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Yue Liu, Glenn R Gibson, Gemma E Walton #

Department of Food and Nutritional Sciences

Whiteknights, PO Box 226

University of Reading,

Reading

United Kingdom

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Address correspondence to Gemma E Walton, g.e.walton@reading.ac.uk

Glenn R Gibson, Yue Liu, Gemma E Walton designed the study.

Yue Liu conducted acquisition, analysis and interpretation of data.

Yue Liu drafted the manuscript, Glenn R Gibson, Gemma E Walton conducted critical revision.

Abstract

This study aimed to determine the impact of fat intake combined with *Bacillus coagulans* or *trans*-galactooligosaccharides (B-GOS) on bacterial composition and immune markers in an *in vitro* model. A three-stage continuous gut model system was used to simulate specific human colonic regions. Peripheral blood mononuclear cells were exposed to cell free supernatants and subsequent levels of inflammatory cytokines were measured by flow cytometry. Although fat addition decreased bifidobacteria from 8.76±0.12 to 8.63±0.13 and from 8.83±0.08 to 8.67±0.07 in pre- and probiotic models respectively, the changes were not significant. Fat addition also did not impact on cytokines induced by LPS. Under high fat conditions, numbers of bifidobacteria significantly increased by *B. coagulans* or B-GOS. In addition, *B. coagulans* or B-GOS significantly suppressed TNF-αproduction induced by LPS. Under high fat conditions, either *B. coagulans* or B-GOS led to potentially beneficial effects by targeting specific bacterial groups and modulating immune markers.

Abbreviations

B-GOS, *trans*-galactooligosaccharides; IL-6, interleukin-6; IL-1β, interleukin-1β; TNF-α, tumour necrosis factor-α; NK, natural killer; FISH, fluorescent *in situ* hybridisation; SS, steady state; SCFA, short-chain fatty acid; GC, gas chromatography; PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide

Keywords

Probiotics; Prebiotics; Fat; Microbiota; Immune markers

Chemical compounds studied in this article

Galactooligosaccharides (PubChem CID: 165512).

1. Introduction

It has been reported that between the years 2000 and 2050, the percentage of the population aged 60 and over is likely to double from about 11% to 22% (WHO, 2014). Subsequently, requirements for long-term care, including home nursing, are increasing. Compared to young adults, elderly people suffer from some physical and functional changes. For example, poor chewing and swallowing (Castell, 1988; Karlsson, Persson, & Carlsson, 1991; Remond et al., 2015) can result in reduced dietary choice, poor nutrition, digestion and absorption (Remond et al., 2015). In terms of effects on the gastrointestinal tract, Woodmansey, McMurdo, Macfarlane, and Macfarlane (2004) suggested that less gastrointestinal mobility and longer transit time might result in a higher susceptibility to colon disorders.

In addition, elderly populations typically have increased levels of cytokines associated with inflammation such as interleukin-6 (IL-6), IL-1β, and tumour necrosis factor-α (TNF-α), along with decreased phagocytosis and natural killer (NK) cell activity. These immunological changes are loosely termed immunosenescence (Bruunsgaard, Pedersen, Schroll, Skinhoj, & Pedersen, 2001; Butcher et al., 2001; Goodwin, 1995; Schiffrin, Morley, Donnet-Hughes, & Guigoz, 2010; Vulevic, Drakoularakou, Yaqoob, Tzortzis, & Gibson, 2008).

The combination of age-related changes in the gastrointestinal tract, such as decreased transit time and increased mucosal membrane permeability (Hopkins, Sharp, & Macfarlane, 2002), dietary changes on the microbiota (Candela, Biagi, Turroni, Rampelli, & Brigidi, 2015; Rampelli et al., 2013a; Rampelli et al., 2016; Vulevic et al., 2008). Indeed, studies have shown decreased viable counts of *Bacteroides* in elderly compared to younger adults (Hopkins & Macfarlane, 2002; Woodmansey et al., 2004). A reduction of bifidobacteria in numbers and

species diversity is one of the most notable changes in elderly populations and has been reported in studies using traditional culture methods as well as molecular methods (Claesson et al., 2011; Gavini et al., 2001; Hopkins & Macfarlane, 2002; Hopkins, Sharp, & Macfarlane, 2001; Mitsuoka, 1992; Mueller et al., 2006; Woodmansey et al., 2004). In addition, the age-related microbiota changes in elderly were associated with decreased short-chain fatty acid (SCFA) production, 90-95% of which are acetate, propionate, and butyrate and originate from fermentation of non-digestible carbohydrates (Puddu, Sanguineti, Montecucco, & Viviani, 2014). Elderly individuals were characterised with decreased saccharolytic fermentation and increased proteolytic fermentation compared to younger adults (Rampelli et al., 2013c). These age-related changes in microbiota composition may drive some of the immune response changes and could lead to higher disease risk in elderly compared to younger adults.

According to a recent report (Bates, Lennox, Prentice, Bates, & Swan, 2012), a typical elderly person's daily intake of total fat is 36.1% food energy of which 14.2% is saturated fatty acids, these are higher than the UK Dietary Reference Values, of 35% and 11% respectively. In murine studies, a high fat diet has been shown to have negative effects on microbiota composition, including, for example lower *Bacteroides* group and higher Firmicutes phylum in animal models (Cani et al., 2007a; Cani et al., 2007i; Pyndt Jørgensen et al., 2014; Rampelli et al., 2016). A high fat diet may also impact upon *Clostridium* spp. and *Lactobacillus* spp., which have been observed to decrease following high fat treatments (Biagi et al., 2010; Cani et al., 2007a; Druart et al., 2013). High fat diets are also associated with a negative impact on immune function in murine studies. For example, increased levels of TNF-α, IL-1β and IL-6 in plasma and tissues following a high fat diet have been observed in animal models, subsequently resulting in greater inflammatory status (Cani et al., 2007a; Cani et al., 2007i; Chen, Wang, Li, & Wang, 2011; Kim, Gu, Lee, Joh, & Kim, 2012; Lam et al., 2012; Neyrinck et al., 2011; Park et al., 2013). Because elderly people are consuming high fat diets, they may additionally be

more vulnerable to disease risk. Therefore, the impact of high fat consumption on elderly persons may be relevant.

