Galacto-oligosaccharides to counter the side effects of antibiotic treatments

Stéphanie Émilie Ladirat

Thesis committee

Promotors
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Professor of Food Chemistry
Wageningen University

Prof. Dr H.A. Schols
Personal chair at the Laboratory of Food Chemistry
Wageningen University

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Thesis

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Stéphanie Émilie Ladirat
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Abstract

Antibiotic treatments are known to disturb the composition and metabolic activity of the human gut microbiota and, therefore, may lead to gut disorders. In this thesis, it was investigated whether and by which mechanisms galacto-oligosaccharides (GOS), a prebiotic known to stimulate the growth of bifidobacteria and to positively influence human health, may counter the negative effects of antibiotics on the microbiota.

First, a high throughput approach combining the *in vitro* fermentation screening platform with a phylogenetic microarray read-outs was shown to be reliable to simultaneously analyse the effects of several often-used antibiotics on the intestinal microbiota. Then, using the same approach, the recovery of the composition and metabolic activity of the microbiota treated with four selected antibiotics upon GOS addition was shown to be antibiotic and dose dependant. The addition of GOS to an amoxicillin (AMX)-treated microbiota was considered successful as, after a decrease of the level of *Bifidobacterium* species, the recovery of mainly *Bifidobacterium longum*, was observed. The growth of bifidobacteria and the production of the beneficial butyrate tended to be higher upon addition of small GOS (dimers-trimers) than upon large GOS in non-treated microbiota (tetramers to hexamers). On the contrary in AMX-treated microbiota, the growth of bifidobacteria and production of butyrate tented to be higher upon addition of large GOS than upon addition of small GOS. The positive results of GOS on AMX-treated microbiota during *in vitro* experiments were evidenced in a double-blind randomized parallel intervention study involving 12 healthy adults.

Overall, the addition of GOS, especially the large oligosaccharides, allowed the recovery of *B. longum* and, subsequently, stimulated the activity of the microbiota through cross-feeding after an AMX treatment.

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Chapter 1

General Introduction

Project outline

In the beginning of the 20th century, public health improved tremendously due to better sanitation, better hygiene and medical discoveries on the treatment of disease, such as penicillin (1928), the first antibiotic. Nowadays, human health is also a reflection of what people eat. The diet can influence the incidence of certain disorders, such as coronary heart diseases, diabetes and obesity. Recently, it has been shown that a number of these health disorders can be related to the composition and metabolic activity of the bacteria present in the gut, the gut microbiota. The gut microbiota, comprising more bacteria than our body has cells, has become in the last decades a key towards human health to be unveiled.

One occasion of disturbance in the gut microbiota is the use of antibiotics. Despite their positive action to treat infectious diseases, antibiotic also alter the composition and metabolic activity of the gut microbiota and may, therefore, lead to gut disorders. On the contrary, prebiotics that are known to stimulate beneficial bacteria are thought to be a means to prevent the side effects of antibiotics on the microbiota. This thesis focuses on understanding when and how prebiotics can help to counter the side effects of antibiotics on the human gut microbiota.

Gut microbiota

The gut comprises the small intestine and the large intestine, also called colon. The small intestine is the first part of the gut, where most of the food compounds are digested and absorbed into the blood. The colon is the last part of the human gastrointestinal tract. Different regions can be distinguished: The ascending colon, the transverse colon, the descending colon, the sigmoid colon and rectum (Figure 1).[1, 2] The main functions of the colon includes the absorption of electrolytes and water, the accumulation and excretion of waste material[3] as well as protective, trophic and metabolic functions through the presence of a bacterial ecosystem, the gut microbiota.[4, 5] The concentration of bacteria in the gut is increasing from the small intestine (10^4-10^7 cells g^{-1}) to the colon (10^{10}-10^{12} cells.g^{-1}) (Figure 1).[2]

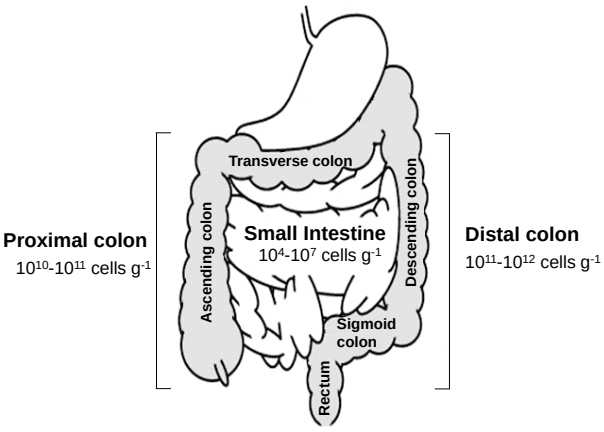

Figure 1: Schematic representation of the human gut and amount of bacteria per gram of intestinal contents (adapted from Payne *et al* [2]).

Microbiota composition

The colonic or gut microbiota is a complex microbial ecosystem, which comprises a large number and variety of bacteria, up to 10^{12} bacteria for every gram of gut content and more than 1000 different species.[2, 6] The microbiota is dominated by anaerobic bacteria, which are usually either saccharolytic or proteolytic.[7] These bacteria mainly belong to two phyla, the *Firmicutes* and *Bacteroidetes,* whereas the *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria, Fusobacteria* and *Cyanobacteria* are present in minor proportions.[8] The main two phyla represent about 90% of the total population. The main phylogenetic cluster belonging to the *Firmicutes* is the class of *Clostridia*, while the main phylogenetic cluster belonging to the *Bacteroidetes* is the genus of *Bacteroides*. Furthermore, the phylum of *Actinobacteria* includes important members of the gut microbiota, such as the beneficial gram positive *Bifidobacterium* genus.[9, 10] *Bifidobacterium* spp. represent 3% to 6% of the total bacterial cell count in adults.[10, 11]

It has been suggested that the human gut microbiota has a dominant core of bacteria.[12, 13] In adults, three clusters, named "enterotypes", have been identified by the variation in the levels of genera and named after the most dominant one: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3).[14] Despite a common core of bacteria, the diversity of the microbiota at species level remains highly individual dependant.[8] For each individual, the microbiota composition is highly resilient and stable over periods of life.[15] The stability is suggested to be due to the functional redundancy of certain groups of bacteria, for instance the *Bacteroides sp.* that show redundancy in their genomes to degrade polysaccharides. [10, 16]

Microbiota metabolic activity

The metabolic activity of the microbiota refers to the degradation and fermentation of the non-digested compounds reaching the colon by the complex microbiota. The main substrates reaching the colon are from dietary origin, including resistant starch, non-starch polysaccharides, non-digestible oligosaccharides,[17] and also minor concentrations of proteins, peptides and amino acids.[4] Due to the diversity of the substrates reaching the colon, a synergy between the bacterial groups is in place to achieve partial or complete degradation of the substrates.[18, 19] Released disaccharides and monosaccharides are further fermented into organic acids (OA), which can be intermediate OA, such as lactate and succinate, and short chain fatty acids (SCFA), such as acetate, butyrate and propionate. Intermediate OA and acetate serve as growth substrate for other bacteria,[18, 20, 21] while SCFA are absorbed into the blood through the portal vein. The SCFA production is strongly dependant on the substrate source, the species and amount of bacteria present and the gut transit.[22] Overall, the different bacterial groups of the gut microbiota appear to work in a coherent and efficient network of cross-feeding species and, therefore, to ensure the functioning of the gut microbiota (Figure 2).[17]

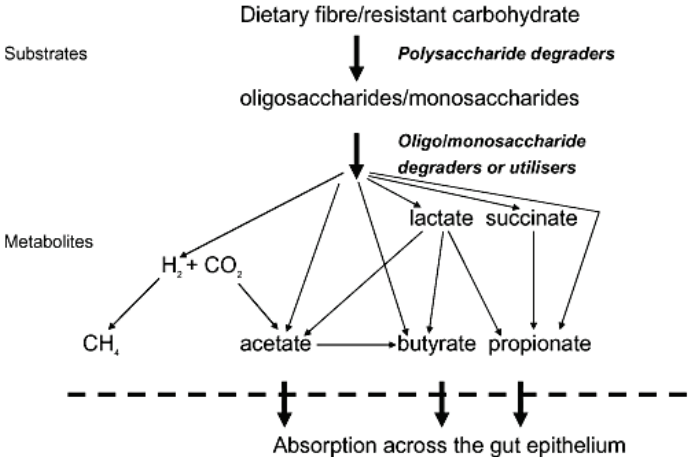

Figure 2: Diagram illustrating the sequential degradation of dietary carbohydrates and the intermediate and final metabolites formed by the gut microbiota (reprinted from Scott *et al*[17]).

Gut microbiota and its relevance to health

The gut microbiota may have an effect on health because of the nature of the bacteria itself. Bacteria have been reported to have either or both a beneficial and a harmful effect on host health (Figure 3)[3]. For example, *Bifidobacterium spp.* are known to have several benefits on health, such as lowering blood cholesterol level, producing vitamins and restoring the normal intestinal microbiota after antibiotic therapy.[23] On the contrary, *Clostridium difficile* is known to induce diarrhoea or severe inflammation of the colon.[24]

Furthermore, compounds resulting from the bacterial fermentation may influence the host's health as well.[3] Saccharolytic fermentation results in end-products that have benefits for the host. Propionate has been shown to inhibit cholesterol synthesis, while butyrate has been proposed to lower the risk of colon cancer and to play a major positive role in the chronic intestinal inflammations.[22] Overall, SCFA decrease the pH in the colon and, therefore, limit pathogen colonisation.[3] On the contrary, proteolytic fermentation results in metabolites, such as ammonomia, phenols, indoles and amines, that are toxic.[22] The proteolytic fermentation mostly takes place in the distal colon, which is a part of the colon reported to be the main site of chronic gut disorders, such as bowel cancer and ulcerative colitis.[4]

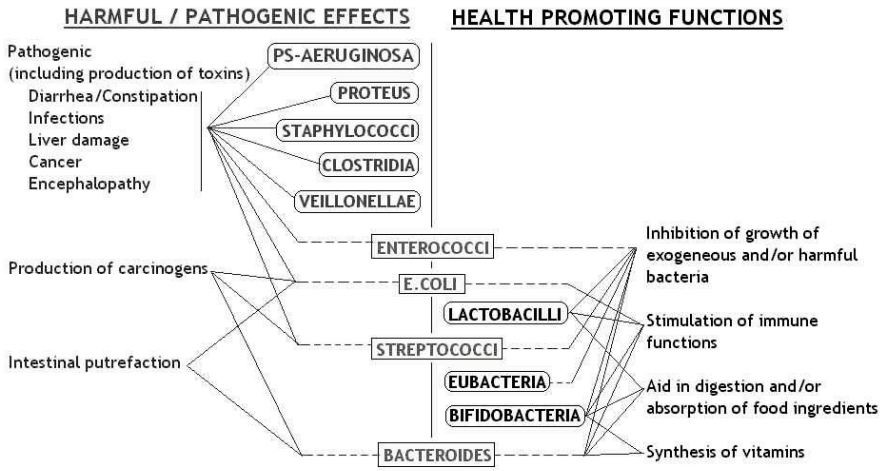

Figure 3: Generalized scheme of the composition and health effects of predominant faecal bacteria (adapted from Gibson *et al*[3]).

Finally, the gut microbiota may have a health impact because it acts as a protective barrier. The microbiota diversity helps to resist to the potential intestinal pathogen colonisation.[4] Possible mechanisms involved in colonisation resistance are e.g. acids production that lower the gut pH, competition for adhesion site and for nutrients, production of toxic metabolites and antagonistic compounds, and enhancement of the immune system.[25] Recent studies have highlighted the fact that an imbalanced gut microbiota is characteristic for many chronic gut-related diseases (e.g. inflammatory bowel disease and colon cancer)[9, 26] and systemic diseases (e.g. allergy, diabetes and obesity).[27, 28] Conversely, certain functional foods reaching the gut microbiota have shown promise in reducing the risk of developing these diseases.[29] Orienting the microbiota composition towards a beneficial microbiota seems to be a key towards human health.

Variation in the microbiota composition

Although the bacterial composition and activity of the human gut microbiota is relatively stable over life-time, age-related changes can be distinguished.[30] Furthermore, the microbiota can vary with factors such as diet and antibiotic therapy.[31]

Influence of age

Bacterial colonisation of the gastrointestinal tract of babies occurs during the delivery process. The first colonisers are facultative anaerobic organisms, such as *Escherichia coli* and streptococci. These bacteria metabolise the oxygen present, thereby reducing the environment into anaerobic conditions.[32] Further colonisation mainly occurs upon feeding. Overall, babies have a lower number of bacteria per gram of faeces and a reduced diversity as compared to adults. One of the main component of this “simple” microbiota is the genus *Bifidobacterium* that represent about 50% of the total bacteria in breast fed babies.[33] The gut microbiota gradually develops during the first year of life upon intake of solid food. The gut microbiota is considered to be mature at the age of 2 years.[34, 35] At adult age, the microbiota is a complex ecosystem, unique to each individual, highly resilient and stable in time.[15, 36] With ageing, the physiology and metabolism of the individual alter, resulting in a shift in the microbiota composition with a decrease in bacterial diversity rather than a decrease in total number of bacteria.[30] These changes are likely the reason for the higher incidence of chronicle gut disorders in elderly.[37]

The relative proportions of the various health beneficial *Bifidobacterium* species also differ with age, and each age group has its characteristic species. Predominant species in breast-fed infants are *Bifidobacterium breve, Bifidobacterium longum* and *Bifidobacterium*

infantis.[32] In adults, *B. longum* and *Bifidobacterium adolescentis* are most often present,[23, 38] while in elderly, the relative proportion of *B. adolescentis* increases considerably.[39]

Influence of antibiotic

Antibiotic treatments are known to disrupt the microbiota and may cause short term side effects, such as Antibiotic-Associated-Diarrhoea (AAD).[40] C*lostridium difficile* infection accounts for nearly one third of the AAD cases. The etiology for the other AAD cases is diverse or unknown.[24] On long term, antibiotic treatments can lead to Inflammatory Bowel Disease (IBD) and Crohn's disease.[41] In infants, the use of antibiotics in the neonatal period may result on a long term impact on the microbiota due to the inadequate gut colonisation and development.[42-44] Gut alterations in early life might even have long term consequences, such as eczema, allergic rhinitis and Inflammatory Bowel Diseases.[28]

Influence of diet and prebiotics

The diet can influence the microbiota composition since the non-digestible parts reach the colon to be fermented by bacteria.[45] A well-known example is the increased diversity of the microbiota of formula-fed babies as compared to breast-fed babies.[32] Modulation of the microbiota through the diet may be of interest in order to achieve a more beneficial intestinal bacterial community. Ingestion of probiotics is a way to increase the concentration of specific beneficial bacteria in the colon. However, the percentage of probiotics that survive the acidic conditions of the stomach and settle in the gut microbiota is rather low. Hence, the use of probiotics is questioned.[3] Another option to specifically stimulate the beneficial bacteria already settled in the host's large intestine is the use of prebiotics. A prebiotic is defined as *"selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits upon host well-being and health"*[46]. Prebiotics are routinely used for their ability to selectively promote bifidobacteria.[47, 48]

In this thesis, we focussed on the changes occurring in adult microbiota due to antibiotics and prebiotics as adults have a mature microbiota, stable within individuals.

Antibiotics

Classification and intake of antibiotics in Europe

Antibiotics are antibacterial agents that are used to treat bacterial infections. The main classes of antibiotics used in Europe are penicillin, tetracycline, quinolone, cephalosporin and macrolide-lincosamide.[49] Antibiotics belonging to these classes can have a specific spectrum of action towards gram positive bacteria or gram negative bacteria, or have a broad spectrum of action. Antibiotics are classified as bactericidal if they kill bacteria and as bacteriostatic if they prevent bacterial growth.[50]

In Europe, the median consumption of antibiotics was 18,97 Defined Daily Doses per 1000 inhabitants per day in 2010. The use of antibiotic differs for out-patient use (ambulatory care) and for hospital use.[49] The distribution of the classes of antibiotic used in the Netherlands in 2009 is shown as example in figure 4.[51]

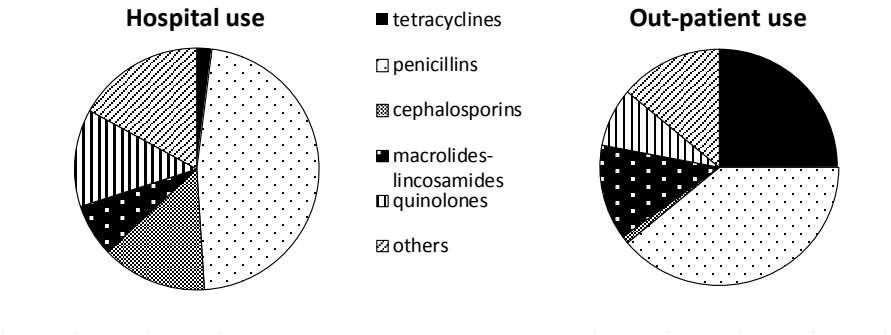

Figure 4: Dutch antibiotic consumption in the Netherlands for hospital and out-patient use (Based on the SWAB/RIVM NethMap report 2009[51]).

