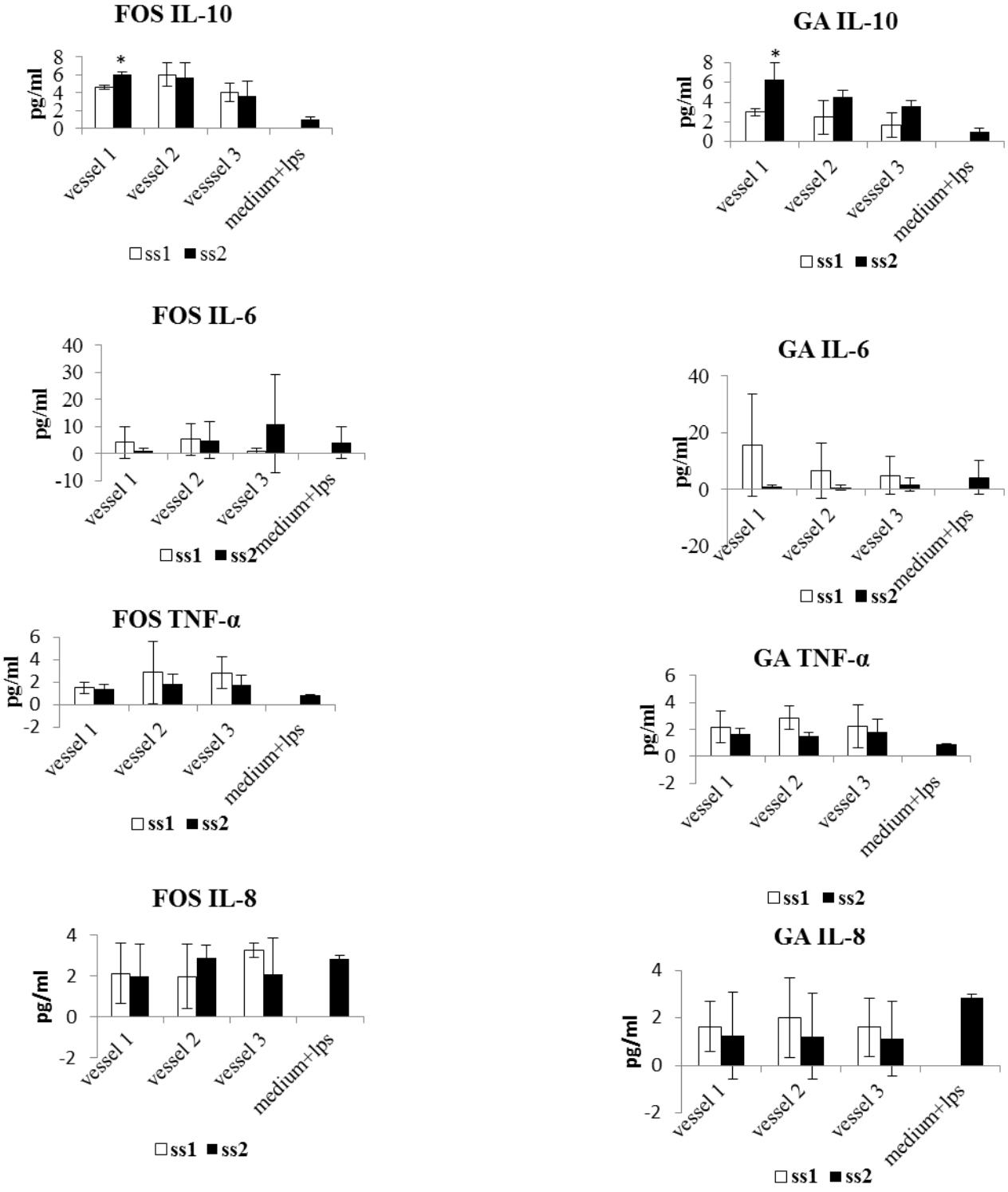

Figure 7: Shows the changes in the cytokines parameters comparison between SS1 steady state before treatment and SS2 steady state after treatment. GA gum acacia and FOS fructooligosaccrides both 1%. Supernatants from fermentation of GA and FOS in gut model system were incubated with PBMC cells.

3.5 Discussion

To date, although several studies have investigated the impact of GA on the gut microbiota of young adults, this is the first study to assess the potential prebiotic effect of GA on the elderly gut microbiota and to determine whether it has anti-inflammatory properties, which are important because of the changes in immune function in the elderly leading to increased incidence of infectious diseases [31].

The aim of this study was to investigate the fermentation properties and prebiotic potential of GA compared with FOS, a known prebiotic, using an *in vitro* continuous culture model of the human colon. Faecal samples from donors aged 65 years or older were used. The impact of the substrates on the modulation of immune function as assessed using inflammatory cytokines was determined using PBMCs stimulated with LPS.

The fermentation of GA led to an increase in *Bifidobacterium* spp. Similarly, FOS fermentation was shown to have a bifidogenic effect. These results might indicate that the gut composition is modulated following treatment with GA. This result was in line with that from previous studies, indicating that GA is a potential prebiotic candidate [32],[26]. An *in vitro* study using a gut model (SHIME) Marzorati *et al* (2015) reported that a blend of FOS, inulin, and GA (41% FOS, 41% GA, 18% inulin) and 3.3 g of fibre given twice daily with the faecal microbiota from a healthy 75-year-old volunteer increased the number of bifidobacteria significantly and subsequently increased the concentration of SCFAs [33]. However, this study involved one volunteer, and the effect could have been due to the volunteer's microbiota; in addition, a blend of two substrates was used, hence the results do not indicate exactly which product was responsible. Another *in vitro* study used arabinogalactan from GA compared it with 5 g daily of FOS in a SHIME model, finding an enhancement of bifidobacteria and lactobacilli and *F. prausnitzii*, which is a microorganism that can exert

anti-inflammatory effects [34]. This positive effect was also observed in a human study by Calame *et al* (2008), demonstrating that the consumption of GA at 10 g daily by healthy adults for 4 weeks increased bifidobacteria and lactobacilli [26]. Moreover, human and animal studies are still the best way to evaluate the ultimate response to nutritional interventions; however, they do not allow the dissection of the individual processes that may lead to the identification of the potential mechanism of action of the tested products.

Overall, in this study, we were able to demonstrate the modulation of the elderly gut microbiota following the fermentation of GA, and this result was supported by the increase in SCFA production. The changes in the gut microbiota in the elderly, due to decreased carbohydrate consumption and as a result reduced levels of SCFA, might result in increased inflammation caused by enteric pathogens.

SCFAs are end-products of the metabolism of gut microbiota; thus, the fact that in the current study we observed a modulation of SCFA levels is evidence of the potentially positive modulation of saccharolytic fermentation. SCFAs also can be assessed; normally, these would be absorbed *in vivo*. Acetate and propionate have been observed to enter the systemic circulation [35], and butyrate has an effect on colonocytes and plays a role in cell differentiation and proliferation [36]. Further more, butyrate has been observed to induce apoptosis in macrophages cells and regulate the immune cell activation and intestinal epithelium [37], [38], [39]. Thus this may relate to enhance the antiinflammatory cytokines in elderly people.

Furthermore, increased acetate and propionate levels and an increasing trend for butyrate production were observed. Although the increase in butyrate was not significant, butyrate has been shown to have anti-inflammation properties related to cytokines by reducing the

expression of TNF-α and upregulating IL-10 in mice [40]. The possible mechanism of this effect is through the inhibition of the nuclear translocation of NF-Kb [41]. Propionate has previously been observed to increase following GA treatment. This result is in line with that of previous studies, indicating that GA could potentially play an important role as an anti-obesity agent, due the possible mechanism that after 180 days of GA consumption (1%w/v), that a down regulation of TNF-α in β3-adrenergic stimulation of adipocytes and related to the modelation in the microbiotia which produce the organic acids that could contributes to a reduction in proinflammatory cytokines in adipose tissue [32].

In the literature, there is evidence that elderly individuals exhibit a decline in immune function, as indicated by cytokine production; therefore, in the current study, we measured cytokine production in order to observe the modulation of immune function. The modulation of the immune cytokines IL-10 and TNF-α was observed following the fermentation of GA. In an *ex vivo* study, similar results were reported, where GA resulted in reduced inflammatory potential by increasing IL-10 Xuan *et al* (2010) reported that GA fermentation by mice led to the stimulation of DCs and increased IL-10 levels through the formation of SCFAs, in particular butyrate, which have been shown to inhibit the functional differentiation of DCs [18]. Several studies have shown that prebiotics can increase IL-10 production in cells exposed to LPS, and an effect of GA on IL-10 has been observed previously in Ali *et al* (2013) in plasma following GA treatment in chronic renal failure (CRF), and TNF-α inhibition has also been observed in plasma and urine [19]. Additionally, in the same study the researcher reported that the daily intake of 15% GA in drinking water by rats up-regulated their immune function. Additionally, Bliss *et al* (1996) performed a human trial on 16 chronic renal failure CRF patients who consumed 50 g of GA daily and observed increased serum butyrate levels [42]. Further more, Glover *et al* (2009) in a (unblinded study

with a washout period and no placebo arm) study on type 2 diabetic subjects (n = 23 for 12 weeks) found that GA led to increased SCFA plasma levels after supplementation with the fibre [27]. However, they did not perform a bacterial SCFA analysis, which could have confirmed that the production of serum SCFAs resulted from the SCFAs produced by intestinal bacteria.

IL-10 is an anti-inflammatory cytokine mainly produced by monocytes and, to a lesser extent, by T cells [40], [43]. In the current study, both FOS and GA fermentation metabolites were shown to enhance IL-10 production induced by LPS. The up-regulation of IL-10 indicated that GA could positively modulate the immune response in the elderly population. Other fermentation metabolites have an inhibitory effect on TNF-α. GA induced anti-inflammatory effects by down-regulating pro-inflammatory cytokines and enhancing anti-inflammatory cytokines induced by LPS. Furthermore, the fermentation metabolites of the prebiotic FOS increased anti-inflammatory cytokines. Previously, Likotrafiti *et al* reported that FOS can modulate the elderly gut microbiota in a three-stage gut model system, resulting in increased acetate production [44]. During the fermentation, bifidobacteria were stimulated by FOS and GA, and increased SCFA concentrations were observed. Matsumoto *et al* (2006) evaluate the effect of GA consumption in an *in vivo* study on 10 healthy volunteers for 8 weeks at 25 g/day resulted in increased serum butyrate levels, and *in vitro* in the same study results indicate that GA has beneficial effects on renal pro-fibrotic cytokine generation [28]. It has been proposed that butyrate production may exert an anti-inflammatory effect. Moreover, several studies have reported that butyrate inhibits pro-inflammatory cytokine production by inhibiting LPS-induced NF-kB activation *in vitro* [41]. NF-kB plays an important role in immune and inflammation responses including TNF-α. After LPS induction, IkB is phosphorylated and degraded, and transcription is induced. Acetate also can down-regulate

pro-inflammatory cytokine production by lipoxygenase activation without inhibition of NF-kB. Murine studies have associated the increase in bifidobacteria with the increase in interleukin IL-10, which reduces inflammation [45].

In the current study, FOS showed a prebiotic effect by enhancing bifidobacteria and inhibiting *Clostridium histolyticum* spp. FOS fermentation led to a bifidogenic effect in vessel 1, which was associated with modulation of the anti-inflammatory cytokine IL-10. Although there was no association between SCFA production in vessel 1 and FOS, in vessel 2, an increasing trend for acetate and butyrate was observed. FOS also showed anti-inflammatory properties in vessels 1 and 2. In addition, FOS has bifidogenic activity [46]. In this study, we demonstrated a direct immune modulatory effect of the end products of the fermentation of GA on PBMCs through the modulation of cytokine production *in vitro*. Capita´n-Can˜adas *et al* (2014) reported that prebiotics can enhance IL-10 production [25], this can be due to the enhancement of the numbers of the beneficial bacteria and the selectivity of prebtioics.