Several murine studies have shown that prebiotics and probiotics can positively modulate the gut microbial composition and immune markers following a high fat diet (Cani et al., 2007i; Chen et al., 2011; Park et al., 2013). *Trans*-galactooligosaccharides (B-GOS) are promising prebiotics that have been observed to positively improve microbiota composition and immune function in the elderly (Vulevic et al., 2008; Vulevic et al., 2015). The impact of B-GOS following a high fat diet has not been investigated. However, B-GOS was shown to increase the number of faecal bifidobacteria and decrease *C. histolyticum* group in overweight adults (Vulevic, Juric, Tzortzis, & Gibson, 2013). B-GOS may have promising potential to modulate microbiota composition and immune markers in elderly under high fat intakes. *Bacillus coagulans* GBI-30, 6086 (GanedenBC^{30} (BC30)) has the potential to suppress the growth of pathogens (Honda, Gibson, Farmer, Keller, & McCartney, 2011). *B. coagulans* can also modulate the microbiota with significant increases observed in *Bacillus* spp., *C. litsueburense* and *F. prausnitzii* after 28-day supplementation in healthy elderly adults (65 and 80 years) (Nyangale, Farmer, Keller, Chernoff, & Gibson, 2014). In an *in vitro* study, both the cell wall and metabolite fractions of *B. coagulans* induced IL-10 production, thereby demonstrating anti-inflammatory potential. This suggests immune modulating effects of BC30 *B. coagulans* (Jensen, Benson, Carter, & Endres, 2010) which could help to combat both ageing and high-fat diet effects. In addition, the safety of *Bacillus coagulans* GBI-30, 6086 has been confirmed including exerting no mutagenic, clastogenic or genotoxic effects (Endres et al., 2009), harbouring no risk-associated genes and producing no biogenic amine (Salvetti et al., 2016).

Recent studies have provided a good indication about prebiotic and probiotic effects on elderly populations. However, the influence of prebiotic and probiotic (B-GOS and *B. coagulans*) on elderly persons in the presence of high fats has not been well studied. Therefore, the aim of the

current study was to assess the impact of *B. coagulans* and B-GOS on bacterial composition and immune markers in senior individuals consuming a high fat diet using a three-stage gut model.

2. Materials and methods

2.1. Chemicals and bacteria

Unsalted butter (Sainsbury's basics, UK), sunflower oil (Sainsbury's SO Organic, UK) and olive oil (Filippo Berio, UK) were from Sainsbury's supermarket (Reading, UK). Coconut oil was from Holland and Barrett (Reading, UK). B-GOS mixture was from Clasado Ltd (Milton Keynes, UK), the degree of polymerisation ranged from 2 to 5, average molecular weight was 496.8 kDa, and galactooligosaccharide content of B-GOS mixture was 58% (w/w). *Bacillus coagulans*: GBI-30 (PTA-6086, GanedeBC^{30}TM) was sourced from American Type Culture Collection (Manassas, United States) and was used as spores in the current study. All nucleotide probes used for fluorescent *in situ* hybridisation (FISH) were commercially synthesised and labelled with the fluorescent dye Cy3 at the 5′ end (Sigma Aldrich Co. Ltd., Spain). Sterilisation of media and instruments was done by autoclaving at 121 °C for 15 min.

2.2. Three-stage continuous culture system

The physicochemical conditions in the colon were replicated in the three-stage continuous system, as validated by Macfarlane, Macfarlane, and Gibson (1998). It was set up simulating, the proximal, transverse and distal colon, which were present as three connected fermentation vessels (V1, V2, and V3). Faecal samples were donated by three healthy persons aged over 60 years old (2 female and 1 male). Volunteers were not regular consumers of pre or probiotics and had not received antibiotic treatment in the previous three months. A different faecal donor was used for each of the three repetitions. A 20% (w/v) faecal slurry (28.57ml (V1), 33.33 ml (V2), 37.5 ml (V3)) was inoculated into culture medium (51.43 ml (V1), 66.67 ml (V2), 82.5

ml (V3)) and left to equilibrate for 24 hours as a batch culture system. Briefly, culture medium was prepared in distilled water and consisted of (litre^{-1}): 5g starch, 5g peptone water, 5g tryptone, 4.5g yeast extract, 4.5g NaCl, 4.5g KCl, 4g mucin (porcine gastric type III), 3g casein, 2g pectin (citrus), 2g xylan (oatspelt), 2g arabinogalactan (larch wood), 1.5g $NaHCO_3$, 1.25g $MgSO_4.7H_2O$, 1g guar gum, 1g inulin, 0.8g cysteine, 0.5g KH_2PO_4, 0.5g K_2HPO_4, 0.4g bile salts No. 3, 0.15g $CaCl_2.6H_2O$, 0.005g $FeSO_4.7H_2O$, 0.05g hemin, 10µl Vitamin K and 1ml Tween 80. Following this, the medium flow was turned on with a system retention time of 48 hours, culture temperature was 37°C and pH in each vessel was 5.5 (V1), 6.2 (V2), 6.8 (V3), these were maintained using a pH pump (Electrolab, UK) with 0.5M NaOH and HCl solutions as appropriate. An anaerobic environment was maintained by continuous sparging of oxygen-free nitrogen supply (15 mL/min).

During *in vitro* fermentation, the initial steady state (SS1), when equilibrium was reached, was at day 16. This was confirmed by stabilisation of SCFA profiles after three consecutive days. Following SS1, high fat mixture (4.78g), (unsalted butter 2g, coconut oil 0.83g, sunflower oil 1.17g and olive oil 1.33g) was added into vessel 1 daily (Table 1). A second steady state (SS2) was reached after 35 days. SS3 involved daily addition of the same amount of fat mixture as SS2 and either probiotic (*B. coagulans*) or prebiotic B-GOS mixture treatment until equilibrium at 53 days. *B. coagulans* (GanedenBC30) was added into V1 at a dose of 3.3×10^8 cfu daily (Table 1). Another 3 gut systems were fed 2.5g of B-GOS mixture in V1 daily. This B-GOS mixture consisted of the following ingredients: glucose 0.394g; galactose 0.017g; lactose 0.348g; B-GOS 1.063g; protein 0.003g; ash 0.008g and moisture 0.611g, in which the B-GOS content of B-GOS mixture was 58% (w/w). The same amounts of glucose, galactose and lactose were also added to non-prebiotic fed gut models daily (Table 1). Samples (5ml) were collected from each vessel at each steady state over three consecutive days for further analysis.

2.3. Sample preparation

A sample of 375µl (in duplicate) was taken for FISH analysis. This sample was fixed immediately in 4% (w/v) paraformaldehyde solution (1125µl) at 4°C for 4 hours, then centrifuged for 5 minutes at 11337 g (Eppendorf centrifuge minispin, Eppendorf, UK) at room temperature. The supernatant was carefully removed and discarded. The pellet was re-suspended in 1 ml of cold 1×PBS by aspirating carefully using a pipette. Again, the sample was centrifuged for 5 minutes at 11337 g at room temperature and the supernatant discarded. The sample was washed again in 1 ml cold PBS as above and centrifuged. All supernatant was carefully removed. Finally, the pellet was re-suspended in 150 µl cold 1×PBS and 150 µl ethanol. The sample was mixed by vortexing and then stored at -20°C, for FISH based analysis of bacterial counts.