The Netherlands is one of the countries where the least antibiotics are prescribed (11.39 Defined Daily Doses per 1000 inhabitants per day in 2009). The type of antibiotic used is, nevertheless, representative for European countries.[51] The most commonly used antibiotics are penicillin, macrolide—lincosamide and quinolone for both out-patient and hospital use. Tetracycline is most often used for out-patient, while cephalosporin is mostly used for hospital use. The use of cephalosporin for out-patient is, however, lower in the Netherlands than in most other European countries. In the Netherlands, the mostly used antibiotics per class are doxycycline for the tetracycline class, amoxicillin for the penicillin class, ciprofloxacin and norfloxacin for the quinolone class and clarithromycin, azithromycin and clindamycin for the macrolide-lincosamide class.[51]

Impact of antibiotics on the gut microbiota

Even though antibiotics are important to treat infectious diseases all over the body, a side effect is that antibiotics kill or inhibit bacteria that are not targeted, especially the ones being part of the gut microbiota.[40, 52] The impact on the non-targeted microbial populations depends on the spectrum of action, the mode of administration (oral vs. intravenous), the dose and the absorption rate in the upper gastrointestinal tract.[53] Antibiotic treatments induce a decrease in the total amount of bacteria[54-56] and/or a shift in the microbiota composition.[57, 58] Antibiotics affect directly certain bacteria, but other species that depend on the secondary metabolites may be affected indirectly as well.[52] The disruption of the microbiota due to antibiotic treatments therefore result in metabolic changes, such as decrease of SCFA and a reduced fermentation of carbohydrates.[59] Even though antibiotics are important tools against infectious diseases, strategies are required to counter the side effects on the gut microbiota.

Resilience of the gut microbiota

After disruption, the microbiota has the ability to return to its initial composition, so called "resilience". Several studies showed that a resilience of the microbiota within a month in healthy adults.[41, 54] Another study, which followed the evolution of the microbiota per individual, also showed a recovery of 89% of the initial microbiota composition in 60 days.[40] One subject, however, still had less than 70% similarity to the initial microbiota composition after 2 months. Long term impact on the microbiota has also been suggested elsewhere.[60] The latter study reported a difference in the level of *Bacteroides* upon a 7-day clindamycin treatment that was still detected after 2 year post-treatment.

Galacto-oligosaccharides as prebiotics

Many food oligosaccharides and polysaccharides are prebiotic candidates, but only inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), lactulose and recently xylo-oligosaccharides have been accepted as prebiotics.[61, 62] In this thesis, the focus is on GOS.

GOS structure

Commercially available GOS are produced by trans-galactosylation of lactose by β-galactosidases from yeast, fungi or bacteria.[63] During the trans-galactosylation process, a complex mixtures of oligosaccharides is produced with different degrees of polymerisation (DP) and glycosidic linkages.[63] The DP varies from 2 to mostly 8. Possible linkages are β(1-2), β(1-3), β(1-4) and β(1-6) and β(1-1). Due to the diversity of glycosidic linkages, several isomeric oligosaccharides are present in each DP fraction[64] (Figure 5).

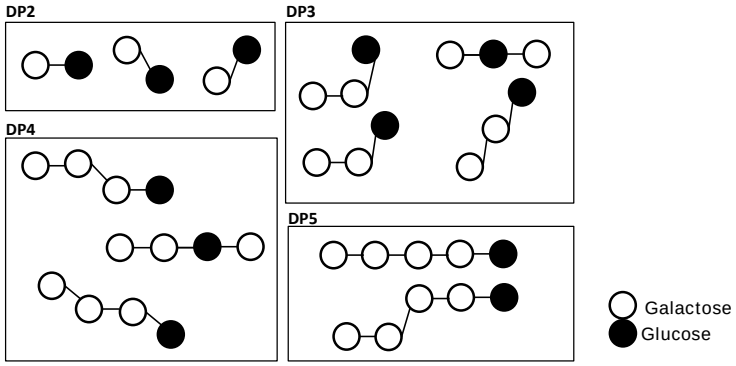

Figure 5: Example of possible GOS isomers per degree of polymerisation (DP).[65]

The enzymes and conditions used during GOS production determine the abundance of the different oligosaccharides in the final product. Enzymes originating from *Aspergillus oryzae* mainly form β-(1-6)-linkages (Oligomate products); enzymes from *Bifidobacterium bifidum* mainly form β-(1-3)-linkages (Bimuno) and enzymes from *Bacillus circulans* and *Cryptococcus laurentii* mainly form β-(1-4)-linkages (Vivinal® GOS and Cup-Oligo)[64, 66]

With advanced analytical tools, all dimers and a number of trimers structures of the Vivinal® GOS (FrieslandCampina Domo) have been identified and quantified (Table 1).[65] The galacto-oligosaccharides present in the mixture are predominantly reducing oligosaccharides, with [β-D-Gal-(1-4)-β-D-Gal-(1-4)]$_n$-D-Glc being the most abundant representatives for oligosaccharides of DP≥3.[65]

Table 1: Structures and relative abundance of DP2 and DP3 compounds in Vivinal® GOS.[65]

DP 2 (37.4 % (w/w) in Vivinal® GOS)	
Compound	**% (w/w) in DP2**
β-D-Gal-(1<->1)-β-D-Glc	7
β-D-Gal-(1->4)-D-Glc (lactose)	27
β-D-Gal-(1->6)-D-Glc (*allo*-lactose)	15
β-D-Gal-(1->4)-D-Fru (lactulose)	5
β-D-Gal-(1->3)-D-Gal	1
β-D-Gal-(1->4)-D-Gal	3
β-D-Gal-(1->3)-D-Glc	26
β-D-Gal-(1->2)-D-Glc	16
DP 3 (22.1 % (w/w) in Vivinal® GOS)	
Compound	**% (w/w) in DP3**
β-D-Gal-(1->4)-β-D-Gal-(1->4)-D-Glc *+* β-D-Gal-(1->4)-β-D-Gal-(1->4)-Fru	45
β-D-Gal-(1->4)-β-D-Gal-(1->6)-D-Glc *or* β-D-Gal-(1->6)-β-D-Gal-(1->4/6)-D-Glc	15
β-D-Gal-(1->4)-β-D-Gal-(1->6)-D-Glc *or* β-D-Gal-(1->6)-β-D-Gal-(1->4/6)-D-Glc	9
β-D-Gal-(1->4)-β-D-Gal-(1->3)-D-Glc	8
β-D-Gal-(1->6)-[β-D-Gal-(1->2]-D-Glc	5
β-D-Gal-(1->4)-β-D-Gal-(1->2)-D-Glc	9
other	9

Gal: galactose, Glc: glucose, Fru: Fructose

The typical structure of β-glycosidic linkages between the saccharide units prevents GOS from being hydrolysed by the human digestive enzymes secreted in the upper gastrointestinal tract.[67] GOS are, therefore, non-digestive oligosaccharides and reach the colon where they can be fermented by the gut microbiota and reveal their prebiotic properties.

Prebiotic application

GOS is added to products mostly for their health promoting properties. The main market for the health promoting properties of GOS is in infant milk formula and infant food.[68] The most known health promoting effect of GOS is its bifidogenic property. GOS was proven during *in vitro* fermentations and *in vivo* trials to be a selective substrate for modulating specifically bifidobacteria within the gut microbiota.[69, 70] A recent study using a ^{13}C-labelling technique unambiguously showed that *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium catenulatum, Lactobacillus gasseri* and *Lactobacillus salivarius*

specifically fermented ^{13}C-GOS.[71] The bifidogenic effect was reported to be observed between 5g to 15g per day.[48, 61] An excess of GOS consumption (estimated to >20g/human body) might result in transient osmotic diarrhoea.[66] Besides specific stimulation of bifidobacteria and to a lesser extent lactobacilli, GOS fermentation is reported to reduce the growth of pathogens[69, 72] and the adherence of pathogens to tissue cells.[73] *In vivo* studies reported other health related impacts in humans, such as enhance calcium absorption and protection against gut infection.[61, 74, 75]

Prebiotics to counter the side effects of antibiotics

The use of prebiotics, alone or in combination with probiotics, to prevent from antibiotic-associated gut diseases or to recover from an antibiotic treatment has already been suggested.[31, 61, 76] However, only a few studies have been performed and the results are contradictory in nature regarding the efficacy to limit the risk of antibiotic-associated diarrhoea and the impact on the microbiota composition (Table 2). Intake of a synbiotic (FOS + *B. longum* or *L. acidophilus*) during a treatment of cefpodoxime proxetil successfully limited the decrease of lactobacilli and prevented the growth of *Clostridium difficile*.[77] Also, supplementation of FOS during and after an various antibiotic treatments (30 days) resulted in an increase of bifidobacteria and in a reduced risk of relapse of *C. difficile*-associated-diarrhea.[78] In contradiction to these positive results, addition of Inulin, FOS and GOS during an *in vitro* fermentation using clindamycin treatment were shown to induce a loss of bifidobacteria and a loss of colonisation resistance against *C. difficile.*[79] Also, FOS supplementation (12g/day) during and after various antibiotic treatments did not help to prevent AAD in elderly even though the number of bifidobacteria remained stable in the supplemented groups while it dropped in the placebo group.[80] At last, non-conclusive results have also been reported: No change in the diarrhoea frequency was reported in infants treated with amoxicillin and receiving FOS and Inulin (70:30 w/w ratio), although a promising increase of bifidobacteria was reported.[56] Discrepancies in literature (Table 2) regarding the effect of prebiotic addition on antibiotic-treated microbiota might be explained by the variability in study parameters among the studies. Parameters, such as type of prebiotics and antibiotics, dosages and age of subjects are known to have a high impact on the microbiota composition.[81]

Table 2: Overview of studies involving prebiotics to prevent/recover from antibiotic-associated diarrhoea.

AIM	Outcome	Antibiotic		Prebiotic		
& Reference	**regarding aim**	**Type + dosage**	**Duration**	**Type + dosage**	**Duration**	**Target**
Decrease diarrhoea frequency[56]	Failure	Amoxicillin (50mg/kg/day)	7 days	FOS:Inulin (70:30) (2.25 g/d)	3 weeks after AT	Infants *In vivo*
Prevention of AAD[80]	Failure	Broad spectrum	Varies	FOS (12g/d)	During AT + 1 week after	>65y *In vivo*
Prevention of relapse of CDAD[78]	Success	Not applicable	Not applicable	FOS (12g/d)	30 days after AT	Ill subjects *In vivo*
Prevention of CDAD[77]	Success	Cefpodoxime proxetil (200mg/day)	7 days	*B. longum*/ *L. acidophilus* + FOS (15g/d)	During AT + 2 weeks after	Healthy adults *In vivo*
Prevention of CDAD[77]	Failure	Cefpodoxime proxetil (200mg/day)	7 days	FOS (15g/d)	During AT + 2 weeks after	Healthy adults *In vivo*
Colonisation resistance against *C.difficile*[79]	Failure	Clindamycin (20µg/ml)	48h	FOS GOS Inulin (10g/L)	During AT	Healthy adults inoculum *In vitro*

AAD: antibiotic-associated diarrhoea, CDAD: *Clostridium difficile* associated diarrhoea, AT: Antibiotic treatment, GOS: Galacto-oligosaccharides, FOS: Fructo-oligosaccharides

Fermentation models

Investigating the human microbiota composition and activity requires digesta from different locations of the colon. The access to the colon is, however, limited for practical and ethical reasons. Several model systems have, therefore, been developed to study the microbiota.[1] *In vitro* models range from simple batch system to pH-controlled multistage continuous culture systems and use pure cultures, defined mixed-cultures and/or faecal material as inoculum. Main limitations are the overgrowth of non-representative microbial population over fermentation time and the difficulty to reproduce the dynamic conditions of the colon, such as absorption of compounds and interactions with the host cells.[9] Nevertheless, *in vitro* models are often used as they are relatively inexpensive and high-throughput. Furthermore, *in vitro* models allow studying the microbial activity, such as dietary component fermentation and SCFA production in time, and give a first indication of the impact on the microbiota composition.[2, 82]

Because the *in vitro* models do not allow to mimic the colon conditions, the effects studied in *in vitro* models need to be confirmed in *in vivo* models.[1] Animal models are of great value to investigate the effect of controlled diet or a specific compound, such as drugs and toxins, on the microbiota and subsequently its host. The main limitation is, however, that the intestinal tracts of animals differ from that of humans. Human studies using healthy volunteers, patients or sudden death victims remain the best model to study the microbiota in relation to the host health.[1]

Diagnostic tools to describe the gut microbiota

The most commonly used techniques with their advantages and limitations are described below and summarized in table 3. Bacteria from the gut microbiota were first studied by plate count analysis. Culturing on specific media in combination with phenotypic characteristics enables identification of some bacteria. However, this method is time consuming and around 80% of the bacteria are not cultivable.[9, 83] New methods based on nucleic-acid-based analysis have been developed. They provide evidence that bacteria numbers and diversity are underestimated by the plate count method. These molecular approaches focus on 16S ribosomial RNA (rRNA) gene as target molecule. This sequence is common to bacteria, while it presents variation depending on the species or groups. Therefore, specific primers can be designed on the basis of variable regions of this sequence to detect specific species or larger groups of bacteria.[84-86] These new

technologies give opportunity to elucidate the complexity of the microbiota ecosystem by providing powerful tools able to study diversity and dynamic of the microbiota.[9] Sequencing techniques (cloning and sequencing of SSU rRNA, and pyrosequencing) provide the most detailed phylogenetic information. However, these techniques require extensive bioinformatic analyses and are costly.[87] Fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE), are often used to compare microbial communities and monitor their dynamics. The main drawbacks of these methods are the poor detection of low abundant bacteria and a difficult quantification of the detected bacteria due to the comigration of many phylotypes, especially in case of complex ecosystem as the gut microbiota.[87] More suitable methods for quantification are fluorescence in situ hybridisation (FISH) and quantitative polymerase chain reaction (qPCR). They can be used to target either large or specific bacterial groups. One limitation of the techniques is the specificity of the primers used and the limitation to detect only known bacterial groups.[87]

All these above approaches are relatively low throughput. In contrast, phylogenetic microarray microarrays allow high-throughput analyses of thousands of microbes and provide insights on the microbiota composition in relation to gut health and disease.[9] The limitations of phylogenetic microarray analyses, as for other culture-independent technologies, depend on the isolation of nucleic acids and subsequent polymerase chain reaction (PCR) amplification of SSU rRNA genes. Another limitation of the phylogenetic microarrays is that no link between microbiota diversity and functionality can be established.[9] New tools, including stable isotope probing and meta'omics approaches, have been developed to address knowledge gaps on gut microbiota composition and have started to reveal core metabolic functions of the gut microbiota. These tools remain, however, costly and required laborious data analysis.[9, 87]

Regarding the aim this thesis, we have chosen to screen the changes in the microbiota composition using a high-throughput tool, namely the phylogenetic microarray, and to confirm changes in most influenced group using a quantitative tool, namely the qPCR.

Table 3: Advantages and limitations of the diagnostic tools to describe the microbiota.[9,13]

Diagnostic tools		Advantages	Limitations
Bacteria counting		Viable cultures are counted	Low throughput Low percentage of cultivable bacteria
Molecular methods	Cloning and sequencing of 16S rDNA gene	Detailed phylogenetic information	Clone library required Low throughput, Costly
	PCR-DGGE	Profile of bacterial diversity	Difficult quantification, Co-migrating phylotypes Bias due to PCR amplification, High detection thresholds
	Fluorescence *in situ* hybridization (FISH)	Results *in situ* Quantitative	Low throughput Detection limited to known phylotypes Time consuming data analysis
	Quantitative PCR (qPCR)	Specific bacteria targeted Quantitative	Bias due to PCR amplification Detection limited to known phylotypes No difference between viable and dead bacteria
	DNA microarrays	High-throughput Diversity and abundance measured Link gut microbiota and health or disease	Semi-quantitative Bias due to PCR amplification Detection limited to known phylotypes
	Pyrosequencing	Detailed phylogenetic information No cloning bias introduced High-throughput	Extensive bioinformatic analysis required Costly
	Stable isotope probing (SIP)	Microbiota diversity and activity *in situ*	Incorporation of the label Costly
	Meta'omic approaches	Microbial composition and pathway : link diversity to functionality	Extensive bioinformatic analysis required Costly

Thesis outline

As stated above, only a limited number of studies have been performed to investigate the effect of prebiotics on antibiotic-treated microbiota and results were not consistent. Therefore, the aim of this thesis was to understand whether and by which mechanisms the prebiotic GOS could help to counter the side effects of antibiotics on the human gut microbiota. We hypothesised that the recovery of the microbiota upon GOS addition will be antibiotic dependant since antibiotics have different spectrum of actions. Furthermore, the prebiotic GOS will help to recover the balance of the human microbiota by stimulating bifidobacteria, and, subsequently, stimulating other bacterial groups through cross-feeding.