Previous *in vitro* studies focused on ageing as a possible target for prebiotic intervention [23, 47]. Therefore, in the current study, we demonstrated the modulation of cytokine secretion following the fermentation of GA and FOS. Additionally, although using a three-stage continuous culture gut model system is different from using a closed batch culture system, the current model allows changes in microbial groups to be approximated in different parts of the colon based on differences in pH and nutrient availability.

3.6 Conclusion

In conclusion, GA modulated the immune response in vitro by affecting cytokine production in elderly monocyte cells induced with LPS. Although the gut microbiota was not affected, an increase for bifidobacteria was found following GA treatment. GA had a stronger anti-inflammatory effect than FOS, even though FOS was shown to modulate the gut microbiota. Further studies are needed to investigate the effect of GA on immune markers in the elderly population *in vivo*. Additional investigations are also needed to determine which intestinal bacterial groups are responsible for this positive effect of GA. This study suggests that the interaction between the changes in the gut composition and the mucosal immune system in the gut is related to the fermentation of the potential prebiotic GA and FOS.

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4. Chapter (4) Gum acacia enrichment culture to extract probiotic microorganisms

Abstract

The aim of this study was to determine key utilisers of gum acacia (GA) and isolate putative probiotics using GA enrichment culture experiment for 14 days from healthy elderly faeces then investigate the antimicrobial activity of the isolated probioitcs *in vitro* using agar spot test. The putative probiotics were then assessed for their probiotic potential via antipathogenic activities against pathogenic strains of *Escherichia coli*, *Salmonella* Typhimurium and *Enterococcus faecalis*. Results shows that probiotics from three volunteers were able to produce active compounds on solid media with antimicrobial activity and the most effective putative probiotic was from volunteer A. These were also confirmed when cell-free culture supernatants (CFCS) from the putative probiotics were used in agar well diffusion assay. These experiments were able to confirm the capacity of potential probiotics to inhibit selected pathogens. One of the main inhibitory mechanisms may result from the production of organic acids from utilising the GA and consequent lower of culture pH. These observations could lead to the end products of GA metabolism. Moreover, microbial community composition was analysed using next generation sequencing (NGS) and whole genome sequencing (WGS), increased in Firmicutes phylum and Ruminococcaceae in family level and the isolated microorganism was identified as lactobacilli at genus level after GA enrichment. In conclusion, the potential probiotics shows a remarkable antimicrobial activity, this is relevant to elderly population to decrease infections caused by pathogens bacteria. Further investigation to evaluate the probiotic bacteria capacity using *in vitro* mixed cultures.

4.1 Introduction

Aging is associated with decreases in potentially beneficial bacteria such as bifidobacteria [1]. Bifidobacteria are associated with improved immune function of elderly population [2]. Addtionally the genera *Lactobacillus* have been linked to improved gut health and wellbeing and can act to inhibit the growth of pathogens such as *E. coli* and *Salmonella* [3], [4]. The changes in the gut microbiota in aged people are also associated with an increased inflammatory status that might be associated with increased infectious diseases and reduced immunity [5]. Previous work showed a potential prebiotic effect of GA in an *in vitro* batch culture study (chapter 2). Furthermore, in a complex *in vitro* gut model system using elderly gut microbiota, potential anti-inflammatory properties were observed with both GA and fructooligosacchrides (FOS). These results imply that the GA is able to impact on the negative changes associated with ageing.

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer to a health benefit on the host" [6]. Furthermore, probiotics might reduce gastrointestinal infections through the ability to produce substances with antimicrobial properties such as organic acids [7]. Likotrafiti et al., (2004) demonstrated in an *in vitro* study that probiotics could inhibit *Clostridium difficile*, enteropathogenic *Escherichia coli* (EPEC), vero-cytotoxigenic *E. coli* (VTEC) and *Campylobacter jejuni*. In addition, prebiotics and synbiotics in a nutritional intervention study with elderly led to increases in organic acids in the colon, thus indicating some anti-pathogenic potential [8].

Studies have reported that GA has prebiotic activity through fermentation leading to increases in organic acids [9], [10]. Furthermore, in Sudan which GA can be used traditionally, Salih *et*

al (2012) observed a reduction in acute diarrhoea in children an intervention randomised controlled study following GA consumption and anti-diarrhoeal properties could have attributed to antimicrobial activity, however, the mechanism is uncertain and the clinical use of GA is still at a very early stage [11] . *In vitro* studies provide a rapid method to identify bacteria with the ability to inhibit pathogens and hence with the potential to reduce the risk of enteric infections in vivo, this could be relevant to hospitalised elderly [8]. Additionally, in previous studies on the GA effect on the intestinal bacteria, an *in vitro* pig model, indicated that the intestinal bacteria likely to be responsible for fermentation of high molecular weight gum acacia was *Prevotella ruminicola*-like bacterium, as determined by 16S rRNA gene sequencing[10]. Although there are similarities in the gut composition in between humans and pigs, more studies are needed to investigate which predominant bacteria that can ferment GA.

The use of an enrichment culture with known substrate offers a simplified approach to assess from within a mixed microbial community some of the main utilisers of the substrate. Furthermore, if isolates can be found with probiotic potential there exists the possibility of finding novel probiotics, ideal for use in synbiotic combination. This provides a novel approach to isolating potential probiotics that are key utilisers of GA. As the field of prebiotics has developed, the methodology for assessing the modulation of gut microbiota has changed to assess particular microbiota compositional changes as a response to fermentation. In this study NGS has been used as a way of sequencing millions of fragments even in a complex environments like the intestinal microbiota [12]. Here the aim of the present study was firstly to isolate probiotics bacteria from GA enrichment culture with evaluation *in vitro* of the ability of bacteria isolated from elderly faecal microbiota to inhibit selected Gram-

positive and Gram- negative pathogenic strains. Secondly, examine the ability of gut bacteria to utilise GA.

4.2 Material and method

4.2.1 Enrichment culture

All chemicals unless otherwise stated were purchased from Sigma Aldrich. Pre-reduced basal medium (peptone water (2 g/l), yeast extract (2 g/l), NaCl (0.1 g/l), $K2HPO_4$ (0.04 g/l), $KH2PO_4$ (0.04 g/l), NaHCO3 (2 g/l), MgSO4•7H2O (0.01 g/l), CaCl2•$6H_2O$ (0.01 g/l), tween 80 (2 ml/l), hemin (50 mg/l), vitamin K1 (10 ml/l), L-cysteine (0.5 g/l), bile salts (0.5 g/l), 0.5 ml of 10% cysteine –HCL, resazurin (1 mg/l) with 1% pre-digested GA was added into hungate tubes (9 ml) autoclaved. Faecal slurry (1ml, 1%) was inoculated into the hungate tubes for each volunteer, aged over 65 years, in good general health who had not consumed antibiotics in the previous 6 months. The hungate tubes were incubated anaerobically at 37^{o}C. Every 24 h 1ml of supernatant was transferred into 9 ml of fresh growth medium, this continued for 14 days. Samples were taken after 24 h then after the first week and second week of the experiment. Organic acids were analysed using GC. Additionally, DNA was extracted from the pellet of the samples to be used to assess the microbes present during the enrichment procedure.

4.2.2 Isolation of gum acacia fermenting bacteria

After 14 days of the enrichment experiment, i.e. incubation of faecal bacteria from three volunteers with gum acacia samples were plated (100µL) on MRS agar under anaerobic conditions at 37^{o}C at10^{-1} -10^{-8} dilutions within PBS 1%. After 48 h colonies were countable on 10^{-4}, 10^{-5}, 10^{-6} dilutions. A commonly occurring (based on morphology) colony was selected to investigate the antimicrobial properties using an agar spot test.

4.2.3 Bacterial strains and culture conditions

The bacterial strains used in the study are listed in Table (1), *Escerichia coli, Salmonella enterica* Typhimurium, *Entrococcus faecalis*, these strains were maintained at -70^{o}C in 15% (w/w) glycerol onto Cryobank cryogenic beads (Prolab Diagnostics,UK). Bacterial strains were cultured aerobically with 10 ml LB broth in Bijou bottles at 37 ° C in a shaking incubator for 24h.

Table 14: Bacterial strains used in this study

Bacterial species	Strain
Escherichia coli	NCFB1989
Salmonella Typhimurium	LT2
Enterococcus faecalis	NCTC 775

4.2.4 Antimicrobial activity by an agar spot test

Antimicrobial activity was investigated as by Barbosa et al (2005) using a colony overlay assay [13]. Pure culture microorganisms isolated from enrichment culture at 14 days were inoculated as 5 µL spots on MRS agar plates (3 spots/plate) and incubated at 37°C for 24 h under anaerobic conditions, to allow growth to occur. Subsequently, the growth that had occurred was exposed to chloroform vapour for 30min. the plates were aerated for 20min before overlaying with 10 ml of 0.7% (w/v) LB agar at 45oC, of an overnight culture previously inoculated with 10 µL of the indicator pathogen strain. MRS agar plates without any culture spot were also poured with 0.7% (w/v) LB agar containing 10 microliters of each indicator pathogenic. The plates were incubated at 37°C aerobically. Zones of inhibition around the spots at any of the incubation time points (8, 24 and 48 h) were examined and

scored. The experiment was conducted using isolated from the enrichment culture experiments.

4.2.5 Preparation of cell-free culture supernatants (CFCS)

2ml of putative probiotic overnight pre-cultures (10ml) was transferred to 100 ml of MRS broth and incubated for 24 h under the same conditions. Then, an aliquot of the cultures was centrifuged at 12,000 g for 10 min at 4 °C. the pellet was discarded and the CFCS aliquot into 4 tubes of 15 ml. two of them were neutralised to pH 7 by addition of 4 M NaOH and all of them were filter-sterilised through a sterile 0.20 µm-pore-size filter (Sartorius Stedim Biotech S.A., Aubagne Cedex, France). CFCS were concentrated by freeze-drying (~3days) (Christ-Gamma 2-16 LSC-Germany) and reconstituted with sterile high –performance liquid chromatography (HPLC) water (Fisher Scientific Ltd, Loughborough, Leicestershire, UK) to achieve 10-fold concentration.

4.2.6 Antimicrobial activity by well diffusion assay

Potential mechanisms involved in the inhibition of pathogenic bacterial growth were investigated by a well diffusion assay. This experiment was run in order to test if the inhibitory effect of the supernatants was exclusively due to its acidic pH or whether other mechanisms were involved. Twenty millilitres of 1.2% (w/v) of LB agar at 45 °C was mixed with 20 µL of an overnight culture of the indicator pathogen strain during the stationary phase and poured into petri dishes for the well diffusion assay according to Delgado et al., (2007) [11]. The agar was left to set for 30 min and 3 separate 6-mm diameter wells punched into the agar with sterile metal cylinder. Then, 60 µL of non –concentrated CFCS, were added into each well. The same procedure was carried out using CFCS non –concentrated and concentrated to 10 folds adjusted to pH 7 by addition of 4 M NaOH. The plates were incubated under appropriate conditions at 37°C for 24-48 h and antimicrobial activity

recorded as growth–free inhibition zones around the wells. Inhibition zones were measured in mm from the edge of the wells. As the controls, non-concentrated fresh MRS broth (pH6) as well as non-concentrated fresh MRS broth, adjusted to pH 7 by the addition of 4 M NaOH, were used. This experiment was carried out in triplicate and for each biological replicate, three technical repeats were used.

4.2.7 Short chain fatty acid production

To quantify the SCFA at different stages of the enrichment experiment samples were acidified to transfer the SCFA into the liquid phase to enable detection. 100 µl of sample supernatant mixed with 260 µl of H2SO4 solution (20 µ l of concentrated sulphuric acid with 100 m of distil water) to the sample was incubated for 10 minutes. After that, 50µl of internal standard was added 4 mM. inject volume 1 microl, inlet temperature 240 and detect 240, oven initial temperature 14^0C 5 min, the ratio 1^0C /min to 24^0C 5min.