In preparation for SCFA analysis, 1 ml duplicate samples were taken and centrifuged (11337 g, 10 min) at room temperature. The supernatant was stored at -20°C for future analysis.

In preparation for *in vitro* immunoassays, 1 ml of gut model supernatant was sampled in triplicate, centrifuged for 10 minutes at 11337 g at room temperature and then filtered through a 0.22 µm filter device (Millipore, Schwalbach, Germany). The cell-free supernatant was stored at -20°C.

2.4. Bacterial enumeration

FISH analysis involved the use of fluorescently labelled oligonucleotide probes (using Cy3), targeting specific 16S rRNA sequences (Daims, Stoecker, & Wagner, 2005). The probes used in this study were: Ato 291 for *Atopobium* cluster (*Atopobium, Coriobacterium, Collinsella* spp.) (Harmsen et al., 2000), Lab 158 for lactobacilli/enterococci (Harmsen, Elfferich, Schut, & Welling, 1999), Bif 164 for bifidobacteria (Langendijk et al., 1995), Erec 482 for *Eubacterium rectale – Clostridium coccoides* group (Franks et al., 1998), Chis 150 for the *Clostridium histolyticum* group (Franks et al., 1998), Bcoa 191 for *Bacillus* spp. (Sakai & Ezaki,

2006), Bac 303 for *Bacteroides–Prevotella* spp. (Manz, Amann, Ludwig, Vancanneyt, & Schleifer, 1996), and EUB 338 mixture consisting of EUB338, EUB338II and EUB338III for total bacteria (Daims, Bruhl, Amann, Schleifer, & Wagner, 1999). Conditions of hybridisation and washing for individual probes are given in Supplementary 1. Hybridisation of samples was performed as described by Daims et al. (2005).

Briefly, fixed samples were kept on ice and diluted with PBS solution, then 20 µl diluted suspension was evenly placed onto a 5mm diameter well in Teflon- and poly L-lysine-coated slide (Tekdon Inc, Myakka City, FL). These slides were dried for 15 min at 46-50°C and washed in 50%, 80% and 96% (v/v) ethanol solution for 3 minutes respectively, then dried for 2 min. Some Gram-positive bacteria needed lysozyme treatment prior to hybridisation, such as bifidobacteria and lactobacilli/enterococci. 20 µl of lysozyme (1 mg/ml) was therefore added to each well before dehydration in ethanol. Following this, 50µl pre-warmed hybridisation buffer (0.9 M NaCl, 0.02 M Tris/HCl (pH 8.0), (Supplementary 1), 0.05µl 10 % (w/v) sodium dodecyl sulphate, 39.95µl HPLC water and 4.55 ng ml^{-1} probe) were added to each well, and slides placed on a tray, which was sealed and put in a hybridisation oven for 4h at probe specific hybridisation temperature (Supplementary 1). 20 µl nucleic acid stain 4', 6-diamidino-2-phenylindole (DAPI; 50 ng µl-1) was added to the wash buffer before hybridisation finished. Once the hybridisation was complete, slides were placed into wash buffer (0.9 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.005 M ethylenediaminetetraacetic acid (EDTA) solution (pH 8.0, Supplementary 1), H_2O), warmed at the appropriate temperature for each probe (Supplementary 1) for 10-15 minutes. After washing, slides were dipped into ice-cold distilled water for 2-3 seconds and dried by a stream of compressed air. Finally, antifade solution (Dabco) was added to each well, a cover slip applied and slides examined using fluorescence microscopy (Nikon Eclipse E400; Nikon, Tokyo, Japan).

2.5. Organic analysis

SCFAs were determined by gas chromatography (GC) following N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide derivitisation (Richardson, Calder, Stewart, & Smith, 1989). Each sample was vortexed and 1 ml of sample or a standard solution transferred into a labelled 100 mm×16 mm glass tube (Fisher Scientific UK Ltd, Loughborough) with 50 µl of 2-ethylbutyric acid (0.1 M internal standard solution), 500 µl concentrated HCl and 2 ml diethyl ether. All samples were vortexed for 1 min and centrifuged at 2000 g for 10 min at room temperature (SANYO MSE Mistral 3000i, Sanyo Gallenkap PLC, UK) twice (1 ml of diethyl ether added in second extraction), aiming to completely extract the SCFAs. 400 µl of the pooled ether extract and 50 µl N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) were added into a GC screw-cap vial. Samples were heated at 80°C for 20 min and kept at room temperature for 48h to enable further derivatisation.

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 SCFA analysis. Temperature of injector and detector was 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 SCFA external standard was run every 20 samples to update the calibration as necessary. The SCFA external 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. This standard was treated the same as the samples and derivatised with added internal standard. Peak areas of the standard solution, to which internal standard was added, were used to calculate the response factors for each organic acid with respect to the internal standard. Response factor and peak areas within samples were calibrated and calculated using Chemstation B.03.01

(Agilent Technologies, Cheshire, UK). Response factors were calculated using Equation 1. The amount of organic acids with the samples was calculated using Equation 2.

$$Internal\ Response\ Factor = \frac{areaIS \times amountSC}{amountIS \times areaSC}$$

Equation 1 IS=Internal Standard; SC=Specific Compound of Interest

$$Amount\ of\ Specific\ Compound = \frac{amountIS \times areaSC \times IRFSC}{areaIS}$$

Equation 2 IS=Internal Standard; SC=Specific Compound of Interest; IRFSC=Internal Response Factor for Specific Compound of Interest

2.6. Preparation of peripheral blood mononuclear cells

Fasted blood samples were taken from six healthy volunteers aged 60–75 years, 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 University of Reading. Written informed consent forms was obtained from all subjects. Blood was layered over an equal volume of lympholyte (Cedarlane Laboratories Limited, Burlington, Ontario, Canada) and centrifuged at 930 g for 15 min at room temperature. Peripheral blood mononuclear cells (PBMCs) were harvested from the interface, washed once with PBS and then resuspended in Roswell Park Memorial Institute (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. The pellet was resuspended in RPMI 1640 medium and cell numbers counted using a cell counter (Coulter, Fullerton, CA, USA) and trypan blue.

2.7. Viability assays

To determine the appropriate supernatant concentration, PBMC viability, at different supernatant concentrations was determined using the trypan blue test. PBMC, adjusted to 2×10^6 cells/ml, incubated in twenty-four-well plates in the presence of RPMI 1640 medium, pure gut model medium supernatant, SS1V1, SS2V1 and SS3V1 fermentation supernatant from B-GOS and *B. coagulans* treated vessels, separately for 24 h at 37°C in an air–CO_2 (19:1) atmosphere incubator. The tested supernatant amount of each treatment was 1%, 1.5%, 2%, 3%, 4%, 5% and 10% (v/v) of 2ml (final working volume). At the end of the incubation, cell numbers were counted using trypan blue test. According to viability results, only 1% (v/v) was appropriate to use for different treatment supernatants.