In **chapter 2**, a new high-throughput approach is introduced to monitor the impact of antibiotics on the human microbiota: *In vitro* fermentations were run using a screening-platform and the microbiota composition was determined using a phylogenetic microarray. The reliability of the method was first verified and, subsequently, an overview of the effects of seven antibiotics on adult intestinal microbiota was obtained. Four antibiotics were selected based on their mode of action, classification and specific impact on the microbiota.

In **chapter 3**, the impact of addition of the prebiotic GOS on the adult gut microbiota treated with the 4 selected antibiotics was investigated *in vitro* on both the microbiota composition and activity. The results were compared using the high-throughput approach described in chapter 2.

A specific combination of prebiotic-antibiotic, GOS-Amoxicillin, was further studied in chapters 4 and 5. In **chapter 4**, GOS was fractionated based on the size of the oligosaccharides and each fraction was *in vitro* fermented using amoxicillin-treated microbiota. The contribution of each fraction to the recovery of the microbiota composition and activity was investigated. In **chapter 5**, the combination of GOS-Amoxicillin was studied in a human study, including 12 healthy adults. This preliminary trial investigated whether results obtained *in vitro* so far could also be observed *in vivo*.

In **chapter 6**, the results obtained in the research are discussed and their impact on future research on gastro-intestinal health after antibiotic treatment is reflected.

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Chapter 2

High-throughput analysis of the impact of antibiotics on the human intestinal microbiota composition

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Abstract

Antibiotic treatments can lead to a disruption of the human microbiota. In this *in vitro* study, the impact of antibiotics on adult intestinal microbiota was monitored in a new high-throughput approach: a fermentation screening-platform was coupled with a phylogenetic microarray analysis (Intestinal-chip). Faecal inoculum from healthy adults was exposed in a fermentation screening-platform to seven widely-used antibiotics during 24h *in vitro* fermentation and the microbiota composition was subsequently determined with the Intestinal-chip. Phylogenetic microarray analysis was first verified to be reliable with respect to variations in the total number of bacteria and presence of dead (or inactive) cells. Intestinal-chip analysis was then used to identify and compare shifts in the intestinal microbial composition after exposure to low and high dose (1 $\mu g.ml^{-1}$ and 10 $\mu g.ml^{-1}$) antibiotics. Observed shifts on family, genus and species level were both antibiotic and dose dependent. Stronger changes in microbiota composition were observed with higher doses. Shifts mainly concerned the bacterial groups *Bacteroides, Bifidobacterium, Clostridium, Enterobacteriaceae,* and *Lactobacillus*. Within bacterial groups, specific antibiotics were shown to differentially impact related species. The combination of the *in vitro* fermentation screening platform with the phylogenetic microarray read-outs has shown to be reliable to simultaneously analyse the effects of several antibiotics on intestinal microbiota.

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Introduction

The human intestinal microbiota, a complex ecosystem mainly dominated by anaerobic bacteria, plays an important role in the health of its host.[1] Under normal conditions, the composition of the microbiota is relatively stable for long periods of time,[2] but this can change due to external factors, such as antibiotic treatments.

Antibiotics are used to treat specific bacterial infections. However, these agents are also known to kill or inhibit bacteria which are not primarily targeted, such as resident commensal gut microbiota.[3] The extent of the impact on the non-targeted microbial populations depends on the spectrum of action, the mode of administration (oral vs. intravenous), the dose and the absorption rate.[4] A disruption of the microbiota due to antibiotic treatments can lead in 5-35% of the cases to Antibiotic-Associated-Diarrhoea (AAD), with Amoxicillin and Clindamycin having the highest impact.[5]

Increasingly, changes in the microbiota composition are correlated with health disorders.[6-8] Such correlations between microbiota composition and health disorders can be detected from *in vivo* studies. These studies, however, are rather expensive and have a low-throughput. Moreover, comparison of the outcomes of different studies is difficult since many parameters in the study designs differ. In this perspective, *in vitro* screening-platforms are considered useful tools to perform multiple fermentations in a high-throughput. Furthermore, to establish correlations between microbiota composition and human gut diseases, it has been suggested to use new high-throughput analytical tools like phylogenetic microarrays.[8] Such phylogenetic microarray have been developed and validated for e.g. oral and intestinal microbiota.[9, 10] Combining the use of *in vitro* screening-platforms with intestinal microarray analysis appear to be promising to increase fermentation-throughput and compare straightforwardly the resulting bacterial fingerprints obtained under similar conditions.

Phylogenetic DNA microarrays enable quick determination of microbiota composition. However, the DNA array read-outs might be influenced by factors induced by the antibiotic treatment, such as variation in the total cell number[11] and presence of nucleic acids derived from dead (or inhibited) cells.[12] Reliability of the microarray read-outs still has to be tested on complex human microbiota in case of antibiotic treatments. In this study, we addressed the use of a phylogenetic microarray to monitor the changes in the human intestinal microbiota after antibiotic treatment during 24h *in vitro* fermentation in screening-platforms. First, the reliability of the I-chip readouts was addressed with respect to the variation in cell numbers and presence of dead cells. Secondly, an overview of the

impact of seven widely-used antibiotics on the microbiota composition was shown under comparable conditions.

Material and methods

Antibiotics

Amoxicillin (AMX) (≥90%), Azithromycin (AZM) (≥98% HPLC), Cefadroxil (CFR), Ciprofloxacin (CIP) (≥98% HPLC), Clindamycin hydrochloride (CLI) (≤2 mol/mol EtOH), Doxycycline (DOX) (≥98% TLC), and Erythromycin (ERY) (≥85%) were obtained from Sigma-Aldrich (St Louis, MO, USA). Their characteristics are presented in table 1.

Table 1: Characteristics of antibiotics used including class, type, mode of action and impact on antibiotic-associated-diarrhoea.

Class	Name	Type	Mode of action	AAD[a] frequency
Penicillin	Amoxicillin	Bacteriolytic	Inhibition peptidoglycan biosynthesis[3]	High[5]
Tetracycline	Doxycycline	Bacteriostatic	Translation inhibition[3]	Low[5]
Macrolide-Lincosamide	Erythromycin	Bacteriostatic	Translation inhibition[3]	Low[13]
	Azithromycin	Bacteriostatic	Translation inhibition[3]	Low[13]
	Clindamycin	Bacteriostatic	Translation inhibition[3]	High[5]
Quinolone	Ciprofloxacin	Bacteriolytic	Replication and transcription inhibition[3]	Low[5]
Cephalosporin	Cefadroxil	Bacteriolytic	Inhibition of peptidoglycan biosynthesis[14]	Medium / High[5]

[a] AAD: Antibiotic-Associated-Diarrhoea

Microarray construction and validation of the intestinal microbiota representing microarray

The intestinal microbiota representing microarray was constructed as described for the oral microbiota microarray by Crielaard *et al*.[9] Instead of primers for oral bacterial species, primers for intestinal bacterial species were selected based on scientific literature, sequence databases and 454 sequencing of faecal material, resulting in a DNA based microarray enabling the detection of more than 400 bacterial targets from the human large intestinal microbiota. The selected targets included primers that are specific at family, genus and species level. Several groups of bacteria, e.g. *Bacteroides, Bifidobacterium, Enterobacteriaceae, Clostridium and Lactobacillus* are targeted. These groups are known to be the main bacterial groups in the human intestinal microbiota.[15] The intestinal microarray (I-chip) performance was validated for the same criteria as mentioned by Crielaard *et al*.[9]

Experimental set up and sampling

Intestinal microbiota were cultured by *in vitro* fermentations in microtiterplate (96 wells - 1.5 ml volume per well). The culture medium was based on the modified standard ileal efflux medium (SIEM) composition[16] and modified as follow ($g.l^{-1}$): pectin (0.047), xylan (0.047), arabinogalactan (0.047), amylopectin (0.047), starch (0.392), casein (24.0), Tween 80 (17.0), bactopepton (24.0), ox-bile (0.4) and cysteine (0.2). All medium components were provided by Tritium Microbiology (Veldhoven, The Netherlands). The pH was adjusted to 5.8.

A standardized pool of adult faecal inoculum was prepared as validated by Minekus *et al*.[16] This pool approach was especially relevant in our study since it limited inter-individual variations and increased the probability to have a larger representation of potential bacterial species in the human colon. The faecal samples used to produce the standardized inoculum were from eight healthy European adults (25-45 years old) who neither received antibiotic treatments in the 2 months before donation nor consumed prebiotics or probiotics the week before donation. After storage at -80°C in 12% glycerol, the standardized faecal inoculum was incubated in the adapted SIEM under anaerobic conditions overnight (37°C; 300 rpm) in order to activate the bacteria. This pre-culture step was found not to significantly modify the microbiota composition and activity as determined by I-chip analysis (data not shown).

For antibiotic exposure experiments, SIEM, antibiotics (1 µg.ml^{-1} or 10 µg.ml$^{-1)}$ and pre-cultured inoculum (0.1% v/v) were mixed in each well. The fermentation was conducted under anaerobic conditions at 37° for 24h. Inoculated SIEM without antibiotic was used as a blank. Each specific fermentation condition was performed 5 times. After 24h of fermentation, collected samples were split in three parts. One part was directly stored at -20°C for DNA isolation. A second part was immediately treated with propidium monoazide (PMA) (Biotium, Hayward, CA, USA) as described by Nocker *et al*[12] with a final concentration of 50 µM, and stored at -20°C. The third part was directly stored at -80°C for RNA isolation.

DNA isolation

Total faecal DNA from collected samples was isolated as described by Crielaard *et al* [9] with some minor adjustments: The samples were initially mixed with 250 µl lysis buffer (Agowa, Berlin, Germany), 250 µl zirconium beads (0.1 mm), and 200 µl phenol, before being introduced to a BeadBeater (BioSpec Products, Bartlesville, OK, USA) for twice 2 min.

Faecal RNA isolation and cDNA synthesis.

Next to DNA isolation, RNA isolation was necessary to investigate the activity of bacteria present in the samples. Isolation of RNA and cDNA synthesis was carried out for one replicate out of 5. RNA isolation through bead beating in phenol/chloroform extractions was performed following the protocol described by Kort *et al*.[17] Isolated RNA was purified from DNA using the Invitrogen Kit (Invitrogen, Breda, The Netherlands) with a modified buffer (100 mM Tris/HCl pH7.5, 25 mM $MgCl_2$, 2.5 mM $CaCl_2$). RNA purity and concentration were determined on a 1.5% (w/v) agarose gel stained with ethidium-bromide (Sigma-Aldrich).

DNA copies (cDNA) were synthetized by incubating 2.5 µl RNA for 5 min at 60°C with 10 µl anneal mix containing 5 µl dNTP's [2mM] (Invitrogen), 0.1 µl RNAsin (Promega, Leiden, The Netherlands), 23 nl 1061-R primer (TCA CGR CAC GAG CTG ACG AC), 0.15µl 0,1 M DTT (Invitrogen) and RNAse free water. After cooling the samples on ice, 8 µl RT mix containing 1 µl RNAsin (10 U.µl^{-1}, Promega), 4 µl First Strand Buffer 5× (Invitrogen), 2 µl 0,1 M DTT (Invitrogen) and 0.5 µl Superscript II enzyme (200 U.µl^{-1}, Invitrogen) were added. The reverse transcription took place at 42°C during 2h. Samples were inactivated at 70°C for 10 min and stored at -20°C. cDNA was amplified and labelled with PCR as described further.

PCR amplification and PCR product purification

Significant amounts of DNA or cDNA are needed for analysis on the microarray. A multiplex PCR was therefore performed on each sample (DNAs and cDNAs) with a 25 µl reaction mixture containing 12.5 µl 2x Multiplex PCR mix (Qiagen GmbH, Hilden, Germany), 0.5 µl of 16s-8-F/unibifi [25-2.5 pmol.µl^{-1}] (AGA GTT TGA TCH TGG YTC AG / TGG CTC AGG ATG AAC GCT G), 1 µl 16s-1061-R [25 pmol.µl^{-1}] (TCA CGR CAC GAG CTG ACG AC), 0.25 µl Entero(Hsp60)-F- [25 pmol.µl^{-1}] (GGT AGA AGA AGG CGT GGT TGC), 0.5 µl Entero(Hsp60)-R- [25 pmol.µl^{-1}] (ATG CAT TCG GTG GTG ATC ATC AG), 5 µl of isolated DNA and 5,25 µl milli-Q water. The forward primers contained a 5'phospho modification while the reverse primers contained a 5'–C6 Cy3 modification. Only 16s-8-F/unibifi and 16s-1061-R were used to amplify cDNAs. The program used for amplification was as follows : 94°C for 15 min, 30 cycles of 94°C for 30 seconds, 50°C for 90 seconds, 72°C for 80 seconds, 1 cycle of 72°C for 2 minutes and cooled to 4°C. The PCR products were analysed on a 1.2% agarose gel (100V; 45 min) and stained with Serva-G (SERVA Electrophoresis, Heidelberg, Germany).

The PCR products from DNA were purified using a SigmaSpin Post-Reaction Clean-up plate (Sigma-Aldrich) while the PCR products from cDNA were purified using autoseq G50 columns (GE Healthcare), as described by the manufacturers. The samples obtained were dried by vacuum centrifugation at 60°C. A mixture of 0.5 µl lambda exonuclease (BioLabs inc. Frankfurt, Germany), 2 µL lambda exonuclease buffer and 17.5 µL water was added. Incubation took place for 30 minutes at 37°C, and inactivation during 10 minutes at 75°C. DNA was purified again with a SigmaSpin-2-Post-Reaction Clean-up plate (Sigma-Aldrich) and dried. The single-stranded products were analysed on a 1.2% agarose gel (100V; 45 min) and stained with Serva-G (SERVA Electrophoresis).

Hybridization

Hybridization of the PCR products on the I-chip was performed as described by Crielaard *et al*[9] with minor changes. Dried single-strand DNA was suspended in 45 µl DIG Easyhyb (Roche, Basel, Switzerland) for 20 min at 37°C and denatured for 2 min at 95°C before being placed on the pre-warmed microarray. Hybridization, cleaning steps and scanning were carried out according to the described procedure.

Data analysis

Imagene 5.6 software (BioDiscovery, Marina del Rey, CA, USA) was used to analyse the results. Signals were quantified by calculating the mean of all pixel values of each spot and calculating the local background around each spot. For each spot a signal to background ratio (S/B), namely signal intensity, was calculated and used for further analysis. Only the spots with a S/B ratio larger than two were used for further analysis. This cut-off was selected based on the observation that negative control spots never resulted in signals above this cut-off (data not shown). When comparing data from all experiments, the minimal number of observations higher than three times above its local background for each spot was set to 10. This criterion was mainly used to discard data resulting from technical noise. The data matrix (116 targets out of 400) was analysed with Significant Analysis Microarray (SAM) to identify markers significantly different between predefined groups programs (TM4 software) and with a hierarchical clustering based on Euclidian distances. A Principal Component Analysis (PCA) was performed using the TM4 software to investigate the correlation among the bacterial fingerprints.[18]

Quantitative PCR

Quantitative PCR was performed to investigate the variation in the total amount of bacteria after different antibiotic exposures. Quantification of the total amount of DNA present in the samples was performed using the universal primers 16S-uni-II-F [10 pmol.µl^{-1}] (TCCTACGGGAGGCAGCAGT) and 16S-uni-II-R [10 pmol.µl^{-1}] (GGACTACCAGGGTATCTA ATCCTGTT), and probe 16S-uni-II [5 µM] (6FAM-CGTATTACCGCGGCTGCTGGCAC-TAMRA) (Applied Biosystems, Bleiswijk, The Netherlands) The amplification was performed with 5 µl DNA sample and 25 µl q-PCR mixture that contained 15 µl 2x FastStart Universal Probe Mastermix (Roche, Mannheim, Germany), 1 µl of each primers and probe, and 7 µl MilliQ water. Total microbial faecal DNAs were diluted 1:10 before use in the q-PCR assay.