4.2.8 DNA extraction

The DNA was extracted initially according to Yu and Morrison (2004) [12]. Following this a QIAamp DNA Minikit was used according to the manufacturer's instructions. (Qiagen,51304). Briefly 0.5ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 Mm EDTA and 4% sodium dodecyl sulphate) was added to samples in 2 ml screw –cap tubes. The samples were homogenised for 3 minutes in a Mini-Bead beater™ (Bio Spec Products, Bartlesville, OK, USA) for 1 min pulses with 2 min rest on ice between pulses. After that, samples were incubated for 15 min at 95 °C. For precipitation of DNA 130 µl of 10 M ammonium acetate was added and incubated for 5 min and then was centrifuged at 4°C for 10 min at 16,000× g and transferred to 1.5 tubes with 750 µl iso-propanol and incubated on ice for 30 min. Spectrophotometer Nano Drop (ND-1000) was used to check the extraction DNA quantity.

4.2.9 PCR Amplification and Sequencing

Aliquots of extracted DNA were amplified with universal primers for the V4 and V5 regions of the 16S rRNA gene. The primers U515F (5'-GTGYCAGCMGCCGCGGTA) and U927R (5'-CCCGYCAATTCMTTTRAGT) were designed to permit amplification of both bacterial and archaeal ribosomal RNA gene regions [13].

Forward and reverse fusion primers consisted of the Illumina overhang forward (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and reverse adapter (5'-GTCTCGTGGGCTCGGAGATGTGTAATAAGAGACAG) respectively. Amplification was performed with FastStart HiFi Polymerase (Roche Diagnostics Ltd, UK) using the following cycling conditions: 95 °C for 3 min; 25 cycles of 95 °C for 30 s, 55 °C for 35 s, 72 °C for 1 min; followed by 72 °C for 8 min. Amplicons were purified using 0.8 volumes of Ampure XP magnetic beads (Beckman Coulter). Each sample was then tagged with a unique pair of indices and the sequencing primer, using Nextera XT v2 Index kits, and KAPA HiFi Hot Start ReadyMix using the following cycling conditions: 95 °C for 3 min; 12 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; followed by 72 °C for 5 min. Index-tagged amplicons were purified using 0.8 volumes of Ampure XP magnetic beads (Beckman Coulter). The concentration of each sample was measured using the fluorescence-based Picogreen assay (Invitrogen). Concentrations were normalized before pooling all samples, each of which would be subsequently identified by its unique MID. Sequencing was performed on an Illumina MiSeq with 2 x 300 base reads according to the manufacturer's instructions (Illumina Cambridge UK).

4.2.10 Bioinformatics and Data Analysis

The sequence reads obtained were processed according to the microbiome-helper pipeline (https://github.com/mlangill/microbiome_helper/wiki/16S-standard-operating-procedure).

Essentially paired end reads were merged based on overlapping ends using PEAR (http://sco.h-its.org/exelixis/web/software/pear/), before filtering the data for base-calling quality. The processed sequences were then classified using the pick open reference, the operational taxonomic units (OTUs), process implemented in QIIME v1.9.0 softwares against the Green genes 16S rRNA gene database (http://greengenes.secondgenome.com/). The resulting distribution of OTUs across the multiple samples was further analysed using QIIME v1.9.0. to summarise the distributions and explore alpha and beta diversity. Sequence data were assembled and quality filtered. Further data analysis was performed using the open-source software system Quantitative Insights into Microbial Ecology (QIIME). Operational taxonomic units (OTU) were selected using a closed-reference OTU selection protocol.

4.3 Statistical analysis

All data from organic acids production and bacterial changes were analysed by repeated measures one-way Anova, p value of less that 0.05 was deemed as statistically significant.

4.4 Results

4.4.1 Antimicrobial activity by an agar spot test

All of the cultures tested resulted in a zone of inhibition against all pathogens at 8, 24 h and 48h as shown in Table 15. The potential probiotic from volunteer A resulted in greater antimicrobial activity in which more inhibition of the pathogens in 8 h *Enteroococcus faecalis* and in 24 and 48 h with *Salmonella* typhimurium and finally with *Escherichia coli* in 8, 24 and 48 h compared with other potential probiotics from volunteers A, B and C. figure (10) shows example of the inhibition zone of agar spot test.

4.4.2 Whole genome sequencing

As such this organism was sequenced using whole genome sequencing to identify the organism. The putative probiotic from volunteer A was seen to be more effective than others

and the results show that 99.4% chance to be *Lactobacillus rhamnosus* ATCC 8530 at strain level.using DNA STAR.and by MR-RAST assembled shotgun metagenome which identified the isolated microorganism as lactobacilli at genus level as shown in figures 8 and 9 .

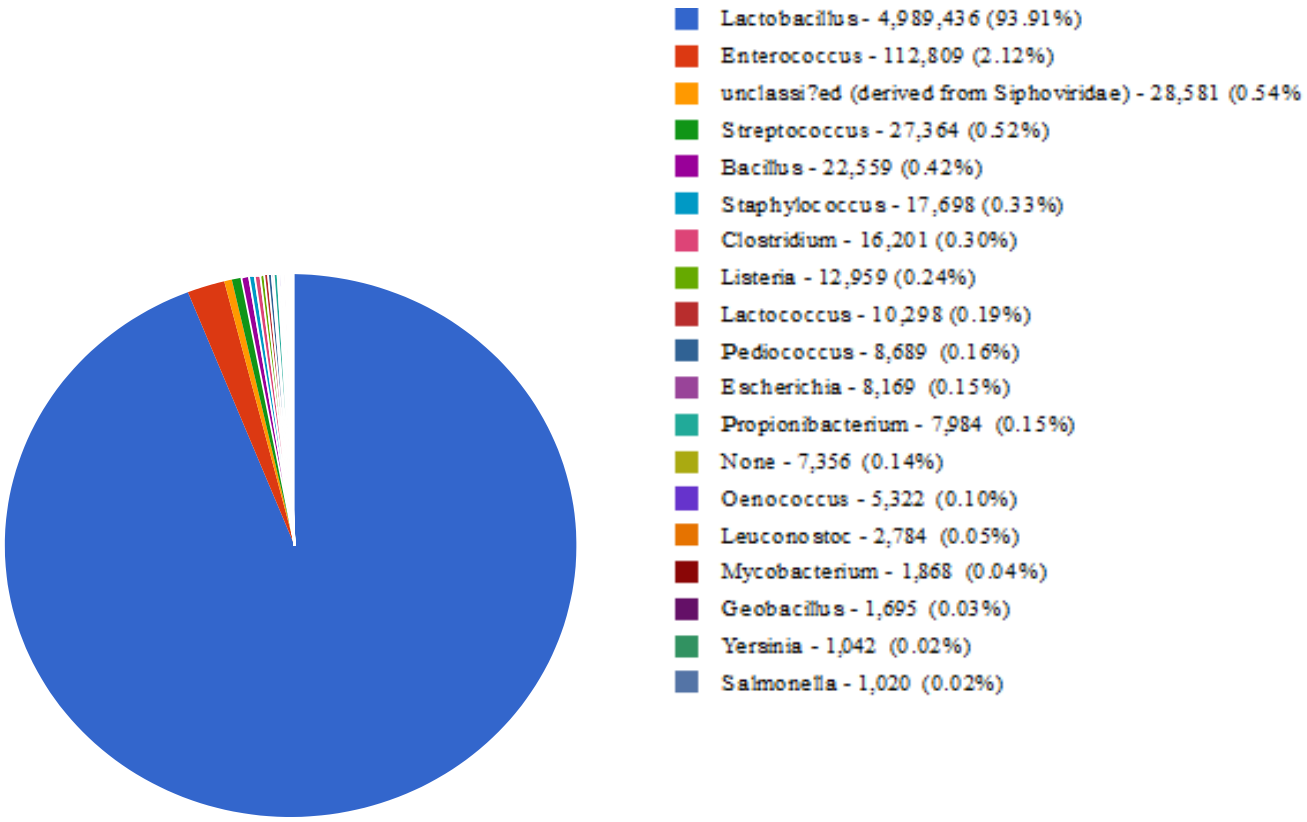

Figure 8: This chart illustrates the distribution of taxonomic domins , genus for the annotations , each slice indicates the percentage of reads with predicted and ribosomal RNA genes annoted to the indicated taxonomic level. This is based on all annotation source databases used by MG-RAST.

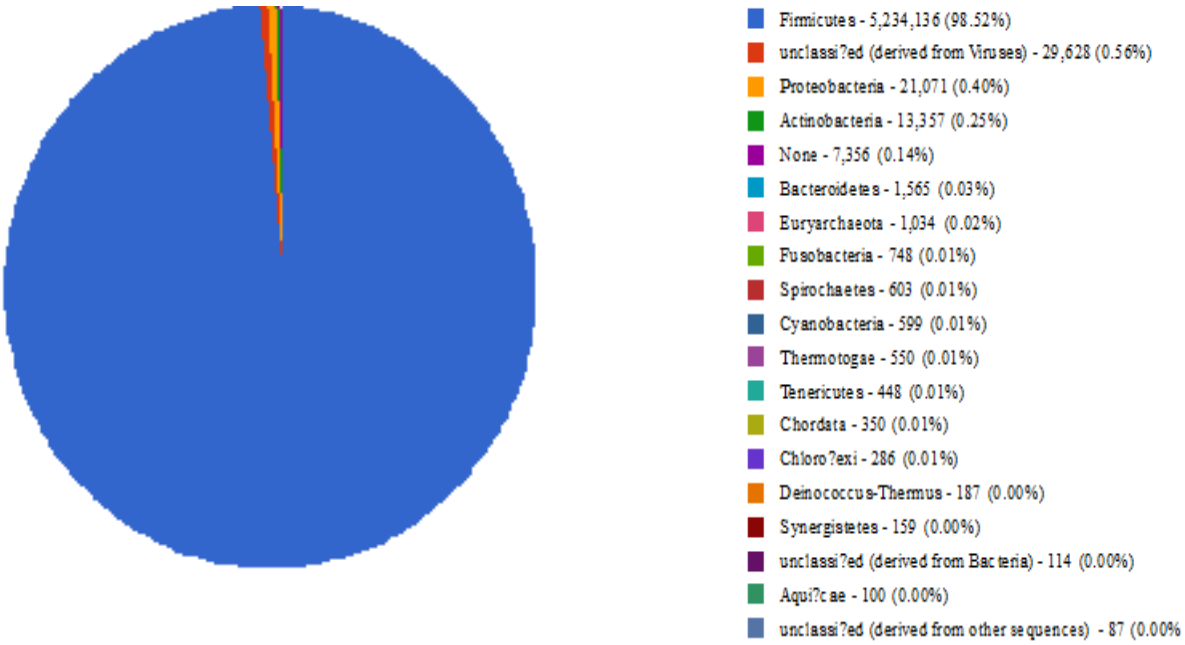

Figure 9: This chart illustrates the distribution of taxonomic domins , phyla for the annotations , each slice indicates the percentage of reads with predicted and ribosomal RNA genes annoted to the indicated taxonomic level. This is based on all annotation source databases used by MG-RAST.

4.4.3 Antimicrobial activity by a well diffusion assay

Table 16 shows the putative probiotics from three different volunteers resulted in inhibition against all pathogens in the spot test assays. Subsequent well diffusion agar test resulted in halos too small to measure with the non-concentrated supernatant. The 10-fold concentrated supernatants were subsequently used. It was seen that the strongest inhibition was with non pH adjusted supernatants from all of the putative probiotics and this was accordance with agar spot test results. Furthermore, well diffusion assay when the CFCSs were more concentrated, larger inhibition zones were observed as shown in Table 16. On the other hand, when the supernatants were pH adjusted, the inhibition activity was difficult to quantify. Though, around the wells observation of a yellow colouration, likely due to some anatanogestic effect comparing with controls that did not show any inhibition. Figures 11 shows inhibition zone of the adjusted pH and non adjusted examples.

4.4.4 Determination of organic acid production

There were no significant differences between day 0 and day 14 although there are notably changes with individual data in Table 17.