2.8. Cytokine stimulation and detection

PBMCs were adjusted to yield 2×10^6 cells/ml. PBMCs were incubated in twenty-four-well plates in the presence of 1 mg/ml lipopolysaccharide (LPS; Sigma Aldrich Co. Ltd., Spain), 1% (v/v) pure gut model medium, 1 mg/ml LPS with 1% (v/v) pure gut medium or 1 mg/ml LPS with 1% (v/v) supernatants from each steady state vessel of B-GOS and *B. coagulans* gut models for 24 h at 37° C in an air–CO_2 (19:1) atmosphere. At the end of the incubation, cell culture supernatants were collected and stored at -20°C for later analysis of cytokine production. Non-stimulated cultures were used as blank.

The production of IL-1β, IL-6, IL-8, IL-10 and TNF-α were measured using BD™ Cytometric Bead Array (CBA) Human Soluble Protein Master Buffer Kit (BD Biosciences, Oxford, UK) and corresponding BD™ Cytometric Bead Array (CBA) Human Flex Set (BD Biosciences, Oxford, UK) by BD Accuri™ C6 flow cytometer according to the manufacturer's instructions. BD™ CBA analysis software FCAP Array v3.0.1 (BD Biosciences, Oxford, UK) was used to perform data analysis.

2.9. Statistical analysis

All statistical tests were performed with the use of SPSS version 18 (SPSS Inc, Chicago, IL). Results are presented as means ± SD.

Changes in specific bacterial groups, SCFA variation and cytokine production were assessed between the three steady states using a one-way ANOVA. Significant differences were assessed by *post hoc* Tukey HSD (Honestly Significant Difference) test. A value of $P < 0.05$ indicates there was a significant difference. In addition, for cytokine production, differences from LPS control values were assessed using an independent t test. Furthermore, independent t test was used to analyse the difference between *B. coagulans* and B-GOS treatments at SS3. A value of $P < 0.05$ was used to indicate a significant difference.

3. Results and Discussion

Previous studies using animal models have found a negative impact of high fat diets on gastrointestinal health; however, the mechanisms are still not clear (de La Serre et al., 2010; Everard et al., 2013; Fava et al., 2013; Kim et al., 2012; Lam et al., 2012). Therefore an *in vitro* gut model combined with an *ex-vivo* approach has been used to investigate the impact of high fat mixture, comparing both prebiotics and probiotics, inoculated with faeces from elderly persons.

3.1. Viability results

After 24h incubation of PBMCs with supernatants, PBMC viability was measured by trypan blue. Viability was 91% with RPMI 1640 medium, 75% with 1% (v/v) pure gut model medium, 70% and 62% with 1% (v/v) SS1V1 and SS2V1 supernatants, 58% with 1% (v/v) *B. cogulans* SS3V1 supernatant, and 60% with 1% (v/v) B-GOS SS3V1 supernatant. Viability at other concentrations (1.5%, 2%, 3%, 4%, 5% and 10% v/v) was lower than 40%. Differences in viability may have an impact on cytokine production; therefore 1% (v/v) supernatant was the

most appropriate choice.

3.2. *In vitro* effect of fat on bacterial composition, SCFA and cytokine production

In the current study, using an *in vitro* approach, high fat supplementation was not observed to lead to significant changes in bacterial composition (Figure 1 and Supplementary 2), cytokines induced by LPS *ex vivo* (Figure 2 and Supplementary 3) or organic acids (Figure 3 and Supplementary 4). The results observed are in contrast to murine studies, where a reduction in *Bacteroides* has been observed (Cani et al., 2007a; Cani et al., 2007i; Mozes, Bujnáková, Sefcíková, & Kmet, 2008; Neyrinck et al., 2011; Patrone et al., 2012). Additionally, a reduction in bifidobacteria following a high fat diet has previously been observed (Cani et al., 2007a; Cani et al., 2007i; Druart et al., 2013), however, these findings are not always reproducible. In the studies, high fat diets had low carbohydrate (Cani et al., 2007a; Cani et al., 2007i; Druart et al., 2013). Therefore, a decrease in bifidobacteria may stem from reduced carbohydrate availability in such approaches. In animal models, a high fat diet has also been observed to upregulate proinflammatory cytokines, such as TNF-α and IL-1β, leading to inflammation and a dysregulated immune response (Cani et al., 2007a; Cani et al., 2007i; Chen et al., 2011; Kim et al., 2012; Lam et al., 2012; Neyrinck et al., 2011; Park et al., 2013). However, there are few studies using human subjects to investigate effects of high fat diets on microbiota composition and immune parameters. In the human study of Fava et al. (2013), subjects were fed a high saturated fat diet for 4 weeks (baseline), and then high monounsaturated fat diets for 24 weeks. High monounsaturated fat diets did not have any significant effect on individual bacterial numbers compared to baseline. Notably, this study recruited subjects who were at increased risk of metabolic syndrome (MS) for a high fat diet treatment. It is suggested that the effect of dietary fat on microbiota composition may be indirectly related to conjugated bile acids (bile salts). With increasing fat intake, hepatic production and release of bile acids from the gallbladder to small intestine, and the amount escaping enterohepatic recycling and entering

into the gut is increased. Some members of gut microbiota can convert 7 α-dehydroxylate primary bile acids into secondary bile acids, which are potentially carcinogenic and related to colon cancer and gastrointestinal diseases (Ou, DeLany, Zhang, Sharma, & O'Keefe, 2012; Ridlon, Kang, & Hylemon, 2006). These bacteria normally represent a small proportion in the gut and consist of species belonging to the genus *Clostridium* (Wells, Williams, Whitehead, Heuman, & Hylemon, 2003). Because, in the current study, only 2g/5L bile salts were added to gut model medium and upper gut secretions are not mimicked, which might explain no significant microbial changes due to fat intake were observed. The combination with other *in vitro* gastric and small intestinal models could provide more valuable insights into the assessment of high fat diet on microbiota changes and immune function in future (Guerra et al., 2012; Payne, Zihler, Chassard, & Lacroix, 2012).