The experiment was performed using the 7500 Fast Real Time PCR system (Applied Biosystems) at the following settings: 1 step of 2 min at 50°C, 1 step of 10 min at 95°C, 40 cycles of 15 seconds at 95°C and 1 min at 60°C. Dilution of the control microbiota was used as quantitative standards (5 fg.µl^{-1} to 5 ng.µl^{-1}).

Corrections of the I-chip readout

To address the reliability of the I-chip readouts with respect to the variation in cell numbers, the signal intensity (S/B) obtained directly from the I-chip needed to be corrected based on the variation of total cells in the samples. According to literature, the relative signal intensity of each target [1] (S/B of one target : total S/B) is directly proportional to the relative quantitative changes of the target.[10] Because of this correlation, a correction factor [2] was calculated in our experiment on the basis of the total number of bacteria measured with qPCR (total number of bacteria in one sample: total number of bacteria in the control) and applied to the relative signal intensity of each target to calculate the corrected relative signal [3]:

$$\text{Corrected relative signal intensity [3]} = \text{Relative signal intensity [1]} \times \text{Correction factor [2]}$$

$$[3] = \frac{\text{Signal intensity of one target}}{\text{Total signal intensity}} \times \frac{\text{Total number of bacteria in one sample}}{\text{Total number of bacteria in the control}}$$

This correction factor enabled comparison of the samples in absolute abundance per target.

Statistical analysis

To evaluate whether two qPCR data sets were significantly different, a Student t-test was performed. P-values were calculated assuming equal variance and two-tailed distribution. Correlations were considered significant at a P-value lower than 0,001.

Results and discussion

In order to investigate the impact of antibiotics on a healthy adult intestinal microbiota, *in vitro* fermentations were performed using a screening-platform, allowing up to 96 experimental variations at once in 1.5ml volumes. Downscaling the fermentation did not influence the outcomes regarding the impact of antibiotics on the microbiota as similar results were observed in fermentation flasks of 120ml (data not shown).

Seven antibiotics widely-used in The Netherlands[19] and in Europe[20] were selected based on their classification and their mode of action (Table 1). A low dose (1 $\mu g.ml^{-1}$) and a high dose (10 $\mu g.ml^{-1}$) antibiotic were selected on the basis of a dose-series test performed on

the screening platform. The low dose influenced either gram positive or gram negative bacteria, while the high dose mostly influenced both gram positive and gram negative bacteria (data not shown). This high dose, however, did not suppress all bacteria allowing a recovery of the non-affected bacteria during the fermentation time. The actual antibiotic concentrations used in the experiments were below the concentrations that could reach the colon considering an adult receiving 0.5 g to 1 g antibiotic per day and an absorption rate of 70 % to 90% (25 $\mu g.ml^{-1}$ to 150 $\mu g.ml^{-1}$). The two selected doses were, however, in the range of MIC-values of currently used antibiotics[21] and relevant for *in vitro* fermentations with 0.1% faecal inoculum.

After 24h fermentation, the changes in the microbiota composition due to 1 $\mu g.ml^{-1}$ and 10 $\mu g.ml^{-1}$ antibiotic treatments were monitored with a phylogenetic microarray, the I-chip (Figure 1). The reproducibility of the impact of antibiotics (n=5), as determined by Pearson's correlation (r), was good (r=0.8) to very good (r=0.98). The level of impact of the antibiotics was shown by a principal component analysis (Figure 2). For the 1 $\mu g.ml^{-1}$ treatments, data for cefadroxil, clindamycin and erythromycin predominantly clustered with the non-treated samples, indicating minor changes in the bacterial fingerprints. Data for amoxicillin, azithromycin, ciprofloxacin and doxycycline clustered separately from the non-treated samples, hence a stronger impact on the microbiota was present. For the 10 $\mu g.ml^{-1}$ treatments, 8 separate clusters could be distinguished, with each antibiotic treatment resulting in a separate cluster and all antibiotics clearly differing from the control situation. These results thus show the strong impact of these antibiotics on the microbiota composition.

A more detailed description of the impact of each individual antibiotic on microbiota composition is presented later, but first the influence of a potential experimental bias due to variations in total bacterial cell numbers and the influence of nucleic acids derived from dead (or inhibited) cells present in the antibiotic treated samples are addressed.

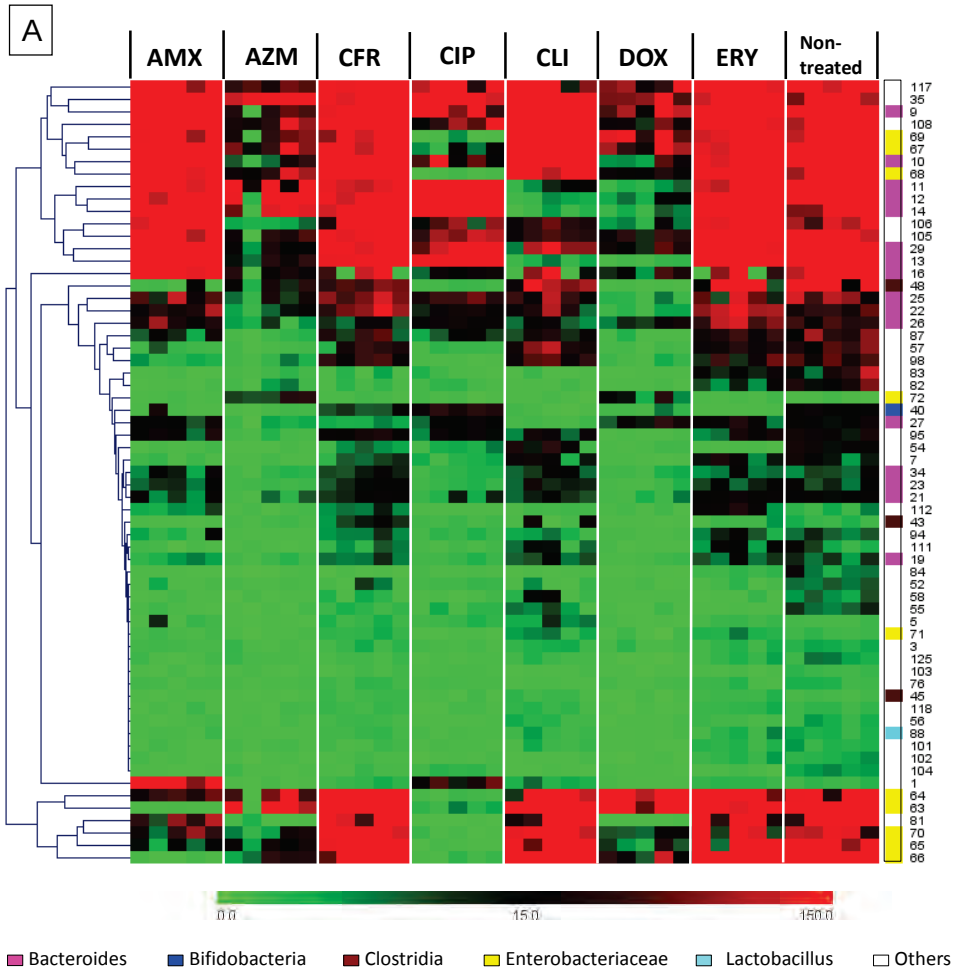

Figure 1: Bacterial fingerprints of the non-treated and antibiotic-treated adult inoculum obtained with the I-Chip after 24h *in vitro* fermentation. Seven antibiotics were used in concentrations of 1 µg.ml^{-1} (A.) and 10 µg.ml^{-1} (B).Targets presented in the figures have intensities which were found to be significantly different among sets of samples (sets based on treatment) by SAM analysis (TM4 software). Main groups of bacteria are highlighted in colour and full name of targets can be found in table 2 based on their numbering. Signal compared to the background (S/B): Green: below detectable level, Black: medium abundance, Red: high abundance.

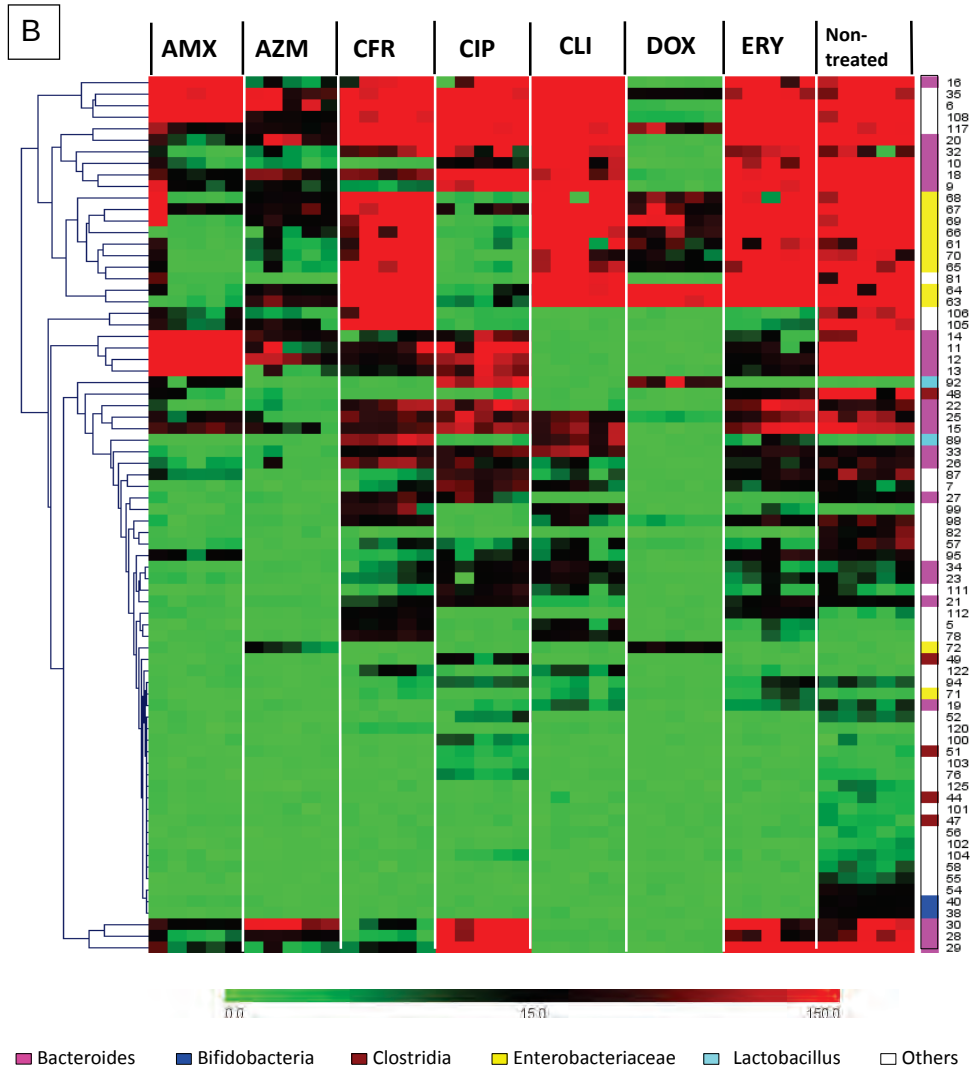

Figure 1 (continued): Bacterial fingerprints of the non-treated and antibiotic-treated adult inoculum obtained with the I-Chip after 24h *in vitro* fermentation. Seven antibiotics were used in concentrations of 1 µg.ml^{-1} (A.) and 10 µg.ml^{-1} (B).Targets presented in the figures have intensities which were found to be significantly different among sets of samples (sets based on treatment) by SAM analysis (TM4 software). Main groups of bacteria are highlighted in colour and full name of targets can be found in table 2 based on their numbering. Signal compared to the background (S/B): Green: below detectable level, Black: medium abundance, Red: high abundance.

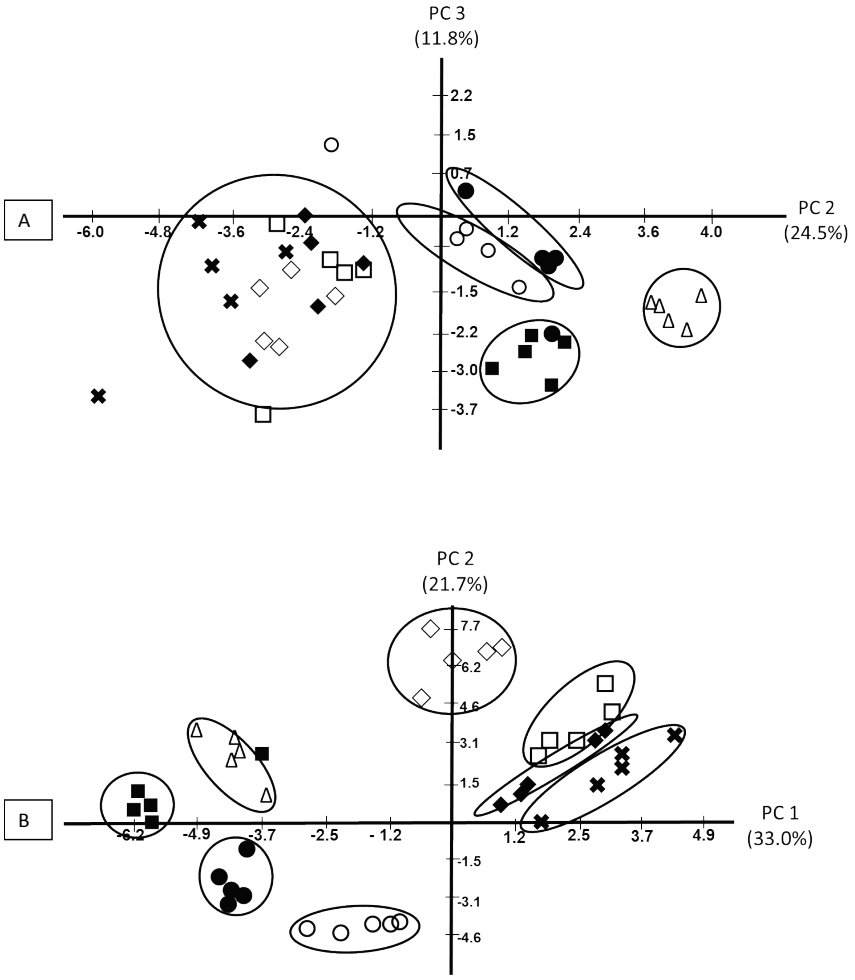

Figure 2: Representation of the non-treated or antibiotic-treated samples in the plane defined by two principal components resulting from a PCA of bacterial fingerprints obtained with the I-Chip after 24h *in vitro* fermentation using adult inoculum. Seven antibiotics were used in a concentration of 1 µg.ml^{-1} (A) or 10 µg.ml^{-1} (B) : AMX (■), AZM (●), CFR (◆), CIP (Δ), CLI (✖), DOX (○), ERY (□), non-treated (◊).

Reliability of the I-chip readouts when using antibiotics

Influence of the variations in total cell numbers

In order to investigate the influence of variations in total bacterial cell numbers on the I-chip readout, the total number of bacteria after 24h fermentation with antibiotics was determined with qPCR. Figure 3 shows that the total number of cells was significantly reduced in case of 10 µg.ml^{-1} of amoxicillin, ciprofloxacin and doxycycline ($p < 0{,}001$). In these cases, relative comparisons between I-chip readouts from different treated-samples might lead to misinterpretations and, as a result, conclusions about changes in abundance might be incorrect.

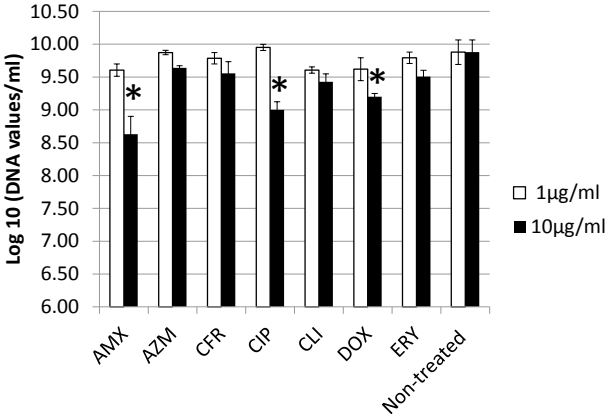

Figure 3: Total amount of DNA in non-treated and antibiotic-treated inoculum from healthy adults after 24h *in vitro* fermentation measured with qPCR. Antibiotics were applied in concentration of 1 µg.ml^{-1} □ or 10 µg.ml^{-1} ■. Standard deviation (n=5) is shown with the error bars. ***** Significant difference versus the non-treated microbiota ($p < 0.001$).