Table 15: Growth inhibition of of pathogens by probiotics potential by an agar spot test, *S.*Typhimurium, *E. coli*, *E. faecalis*.

Pathogenic strain	**Time**		
***Salmonella* Typhimurium**	**8 h**	**24 h**	**48 h**
Volunteer A	23.33±5.77	21.33±2.30	20±0.0
Volunteer B	22.66±1.15	18.66±2.30	17.33±2.30
Volunteer C	22.66±1.15	20±0.0	16±2.0
***Escherichia* Coli**	**8 h**	**24 h**	**48 h**
Volunteer A	30±0.0	24.66±1.15	20.66±1.15
Volunteer B	20±0.0	20.66±1.15	19.33±1.15
Volunteer C	20±0.0	18±2.0	16.66±3.05
Enterococcus faecalis	**8 h**	**24 h**	**48 h**
Volunteer A	28±3.46	14.66±4.61	10±2.0
Volunteer B	20±0.0	14.66±1.15	12.66±1.15
Volunteer C	16±0.0	11± 1.0	10±2.0

The different degree of the growth inhibition is expressed in mm as the mean of three replicates ±SD

Table 16: well diffusion assay. Inhibitory effects of non –adjusted pH of cell-free culture supernatants of selected putative probiotics against pathogenic bacteria *Salmonella* Typhimurium, *Escherichia coli*, *Entrococcus faecalis* (non concentrated) and 10-fold concentrated.

non –adjusted pH of cell-free culture supernatants						
Pathogens strain	***E. coli***		***S.* Typhimurium**		***E. faecalis***	
	NC	**10-fold**	**NC**	**10-fold**	**NC**	**10-fold**
Volunteer A	2.66±0.57	6.4±0.34	3.00±0.0	6.00±0.0	2±0.0	6.33±1.52
Volunteer B	1.50±0.0	5.33±0.57	1.16±0.28	5.00±0.0	1.73±0.46	5.33±0.57
Volunteer C	2.25±0.57	5.00±0.0	2.00±0.0	5.30±0.57	2.33±0.57	5.00±0.0
adjusted pH of cell-free culture supernatants						
Volunteer A	0.0±0.0	1.06±0.11	0.0±0.0	0.93±0.11	0.0±0.0	0.86±0.23
Volunteer B	0.0±0.0	1±0.0	0.0±0.0	1±0.0	0.0±0.0	1±0.0
Volunteer C	0.0±0.0	1±0.0	0.0±0.0	0.93±0.11	0.0±0.0	0.86±0.23

The different degree of the growth inhibition is expressed in mm as the mean of three replicates ±SD

Table 17: SCFA concentration using acidification method for three volunteers A, B, C

	Acetate	**Propionate**	**butyrate**
		Volunteer A	
Day 0	0	6	0
Day 7	39.53	7.54	11.28
Day 14	28.31	4.47	9.78
		Volunteer B	
SAMPLES	**Acetate**	**Propionate**	**butyrate**
Day 0	0	8.61	11.36
Day 7	17.86	11.95	15.51
Day 14	22.29	14.84	5.92
		Volunteer C	
SAMPLES	**Acetate**	**Propionate**	**butyrate**
Day 0	9.62	4.62	17.68
Day 7	19.71	11.27	15.01
Day 14	18.18	11.17	4.52

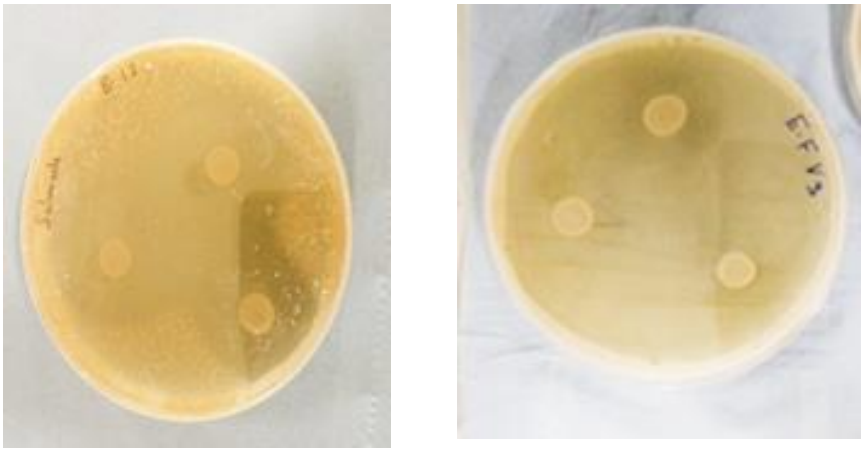

Figure 10: : Agar spot test isolated probiotics from three volunteers A, B and C the growth of inhibition zone, the three isolated probiotics have shown an anti-pathogenic effect against pathogens *S.*Typhimurium, *E. coli, E. faecalis* the plates shows the inhibition zone of agar spot test within, *S.* Typhimurium as an example

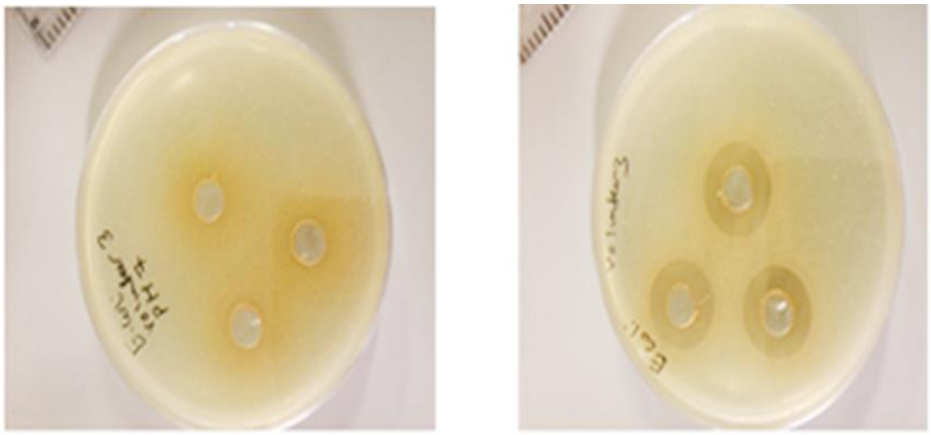

Figure 11: Well diffusion assay**,** the inhibition zone adjusted pH on the left hand and non-adjusted samples on the right hand, *S.* Typhimurium, *E. coli, E. faecalis*. 10-fold concentrated CFCS.

4.4.5 Relative abundance phyla, family levels and genera

In this present study, samples were obtained from enrichment cultures on day 0 and day 14 from three volunteers. The microbial communities were characterised regarding to the different fermentation times, between 0 and 14 days in the presence of single carbohydrate source, GA at 1%.

The phyla *Bacteroidetes, Firmicutes* and *Proteobacteria* were the most abundant during the experiment, which are the most abundant in the human gut composition. After the fermentation of GA for two weeks the phyla *Firmicutes* showed a trend to increase using repeated measures one-way ANOVA between different times ($p=0.08$). There were increases (15.96, 29.30, 31.24) % for the three volunteers respectively compared to (9.77, 11.38, 10.19) % at the beginning of the inoculum. Figure (13) shows the changes in phyla.

Figure (12) shows the beta diversity analysis PCoA shows the diversity of microbial composition between three samples and shows the bacteria has clustered and moved toward a specific composition for the three volunteers after fermentation for 14 days.

Family level Ruminococcaceae, which belongs to Firmicutes phylum, as showns in Figure (14) , was enhanced from (0.0, 0.50, 1.75) % (9.09, 18.67, 15.64) % for the three volunteers.

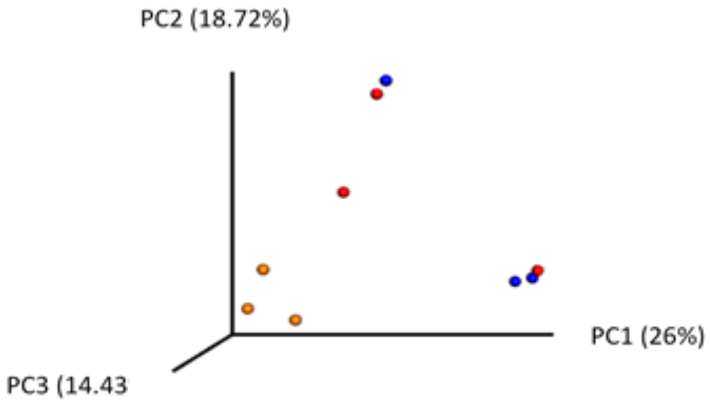

Figure 12: PCoA plot: Investigation of the effect of GA enrichment culture on gut microbiota reads of the three volunteers A, B and C.(bacterial diversity and relative abundance) Beta diversity analysis in different times of individual volunteers different times were represented as spot with red (day 0) baseline, blue (day 7), orange (day14).

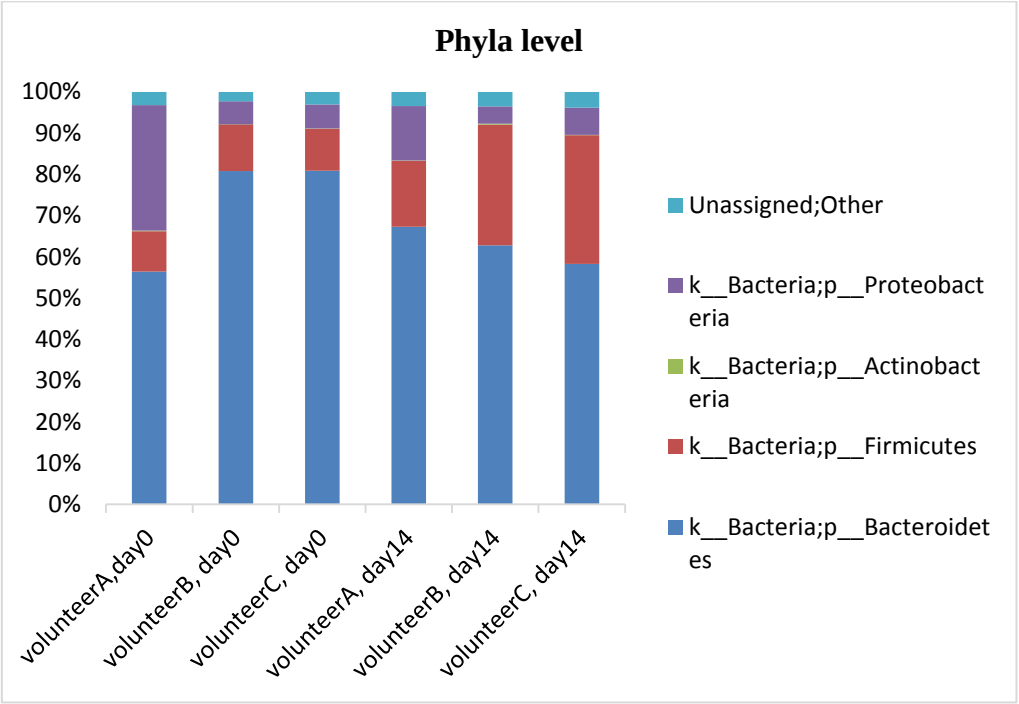

Figure 13: Relative abundance of bacterial reads at phyla level for different three volunteers at time 0 baseline day and 14 day. Investigation of the effect of GA enrichment culture on gut microbiota (bacterial diversity and relative abundance) at phylum level between three volunteers, A, B, C in enrichment culture experiment for 14 days at the beginning of the experiment compared with the end of the experiment. Firmicutes has greater increase within the three volunteers in 14 days and a decrease in bacteroidetes correlate with the increase in firmicutes. A thin green line presents Actinobacteria not clear in all samples.