Inflammation induced by a high fat diet may be related to microbiota changes *in vivo*. It has been suggested that alterations in the microbiota could lead to increased gut permeability following a high fat diet by decreasing mRNA expression of tight junction proteins including zona occludens-1 (Cani et al., 2008; Lam et al., 2012). With impaired gut barrier function these would lead to increased LPS translocation to plasma, which in turn leads to inflammation associated with metabolic disorder (Cani et al., 2007a; de La Serre et al., 2010; Kim et al., 2012). Chylomicrons, which transport dietary fat, also have a high affinity with LPS; and then can carry and move LPS from the gut lumen into the circulatory system (Ghoshal, Witta, Zhong, de Villiers, & Eckhardt, 2009). In this study, because high fat mixtures did not change the microbiota composition and their consequent fermentation metabolites, immune markers were not influenced by high fat mixture fermentation supernatants *in vitro*. Results indicate that this high fat does not directly influence microbiota composition or immune response *in vitro*. Therefore a high fat diet did not directly serve as growth substrate for most microbiota.

3.3. *In vitro* effect of B-GOS on bacterial composition

B-GOS is a novel *trans*- galactooligosaccharide mixture, produced by *β*- galactosidase activity of *Bifidobacterium bifidum* NCIMB 41171 on lactose (Tzortzis, Goulas, & Gibson, 2005). According to Figure 1 and Supplementary 2, B-GOS supplementation with high fat at SS3 led to significantly increased numbers of lactobacilli/enterococci ($p<0.01$, ANOVA), bifidobacteria ($p<0.001$, ANOVA), *Eubacterium rectale – Clostridium coccoides* ($p<0.05$, ANOVA), *Bacillus* spp. ($p<0.001$, ANOVA) and total bacteria ($p<0.05$, ANOVA) in three vessels of gut system compared to SS1 and SS2 ($p<0.05$, ANOVA). In addition, growth of *Clostridium histolyticum* group ($p<0.001$) and *Bacteroides–Prevotella* spp. ($p<0.001$, ANOVA) in the three vessels was also significantly inhibited by B-GOS supplementation with high fat at SS3 compared to SS1 and SS2. In the current study, under high fat conditions, B-GOS may confer a positive impact on the aged colon by conferring positive effects on beneficial bacteria at the expense of potentially negative bacteria. Similar results were also found in murine studies using fructo-oligosaccharides (Cani et al., 2007i; Respondek et al., 2013) and inulin (Druart et al., 2013) as prebiotics following a high fat diet. B-GOS has also been shown to confer positive effects on microbiota composition in healthy elderly persons (Vulevic et al., 2008; Vulevic et al., 2015) and overweight adults (Vulevic et al., 2013). The results of these human trials are in agreement with microbial variations in this *in vitro* study.

3.4. *In vitro* effect of *B. coagulans* on bacterial composition

According to Figure 1 and Supplementary 2, *B. coagulans* supplementation with high fat at SS3 significantly stimulated the growth of lactobacilli/enterococci in vessel 2 ($p=0.013$, ANOVA) and *Bacillus* spp. in all three vessels ($p<0.001$, ANOVA) when compared to SS1. *B. coagulans* supplementation also significantly decreased numbers of *Clostridium histolyticum* group in vessels 1 ($p=0.016$, ANOVA) and 3 ($p=0.021$, ANOVA) and numbers of *Bacteroides–Prevotella* spp. in vessel 1 ($p=0.017$, ANOVA) when compared to SS1. *B. coagulans*

385 supplementation significantly stimulated the growth of lactobacilli/enterococci in vessels 1
386 (p=0.025, ANOVA) and 2 (p=0.003, ANOVA) when compared to SS2. Additionally, growth of
387 bifidobacteria (p<0.05, ANOVA), *Eubacterium rectale – Clostridium coccoides* (p<0.05,
388 ANOVA) and *Bacillus* spp. (p<0.001, ANOVA) were stimulated in all 3 vessels. In addition,
389 the growth of *Clostridium histolyticum* group in the 3 vessels was significantly inhibited by *B.*
390 *coagulans* supplementation compared to SS2 (p<0.05, ANOVA).
391 *B. coagulans* could exert positive effects in the aged colon by modulating microbiota
392 composition and organic acid production. Although *B. coagulans* has not been used to
393 modulate the impact caused by high fat in previous studies, *B. coagulans* can modulate the
394 microbiota with a significant increase in *Bacillus* spp., *C. litsueburense* and *F. prausnitzii* after
395 28-day supplementation in healthy elderly persons (Nyangale et al., 2014). In addition,
396 metabolites produced by *B. coagulans* are beneficial to gastrointestinal health via production
397 of nutrients such as organic acids, vitamin K2 and some B vitamins including folate and B12
398 (Jensen et al., 2010).

399 **3.5. *In vitro* effects of B-GOS and *B. coagulans* on SCFA and immune markers**

400 Supernatants from PBMCs cultured without gut model fermentation supernatant were used as
401 controls (+/-). In the absence of LPS, there was no cytokine stimulation, with the exception of
402 pure gut model medium that significantly stimulated production of IL-1β, IL-6, IL-10 and TNF-
403 α compared to blank (Figure 2 and Supplementary 3). Pure gut model medium did not change
404 production of IL-1β, IL-6, IL-8, IL-10 and TNF-α induced by LPS (p<0.05, independent t test).
405 High fat fermentation supernatants had no significant impact on tested cytokines induced by
406 LPS (Figure 2 and Supplementary 3). Under high fat conditions, either B-GOS or *B. coagulans*
407 fermentation supernatants suppressed LPS-induced TNF-α production (p<0.05, independent t
408 test) and enhanced LPS-induced IL-10 production (p<0.05, independent t-test). In vessel 3, B-
409 GOS significantly increased IL-10 compared to high fat fermentation supernatant (p=0.049,