Table 2 deals with the hypothesis that relative values from a microarray might not be comparable in case of antibiotic treatment as the total number of bacteria may differ per sample. Based on the qPCR measurements of the total bacteria numbers, the signal intensity (S/B) obtained directly from the I-chip was corrected as described in the material and methods section. Treatment with 10 µg.ml^{-1} doxycycline was taken as an example. The three obtained readouts (S/B, relative S/B [1] and corrected relative S/B [3]) showed similar trends of changes in the microbiota composition. Therefore, modification of the microarray data is concluded not to be necessary. Similar conclusions were drawn for the 10 µg.ml^{-1} amoxicillin and ciprofloxacin treatments (data not shown).

Table 2: Impact of doxycycline (10 µg.ml-1) after 24h *in vitro* fermentation on bacterial groups of an adult inoculum as evaluated with the signal intensity, the relative signal intensity and the corrected relative intensity. Formula [1], [2] and [3] are described in the material and method section.

				Signal to Background (S/B)		Relative S/B [1]		Corrected relative S/B [3]*	
	Family	Genus	species	Average	StDev	Average	StDev	Average	StDev
1	Alcaligenaceae	unclassified_		1.2	0.3	0.1	0.0	0.1	0.0
2	Archaea_domain			2.9	0.6	0.2	0.0	0.2	0.0
3	Archea			2.2	0.4	0.2	0.0	0.2	0.0
4	Archea			1.4	0.2	0.1	0.1	0.1	0.0
5	Bacillaceae	Bacillus	group	1.2	0.2	0.1	0.0	0.1	0.0
6	Bacteriales_order			2.3	0.6	0.2	0.0	0.2	0.0
7	Bacteriales_order			1.3	0.2	0.1	0.0	0.1	0.0
8	Bacteriales_order			1.2	0.1	0.1	0.0	0.1	0.0
9	Bacteroidaceae	Bacteroides	dorei	1.6	0.5	0.1	0.0	0.1	0.0
10	Bacteroidaceae	Bacteroides	dorei	1.1	0.2	0.1	0.0	0.1	0.0
11	Bacteroidaceae	Bacteroides	fragilis	1.2	0.2	0.1	0.0	0.1	0.0
12	Bacteroidaceae	Bacteroides	fragilis	1.1	0.2	0.1	0.0	0.1	0.0
13	Bacteroidaceae	Bacteroides	fragilis	1.4	0.3	0.1	0.0	0.1	0.0
14	Bacteroidaceae	Bacteroides	fragilis/uncultured	1.1	0.1	0.1	0.0	0.1	0.0
15	Bacteroidaceae	Bacteroides	group	1.1	0.2	0.1	0.0	0.1	0.0
16	Bacteroidaceae	Bacteroides	group	1.3	0.2	0.1	0.0	0.1	0.0
17	Bacteroidaceae	Bacteroides	massiliensis	1.3	0.2	0.1	0.0	0.1	0.0
18	Bacteroidaceae	Bacteroides	ovatus	2.3	0.7	0.2	0.0	0.2	0.0
19	Bacteroidaceae	Bacteroides	stercoris	1.1	0.1	0.1	0.0	0.1	0.0
20	Bacteroidaceae	Bacteroides	thetaiotaomicron	1.6	0.2	0.1	0.0	0.1	0.0
21	Bacteroidaceae	Bacteroides	thetaiotaomicron	1.4	0.2	0.1	0.1	0.1	0.0
22	Bacteroidaceae	Bacteroides	thetaiotaomicron	1.2	0.2	0.1	0.0	0.1	0.0
23	Bacteroidaceae	Bacteroides	thetaiotaomicron	1.3	0.2	0.1	0.0	0.1	0.0
24	Bacteroidaceae	Bacteroides	uncult	1.2	0.3	0.1	0.0	0.1	0.0
25	Bacteroidaceae	Bacteroides	uncultured	4.0	0.9	0.3	0.1	0.3	0.1
26	Bacteroidaceae	Bacteroides	uncultured	1.1	0.1	0.1	0.0	0.1	0.0
27	Bacteroidaceae	Bacteroides	uncultured	1.2	0.0	0.1	0.0	0.1	0.0
28	Bacteroidaceae	Bacteroides	uniformis	1.4	0.3	0.1	0.0	0.1	0.0
29	Bacteroidaceae	Bacteroides	uniformis	1.2	0.2	0.1	0.0	0.1	0.0
30	Bacteroidaceae	Bacteroides	uniformis	1.3	0.2	0.1	0.0	0.1	0.0
31	Bacteroidaceae	Bacteroides	uniformis	1.4	0.3	0.1	0.0	0.1	0.0
32	Bacteroidaceae	Bacteroides	xylanisolvens	1.4	0.4	0.1	0.1	0.1	0.1
33	Bacteroidaceae	Bacteroides	xylanisolvens/ finegoldii	1.2	0.1	0.1	0.0	0.1	0.0
34	Bacteroidaceae	Bacteroides	xylanisolvens/finegoldii	1.1	0.2	0.1	0.0	0.1	0.0
35	Bacteroidetes_phylum			25.5	3.5	2.2	0.5	2.1	0.4
36	Bifidobacteriaceae	Bifidobacterium	catenulatum / angulatum/	1.2	0.1	0.1	0.0	0.1	0.0
37	Bifidobacteriaceae	Bifidobacterium	gallinarum	3.9	6.1	0.3	0.5	0.3	0.4
38	Bifidobacteriaceae	Bifidobacterium	longum	1.3	0.2	0.1	0.0	0.1	0.0
39	Bifidobacteriaceae	Bifidobacterium	longum	1.1	0.1	0.1	0.0	0.1	0.0
40	Bifidobacteriaceae	Bifidobacterium	species	1.2	0.2	0.1	0.0	0.1	0.0
41	Carnobacteriaceae	Carnobacterium	divergens	1.1	0.1	0.1	0.0	0.1	0.0
42	class "Clostridia"			1.2	0.2	0.1	0.0	0.1	0.0
43	Clostridiaceae	Clostridium	carnis	1.3	0.5	0.1	0.1	0.1	0.1
44	Clostridiaceae	Clostridium	carnis/ tertium/ sardiniense/ vincentii	1.2	0.1	0.1	0.0	0.1	0.0
45	Clostridiaceae	Clostridium	disporicum	1.2	0.1	0.1	0.0	0.1	0.0
46	Clostridiaceae	Clostridium	disporicum	1.1	0.1	0.1	0.0	0.1	0.0
47	Clostridiaceae	Clostridium	group	1.3	0.3	0.1	0.0	0.1	0.0
48	Clostridiaceae	Clostridium	group (7)	1.3	0.5	0.1	0.0	0.1	0.0
49	Clostridiaceae	Clostridium	group 2	1.2	0.1	0.1	0.0	0.1	0.0
50	Clostridiaceae	Clostridium	paraputrificum	1.5	0.2	0.1	0.1	0.1	0.1
51	Clostridiaceae	Clostridium	uncultured	1.1	0.1	0.1	0.0	0.1	0.0
52	Clostridiales_order			1.2	0.4	0.1	0.0	0.1	0.0
53	Clostridiales_order			1.2	0.0	0.1	0.0	0.1	0.0
54	Coriobacteriaceae	Collinsella	aerofaciens	1.3	0.4	0.1	0.0	0.1	0.0
55	Coriobacteriaceae	Collinsella	aerofaciens	1.2	0.3	0.1	0.0	0.1	0.0
56	Coriobacteriaceae	Olsenella	profusa	1.2	0.2	0.1	0.0	0.1	0.0
57	Coriobacteriaceae			2.1	0.5	0.2	0.0	0.2	0.0
58	Coriobacteriaceae			1.1	0.1	0.1	0.0	0.1	0.0
59	Desulfovibrionaceae	Desulfovibrio		1.2	0.2	0.1	0.0	0.1	0.0

*** Correction factor [2] = 0.91**

below detectable level	<2	<0,1 %	<0,1 %
low abundance	2< <4	0,1% < < 0,2%	0,1% < < 0,2%
medium abundance	4 < < 50	0,2% < < 4%	0,2% < < 4%
high abundance	>50	>4%	>4%

Table 2 (continued)

				Signal to Background (S/B)		Relative S/B [1]		Corrected relative S/B [3]*	
	Family	**Genus**	**species**	**Average**	**StDev**	**Average**	**StDev**	**Average**	**StDev**
60	Enterobacteriaceae	Cronobacter	sakazakii/ turicensis	1.3	0.2	0.1	0.0	0.1	0.0
61	Enterobacteriaceae	E.coli/ Shigella		48.9	28.9	3.6	1.9	3.4	1.8
62	Enterobacteriaceae	Enterobacter	cloacae/ asburiae	1.1	0.2	0.1	0.0	0.1	0.0
63	Enterobacteriaceae	Escherichia /shigella	E.coli/shigella	245.0	13.3	18.9	2.4	18.1	2.7
64	Enterobacteriaceae	Escheriachia /shigella	E.coli/shigella	246.9	13.8	21.5	5.4	20.1	4.6
65	Enterobacteriaceae	Escheriachia /shigella		21.9	6.1	1.8	0.3	1.7	0.3
66	Enterobacteriaceae	Escherichia /shigella	E.coli/ Shigella	49.2	9.4	4.6	1.7	4.3	1.5
67	Enterobacteriaceae	Escherichia /shigella	E.coli/shigella	48.3	24.5	4.0	1.2	3.7	1.2
68	Enterobacteriaceae			65.6	24.7	5.9	3.2	5.5	2.8
69	Enterobacteriaceae			136.1	90.3	10.4	5.6	9.9	5.4
70	Enterobacteriaceae			20.0	13.2	1.7	1.0	1.6	0.9
71	Enterobacteriaceae			1.5	0.2	0.1	0.0	0.1	0.0
72	Enterobacteriaceae			25.2	8.1	2.2	1.0	2.1	0.9
73	Enterobacteriaceae			1.3	0.3	0.1	0.0	0.1	0.0
74	Erysipelotrichaceae	Coprobacillus		1.3	0.2	0.1	0.0	0.1	0.0
75	Erysipelotrichaceae	Turicibacter		1.4	0.3	0.1	0.0	0.1	0.0
76	Erysipelotrichaceae			1.1	0.1	0.1	0.0	0.1	0.0
77	Erysipelotrichaceae			1.6	0.1	0.1	0.0	0.1	0.0
78	Fibrobacteraceae	Fibrobacter	succinogenes	1.2	0.3	0.1	0.0	0.1	0.0
79	Fusobacteriaceae	Fusobacterium	group	1.3	0.1	0.1	0.0	0.1	0.0
80	Fusobacteriaceae	Fusobacterium		7.1	5.9	0.5	0.4	0.5	0.4
81	Gammaprotein_class			1.2	0.2	0.1	0.0	0.1	0.0
82	Incertae Sedis XI	Peptoniphilus	harei	0.9	0.3	0.1	0.0	0.1	0.0
83	Incertae Sedis XI	Peptoniphilus	harei	1.2	0.1	0.1	0.0	0.1	0.0
84	Incertae Sedis XI	Peptoniphilus	harei	1.1	0.1	0.1	0.0	0.1	0.0
85	Lachnospiraceae	Incertae Sedis	xylanophilum	2.1	1.9	0.2	0.1	0.2	0.1
86	Lachnospiraceae	Roseburia	cecicola/intestinalis	1.1	0.1	0.1	0.0	0.1	0.0
87	Lachnospiraceae	unclassified		1.2	0.1	0.1	0.0	0.1	0.0
88	Lactobacillaceae	Lactobacillus	acidophilus	1.3	0.3	0.1	0.1	0.1	0.1
89	Lactobacillaceae	Lactobacillus	brevis / hammesii/ parabrevis	1.2	0.2	0.1	0.0	0.1	0.0
90	Lactobacillaceae	Lactobacillus	group	1.2	0.1	0.1	0.0	0.1	0.0
91	Lactobacillaceae	Lactobacillus	group 6	1.2	0.1	0.1	0.0	0.1	0.0
92	Lactobacillaceae	Lactobacillus	johnsonii / gasseri / taiwanensis	79.3	36.0	6.6	2.2	6.2	2.1
93	Lactobacillaceae	Lactobacillus	plantarum/ paraplantarum/	30.5	13.6	2.6	1.0	2.5	1.0
94	Leuconostocaceae	Leuconostoc	group 2	1.3	0.1	0.1	0.0	0.1	0.0
95	Leuconostocaceae			1.3	0.2	0.1	0.0	0.1	0.0
96	Micrococcaceae	Arthrobacter	globiformis	1.5	0.2	0.1	0.0	0.1	0.0
97	Moraxellaceae	Acinetobacter	group	2.3	1.3	0.2	0.1	0.2	0.1
98	Nitrospiraceae			3.8	1.2	0.3	0.1	0.3	0.1
99	order "Lactobacillales"			1.1	0.2	0.1	0.0	0.1	0.0
100	order Bacillale			1.2	0.2	0.1	0.0	0.1	0.0
101	order Clostridiale			1.3	0.3	0.1	0.0	0.1	0.0
102	Peptococcaceae	Peptococcus	uncultured	1.2	0.1	0.1	0.0	0.1	0.0
103	Peptostreptococcaceae	Sporacetigenium		1.1	0.1	0.1	0.0	0.1	0.0
104	Peptostreptococcaceae			1.4	0.3	0.1	0.0	0.1	0.0
105	Porphyromonadaceae	Parabacteroides	distasonis	1.2	0.3	0.1	0.0	0.1	0.0
106	Porphyromonadaceae	Parabacteroides	uncultured/ distasonis	1.2	0.4	0.1	0.0	0.1	0.0
107	Porphyromonadaceae	Parabacteroides	uncultured/ distasonis	1.2	0.1	0.1	0.0	0.1	0.0
108	Prevotellaceae	Prevotella	group	3.7	0.6	0.3	0.1	0.3	0.1
109	Prevotellaceae	Prevotella	group 3	1.1	0.2	0.1	0.0	0.1	0.0
110	Prevotellaceae	Prevotella	group 5	1.2	0.1	0.1	0.0	0.1	0.0
111	Prevotellaceae	Prevotella	group 7	1.2	0.1	0.1	0.0	0.1	0.0
112	Rikenellaceae	Alistipes	onderdonkii/shahii	1.2	0.3	0.1	0.0	0.1	0.0
113	Ruminococcaceae	group		1.0	0.3	0.1	0.0	0.1	0.0
114	Ruminococcaceae	Ruminococcus	albus	1.2	0.2	0.1	0.0	0.1	0.0
115	Ruminococcaceae	Ruminococcus	flavefaciens/ callidus	1.4	0.5	0.1	0.0	0.1	0.0
116	Ruminococcaceae	unclassified		1.3	0.2	0.1	0.1	0.1	0.0
117	Sphingobacteriaceae			74.8	39.1	6.1	2.6	5.7	2.6
118	Sphingobacteriales	Chitinophaga		1.3	0.3	0.1	0.0	0.1	0.0
119	Staphylococcaceae	Staphylococcus	caprae	1.3	0.2	0.1	0.0	0.1	0.0
120	Staphylococcaceae	Staphylococcus	group	1.1	0.1	0.1	0.0	0.1	0.0
121	Streptococcaceae	Streptococcus	agalactiae/ equi	1.3	0.1	0.1	0.0	0.1	0.0
122	Streptococcaceae	Streptococcus	group	1.2	0.1	0.1	0.0	0.1	0.0
123	Streptococcaceae	Streptococcus	group	1.2	0.2	0.1	0.0	0.1	0.0
124	Streptococcaceae	Streptococcus	oligofermentas/ infantarius	1.5	0.5	0.1	0.0	0.1	0.0
125	Streptococcaceae	Streptococcus	thermophilus	1.2	0.4	0.1	0.0	0.1	0.0
126	Veillonellaceae	Phascolarctobacterium	faecium	1.7	0.9	0.1	0.1	0.1	0.1
127	Verrucomicrobiaceae	Akkermansia	muciniphila	1.5	0.4	0.1	0.1	0.1	0.1
128	Yeast	Galactomyces	geotrichum	1.2	0.1	0.1	0.0	0.1	0.0

*** Correction factor [2] = 0.91**

below detectable level	<2	<0,1 %	<0,1 %
low abundance	2< <4	0,1% < < 0,2%	0,1% < < 0,2%
medium abundance	4 < < 50	0,2% < < 4%	0,2% < < 4%
high abundance	>50	>4%	>4%

Influence of nucleic acids derived from dead or inactive cells

I-chip results were based on isolated DNA. DNA derived from both dead and inactive cells was potentially present after antibiotic-treatments and this could result in false positive results in our experiments. Hence, discrimination between dead and viable cells and between active and inactive cells was supportive in drawing reliable conclusions.