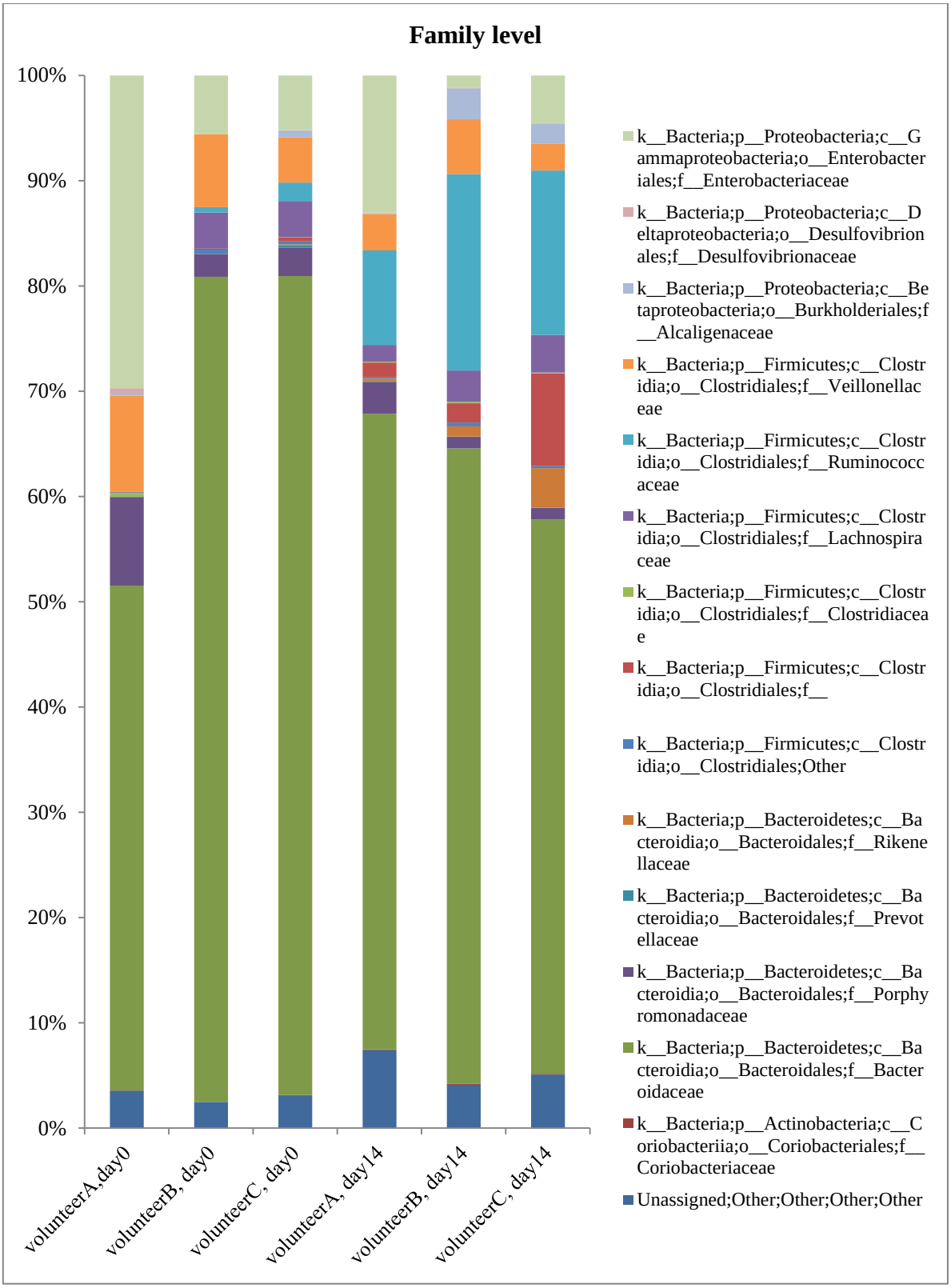

Figure 14: Relative abundance of bacterial reads at family level for different three volunteers at time 0 baseline day and 14 days. Investigation of the effect of GA enrichment culture on gut microbiota (bacterial diversity and relative abundance) at family level between three volunteers in enrichment culture experiment for 14 days, The first three columns are the three volunteers gut composition at the beginning of the experiment compared with the end of the experiment, the last 3 columns representing day14 for each. Ruminococcaceae family has the greatest increase within the three vounteers in 14 days.

4.5 Discussion

An *in vitro* enrichment culture study was used with the aim of identifying the microorganisms within the elderly faecal microbiota that utilise GA. Furthermore putative probiotics were isolated from these enrichment experiments and tested for antimicrobial activity against three pathogenic strains *S.* Typhimurium, *E. coli, E. faecalis*. Bacterial populations were characterised at days 0, and 14 by community profiling (NGS).

Previous investigations have shown that bifidobacteria, bacterioides and lactobacilli are increased following the fermentation of GA [14]. In our previous work GA shows anti-inflammatory properties in a gut model system, whilst the impact on the microbiota was not so marked. As a further investigation to seek other members of microbiota which might be responsible for GA fermentation was undertaken. NGS community profiling was employed to discover this.

At the phyla level during all experimental stages *Bacteriodetes, Firmicutes* and *Protobacteria* were the most abundant bacterial groups. Similar results have been seen in other studies, among elderly populations [15],[16]. Increases in the *Firmicutes* have been observed in the end of the experiment, yet this change was not significant, this result is relevant to maintain the gut microbiota balance in elderly population. Furthermore, within the family level it was seen that Ruminococcaceae, which might be a butyrate producer, utilised GA as a carbohydrate source. This result is important as could improve the elderly health in producing butyrate. However, within this enrichment experiment SCFA were not observed to significantly increase, In the current study GA did not show lead to significant increases in bifidobacteria and / or lactobacilli at genus level, however, it was still posible to isolate out lactobacilli at the end of the enrichment experiment. This means that lactobacilli were able to use GA, although, they may have not been a predominant utiliser. Wyatt et al (1986)

observed using human faecal inoculum in basal medium with 2% of GA resulted in isolation of *Bifidobacterium longum* and *Bacteroides ovatus, Bacteroides oris, Bacteroids buccae* and prevotella ruminicola-like bacterium [17].

SCFA were assessed during the enrichment experiments, however, these did not significantly increase. This could possibly be because that after the fermentation for 24 h a less diverse bacteria consortium is being transferred to the subsequent tube therefore, the production of the SCFA may be lower than the previous cultured inoculum, depending on which microorganisms were selected for by the GA and therefore the more the samples were diluted the lowest bacterial numbers composition resulted.

In this current study, microorganisms were isolated onto MRS agar following the enrichment culture. These isolates were assessed for anti-microbial activities against known pathogens. Three separate microorganisms were isolated from the enrichmentment culture experiments. The microorganism that showed the greatest anti-pathogenic potential was further identified by whole genome sequencing to be *Lactobacillus rhamnosu*s (WGS) 99.4 %. *Lactobacillus* bacteria is known to have antipathogenic and probiotic properties, therefore the resultant microorganism was further studied for its probiotic potential [18]. The premise of this experiment being to isolate GA utilisers that show probiotic potential. The antagonistic activities of isolated putative probiotics could not be attributed to competition for the growth substrates between potential probiotic and pathogens as probiotic cells were killed with chloroform before overlaying the pathogen, thus another factor produced by these organisms was involved.

Moreover, the pH of the cell-free culture was between 4 and 5, therefore, correspondingly suggesting that the mechanism of this effect may be attributed to the low culture pH. Other mechanisms that could be attributed to this effect associated with lactobacilli for example bacteriocins that cause of the extension of the lag phases and the lower growth rate of pathogens. Further, these studies suggest that growth-inhibiting activity was attributed to the lower pH associated with production of organic acids. *E. coli* is a Gram-negative bacterium which is a member of Enterobacteriaceae family. *E. coli* can colonise in the body, especially in the lower intestine and be transmitted through the faecal-oral route. Pathogenic strains of the bacterium can cause diseases from gastroenteritis to extra-intestinal infections of the urinary tract, pulmonary and nervous system [19]. The antimicrobial potential by each probiotic used here could indicate to the ability of probiotics to to inhibit pathogens within the colon and in particular elderly gut microbiota. The bacterium that led to the greatest antimicrobial activity belonged to volunteer A , this microorganism was identified by whole genome sequencing. Following neutralising of the supernatants within the well diffusion assays a large reduction in the antipathogenic properties was observed. Therefore it is likely that SCFA and organic acids were key to these effects. It is well known that lactobacilli can produce organic acids which lead to a decrease in pH [20]. In another study, De Keersmaecker and others, reported strong antimicrobial activity of *L. rhamnosus* GG against Salmonella that was mediated by lactic acid [21].

When culturing the isolated probiotics from volunteer A lactobacilli was identified confirming that lactobacilli is able to use GA. Therefore, the reason for the lack of observed selectivity could be attributed to a limitation of the DNA sequencing method, whereby the choice of primers can result in lost information. Moreover, the potential probiotics were able to grow well in selective medium. On the other hand, this could mean that FISH analysis

could provide an important tool for assessing probiotic selectivity, as NGS is a not fully quantitative method. As such as *Bifidobacteirum* and *Lactobacillus* could be underestimated by 16S rRNA gene sequencing analysis if the correct primers are not used and this had been reported in a previous study [22].

4.6 Conclusion

Overall, we were able to observe the probiotic potential of isolated bacterial microorganisms. These results could establish the basis for further experiments, e.g. the design of *in vitro* mixed batch culture to test the potential probiotic and synbiotic properties using GA as a prebiotic.

4.7 References

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5. Chapter (5) An *in vitro* study exploring a synbioitc on elderly gut microbiota against *Escherichia coli*

Abstract

The aim of this study was to evaluate *in vitro* the effect of a putative probiotic, (*Lactobacillus rhamnosus*), potential prebiotic gum acacia (GA) individually and in a synbiotic combination against the survival *E. coli* (NCFB1989) in a mixed community using pH-controlled anaerobic batch cultures inoculated with faeces from elderly volunteers. Population changes of the bacterial groups were enumerated using fluorescence *in situ* hybridisation (FISH) and the short chain fatty acids concentration were determined using gas chromatography (GC). The Synbioitc significantly increased *Lactobacillus* spp. numbers at 10 h and reduced numbers of bacteria in the *Clostridium histolyticum* group. Butyrate significantly increased within the synbiotic vessel. GA alone also led to significant reductions in numbers of bacteria in the *Clostridium histolyticum* group. The probiotic led to increase in acetate and propionate production. *E. coli* growth was not affected by the pro-pre biotic or synbitoic addition the microbiota was however modulated by the treatments. Overall, the synbiotic treatment gave rise to the greatest impact on the intestinal microbiota.

5.1 Introduction

There is variation in the gut microbial composition between young adults and aged people. In a study comparing young and aged people in India, concluded that the gut microbiota of the elderly harboured a greater proportion of *Bacteroidetes* and a distinct abundance in *Firmicutes* numbers [1]. Moreover, a research found similar results when comparing elderly and younger populations gut microbiota [2]. Furthermore, two studies observed less *Bifidobacterium* and *Clostridium* cluster IV alongside increases in enterobacteria and *Escherichia coli* in elderly, when compared to middle age subjects [3],[4]. The differences between young adults and elderly in the gut composition could contribute in part to the 400 times more gastrointestinal infections observed in the elderly than in younger adults [5]. Previous studies have been shown that probiotics have a protective effects against the intestinal infection by *E.coli* O157:H7 in animals models [6, 7] also in human trails FOS has been associated with the reduction of the traveler's diarrhoea symptoms [8]. *E.coli* for example can colonise the large intestine after passing the small intestine which and can act as a potnetial pathogen within, particularly in the case of dysbiosis [9]. Concluding that people with weak immune system are at increased risk for developing these complications such as elderly population.Dietary intervention is one approach that could be used to promote healthy ageing. Indeed, probiotics and prebiotics have been observed to modulate the microbiota and enhance the immune function in elderly volunteers [10], [11]. To increase the efficacy of probiotics, prebiotics could be used in conjunction to selectively enhance the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits to host health [12]. A combination of pro-prebiotic, which is known as a synbiotic identified as "a mixture of probiotics and prebiotics that beneficially affects the hosts by improving the survival and implantation of live microbial dietary supplements in gastrointestinal (GI) tract, by

selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health –promoting bacteria and thus improving host welfare" [13], should achieve the requirements of both pro- and prebiotics.