410 ANOVA). In vessels 2 and 3, under high fat condition B-GOS supernatant reduced IL-6
411 ($p<0.05$, independent t test) and IL-1β ($p<0.05$, independent t test) induced by LPS.
412 Under high fat conditions *in vitro*, either B-GOS or *B. coagulans* led to an anti-inflammatory
413 impact by down-regulating proinflammatory cytokines and enhancing anti-inflammatory
414 cytokines induced by LPS. The anti-inflammatory impact of B-GOS or *B. coagulans* have not
415 been tested before in animal models under high fat conditions. However, in a few murine
416 studies, some other prebiotics and probiotics may modulate immune responses by upregulating
417 anti-inflammatory cytokines and down-regulating proinflammatory cytokines (Cani et al.,
418 2007i). This has been the case for fructooligosaccharides, *Bifidobacterium longum* (Chen et al.,
419 2011), *Lactobacillus* spp. (Park et al., 2013).
420 In the current study, the anti-inflammatory impact of prebiotic or probiotics may be related to
421 fermentation metabolites. In either prebiotic or probiotic supplemented *in vitro* gut models,
422 acetate was the predominant fatty acid, followed by butyrate and propionate (Figure 3 and
423 Supplementary 4). Compared to SS1, *B. coagulans* with high fat at SS3 stimulated production
424 of acetate in vessel 3 ($p=0.035$, ANOVA). Compared to SS2, *B. coagulans* significantly
425 stimulated acetate ($p<0.05$, ANOVA) and butyrate ($p<0.05$, ANOVA) in all three vessels.
426 Compared to SS1, B-GOS with high fat at SS3 significantly stimulated the production of
427 acetate in all three vessels ($p<0.01$, ANOVA) and butyrate in vessels 2 and 3 ($p<0.05$, ANOVA).
428 Compared to SS2, B-GOS supplementation significantly stimulated acetate ($p<0.01$, ANOVA)
429 and butyrate ($p<0.01$, ANOVA) in the three vessels.
430 TNF-α production induced by stimuli *in vitro* could be inhibited by SCFAs, especially butyrate
431 and acetate (Liu et al., 2012; Segain et al., 2000; Usami et al., 2008; Vinolo et al., 2011).
432 Butyrate could suppress proinflammatory cytokine production by inhibiting LPS-induced
433 nuclear factor-κB (NF-κB) activation *in vitro* (Liu et al., 2012; Segain et al., 2000; Usami et al.,
434 2008; Vinolo et al., 2011). Acetate could down-regulate proinflammatory cytokine production

by lipoxygenase activation without inhibition of NF-κB (Usami et al., 2008). A variation in TNF-α is in line with SCFA production by B-GOS or *B. coagulans* under high fat conditions. Other fermentation metabolites show an inhibitory impact on TNF-α by blocking NF-κB activation, such as vitamins and unknown fermentation end-products produced by gut microbiota (van Hylckama Vlieg, Veiga, Zhang, Derrien, & Zhao, 2011), although they were not determined in the current study.

IL-10 is an important anti-inflammatory cytokine that could counteract the production of proinflammatory cytokines, such as TNF-α (Cavaglieri et al., 2003; Saemann et al., 2000). In the current study, up-regulation of IL-10 indicated that prebiotics or probiotics could positively modulate the immune response in elderly under high fat conditions, which may be associated with their fermentation end products. A few studies have shown that butyrate and acetate could increase IL-10 production *in vitro* (Cavaglieri et al., 2003; Liu et al., 2012; Segain et al., 2000). IL-6 is mostly considered as a proinflammatory cytokine and proinflammatory activities of IL-6 are mediated by *trans-signaling* (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). The inhibitory effect of B-GOS on IL-6 has been seen previously. In the study of Vulevic et al. (2008), daily intake of 5.5g BiMuno® mixture (2.64g B-GOS) by healthy elderly volunteers was shown to decrease IL-6 production.

In the current study, under high fat conditions, either *B. coagulans* or B-GOS could improve the gut heath and modulate immune markers. However, when comparing *B. coagulans* and B-GOS supplementations at SS3, B-GOS significantly stimulated the growth of lactobacilli/enterococci ($p<0.01$, independent t test), bifidobacteria ($p<0.001$, independent t test) and *Eubacterium rectale – Clostridium coccoides* ($p<0.05$, independent t test) to a greater extent than *B. coagulans* in the three vessels of gut system ($p<0.05$, independent t test); while *B. coagulans* significantly increased numbers of *Bacillus* spp. in vessels 1 and 2 to a greater extent than B-GOS ($p<0.05$, independent t test). In addition, B-GOS led to significantly greater

stimulation of acetate in all three vessels of gut system ($p<0.05$, independent t test). Compared to *B. coagulans* fermentation supernatant, B-GOS under high fat condition had lower levels of IL-6 ($p<0.05$, independent t test). Therefore, the effects of *B. coagulans* could be much enhanced and protected by mixed with prebiotics. The impacts of *B. coagulans* have been shown to be enhanced with the addition of potentially prebiotic β-glucans (Arena et al., 2016) and inulin (Abhari, Shekarforoush, Sajedianfard, Hosseinzadeh, & Nazifi, 2015). The anti-inflammatory impacts can be enhanced by synergistic effects of combination of *B. coagulans* and β-glucans (Arena et al., 2016). Inulin could enhance the survival and growth of *B. coagulans*, with the number of spores significantly higher in synbiotic fed rats compared to probiotic fed (Abhari et al., 2015).

In conclusion, high fat addition did not significantly influence the composition of bacteria and immune responses *in vitro*. However under high fat conditions, either *B. coagulans* or B-GOS may act as health-promoting food supplements to enhance the aged gastrointestinal tract by targeting specific bacterial groups, increasing saccharolytic fermentation and modulating immune markers. Human intervention research would further clarify pre/probiotic effects during high fat diets on elderly gut microbiota and mechanisms of microbial and immune response modulation.

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Table 1 Feeding materials added to three-stage continuous culture daily at stage SS2 and SS3

		Amount (daily)
SS2-high fat treatment	Unsalted butter	2g

	Sunflower oil	1.17g
	Olive oil	1.33g
	Coconut oil	0.83g
SS3-high fat and prebiotic treatment*	glucose	0.394g
	galactose	0.017g
	lactose	0.348g
	B-GOS	1.063g
	Protein	0.003g
	Ash	0.008g
	Moisture	0.661g
SS3-high fat and probiotic treatment*	*B. coagulans*	3.3×10^{8} cfu

699 At stage SS3, same amount of high fat treatment were added to culture daily as SS2. The same
700 amounts of glucose, galactose and lactose were also added to non-prebiotic fed culture daily.

Table 2 Hybridisation and washing conditions for oligonucleotide probes

Probe name	Sequence (5' to 3')	Hybridisation pre-treatment	Formamide (%) in hybridisation buffer	Hybridisation-washing temperature (°C)	Reference
Ato 291	GGTCGGTCTCTCAACCC	Lysozyme	0	50-50	(Harmsen et al., 2000)
Lab 158	GGTATTAGCAYCTGTTTCCA	Lysozyme	0	50-50	(Harmsen et al., 1999)
Bif 164	CATCCGGCATTACCACCC	Lysozyme	0	50-50	(Langendijk et al., 1995)
Erec 482	GCTTCTTAGTCARGTACCG	None	0	50-50	(Franks et al., 1998)
Chis 150	TTATGCGGTATTAATCTYCCTTT	None	0	50-50	(Franks et al., 1998)
Bcoa 191	GCCGCCTTTCCTTTTTCCTCC	Lysozyme	20	46-48	(Sakai & Ezaki, 2006)
Bac 303	CCAATGTGGGGGACCTT	None	0	46-48	(Manz et al., 1996)
EUB338*	GCTGCCTCCCGTAGGAGT	None	35	46-48	(Daims et al., 1999)
EUB338II*	GCAGCCACCCGTAGGTGT	None	35	46-48	(Daims et al., 1999)
EUB338III*	GCTGCCACCCGTAGGTGT	None	35	46-48	(Daims et al., 1999)

***** These probes were used together in equimolar concentrations (all at 50 ng μl^{-1})

Table 3 Bacterial numbers in three different vessels (Vessel 1, Vessel 2, Vessel 3) of *in vitro* colonic models before adding any treatment at steady state (SS) 1, after adding high fat mixture at SS2, and after adding high fat mixture with B-GOS/ *B. coagulans* treatment at SS3.