To discriminate dead from viable cells, a treatment with propidium monoazide (PMA) was applied on replicates of the collected samples. PMA can bind to DNA in case the bacterial membrane is permeable (indicative for dead cells), and thereby inhibiting PCR amplification.[12] The viability of cells was not checked on culturing plate since 50% to 90% of anaerobic bacteria from faecal sample are not cultivable. Culture independent methods are preferred for this type of analysis.[2] Results from PMA-treated samples were compared to the corresponding non-PMA-treated samples. Differences between the two sets of samples were detected neither with qPCR on the total cell numbers (Figure 3) nor with I-chip on the microbiota composition (fingerprints similar to the ones observed in Figure 1). Therefore, potentially present dead cells after antibiotic-treatments did not influence microarray measurements after 24h *in vitro* fermentation. This conclusion is in contrast with results from previous studies.[12, 22] However, Kobayashi *et al*[22] focused on specific species, which do not recover from the antibiotic treatment, while Nocker *et al*[12] performed measurements shortly after antibiotic addition and not after 24h fermentation. In our experimental set-up, the non-affected bacteria grow during 24h fermentation time and the error due to the presence of dead cells apparently becomes negligible.

To discriminate actively growing cells from inactive cells, I-chip hybridizations were performed for both DNA and RNA isolated from the same sample (Figure 4). The differences between bacterial fingerprints based on DNA versus RNA especially concerned the bacteria present in low abundances. The signal intensities based on RNA were most often higher than the ones based on DNA. These differences indicate that these bacteria are active albeit present in low numbers, which is in line with literature reporting that more copies of RNA are present in one cell than copies of DNA.[23] In some occasions, it was observed that the signal intensity based on RNA was lower than the one based on DNA e.g. 1 $\mu g.ml^{-1}$ amoxicillin and 1 $\mu g.ml^{-1}$ clindamycin. Low RNA signals as compared to DNA signals are the differences to be discriminated as the bacteria are present but not active. These differences are not dependent on the mode of action of the antibiotic: Inhibition of activity was also observed with amoxicillin, which is known as a bacteriolytic antibiotic (Table 1). Apparently, antibiotics may be bacteriolytic for the bacteria they target for, but may be bacteriostatic for other bacteria, as also reported earlier.[3]

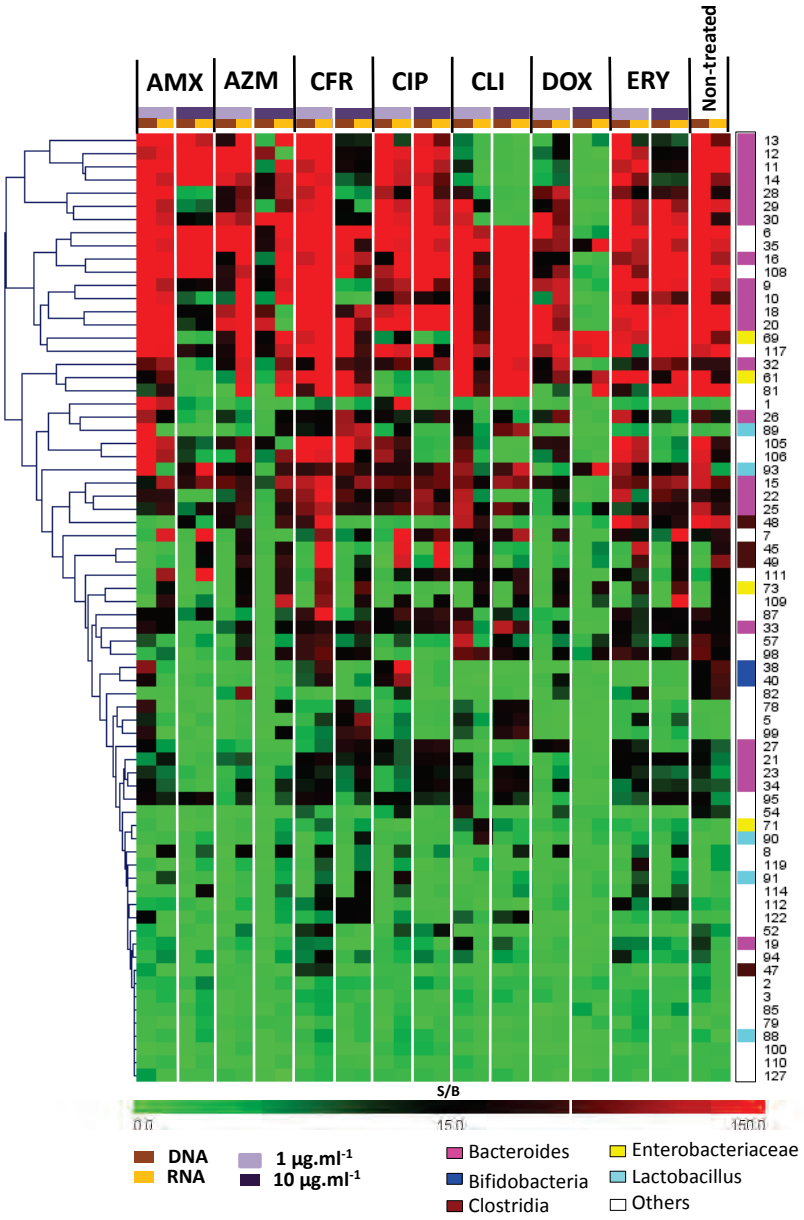

Figure 4: Bacterial fingerprints of non-treated and antibiotic-treated adult inoculum obtained with the I-chip based on DNA vs. RNA after 24h *in vitro* fermentation. Seven antibiotics were tested in concentrations of 1 µg.ml^{-1} and 10 µg.ml^{-1}. Main groups of bacteria are highlighted in colour and full name of targets can be found in table 2 based on their numbering. Signal to the background (S/B): Green: below detectable level, Black: medium abundance, Red: high abundance.

Overall, bacterial fingerprints based on DNA differ from the ones based on RNA. However these differences especially concern a low proportion of the total microbiota. Hence, in our study aiming at screening for major differences in the microbiota composition due to antibiotic treatments, no major misinterpretations are made when ignoring the presence of inhibited cells for any antibiotic mode of actions.

In conclusion, the I-chip readout is not influenced by the variations in the total number of bacteria nor the presence of dead or inactive cells after 24h *in vitro* fermentation. Hence the impact of antibiotics on the adult microbiota can be made with this microarray and conclusions on biological effects can be drawn from figure 1 (p34-35).

Impact of different antibiotics on the intestinal microbiota of healthy adults

Seven antibiotics were tested at low dose (1 $\mu g.ml^{-1}$) and high dose (10 $\mu g.ml^{-1}$). The impact of the antibiotics on microbiota as compared to the non-treated microbiota was determined with the I-chip 24h after starting the exposure.

Amoxicillin (Figure 1 A/B - AMX)

At 1 $\mu g.ml^{-1}$ antibiotic concentration, the abundance of *Bacteroides* remained stable, that of *Enterobacteriaceae* slightly decreased (*Escherichia coli* mainly) while those of *Bifidobacterium, Lactobacillus* and *Clostridium* decreased to an undetectable level. At 10 $\mu g.ml^{-1}$, the abundances of most *Bacteroides* species (except *B. fragilis)* and *Enterobacteriaceae* decreased to undetectable levels, although one outlier did not show significant reduction in the last group of bacteria. The abundance of *Lactobacillus gasseri* slightly increased. These results are in agreement with literature where amoxicillin is described as a broad spectrum antibiotic with an increasing suppression of both anaerobic and aerobic bacteria with increasing doses, although levels of *Enterobacteriaceae* are reported to increase but also to decrease.[4]

Azithromycin (Figure 1 A/B - AZM)

At 1 $\mu g.ml^{-1}$, the abundance of a few *Bacteroides* species (mostly *B. uniformis* and *B. vulgatus*) slightly decreased while *Bifidobacterium* abundance was reduced to undetectable levels. The abundances of the other groups of bacteria remained stable. At 10 $\mu g.ml^{-1}$, the abundances of *Bacteroides* and *Enterobacteriaceae* were slightly lowered

whereas that of *Clostridium* was reduced to an undetectable level. The abundance of *Lactobacillus* remained stable. These results fit with literature indicating a low activity for AZM against gram positive bacteria and an increased activity against aerobic gram negative rods.[24]

Cefadroxil (Figure 1 A/B - CFR)

At 1 µg.ml^{-1}, the abundances of *Bifidobacterium, Clostridium* and *Lactobacillus* slightly decreased, while those of the other groups remained stable. At 10 µg.ml^{-1}, the abundances of *B. uniformis, B. dorei, B. fragilis, Bifidobacterium,* and *Clostridium* decreased to undetectable levels. The abundance of *Enterobacteriaceae* remained stable, while that of *Lactobacillus brevis* increased. No major effects of cefadroxil (1g, 10 days) on the microbiota is reported in literature,[4] indicating that the absorption rate of cefadroxil *in vivo* might be greater than 90% as assumed in the present study.

Ciprofloxacin (Figure 1 A/B - CIP)

At 1 µg.ml^{-1}, the abundances of *Enterobacteriaceae* and *Clostridium* were dramatically reduced and became undetectable. At this low dose, the abundance of *Bifidobacterium* slightly increased and that of *Lactobacillus* remained stable. At 10 µg.ml^{-1}, the abundance of *Bifidobacterium* decreased to undetectable levels and that of *L. gasseri* increased. The abundance of *Bacteroides* remained stable for the two doses tested. Strong activity of 10 µg.ml^{-1} treatment is well known against *Enterobacteriaceae* and *Clostridium*[4] but not the activity against other bacterial groups.

Clindamycin (Figure 1 A/B - CLI)

At 1 µg.ml^{-1}, the abundances of *B. fragilis* and *Bifidobacterium* decreased to an undetectable level, that of *L. brevis* slightly increased and those of the other groups remained stable. At 10 µg.ml^{-1}, the abundances of *B. fragilis*, *B. uniformis and Clostridium* were reduced to undetectable levels, that of *Enterobacteriaceae* remained stable, and that of *L. brevis* increased. In line with the observed results, Clindamycin activity is reported to mainly be active against anaerobic bacteria.[4] Different impact per species of *Bacteroides* has been reported,[25] although our data do not show survival of *B. fragilis*.

Doxycycline (Figure 1 A/B - DOX)

At 1 µg.ml^{-1}, the abundances of *Bifidobacterium* and *Enterobacteriaceae* were slightly reduced while those of *B. fragilis, Clostridium* and *Lactobacillus* decreased to undetectable levels. At 10 µg.ml^{-1}, the abundances of *Bacteroides* and *Bifidobacterium* were reduced to undetectable levels, that of *L. gasseri* increased, while that of *Enterobacteriaceae* did not further decrease as compared to the abundances observed with 1 µg.ml^{-1}. The class of "Tetracycline" is not often studied.[4] Only a decrease of *Bifidobacterium* and of the general microbiota diversity measured by PCR-DGGE have been reported by Saarela *et al*[26].

Erythromycin (Figure 1 A/B - ERY)

At 1 µg.ml^{-1}, the abundance of each group of bacteria remained stable except for the abundance of *Bifidobacterium*, which decreased to an undetectable level. At 10 µg.ml^{-1}, the abundance of *B. fragilis* slightly decreased while the abundance of *Clostridium* decreased under the detectable level. The abundances of *Enterobacteriaceae* and *Lactobacillus* remained stable. These results are according to the trends reported in literature although a stronger impact of erythromycin towards *Enterobacteriaceae* and anaerobes has been reported.[4]

Overview of the antibiotic impact on the human intestinal microbiota

The impact of seven antibiotics on the human microbiota composition was now monitored under similar conditions in *one* experimental set-up. Although the interaction between host and microbiota are not mimicked in *in vitro* system, our findings for specific antibiotic were in general consistent with trends described in previous studies.[4,25,27-30] The outcomes of previous studies are, however, sometimes difficult to compare among each other or with current data due to the use of different conditions and analytical techniques. The advantage of our approach is illustrated in table 3 where the straightforward comparison of the outcomes regarding antibiotic impact on *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterobacteriaceae* and *Lactobacillus* is summarised.

Table 3: Overview of the impact of antibiotics on the main bacterial groups of adult inoculum measured with the I-Chip after 24h *in vitro* fermentation as compared to non-treated microbiota.

	µg.ml^{-1}	***Bacteroides***	***Bifido-bacterium***	***Lacto-bacillus***	***Clostridium***	***Entero-bacteriaceae***
AMX	**1**	=	↓↓	↓	↓↓	↓ *E.coli*
	10	↓↓ *B. dorei* *B. uniformis* *B. thetaiotaomicron*	↓↓	↑ *L. gasseri*	↓↓	↓↓
AZM	**1**	↓ *B. vulgatus* *B. uniformis*	↓↓	=	=	=
	10	↓	↓↓	=	↓↓	↓
CFR	**1**	=	↓	↓	↓	=
	10	↓↓ *B. dorei* *B. uniformis* *B. fragilis*	↓↓	↑↑ *L. brevis*	↓↓	=
CIP	**1**	=	↑	=	↓↓	↓↓
	10	=	↓↓	↑↑ *L. gasseri*	↓↓	↓↓
CLI	**1**	↓↓ *B. fragilis*	↓↓	↑ *L. brevis*	=	=
	10	↓↓ *B. fragilis* *B. uniformis*	↓↓	↑↑ *L. brevis*	↓↓	=
DOX	**1**	↓↓ *B. fragilis*	↓	↓↓	↓↓	↓
	10	↓↓	↓↓	↑↑ *L. gasseri*	↓↓	↓
ERY	**1**	=	↓↓	=	=	=
	10	↓↓ *B. fragilis*	↓↓	=	↓	=

In general, the impact on the microbiota is antibiotic and dose dependent, even if antibiotics belong to the same class, as reported in literature.[27] Details of the description are at family, genus but also species level when appropriate. Within a bacterial group, a specific antibiotic can have different impacts for different species (e.g *Bacteroides*) and the dose of this antibiotic can influence specific species within a bacterial group (e.g *Lactobacillus)*. These differences at species level are not often considered in literature although predominance or absence of certain species might have an influence on the ecosystem and, therefore, on human health. For instance, *L. gasseri* that became predominant upon a treatment with 10 $\mu g.ml^{-1}$ amoxicillin, ciprofloxacin and doxycycline, has been shown to result in a significant reduction of inflammation in IL-10- deficient mice.[31]

For all the antibiotics except ciprofloxacin, the concentration of 1 $\mu g.ml^{-1}$ is below the MIC values for the pathogens targeted. It is interesting to notice in table 3 that this low dose of antibiotic can already influence some bacterial groups of the microbiota. Lactobacilli*,* for instance*,* tends to survive antibiotic treatments. This survival of lactobacilli can be seen as an ability to persist through antibiotic treatment and, therefore, accentuate their probiotic effects or as a means to spread the antibiotic resistance genes within the gut.[32] On the contrary, bifidobacteria seem to be very sensitive micro-organisms. All antibiotics reduced the abundance of this group at 1 $\mu g.ml^{-1}$ except for ciprofloxacin, which reduced it only at 10 $\mu g.ml^{-1}$ concentration. As (minor) changes in the microbiota composition are reported to have consequences for colonic health with respect to development of resistant bacteria[33] and may cause disturbance of colonic fermentation,[34] sub-MIC dose of antibiotics reaching the colon should, therefore, not be under estimated with respect to undesired health effects.

Conclusion

To our knowledge, this study is the first to demonstrate the potential utility of coupling the high-throughput fermentation screening-platform to the I-chip analysis to monitor the effect of antibiotics on the microbiota in comparable conditions. The reliability of the microarray analysis was validated in case of antibiotic exposure. Errors due the variation in cell numbers and presence of dead cells were negligible after 24h *in vitro* fermentation. With this high-throughput approach, a detailed level of information at family, genus and species level was simultaneously obtained for all groups of bacteria whereas most previous studies focus on specific antibiotic or specific bacteria or group of bacteria. Although the interaction between host and microbiota cannot be mimicked completely in

an *in vitro* system, the detailed impact of seven antibiotics as obtained from *one* experimental set-up using a complex ecosystem allows comparison of the different antibiotics. Such detailed high-throughput evaluation could not be reached either in *in vitro* studies using other molecular tools or in *in vivo* studies.