For a probiotic to be classed as such certain criteria need to be fulfilled regarding safety, stability, functionality and physiology. Probiotic organism properties should preferably be of human origin, and be well defined genus/strain, and demonstrate non – pathogenic behaviour and produce antimicrobial substances resulting in antagonistic activities against pathogens [14]. In previous chapter (Chapter 4) studies were reported indicating that elderly populations have increased exposure to pathogenic bacteria which, as a result, could lead to infectious disease [15], [5]. Moreover, previous studies reported that prebiotic FOS was able to reduce symptoms of traveller's diarrhoea, which often associated with *E. coli* strains [8].

Gum acacia (GA) has prebiotic potential observed through modulation of gut microbiota in previous work. Furthermore, a potential probiotic has been isolated from the elderly microbiota using GA as a substrate. The premise being GA should be very specific for this probiotic, thus its activity should be enhanced. The probiotic has shown antimicrobial effects against enteric pathogens *E. coli*, *Salmonella* Typhimurium and *Enterococcus faecalis*. these are desirable properties in probiotics. The inhibition mechanisms includes the production of organic acids and other substrates such as bacteriocins [16], [17].

This study aimed to elucidate the probable effects of synbiotic on the colonic microbiota using a model simulated colonic fermentation in a pH controlled, stirred, batch-culture system, with elderly faecal inoculum, reflecting of the environmental conditions of the distal region of the human large intestine. The putative probiotic used in this combination of

synbiotic with GA has been previously identified to be active against the *S.*Typhimurium, *E. coli*, *E. faecalis*, therefore, *E. coli* was added as an intestinal pathogen.

5.2 Material and Methods

Chemicals

All media constituents were purchased from Oxoid Ltd, Basingstoke, UK and all chemicals were purchased from Sigma- Aldrich, Poole, UK unless otherwise stated. Media used was de Man Rogosa Sharpe (MRS) broth for putative probiotic and LB broth for *E. coli* NCFB 1989 strain.

5.2.1 Bacterial strains and culture preparation

E. coli (NCFB1989) was selected as a pathogen for use in these experiments. The putative probiotic bacteria was isolated from a healthy elderly volunteer (Chapter three). For these bacteria growth curves of optical density (spectrophotometer, Sherwood, UK) (OD660 nm) against colony forming units (CFU) per milliliter by regular sampling of 24 hour cultures was conducted in triplicate. *Lactobacillus rhamnosus*, the putative probiotic was inoculated in de Man–Rogosa–Sharpe (MRS) 10 ml broth and grown at 37°C to late log phase under anaerobic (10:10:80%; H2:CO2:N2) conditions. *E. coli* (NCFB1989) was inoculated in Luria Bertani (LB) broth 10 ml 37°C and grown to late log phase under aerobic conditions. Then the bacterial suspension was centrifuged at 12,000 g for 10 min at 4 °C. According to growth curve, concentration of cells were adjusted to 5×10^8 CFU/ml and 4×10^8 CFU/ml for putative probiotic and *E coli* respectively. The supernatant was removed and cells re-suspend in phosphate –buffered saline (PBS) (1 M, pH 7.4) (Oxoid Ltd, Basingstoke, Hampshire, UK). After that, cells were immediately added to the batch culture vessels according to that the *E. coli* was added to all vessels while putative probioitic was added to the probiotic and

synbiotic vessels. For quantification *E. coli* was plated on Macconkey agar with ampicillin 1μ g/Lfor the survival *E. coli* (Oxoid) each time point.

5.2.2 Antibiotic resistance development for *Escherichia coli*

In order to reduce the effect of some background gut microbiota from donors, the test pathogen strain was developed into an antibiotic resistance strain before being inoculated into pH-controlled anaerobic faecal batch cultures. Antibiotic resistant variant was selected by growing *E. coli* on successive overnight aerobic culture in LB broth whilst increasing ampicillin concentration from 0.100 to 100μg L^{-1}. The resistant colonies were isolated and used within the subsequent experiment.

5.2.3 *In vitro* Upper Gut Digestion

60 g of GA powder was added to 150 ml of distilled - water and the solution was mixed with 20 mg of α –amylase in 6.25 ml $CaCl_2$ (1 mM) and incubated on a shaker at 37 °C for 30 minutes. This simulated the initial oral digestion. Subsequently, 2.7 g of pepsin in 25ml of HCl (0.1M) was used to facilitate gastric breakdown of the sample. The pH was progressively reduced to 2 by adding 6 M HCl, the resulting suspension was incubated on a shaker at 37°C for 2 hours. A further 560mg of pancreatin and 3.5g of bile in 125 ml of $NaHCO_3$ solution was added to simulate the effect of the small intestine on the gum sample. The pH was increased to 7 by adding NaOH (6M) and the suspension was incubated on a shaker at 37°C for 3 hours. Samples were transferred to cellulose dialysis membrane (1 KDa molecular weight), purchased from Cheshire biotech Cheshire, UK, and dialysed against 10mM NaCl solution at 4°C to remove low molecular mass digestion products. After 15 hours, the dialysis fluid was changed and dialysis continued for additional 2 h. The remaining sample was freeze

dried IEC Lyoprep-3000 freeze dryer (Dunstable, UK) (5 days) prior to use in batch culture systems.

5.2.4 Faecal sample preparation

Faecal samples were obtained from three healthy volunteers 65 years of age and above. Volunteers had not been consuming antibiotics for at least 6 months before the experiment and had no history of gastrointestinal disease. Volunteers were not consumers of probiotic or prebiotic supplements. Samples were prepared on the day of the experiment and within 1 hour of production and were diluted to (1:10, w/v) in anaerobic phosphate buffered saline (0.1 M; pH7.4). Samples were homogenised in a stomacher for 2 minutes the resulting slurries were inoculated into seperate batch culture fermenters.

5.2.5 Batch culture

Three independent batch culture experiments were carried out using faeces from a different donor each time. Vessels were autoclaved and then aseptically filled with 135 ml of basal medium (peptone water (2 g/l), yeast extract (2 g/l), NaCl (0.1 g/l), K_2HPO_4 (0.04 g/l), KH_2PO_4 (0.04 g/l), $NaHCO_3$ (2 g/l), $MgSO_4 \cdot 7H_2O$ (0.01 g/l), $CaCl_2 \cdot 6H_2O$ (0.01 g/l), tween 80 (2 ml/l), hemin (50 mg/l), vitamin K1 (10 ml/l), L-cysteine (0.5 g/l), bile salts (0.5 g/l), 0.5 ml of 10% cysteine –HCL, resazurin (1 mg/l)). Vessels were left overnight with nitrogen pumping (15mL/min) through the vessel to provide an anaerobic environment. Before addition of faecal slurry, temperature of basal medium was set at 37 °C and pH was maintained at 6.8 using a pH meter (Electrolab pH controller, Tewksbury, UK) by the addition of 0.5 M HCl or 0.5M NaOH. The vessels were stirred using magnetic stirrers. 0.3 g (1:10 w/v) of pre-digested GA was added to three vessels, a remaining vessel was left as the blank. All vessels were inoculated with faecal slurry. 4×10^8 of *E. coli* suspension was added

to all four vessels and 1 ml of probiotic was added to probiotic and synbiotic vessels. The vessels were left for 48h, with 7 ml samples taken at 0, 5, 10, 24 and 48h. Samples were centrifuged in preparation for GC analysis, or prepared for microbial enumeration by FISH in triplicate.

5.2.6 *In situ* florescent hybridization (FISH) analysis

All nucleotide probes used for fluorescent *in situ* hybridisation were commercially syntheised and labelled with dye Cy3 at the 5' end (Sigma Aldrich Co. Ltd. UK).

375-µl sample obtained from each vessel was fixed for four hours 4˚C in 1125 µL (4% w/v) paraformaldehyde. Fixed samples were then centrifuged at 11,337g (Eppendorf centrifuge mini spin, Eppendorf, UK) at room temperature for 5 minutes. The supernatant was discarded and pellet resuspended in 1 ml of cold 1×PBS by aspirating carefully using a pipette. This step was conducted twice. The washed cells were resuspended in 150 µL of cold 1×PBS, then 150 µL of ethanol (99%) was added and the samples stored at -20˚C.

To assess differences in bacterial numbers, fluorescence *in situ* hybridisation (FISH) was conducted, using oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA. The probes were commercially synthesised and labeled with the fluorescent dye Cy3. The probes used were: Bif164 for bifidobacteria (BIF), Lab158 for lactobacilli/enterococci (LAB), Chis 150 for *Clostridium histolyticum* group (CHIS), Erec 482 for *Eubacterium rectale – Clostridium coccoides* group (EREC), Bac 156 for *Bacteroides–Prevotella* group (BAC). EUB 338 mixture consisting of EUB338, EUB338II and EUB338III for total bacteria (Total) see table 7. Samples were hybridised as described by Daims *et al.,* 1999 [18]. For the hybridisation 20 µ L of diluted sample was pipetted onto a teflon poly-L-lysine-coated six-well slide (Tekdon Inc., Myakka City, FL). The samples were dried onto the slides at 46-

50˚C for 15 minutes and after that dehydrated in an alcohol series 50, 80, and 96%. The ethanol was allowed to evaporate from the slides before hybridisation buffer (0.1 of probe in a 50 μL of (0.9 M NaCl, 0.02 M Tris/HCl (pH 8.0), formamide (if required – Table 5), 10% (w/v) sodium dodecyl sulphate, 4.55 ng ml-1 probe) was applied to permeabilise the cells for use with probes Bif164 and Lab158, samples were treated with 20 μ L of lysozyme at room temperature for 15 min before being washed briefly for 2−3 seconds in water and then dehydrated in the ethanol series. Then the slides were placed in hybridisation oven for 4 hours (ISO20 oven, Grant Boekel). For the washing step, slides were placed in 50 ml of washing buffer (0.9 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.005 M ethylenediaminetetraacetic acid (EDTA) solution (pH 8.0, Table 2)), warmed at the appropriate temperature for each probe and 20 μL of 4;6-diamidino-2-phenylindole di hydrochloride (DAPI) was added to the washing buffer. After 10 minutes slides were briefly washed (2−3 s) in ice-cold water and dried under a stream of compressed air. Five microliters of Pro Long Gold anti-fade re-agent (Invitrogen) was added to each well and a coverslip applied. Slides were stored in the dark at 4 °C until cells were counted under a NikonE400 Eclipse microscope. A DM510 light filter was used to count specific bacteria hybridised with the probes of interest. A minimum 15 random fields of view were counted for each sample. The following formula was used to calculate numbers of bacteria: (0.8 × A1 × 6732.42 × 50 × Dilution factor), where A1 is the average count of 15 fields of view, 6732.42 is the area of the well divided by the area of the field of view, multiplying by 50 takes the count back to millilitre of sample. Results were expressed as Log_{10} bacterial numbers per millilitre batch culture fluid.

5.2.7 Preparation sample for short chain fatty acids

To determine the SCFAs samples were extracted and derivatised as previously described [19]. Samples were defrosted and 1ml of each sample or standard solution was transferred into a labeled 100 mm × 16mm glass tube with the internal standard of 50µl of 2- ethyl butyric acid (0.1M). 0.5 ml concentrated HCL and 2 ml of diethyl ether were added to each glass tube was added to each glass tube and samples were vortex for 1 min. Samples were centrifuged at 2000 g for 10 min (SANYO MSE Mistral 3000i; Sanyo Gallenkap PLC, Middlesex, UK). The diethyl ether (the upper layer) was transferred into a new glass tube. A second extraction was conducted by adding 1 ml of diethyl either followed by vortex and centrifugation. 400 µl of pooled ether extract and 50 µl N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) was added into a GC screw-cap vial. Samples were heated at 80°C for 20 minutes and then left at room temperature for 48 hours to allow lactic acid in the samples to completely derivatise.