		B-GOS			*B. coagulans*		
Group		Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3
		Log_{10} bacteria number/ml					
Lab 158	SS1	8.69± 0.10a	8.43± 0.14a	8.11± 0.12a	8.71± 0.06ab	8.38± 0.07a	8.12± 0.11
	SS2	8.56± 0.14a	8.33± 0.09a	8.04± 0.12a	8.57± 0.08a	8.32± 0.02a	8.07± 0.09
	SS3	9.21± 0.08b*	9.03± 0.05b*	8.79± 0.09b*	8.81± 0.06b*	8.58± 0.04b*	8.24± 0.06*
Bif 164	SS1	8.76± 0.12a	8.68± 0.15a	8.51± 0.13a	8.83± 0.08ab	8.72± 0.14ab	8.53± 0.07ab
	SS2	8.63± 0.13a	8.55± 0.09a	8.39± 0.08a	8.67± 0.07a	8.52± 0.09a	8.35± 0.06a
	SS3	9.91± 0.05b*	9.80± 0.04b*	9.69± 0.04b*	9.05± 0.09b*	8.85± 0.06b*	8.67± 0.11b*
Erec 482	SS1	9.38± 0.06a	9.23± 0.07a	9.13± 0.07a	9.43± 0.03ab	9.32± 0.08ab	9.15± 0.05ab
	SS2	9.12± 0.15a	8.91± 0.11a	8.73± 0.05a	9.13± 0.15a	8.90± 0.10a	8.68± 0.06a
	SS3	9.72± 0.06b*	9.57± 0.09b*	9.47± 0.08b*	9.49± 0.05b*	9.22± 0.07b*	9.12± 0.07b*
Chis 150	SS1	7.35± 0.08b	7.20± 0.06b	6.63± 0.07b	7.25± 0.03b	6.99± 0.19ab	6.54± 0.05b
	SS2	7.41± 0.06b	7.35± 0.09b	7.03± 0.15b	7.42± 0.09b	7.36± 0.11b	6.99± 0.11b
	SS3	6.47± 0.09a*	6.35± 0.09a*	6.13± 0.06a	6.91± 0.10a*	6.83± 0.04a*	6.36± 0.10a
Bcoa 191	SS1	7.00± 0.01a	6.71± 0.10a	6.61± 0.08a	7.01± 0.02a	6.77± 0.04a	6.64± 0.09a
	SS2	6.90± 0.02a	6.65± 0.08a	6.55± 0.07a	7.04± 0.04a	6.73± 0.05a	6.58± 0.02a
	SS3	7.43± 0.06b*	7.34± 0.07b*	7.22± 0.09b	7.90± 0.01b*	7.69± 0.12b*	7.34± 0.07b
Bac 303	SS1	9.48± 0.02b	9.13± 0.07b	8.80± 0.01b	9.46± 0.04b	9.12± 0.07	8.77± 0.03
	SS2	9.38± 0.05b	8.86± 0.04b	8.64± 0.08b	9.41± 0.04ab	8.95± 0.06	8.75± 0.11
	SS3	8.79± 0.09a*	8.14± 0.08a*	7.96± 0.13a*	9.08± 0.09a*	8.39± 0.02*	8.28± 0.02*
Ato 291	SS1	8.85± 0.10	8.43± 0.06	8.20± 0.17	8.88± 0.04	8.50± 0.18	8.20± 0.23
	SS2	8.75± 0.10	8.38± 0.08	8.07± 0.18	8.73± 0.10	8.39± 0.15	8.18± 0.17
	SS3	9.01± 0.13	8.74± 0.32	8.48± 0.28	8.86± 0.09	8.50± 0.17	8.23± 0.20
EUB 338	SS1	10.06± 0.03a	10.01± 0.08a	9.82± 0.08a	10.03± 0.06	9.96± 0.05	9.64± 0.08
mixture	SS2	10.00± 0.08a	9.88± 0.04a	9.69± 0.08a	10.04± 0.05	9.98± 0.09	9.60± 0.13
	SS3	10.26± 0.02b	10.18± 0.03b	10.08± 0.05b*	10.20± 0.06	10.13± 0.04	9.77± 0.05*

Values are based on the average of six gut models using faeces from three elderly subjects± SD. Results are calculated based on the means of data from three consecutive days in each vessel and each steady state.

Lab 158 - lactobacilli/enterococci, Bif 164 - bifidobacteria, Erec 482 - *Eubacterium rectale* – *Clostridium coccoides*, Chis 150 - the *Clostridium histolyticum*, Bcoa 191 - *Bacillus* spp., Bac

303 - *Bacteroides–Prevotella* spp., Ato 291 - *Atopobium*, and EUB 338 mixture - total bacteria.

* Significance difference between *B. coagulans* and B-GOS treatment at SS3, $p<0.05$. Significant differences ($p<0.05$) among SS1, SS2 and SS3. in the same vessel are indicated with different letters.

Table 4 Short chain fatty acids concentration analysed by GC for three different vessels (Vessel 1, Vessel 2, Vessel 3) of *in vitro* colonic models before adding any treatment at steady state (SS) 1, after adding high fat mixture at SS2, and after adding high fat mixture with B-GOS/ *B. coagulans* treatment at SS3.