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Chapter 3

Impact of galacto-oligosaccharides on the gut microbiota composition and metabolic activity upon antibiotic treatment during *in vitro* fermentation

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Abstract

Prebiotics are considered to have potential to reduce disturbances in the gut microbiota induced by antibiotics. Results in literature are, however, not consistent. The current *in vitro* study conducted in a fermentation screening-platform allowed to unambiguously compare the impact of galacto-oligosaccharides (GOS) on adult gut microbiota composition and activity upon treatment with four antibiotics at two doses. The changes in relative abundance of bacteria upon antibiotic treatment and the growth of *Bifidobacterium* and *Lactobacillus* species upon GOS addition was antibiotic and dose dependant. This conclusion explains discrepancies in literature and indicates that particular combinations of GOS-antibiotic should be studied. The combination GOS-Amoxicillin was especially of interest as, after decrease of the level of *Bifidobacterium* spp., a recovery of mainly *Bifidobacterium longum* was observed and could be correlated to specific degradation patterns of GOS. Next to different degradation profiles of individual GOS oligosaccharides, an accumulation of monosaccharides and intermediate organic acids was observed in antibiotic-treated microbiota as compared to non-treated microbiota. This showed that although GOS was utilised and beneficial bacteria could grow for 3 out of 4 antibiotics tested, the metabolic activity of an antibiotic-treated microbiota was still disturbed as compared to the non-treated microbiota

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Introduction

Fermentation of non-digested compounds reaching the human colon requires the cooperation of different bacterial groups and results in the production of gases and Short Chain Fatty Acids (SCFAs).[1] The complex microbiota and its metabolic activity are relatively stable in the various stages of life for each individual, but can be disrupted by e.g. antibiotic treatments or modulated with prebiotic supplementations.[2]

In Europe, the median consumption of antibiotic was 18.3 Defined Daily Doses per 1000 inhabitants per day in 2010. The most commonly used antibiotics were penicillins followed by macrolides and tetracyclines.[3] These antimicrobial agents are used to treat specific bacterial infections, but can also kill or inhibit bacteria that are not primarily targeted, such as bifidobacteria and lactobacilli, within the gut microbiota.[4] The extent of the impact of an antibiotic on the non-targeted microbial populations depends on its spectrum of action, mode of administration (oral vs. intravenous), dose and absorption rate.[5] The resulting disruption of the microbiota can be the basis for the occurrence of metabolic dysfunctions, leading to a decrease of beneficial SCFA levels and to an increase of unfermented oligosaccharides.[6] In 5-35% of the cases, it can also lead to antibiotic-associated diarrhoea (AAD), with the antibiotics amoxicillin and clindamycin having the highest incidence.[7]

Prebiotics are defined as "*selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits upon host well-being and health*".[8] Most known prebiotics are fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS). The use of prebiotics to prevent non-antibiotic associated gut diseases is promising.[9] With respect to the prevention of AAD, only a few studies have been performed. The results are contradictory regarding the efficacy to limit the risk of AAD and the impact on the microbiota composition.[2, 10, 11] Main focus was on the number of bifidobacteria, as these bacteria are expected to be stimulated by the prebiotic addition. The number of bifidobacteria was reported to either increase,[12] decrease[13] or remain stable.[14] Discrepancies regarding the effect of prebiotic addition on antibiotic-treated microbiota might be explained by the variability in parameters among the studies. Parameters, such as prebiotics, antibiotics, dosages and age of subjects are known to have a high impact on the microbiota composition.[15] Using a fermentation screening-platform, this study aims to unambiguously compare the potential beneficial impact of a commercially available prebiotic, galacto-oligosaccharides (GOS), on an adult microbiota treated with four antibiotics at two doses. The microbiota composition was investigated as well as the microbiota metabolic activity, specifically oligosaccharide degradation and organic acid production.

Material and methods

Antibiotics

Amoxicillin (AMX) (≥90%), ciprofloxacin (CIP) (≥98% HPLC), clindamycin hydrochloride (CLI) (impurities ≤2 mol/mol EtOH) and doxycycline (DOX) (≥98% TLC) were obtained from Sigma-Aldrich (St Louis, MO, USA). These antibiotics belong to four of the most widely-used classes of antibiotics used in Europe[3] and were selected based on their different modes of action (Table 1).

Table 1: Characteristics of mostly used antibiotics in main classes frequently used in Europe.

Class	*Name*	*Type*	*Mode of action*[4]	*AAD*[a] *risk*[7]
Penicillin	Amoxicillin (AMX)	Bacteriolytic	Inhibition peptidoglycan biosynthesis	High
Tetracycline	Doxycycline (DOX)	Bacteriostatic	Translation inhibition	Low
Macrolide-Lincosamide	Clindamycin (CLI)	Bacteriostatic	Translation inhibition	High
Fluroquinolone	Ciprofloxacin (CIP)	Bacteriolytic	Replication and transcription inhibition	Low

[a]AAD : Antibiotic Associated Diarrhoea

Prebiotic

Purified Galacto-oligosaccharides (GOS) with <3% (w/w dry matter) monomers and lactose (purified from the lactose-based prebiotic Vivinal® GOS, FrieslandCampina Domo, Borculo, The Netherlands) were used. Vivinal® GOS was purified because monosaccharides and lactose are digested and absorbed in the small intestine. For the purification, Vivinal® GOS was enzymatically treated with a lactase to hydrolyse the lactose into glucose and galactose, after which the monosaccharides were removed by nanofiltration. The degree of polymerisation (DP) of the purified GOS ranged from 2 to 8.

Experimental set up

In a fermentation screening-platform (96 wells of 1.5ml), selected type and dose of antibiotics (1 $\mu g.ml^{-1}$ or 10 $\mu g.ml^{-1}$) and GOS (4,2 $mg.ml^{-1}$) were simultaneously added to the Standard Ileal Efflux Medium (SIEM) and *in vitro* fermented (37°C; pH 5.8) using

healthy adult faecal inoculum (0.1% v/v) under anaerobic conditions.[16] Selection of volunteers (n=8) and methodology to obtain and store the faecal samples were performed as described previously.[16] Polymeric carbohydrates present in the SIEM were used as non-prebiotic substrate in the control.[16] Each fermentation was performed in triplicate. To investigate the microbiota metabolic activity, samples (70 µl) were collected 6 times during fermentation. The collection times were chosen based on a test experiment revealing the time range of GOS degradation. This range was between 8h and 24h fermentation in the control, between 16h and 32h and between 24h and 48h in the fermentations using 1 $\mu g.ml^{-1}$ and 10 $\mu g.ml^{-1}$ antibiotic, respectively (data not shown). The collected samples were then boiled (5 min) and stored at -20°C. To investigate the changes in the microbiota composition, additional samples (70 µl) were collected from the same well at 24h, 32h and 48h, since the fermentation rate may be slowed down by antibiotic addition, and stored at -20°C .

Microbiota composition analysis

The Intestinal (I)-Chip, developed at TNO (Zeist, The Netherlands), was used to investigate the composition of the microbiota. This DNA based microarray enabled the detection of more than 400 bacterial targets from the human large intestinal microbiota. Total faecal DNA from collected samples was isolated, amplified, purified and hybridized as described previously.[16] The hybridization took place on a microarray constructed and validated as described before,[17] using intestinal bacteria primers instead of oral primers. Imagene 5.6 software (BioDiscovery, Marina del Rey, CA, USA) was used to analyse the results. Genes above the detectable level having a signal intensity higher than 3 ($>10^5$ bacteria) in more than 10 samples were used to describe the bacterial fingerprint.

Total bacteria and Bifidobacterium spp. quantification using quantitative PCR

Quantitative PCR (qPCR) was performed to investigate the variation in the total number of bacteria and of *Bifidobacterium* spp. during fermentation. Primers used to measure the total number of bacteria were the universal primers 16S-uni-II-R [10 $pmol.\mu l^{-1}$] (GGA CTA CCA GGG TAT CTA ATC CTG TT) and 16S-uni-II-F [10 $pmol.\mu l^{-1}$] (TCC TAC GGG AGG CAG CAG T), and probe 16S-uni-II [5µM] (6FAM-CGTATTACCGCGGCTGCTGGCAC-TAMRA). The primers to measure the number of *Bifidobacterium* spp. were 16S-Bif-F [10 $pmol.\mu l^{-1}$] (GGA GCA TGC GGA TTA ATT CG), 16S-Bif-R [10 $pmol.\mu l^{-1}$] (GAC CAT GCA CCA CCT GTG

AAC), 16S-Bifspec (6FAM-CTG GGC TTG ACA TGT T) (Applied Biosystems, Bleiswijk, The Netherlands). The amplification was performed with 5 µl DNA sample and 25 µl q-PCR mixture.[16] Total microbial faecal DNA was diluted 1:10 to quantify the total number of bacteria and 1:100 to quantify the number of *Bifidobacterium* spp. The experiment was performed using the 7500 Fast Real Time PCR system (Applied Biosystems) at settings previously described.[16]

DNA of the microbiota from the control and *Bifidobacterium longum* was used as quantitative standards for total bacteria and *Bifidobacterium* spp., respectively (5 ng.µl^{-1} to 5 fg.µl^{-1}).

Statistical analysis

To evaluate whether two qPCR data sets were significantly different, a Student t-test was performed. P-values were calculated assuming equal variance and two-tailed distribution. Correlations were considered significant at a P-value lower than 0,01.

GOS degradation pattern using High Performance Anion Exchange Chromatography (HPAEC)

HPAEC was performed to quantify GOS degradation during the experiments. The samples collected were 10× diluted with Millipore water (0.42 mg.ml^{-1}) and analysed using an ICS3000 HPLC system (Dionex, Sunnyvale, CA, USA), equipped with a CarboPac PA-1 column (2 mm ID × 250 mm; Dionex) in combination with a CarboPac PA guard column (2 mm ID × 25 mm) and a ISC3000 ED detector (Dionex) in the PAD mode. A flow rate of 0.3 ml.min^{-1} was used with the following elution profile of 0.1 M sodium hydroxide (solution A) and 1 M sodium acetate in 0.1 M sodium hydroxide (solution B): 0–20 min, 0–20% B; 20–26 min washing step with 100% B; 26–41 min, equilibration with 100% A. Twenty µl of sample was injected each time.

To describe the degradation of GOS over fermentation time, GOS were roughly subdivided based on the degree of polymerisation (DP):[18] dimers (DP<3, incl. 3% monomers) eluting between 4.5 min and 9.7 min, medium-sized DP (3≤DP≤5) eluting between 9.7min and 13.0min, and large DP (DP>5) eluting between 13.0 min and 18.0 min. The peak areas were measured to express the remaining oligosaccharides as an indication of the degradation rate. Degradation patterns were similar for the triplicate samples. The proportion of remaining oligosaccharides was quantified only in one set of samples.

Organic acids production using gas chromatography (GC) and high performance liquid chromatography (HPLC)

A GC analysis was performed on a TRACE™ GC Ultra Gas Chromatograph system coupled with a FID detector (Interscience, Breda, The Netherlands) to quantify the SCFA (acetate, propionate and butyrate) produced during fermentation: 50µl of diluted sample (0.42 $mg.ml^{-1}$) or standards (1 $mg.ml^{-1}$ to 0.125 $mg.ml^{-1}$) mixed with 50 µl of 0.15 M oxalic acid stood for 30 min before addition of 150 µl of water. Samples (1 µl) were injected to a CP-FFAP CB column (25m x 0.53mm x 1.00 µm) (Agilent Technologies, Santa Clara, CA, USA). The temperature profile was as follows: Start at 100°C, increase to 155°C at 5°C.min^{-1}, and hold at this temperature 1 min. GC data was integrated using the Xcalibur® software (Thermo Scientific, Breda, The Netherlands).

HPLC was performed to quantify lactate and succinate on an Ultimate 3000 HPLC (Dionex) equipped with an RI-101 refractive index detector (Shodex, Kawasaki, Japan), an autosampler and an ion-exclusion Aminex HPX – 87H column (7.8 x 300mm) with a guard column (Bio-Rad, Hercules, CA, USA). The mobile phase was 5mM H_2SO_4 and the flow rate was 0.6 $ml.min^{-1}$ at 65°C. Diluted samples (10µl; 0.42 $mg.ml^{-1}$) and standards (10µl; 0.125 $mg.ml^{-1}$ to 1 $mg.ml^{-1}$) were injected onto the column. The concentration of organic acids was quantified only in one set of samples and expressed as $\mu mol.mg^{-1}$ of GOS.

Results

In order to determine the potential beneficial impact of GOS on antibiotic-treated microbiota composition and metabolic activity (substrate degradation and SCFA production), *in vitro* fermentations using adult faecal inoculum treated with four antibiotics (AMX, CIP, CLI, DOX) at two doses (low : 1 $\mu g.ml^{-1}$ or high: 10 $\mu g.ml^{-1}$) were performed in a fermentation screening-platform.

Impact of GOS on the microbiota composition

Total bacteria and Bifidobacterium spp. quantification

The total numbers of bacteria and *Bifidobacterium* spp. in **A**nti**B**iotic-treated **M**icro**B**iota (AB-MB) and in antibiotic-treated microbiota supplemented with GOS (AB/GOS-MB) as measured with qPCR are presented in table 2. Overall, the total number of bacteria remained stable upon GOS addition. The number of *Bifidobacterium* spp. increased upon GOS addition depending on the antibiotic, dose and time.

Table 2: Numbers of total bacteria and *Bifidobacterium spp.* in adult inoculum treated with 1 µg.ml^{-1} (1) and 10 µg.ml^{-1} (10) antibiotics with and without GOS addition as measured with qPCR during *in vitro* fermentation. Values are expressed as mean ± standard deviation of log_{10} (copies.g^{-1} faeces) (n=3).

		Total Bacteria				***Bifidobacterium***			
		no GOS		**GOS**		**no GOS**		**GOS**	
Non-treated	24h	11.2	±0.1	11.0	±0.4	7.2	±0.2	**9.9**	**±0.0$^{\#}$**
	32h	11.0	±0.3	11.4	±0.2	6.9	±0.6	**9.7**	**±0.1$^{\#}$**
	48h	11.3	±0.5	11.1	±0.1	7.8	±0.6	**9.6**	**±0.1$^{\#}$**
AMX 1	24h	11.0	±0.4	10.7	±0.2	6.0	±0.3†	**7.3**	**±0.2$^{\#\dagger}$**
	32h	11.2	±0.3	10.5	±0.3	6.3	±0.2	**9.2**	**±0.2$^{\#}$**
CIP 1	24h	11.5	±0.1	10.3	±0.6	7.3	±0.1	**9.5**	**±0.3$^{\#}$**
	32h	10.8	±0.8	10.6	±0.3	7.2	±0.4	**9.6**	**±0.3$^{\#}$**
CLI 1	24h	10.4	±0.7	10.2	±0.6	4.8	±0.3†	5.1	±0.2†
	32h	10.8	±0.6	10.1	±0.5	4.7	±0.4†	4.9	±0.3†
DOX 1	24h	10.3	±1.0	10.8	±0.4	7.7	±0.8	**9.8**	**±0.2$^{\#}$**
	33h	10.2	±0.5	**11.3**	**±0.2$^{\#}$**	7.5	±0.4	**9.7**	**±0.2$^{\#}$**
AMX 10	24h	10.9	±0.4	10.9	±0.2	4.8	±0.6†	5.8	±0.4†
	32h	10.9	±0.9	10.7	±0.5	6.1	±0.1	**9.8**	**±0.0$^{\#}$**
	48h	10.7	±0.6	10.7	±0.4	6.1	±0.1	**9.6**	**±0.2$^{\#}$**
CIP 10	24h	9.3	±0.4†	10.0	±0.1	5.0	±0.2†	5.1	±0.3†
	32h	9.2	±0.1†	**9.9**	**±0.2$^{\#\dagger}$**	5.3	±0.1	4.6	±0.3†
	48h	8.8	±0.1†	**10.3**	**±0.2$^{\#\dagger}$**	4.7	±0.1†	**6.3**	**±0.2$^{\#\dagger}$**
CLI 10	24h	10.7	±0.1†	10.1	±0.1	4.8	±0.1†	4.8	±0.1†
	32h	10.8	±0.0	10.0	±0.6	4.7	±0.1†	4.8	±0.1†
	48h	10.7	±0.1	10.5	±0.1	4.8	±0.1†	**5.6**	**±0.2$^{\#\dagger}$**
DOX 10	24h	9.7	±0.1†	9.8	±0.4	4.7	±0.1†	4.8	±0.3†
	32h	8.8	±0.8†	9.8	±0.7	5.0	±0.3	5.2	±0.4†
	48h	9.2	±0.8†	10.1	±0.3†	4.9	±0.1†	5.3	±0.3†

$^{\#}$ significant difference between GOS and no GOS addition ($p<0.01$)

† significant difference between non-treated and antibiotic-treated ($p<0.01$)

The increase of *Bifidobacterium* spp. was significant (P<0.01) for CIP/GOS-MB (+1.6 log_{10}) and CLI/GOS-MB (+0.8 log_{10}) applied with a 10 µg.ml^{-1} dose, although the number of *Bifidobacterium* spp. was still below that of the non-treated microbiota ($10^{7.8}$ copies.g^{-1} faeces ±0.6). The number of *Bifidobacterium* spp. upon GOS addition increased significantly (P<0.01) and reached a level that was similar to GOS-MB ($10^{9.6}$ copies.g^{-1} faeces) for CIP/GOS-MB (+2.4 log_{10}) and DOX/GOS-MB (+2.2 log_{10}) applied with a 1 µg.ml^{-1} dose, and for AMX/GOS-MB applied with a both 1 µg.ml^{-1} and 10 µg.ml^{-1} dose (+2.9 log10 and +3.5 log10, respectively). At low dose, the stimulation of *Bifidobacterium* spp. was quicker for CIP/GOS-MB and DOX/GOS-MB (24h of fermentation) than for AMX/GOS-MB (32h of fermentation). The growth of *Bifidobacterium* spp. for AMX/GOS-MB at both low and high dose is of high interest as the level of *Bifidobacterium* spp. was first decreased due to the AMX action.