A 5890 SERIES II Gas Chromatograph (Hewlett Packard, UK) using an Rtx-1 10m×0.18mm column with a 0.20µm coating (Crossbond 100% dimethyl polysiloxane; Restek, Buckinghamshire, UK) was used for analysis of SCFA. Temperatures of injector and detector were 275°C, with the column programmed from 63°C for 3 minutes to 190°C at 10°C min^{-1} and held at 190°C for 3 minutes. Helium was the carrier gas (flow rate 1.2 ml min-1; head pressure 90 MPa). A split ratio of 100:1 was used. The standard solution contained (mM): sodium formate, 10; acetic acid, 30; propionic acid, 20; isobutyric acid, 5; n-butyric acid, 20; iso-valeric acid, 5; n-valeric acid, 5; sodium lactate, 10; sodium succinate, 20. The sample was injected onto the column, which was maintained at 140 ^{o}C for the first 5 minutes, temperature of the column was increased over 5 minutes to 240 ^{o}C. To maintain

appropriate calibration after injection of every 20 samples an external standard solution, with known concentrations of SCFAs was injected. Peaks and response factors within samples were calibrated and calculated using ChemStation B.03.01 software (Agilent Technologies, Cheshire, UK).

5.3 Statistical analysis

Bacterial numbers were statistically evaluated after transformation to $\log_{10}$ counts per ml using Microsoft Office Excel 2007 (Washington, USA). Subsequently repeated measures one-way analysis of variance (ANOVA) was used to test the effect of time as within the subjects factor, (0 h, 10 h) and to assess the significant differences between the two subjects in the same time points. Significant differences between times points were detected and represented by "*" $p < 0.05$, "**" $p < 0.01$ and "***" $p < 0.00$.

5.4 Results

5.4.1 Bacterial enumeration

Figure (15) shows the bacterial changes after the fermentation of synbioitc, putative probiotic, potential prebiotic. Lactobacilli numbers increased within the synbioitc vessel at 10 h ($p=0.04$). Furthermore, *Clostridium histolyticum* group numbers decreased within synbiotic and GA vessels ($p=0.02$), (0.02) respectively. Additionally, a significant increase in *C. histolyticum* group numbers was observed within the negative control ($p=0.02$) when compared with baseline.

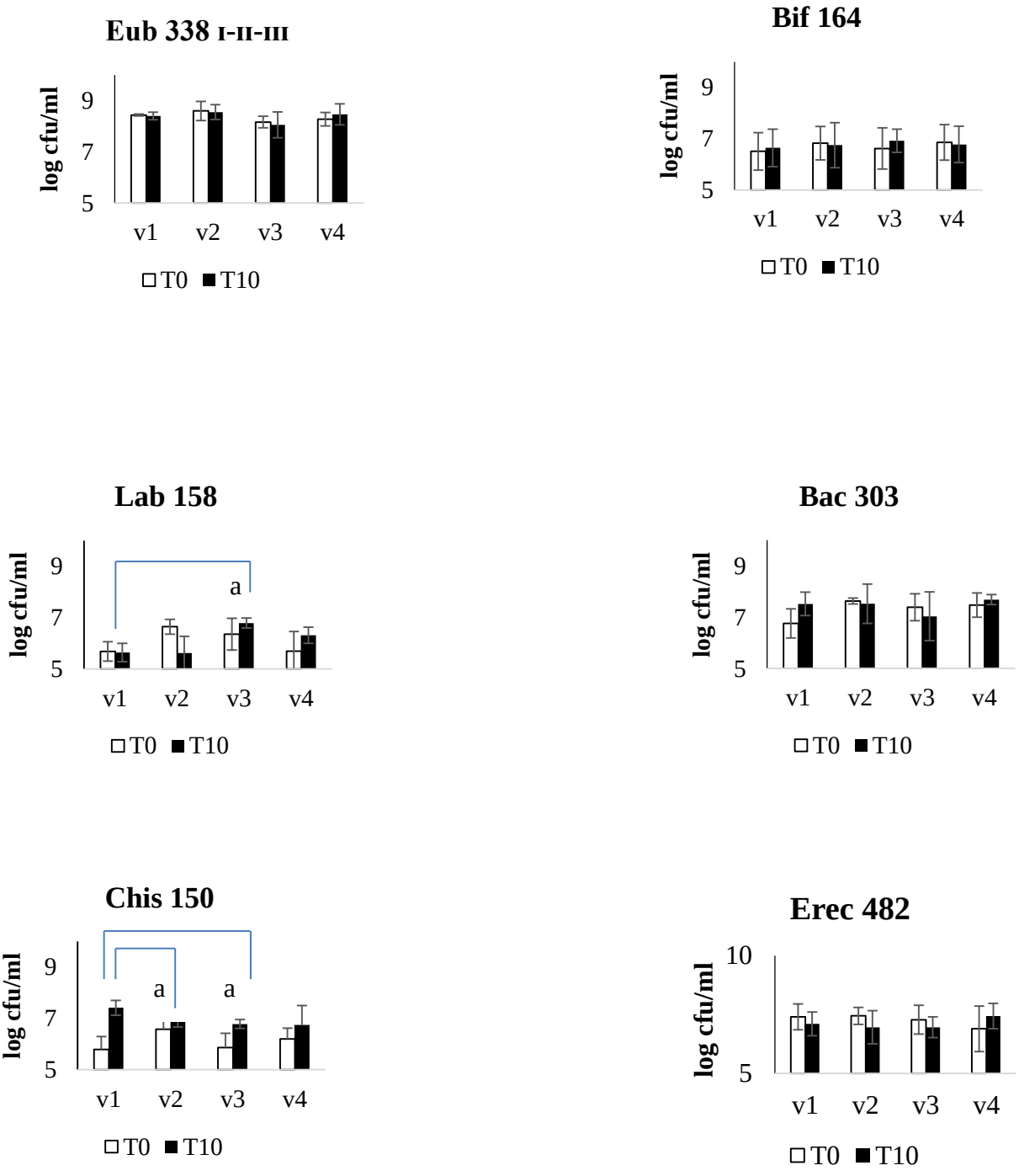

Figure 15: changes in the bacterial composition figures are presenting the mean bacterial populations in pH-controlled batch cultures at 0, 5, 10, 24, and 48h.Values are mean ± SD.*, significant differences from the 0h value within the same treatment, $p<0.05$. small letters differences from the negative control. 1% faecal batch culture inoculated with vessel 1 negative control, vessel 2 GA, vessel 3 synbiotic and vessel 4 is probiotic isolated.

5.4.2 *E. coli* counting

Samples were taken at 0, 5, 10 and 24 h to count the enumerate this bacterial group on selective agar, there were no significant changes however, there were an increase in the numbers throughout the fermentation Table (18). The numbers of *E. coli* were lower than control to some extent at 5 h in the synbiotic and GA vessels.

Table 18: The *E. coli* growth in an *in vitro* batch culture 48h

Substrate	T0	T5	T10	T24
Control	6.54±0.05	9.03±0.62	8.95±0.53	8.77±0.57
GA	6.34±0.15	8.63±0.53	8.81±0.46	9.16±1.08
Synbiotic	6.54±0.11	8.73±0.46	9.35±0.10	10.32±0.94
Probiotic	6.54±0.14	9.14±1.16	9.05±0.48	9.79±0.96

Faecal batch culture (1%) assessing *E. coli* changes following fermentation of GA (gum acacia), *L. rhamnosus* (probiotic) or *L. rhamnosus* and GA (synbiotic) following fermentation at 0, 5, 10 and 24 hours. Values are Log_{10} number of cells/ ml batch culture assessed by culturing on Macconkey agar.

5.4.3 SCFA production

The analysis of SCFA shows that butyrate has been significantly increased within the synbitoic vessel (p=0.03). Acetate was significantly increased in all substrates comparing with baseline (p=0.03, 0.05, 0.00, respectively). Propionate has increased within potential prebiotic and probiotic vessels (p=0.03 for both) as compared with baseline Table (19).

Table 19: SCFA production of the *in vitro* fermentation batch culture 48h

Substrate	Time	Acetate	Propionate	Butyrate
Control	0	1.38±0.52	0.16±0.02	0.12±0.01
	10	7.99±1.27**	2.34±0.34**	1.11±0.79
GA	0	1.54±0.39	0.2±0.06	0.26±0.18
	10	6.25±1.95*	1.73±0.57	1.16±1.74
Synbiotic	0	1.4±0.35	0.17±0.01	0.2±0.1
	10	6.8±1.85*	2.43±0.11*	0.32±0.13*
Probiotic	0	1.34±0.41	0.12±0.00	0.14±0.00
	10	6.51±0.88**	1.65±0.47*	0.38±0.26

*The changes from the control vessel in small letters, the changes from baseline is *. The samples were taken at 0 and 10 hours of faecal batch culture (1%) assessing *E. coli* changes following fermentation of GA (gum acacia), L. rhamnosus (probiotic) or L.rhamnosus and GA (synbiotic). Values are mM concentration.

5.5 Discussion

This study aimed to determine the effect of an isolated probiotic from healthy elderly faecal GA enrichment extract, on the anti-pathogenic properties against *E. coli.* and to test a novel synbiotic by adding GA as selected prebiotic to the probiotic of interest. pH-controlled batch culture fermentation systems were used with elderly gut microbiota. To date, there have been several studies of how pre, pro and synbiotics can shift the gut microbial and immune response in elderly people both *in vitro* and *in vivo* studies [20], [21], [22] however, only

very few study *in vitro* have looked at the effect of probiotic and synbiotic anti-pathogenic effect on elderly people in mixed cultures by isolated probiotics [23], [17].

Prior study (chapter 3) noted the importance of the effect of GA on improving elderly gut microbiota. In this present study, a selective inhibitory effect of GA against the growth of *C. histolyticum* was observed, this is a group of bacteria that have sometimes been associated with inflammation and large bowel disease, thus such a change is potentially of benefit to the host [24, 25]. Moreover, total bacteria, *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides* spp. and *Eubacterium rectale*, increases by GA were not observed. these results were in contrast with our previous work (chapter 2) and *in vivo* studies [26]. In chapter 2 *in vitro* batch cultures GA resulted in significant increases in *Bifidobacterium* spp. and *Lactobacillus* spp. at 10 hours. Therefore this timepoint waas deemed appropriated for monitoring the synbiotic effects. A possible explanation that the beneficial bacterial groups did not significantly increased, for example bifidobacteria was able to utilise GA at the beginning of the fermentation which might explained by suppress the growth of *E. coli* at time 5 h after that a competition of substrates with *E. coli* might occur so bifidobacteria was not able to utilise GA any more while *E. coli* have easier access to metabolise GA while in previous work GA was selectively fermented, another dose to be added could be effective [27]. GA have been previously observed to influence the growth of selected intestinal bacteria such as bifidobacteria and lactobacilli [26] these bacterial groups are able to suppress undesired bacteria such as *E. coli* [13].

Additionally, the numbers of total bacteria, *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides* spp. and *Eubacterium rectale and C. histolyticum* remained constant during the time of fermentation in the presence of the potential probiotic. Nevertheless, the previous work (chapter 4) shows a strong anti-microbial activity against selected pathogens strains (*E.*

coli, *S.* Typhimurium, *E. faecalis*). A possible explanation of the limited effect of probiotic vessel that there was no carbohydrate source to support the probiotic growth, consequently, the effects on the *E. coli* may have been limited.

Moreover, anti-pathogenic effect of lactobacilli strains have been previously observed [28]. Other studies looking at probiotics and their anti-pathogenic effects have observed that lactobacilli isolated from healthy elderly have anti-pathogenic properties against *Clostridium difficile*, enteropathogenic *Escherichia coli* and *Campylobacter jejuni* in solid agar assay [23].

Lactobacilli have been observed to inhibit the binding of *E. coli* with mucosal cells; *E. coli* adherence to mucosal cells is important to induce pathogenisis [29],[30]. Probiotics were expected to inhibit *E. coli* growth yet, the effect was limited. Likewise, lactobacilli have been observed able to modulate the microbial composition and the immune response in elderly people [31], [32]. Tejero-Sariñena *et al* (2013) demonstrated that *in vitro* batch cultures an anti-pathogenic effect was observed when the probiotics were initially able to established while here in this study the aim was to observe the competition between probiotics and *E. coli* in the elderly gut microbiota so they both have been added in the same time to the vessels [28].