	Acetic acid			Propionic acid			Butyric acid			Iso-butyric acid			Iso-valeric acid		
	vessel 1	vessel 2	vessel 3	vessel 1	vessel 2	vessel 3	vessel 1	vessel 2	vessel 3	vessel 1	vessel 2	vessel 3	vessel 1	vessel 2	vessel 3
B-GOS															
SS1	59.43±	70.42±	74.46±	31.38±	36.99±	39.39±	36.00±	40.76±	43.15±	0.88±	2.77±	3.37±	1.06±	3.04±	3.60±
	5.57a	3.95a	5.50a	2.66b	3.47b	3.32b	4.70ab	4.16a	5.31a	0.34b	0.40b	0.21b	0.37b	0.16b	0.26b
SS2	51.46±	63.78±	68.46±	26.17±	32.38±	35.85±	25.19±	29.83±	31.99±	0.66±	2.58±	2.99±	0.86±	2.42±	3.20±
	5.22a	5.21a	6.03a	2.43ab	2.40ab	3.77ab	6.22a	4.91a	4.71a	0.15ab	0.33ab	0.05ab	0.34ab	0.22b	0.32b
SS3	86.51±	102.35 ±	106.70 ±	18.73±	23.92±	27.37±	48.30±	52.84±	57.96±	0.10±	1.45±	1.74±	0.08±	1.33±	1.93±
	4.44b*	6.75b*	6.32b*	3.16a	2.96a	2.58a	2.31b	2.09b*	3.39b*	0.02a	0.39a	0.42a	0.01a*	0.36a	0.48a
B. coagulans															
SS1	56.76±	65.42±	70.62±	29.05±	34.71±	38.54±	35.78±	40.24±	41.75±	0.78±	3.08±	3.38±	1.25±	3.00±	3.49±
	4.50ab	4.72ab	4.45a	3.21	3.82	4.21	6.18ab	3.78ab	3.32ab	0.22b	0.19b	0.30b	0.22b	0.17b	0.23b
SS2	48.24±	61.52±	65.12±	23.65±	28.74±	30.82±	24.57±	30.33±	32.73±	0.71±	2.78±	3.22±	1.12±	2.87±	3.33±
	3.84a	6.04a	4.88a	2.94	1.50	1.93	4.23a	3.27a	3.94a	0.20ab	0.37b	0.27ab	0.24b	0.10b	0.26b
SS3	64.04±	79.08±	87.58±	27.71±	32.85±	37.69±	40.02±	42.70±	44.44±	0.19±	1.54±	2.45±	0.44±	1.95±	2.14±
	4.31b*	4.99b*	5.72b*	4.67	4.06	5.71	4.28b	2.65b*	3.26b*	0.12a	0.38a	0.30a	0.17a*	0.17a	0.21a

Values are based on the average of six gut models from three elderly subjects and reported as mM ± SD. Results from each gut model are calculated

based on the means of data from three consecutive days in each vessel and each steady state. * Significance difference between *B. coagulans* and B-GOS treatment at SS3, $p<0.05$. Significant differences ($p<0.05$) among SS1, SS2 and SS3 in the same vessel are indicated with different letters.

Figure 1 Effect of fermentation supernatants from three different vessels (Vessel 1, Vessel 2, Vessel 3) of *in vitro* colonic models before adding any treatment at steady state (SS) 1, after adding high fat mixture at SS2, and after adding high fat mixture with B-GOS/ *B. coagulans* treatment at SS3 on microbiota composition. Values are mean ± SD. * Significance difference between *B. coagulans* and B-GOS treatment at SS3, $p<0.05$. Significant differences ($p<0.05$) among SS1, SS2 and SS3 from same treatment are indicated with different letter.

Figure 2 Effect of fermentation supernatants from three different vessels (Vessel 1, Vessel 2, Vessel 3) of *in vitro* colonic models before adding any treatment at steady state (SS) 1, after adding high fat mixture at SS2, and after adding high fat mixture with B-GOS/ *B. coagulans* treatment at SS3 on cytokine production by peripheral blood mononuclear cells (PBMC). Values are mean ± SD. #, significant differences from LPS value $p<0.05$. *, significance difference between *B. coagulans* and B-GOS treatment at SS3, $p<0.05$. Significant differences ($p<0.05$) among SS1 (no fat and treatment), SS2 (high fat mixture addition) and SS3 (high fat mixture with *B. coagulans*/B-GOS treatment) in the same vessel were indicated with different letters. Supernatants from PBMCs cultured without gut model supernatant were used as controls (+/-). In addition, cytokines in non-stimulated PBMC (blank) and in only pure gut model medium-treated PBMC (gut) were also determined. There were significant differences between them in cytokines IL-1β, IL-6, IL-10 and TNF-α (not presented in figures). There was also no significant difference between LPS (LPS-stimulated PBMC) and gut+LPS (PBMC incubated with pure gut model medium and LPS).

Figure 3 Effect of fermentation supernatants from three different vessels (Vessel 1, Vessel

2, Vessel 3) of *in vitro* colonic models before adding any treatment at steady state (SS) 1, after adding high fat mixture at SS2, and after adding high fat mixture with B-GOS/ *B. coagulans* treatment at SS3 on SCFA. Values are mean ± SD. * Significance difference between *B. coagulans* and B-GOS treatment at SS3, $p<0.05$. Significant differences ($p<0.05$) among SS1, SS2 and SS3 from same treatment are indicated with different letter.

Supplementary Figure 2 Effect of fermentation supernatants from three different vessels (Vessel 1, Vessel 2, Vessel 3) of *in vitro* colonic models before adding any treatment at steady state (SS) 1, after adding high fat mixture at SS2, and after adding high fat mixture with B-GOS/ *B. coagulans* treatment at SS3 on microbiota composition. Values are mean ± SD. * Significance difference between *B. coagulans* and B-GOS treatment at SS3, $p<0.05$. Significant differences ($p<0.05$) among SS1, SS2 and SS3 from same treatment are indicated with different letter.

Supplementary Figure 3 Effect of fermentation supernatants from three different vessels (Vessel 1, Vessel 2, Vessel 3) of *in vitro* colonic models before adding any treatment at steady state (SS) 1, after adding high fat mixture at SS2, and after adding high fat mixture with B-GOS/ *B. coagulans* treatment at SS3 on cytokine production by peripheral blood mononuclear cells (PBMC). Values are mean ± SD. #, significant differences from LPS value $p<0.05$. *, significance difference between *B. coagulans* and B-GOS treatment at SS3, $p<0.05$. Significant differences ($p<0.05$) among SS1 (no fat and treatment), SS2 (high fat mixture addition) and SS3 (high fat mixture with *B. coagulans*/B-GOS treatment) in the same vessel were indicated with different letters. Supernatants from PBMCs cultured without gut model supernatant were used as controls (+/-). In addition, cytokines in non-stimulated PBMC (blank)

and in only pure gut model medium-treated PBMC (gut) were also determined. There was also no significant difference between LPS (LPS-stimulated PBMC) and gut+LPS (PBMC incubated with pure gut model medium and LPS).

Supplementary Figure 4 Effect of fermentation supernatants from three different vessels (Vessel 1, Vessel 2, Vessel 3) of *in vitro* colonic models before adding any treatment at steady state (SS) 1, after adding high fat mixture at SS2, and after adding high fat mixture with B-GOS/ *B. coagulans* treatment at SS3 on SCFA. Values are mean ± SD. * Significance difference between *B. coagulans* and B-GOS treatment at SS3, $p<0.05$. Significant differences ($p<0.05$) among SS1, SS2 and SS3 from same treatment are indicated with different letter.