The synbiotic treatment in the current study caused a significant increase in the potentially beneficial *Lactobacillus* spp. in relation to the control vessel and a decrease in *C. histolyticum*, which have been assoicated with negative effects on colonic health. The results observed were more potent in the synbiotic vessel, compared to probiotic or prebiotic alone. Prior studies have noted the importance of synbiotic effects on modulation of elderly health. For example, treatment with lactitol and *L. acidophilus*, *B. bifidum*, *B. lactis* and inulin

resulted in increases in *Bifidobacterium* spp. and *Lactobacillus* spp. numbers [33, 34]. Similar observation in a study using synbitoics in a gut model system, Likotrafiti *et al* (2016) demonstrated that in vessel 1 there was inhibition of *E. coli*, however, this did not continue into vessels 2 and 3., It may also might be the case in this study that here a basal medium, with limited nutrients, did not facilitate growth of probiotic [35]. In contrast, Fooks and Gibson (2003) addressed that two synbiotics (*Lactobacillus plantarum* 0407, oligofructose) (*Bifidobacterium bifidum* Bb12, oligofructose and xylo-oligosacchrides) (50:50 w/w) inhibited *E. coli* within *in vitro* batch culture, however, a 1% of starch or FOS were added to the vessels and probiotics established may contributes to the inhibition of pathogens [36].

In vivo and *in vitro* studies have investigated the effect of probiotics on the gut microbiota composition have used a dose of 10^8 or 10^9 probiotics which is similar to this study [37], [20]. The discrepancy in the results of the current study may relate to a diluting effect of the probiotic in the presence of the mixed community of faecal bacteria resulting in a reduced effect on pathogens.

Additionally, In the current study we used a pH appropriate to the distal region of the large intestine. This selection was made because older people often have distal diseases, therefore the use of a pH relevant to this (pH 6.8) was deemed appropriate. Furthermore, in the distal colonic regions fewer sources of carbohydrate are available, thus within this model we can look at the effects of GA in the absense of other substrates. It is noted that the breakdown of prebiotics may occur within more proximal regions, therefore the use of pH 5.5, which may influence the prebiotic effect, would also be appropriate and recommended in future studies [38, 39].Overall, introduction of a synbiotic led to a modulation of bacterial composition, that was more marked than that of the pre and probiotic when used alone – therefore, the enhanced selectivity afforded was observed. Fooks and Gibson (2003) observed, that when

synbiotics were added into an *in vitro* system before a pathogen initial establishment was enabled [36].

In addition, SCFA production was enhanced in GA and probiotics and synbiotic fermentation. In particular, synbiotic gave increased the concentration of butyrate. Increases in butyrate are important as butyrate is as an energy source for the epithelial cells and plays an important role in controlling inflammation and resisting pathogen invasion [40], [41]. therefore, this can be beneficial for elderly gut health.

5.6 Conclusion

In conclusion, it was observed that the synbiotic enhanced the colonic microbiota which highlighted the importance of the combination of pre and probiotic for regulation of the indigenous microbiota to a more beneficial composition. Although no additional benefits were seen in inhibition of *E. coli*. To our knowledge, this is the first time to investigate the effect of a novel synbiotic in mixed culture, that have been challenge tested against pathogenic *E. coli* strains in the batch culture system using faecal samples from elderly volunteers. This emphasises the importance of using mixed cultures to see effects, as this would be more relevant to the *in vivo* situation. Further analysis is needed to study more the different doses of the probiotic potential and develop synbiotic could have more effect on elderly health.

5.7 References

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6. Chapter (6) General discussion

The World Health Organisation reported that the population of those aged 60 years and older is rapidly increasing and over one-third of the UK population will be aged over 65 years by 2050 [1]. Ageing is associated with increases in infections and disease and impaired immunity [2]. There is evidence to link increases in inflammation and gut disorders to the gut microbiota. These changes can be reverse through dietary intervention by modulating the gut microbiota. Pre, pro and synbiotic are therefore potential functional foods that could alter the gut microbiota to enhance the well-being of this population in particular [3], [4]. Therefore, interest in research to find a novel prebiotic recently increased.

The aim of this study was to assess the prebiotic potential of gum acacia (GA) *in vitro* fermentation batch culture then an *ex vivo* approach was used to determine the likely impact on immune function. Moreover, a novel synbiotic was developed using GA to extract an organism that utilises it, whilst offering potential beneficial effects.

Additionally, GA have been used traditionally as food in India and Australia and evidence have been provided that GA have been used in food industry as a stabilizer, thickener and emulsifier, a growth enhancer for probiotic bacteria like other polysaccharides are capable of enhancing viability and enzymes hydrolysis. Moreover, several studies have found that GA can exert several beneficial effects on health. GA has been found to have hypochlesrolemic effect [5]. Also a study found to reduce blood glucose in diabetes patients [6]. Moreover, has anti-obese properties and reduce BMI GA has been investigated in previous data have been focused on other groups such as patients in diabetes, renal failure. Other research has noted anti-diarrhoea properties of GA without measuring the gut microbiota changes. However,

very few studies have been carried out to use GA as prebiotic to enhance the beneficial growth colonic bacteria.

Interest in research using prebiotics in diet as a functional food in this particular group has increased. Prebiotics have been studied in healthy elderly and a positive effect has been observed in terms of modulation of intestinal bacteria and immune function. Moreover, these functional foods can also be applied to other groups such as IBS and IBD patients. The use of different foods as a prebiotics are worth investigate. Other foods such as dates have shown potential prebiotics Eid *et al* (2015) have demonstrated that dates could shift the colonic microbiota in healthy volunteers [7],[8].

The main findings of this thesis include the following:

- GA induced significant increases in *Bifidobacterium* spp. and *Lactobacillus* spp. and increased the production of organic acids in batch culture systems. Furthermore, in a 3-stage *in vitro* continuous culture system *Bifidobacterium* spp. significantly increased upon fermentation of GA, and these changes persisted in the more distal vessels.
- Furthermore, an *ex vivo* PBMC cell assay of these supernatants (from gut model system) led to significant increases in IL-10 an immune anti-inflammatory marker. These changes are potential of benefit to an elderly host, due to changes occurring within their microbiota and immune function.
- Isolated probiotics extracted from GA enrichment culture resulted in anti-pathogenic properties against *Escherichia coli, Salmonella* Typhimurium *and Enterococcus faecalis.*
- A novel synbiotic combination evolved from this work, using GA as the selected fibre for the isolated probiotic. Synbiotic fermentation resulted in significant increases in

Lactobacillus spp and decreases in *Clostridium histolyticum* group – these effects were enhanced as compared to GA and probiotic alone.

- Limited function of GA, probiotic and synbiotic against *E. coli* was observed in *in vitro* batch cultures, with mixed faecal inoculum. This highlight the importance of assessing microbial effects within mixed communities.

Recently, there has been a demand for production of a second generation of a novel prebtioics ingredients Hernandez-Hernandez *et al* (2011) [9]. Such prebioitcs should display greater persistence in the large intestine and have greater selectivity with regards to the intestinal microbiota. Furthermore, an additional biological effect, e.g. by targeting specific physiological functions and or reducing the risk disease such as displacement of pathogens and regulate the function of immune system. In this context GA might represent potential candidates for the second generation prebiotics. Therefore, the current work has *in vitro* models to enhance understanding of GA fermentation and, focussing on an elder population, has determined the cytokine production in PBMC cells from elderly volunteers. GA shows a promising results as it can improve the immune function via increases in IL-10. This is potentially of benefit to an older population who are undergoing increases in inflammatory status.

Additionally, GA have shown to increases in *Firmicutes* which lactobacilli belongs to this phylum have a positive impact and could potentially play an important role in decreasing inflammation and disease in elderly people.

To date, synbiotic studies are of interest, using enrichment culture to isolate probiotics using prebiotics enables a selectivity to be achieved. In the future, this method to develop synbiotic could offer probiotics an enhanced chance of survive in the colon. In the current study the synbiotic results were enhanced when compared to pre-probiotic alone. This shows that the

targeted approach has been successful. However, in the mixed culture environment the effect against *E. coli* was not as potent as in solid agar, therefore, more studies are needed in the mixed cultures, to see the impact of a full consortium of bacteria.

Although the GA, the probiotic and synbiotic did not show antimicrobial effects against *E. coli* in a mixed culture, other potential benefits to the microbial balance were observed. Other studies have isolated probiotics from elderly populations and used them synbiotic combinations, however, the selection of prebiotic was based on randomly screening which synbiotic was more effective, while here in this present study more specific approach to probiotic selection has been used. Previously, studies have isolated probiotics from elderly faeces and tested these in a mixed cultures in order to assess their antimicrobial activity [10], however, in this present study to enhance the viability of the probiotics an enrichment culture was used with GA, furthermore the isolated probiotics had antimicrobial activity, subsequently, a synbiotic combination was in a mixed culture have enhanced the modulation of the gut microbiota.

Further research therefore should concentrate on the investigation of other synbiotics using this technique that developed. These effects are important for maintaining the gut balance in elderly. In terms of the probiotic dose this might be the case in this study therefore, more studies on the dose effect is needed. This study has furthered to our knowledge on the GA metabolite and possesses anti-inflammatory potential. Furthermore, it is utilised by microorganisms that possess possible anti-pathogenic activities leading to a novel synbioitc combination.

Bifidobacteria was observed to increase following GA fermentation in the initial work, this could be due to the bifidobacteia enable to compete with other GA utilisers is reduced. Inverse relationship of increases in *Firmicutes* compared with *Bacteroidetes* phylum

following GA degradation, which enable the beneficial bacteria belongs to the *Firmicutes* to utilise the carbohydrates to saccahrolytic fermentation. The bifidogenic characrestic of GA has been demonstrated in human studies (Calame et al 2008).

These data combined with anti-inflammatory potential shows GA as an appropriate food to study more when considering an elderly population. Further, this work has looked at targeted synbiotics, which could also provide an area for future interesting research. This is especially important for in the clinical setting, were elderly may have altered in their gut microbiota due to changing in diet, medication, inflammation. Prebiotics are commonly added to diet to enhance nutritional status.

6.1 Future direction

GA could be a good candidate as a functional food. Here, in terms of food sector, other food ingredients could potentially be a desirable choice for an elderly group, when compared to using a prebiotic supplement. Gibson (2008) recommended to add prebiotics in beverages and biscuits as convenient food sources. Moreover existing fruits may also be assessed for their prebiotic potential. The results obtained in this thesis show promise in terms of GA impacting on inflammatory status of older volunteers, as such more research into GA using human trials to investigate the potential prebiotic efficacy on the elderly gut microbiota population and to measure cytokines production relevant to the inflammatory status. Furthermore, little is known about the long-term verses the short term effects of prebiotic intervention on the immune function, this would be a useful area of *in vivo* study.

The results of the current study also indicated more potent effects when using a synbiotic, as compared to a prebiotic or probiotic. Looking at the impact of these products side-by-side on immune potential, firstly through *in vitro* approaches, then *in vivo* could highlight the best tools for reducing inflammation in older volunteers.

Moreover, the finding in this project could lead to target the effect of these potential products on the hospitalised elderly patients and whether it has an effect on other pathogens.

Further *in vitro* fermentation in mixed culture is needed to assess the ability of probiotics, pre, synbitoics to modulate the gut microbiota in elderly population and the aim of establishing probiotics with different doses.

Using the novel approach of enrichment culture to isolate probiotics from the candidate prebiotic and screening with *in vitro* approaches provides an effective way of developing a targeted synbiotic product. Furthermore, it could be pertinent to use different sources of potential prebiotics to develop synbiotics that could have stronger characteristic compared to pre or probiotic alone; promising results could then be applied *in vivo*.

6.2 References

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