Microbiota fingerprinting as measured with the I-chip

The impact of GOS on the overall microbiota composition, including specific bifidobacteria species, was determined using the I-chip by comparing AB/GOS-MB and AB-MB (Figure 1). For AB/GOS-MB, the addition of GOS did not influence the abundances of *Bacteroides*, *Clostridium* and *Enterobacteriaceae.* For the latter two bacterial groups, the abundance remained low upon GOS addition, which is preferable as most pathogens belong to these two bacterial groups. In AB/GOS-MB, an impact of GOS addition could be observed on the lactobacilli and bifidobacteria population at each dose of antibiotic. The increase of *Lactobacillus* spp. was dose dependant*:* At low dose (1 µg.ml^{-1}), an increase of *Lactobacillus brevis* was observed upon addition of GOS after 24h of fermentation in most samples (excluding AMX/GOS-MB), while at high dose (10 µg.ml^{-1}), an increase of *Lactobacillus gasseri* was observed in CIP/GOS-MB and DOX/GOS-MB. The increase of *Bifidobacterium* spp. was in line with results measured by qPCR.

The I-chip results also showed that *different* species were stimulated upon GOS addition depending on the antibiotic treatment. For GOS-MB, a broad range of *Bifidobacterium* spp. became more abundant, mainly *B. longum, B. thermophilum* and *B. adolescentis*. For AB/GOS-MB, specific *Bifidobacterium* spp. were stimulated depending on the antibiotic and the dose. Using a low dose of antibiotic, mainly *B. longum* became more abundant for AMX/GOS-MB, while mainly *B. longum, B. animalis* and *B. thermophilum* for CIP/GOS-MB and mainly *B. thermophilum, B. ruminantium* and *B. adolescentis* for DOX/GOS-MB became more abundant. Using a high dose of antibiotic, mainly *B. longum* and *B. thermophilum* became more abundant for AMX/GOS-MB. For CIP/GOS-MB,

Bifidobacterium spp. were stimulated but no preferential growth of specific species were detected.

As the *same* inoculum was used for all fermentations, it is clearly shown that within the same ecosystem the stimulation of specific bacteria upon GOS addition depends on the antibiotic used.

Figure 1: Bacterial fingerprints of the non-treated and antibiotic-treated adult microbiota with and without GOS addition obtained with the I-Chip after 24h, 32h or 48h *in vitro* fermentation. Antibiotics were used in concentrations of 1 µg.ml^{-1} and 10 µg.ml^{-1}. Signal compared to the background (S/B): Green: below detectable level, Black: medium abundance, Red: high abundance.

Metabolic activity of antibiotic-treated microbiota supplemented with GOS

Degradation of the substrate GOS

Since the growth of *Bifidobacterium* and *Lactobacillus* species was observed upon GOS addition in some antibiotic-treated microbiota, the degradation and utilisation of GOS were determined. The HPAEC elution patterns of GOS during fermentation using non-treated microbiota are shown in Figure 2.

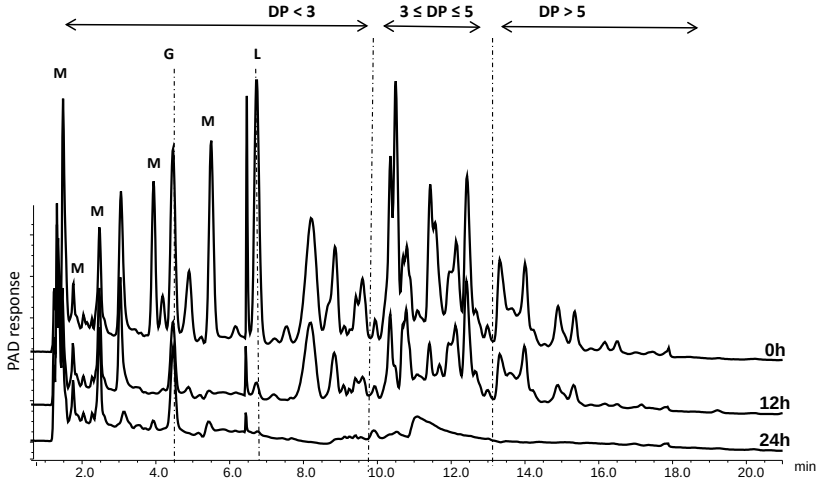

Figure 2: HPAEC elution patterns of GOS during *in vitro* fermentation using non-treated adult microbiota. The complex mixture of GOS was roughly subdivided based on the degree of polymerisation (DP) (6). M: peak from the SIEM medium, G: glucose, L: lactose.

A complex pattern of peaks can be observed at t=0. Few peaks were attributed to compounds present in the growth medium (M) and two peaks were attributed to glucose/galactose (G, Retention time (Rt)= 4.5min) and lactose (L, Rt= 6.3min). Other peaks represented oligosaccharides with different degree of polymerisation (DP) and isomers present in the GOS mixture.[18] Over fermentation time, the different oligosaccharides were degraded at different rates. To visualise the degradation of GOS in non-treated microbiota and 1 $\mu g.ml^{-1}$ or 10 $\mu g.ml^{-1}$ antibiotic-treated microbiota during fermentation, the proportion of remaining oligosaccharides (% of peak area) is presented in Figure 3 for total GOS and for oligosaccharides with a DP<3, 3≤DP≤5 and DP>5.

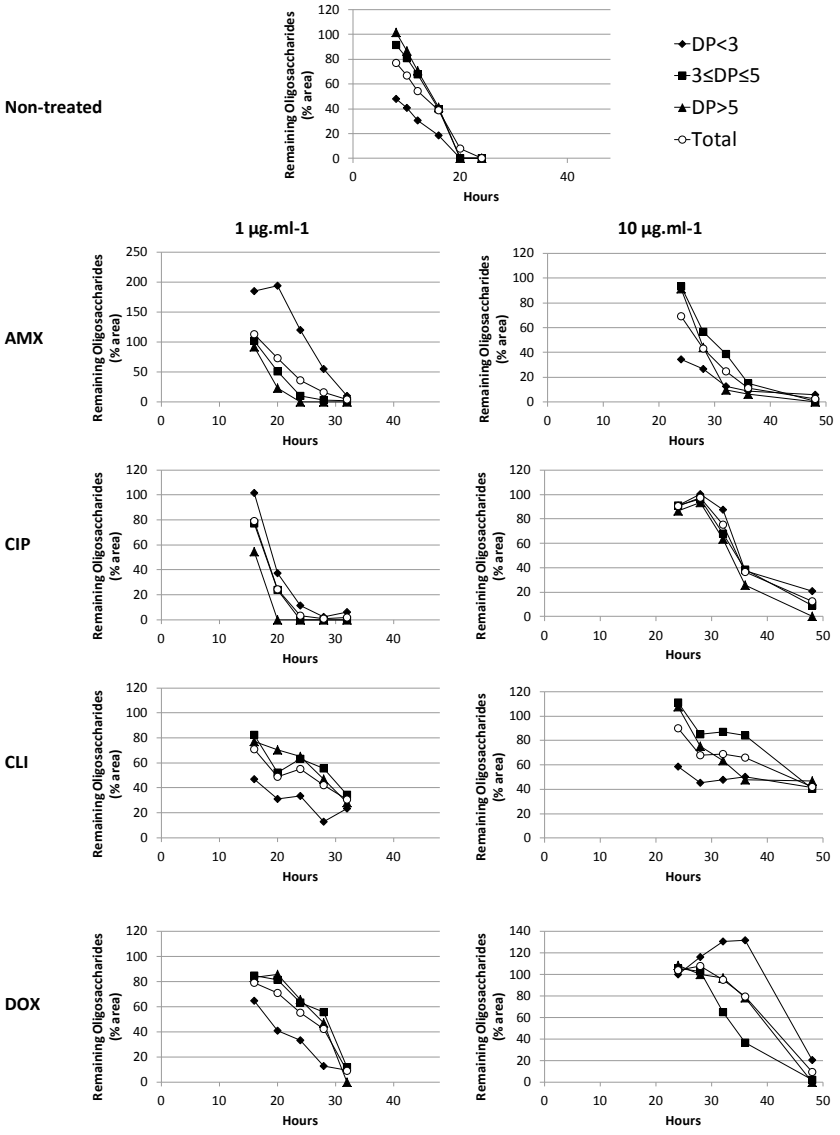

Figure 3: Percentage of remaining oligosaccharides from GOS during *in vitro* fermentation using a non-treated microbiota and an 1 µg.ml^{-1} or 10 µg.ml^{-1} antibiotic-treated microbiota.

In GOS-MB, about 20% of the total GOS was utilised in the first 8h of fermentation and further utilisation occurred gradually until 24h of fermentation. For AB/GOS-MB, a lag-time of utilisation was observed as more than 80% of GOS was still present after 16h and 24h of fermentation when the 1 $\mu g.ml^{-1}$ and 10 $\mu g.ml^{-1}$ doses were used, respectively. The subsequent utilisation of GOS differed per antibiotic and per dose. About 40% of the GOS remained in CLI/GOS-MB after 34h and 48h of fermentation at low and high dose, respectively. On the contrary, the degradation was almost complete (<10% remaining GOS) for CIP/GOS-MB, AMX/GOS-MB and DOX/GOS-MB. Once the degradation started, the 80% remaining GOS were degraded quicker in CIP/GOS-MB (6h), as quick as in AMX/GOS-MB (12h) and slower in DOX/GOS-MB (17h) than in GOS-MB.

Oligosaccharides of **DP<3** were in most cases quickly utilised in 1 $\mu g.ml^{-1}$ treated samples, indicating an easy utilisation by the microbiota. For AMX/GOS-MB, however, oligosaccharides of DP<3 accumulated at the beginning of the fermentation before being fully utilised within 34h of fermentation. For 10 $\mu g.ml^{-1}$ treated samples, oligosaccharides of DP<3 remained present at 48h of fermentation in all cases, which is in contrast to the fermentation pattern of GOS without antibiotic treatment. Oligosaccharides of **3≤DP≤5** decreased gradually in time for each dose of antibiotic. These oligosaccharides were present over time, probably due to a balance between further utilisation and formation. Oligosaccharides of **DP>5** were degraded slowly in DOX/GOS-MB and quickly in AMX/GOS-MB and CIP/GOS-MB for both doses. Overall, it can be concluded that different degradation profiles were observed after a same fermentation time depending on the antibiotic and dose used.

Production of organic acids

The degradation of GOS resulted in the production of organic acids: acetate (A), propionate (P), butyrate (B), succinate (S) and lactate (L) (Figure 4). For GOS-MB, the total amount of organic acids was 14 $\mu mol.mg\ substrate^{-1}$, with a ratio of A:P:B:S:L of 79:6:6:8:1. For 1 $\mu g.ml^{-1}$ antibiotic-treated samples, different concentrations of organic acids were observed per antibiotic used. For AMX/GOS-MB, a lower total amount of organic acids was observed (9 $\mu mol.mg\ substrate^{-1}$), mainly acetate and succinate (A:P:B:S:L = 44:7:5:44.0). For CIP/GOS-MB and DOX/GOS-MB, the amount and relative abundance of organic acids were similar to that of GOS-MB. For CLI/GOS-MB, a rather low amount of organic acids was observed (5 $\mu mol.mg\ substrate^{-1}$), mainly acetate. For 10 $\mu g.ml^{-1}$ treated samples, an overall reduction of organic acids was observed (below 9 $\mu mol.mg\ substrate^{-1}$ after 48h of fermentation). Furthermore, the relative abundance of

organic acids is dramatically changed since it mostly consists of lactic acid and acetate, whereas neither propionate nor butyrate could be detected.

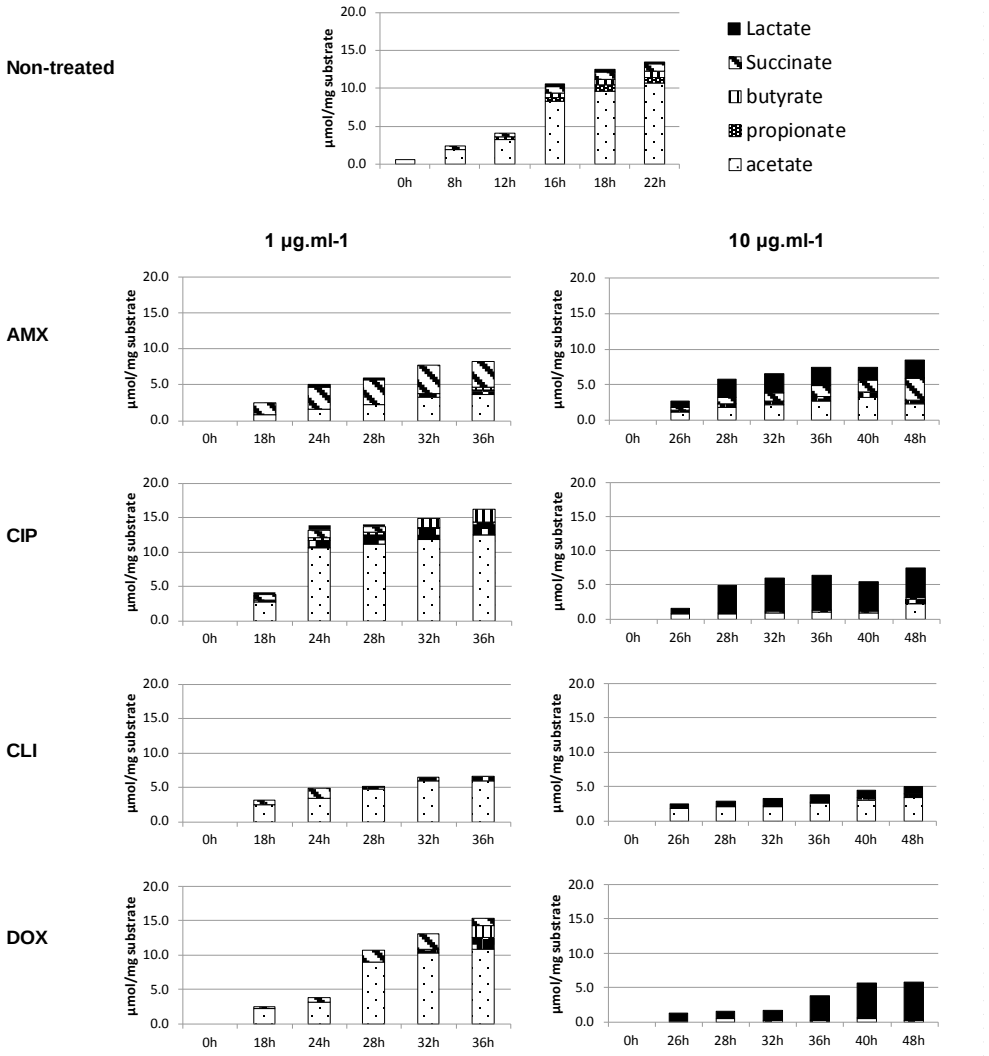

Figure 4: Organic acid amount and relative concentration during *in vitro* fermentation of GOS using a non-treated microbiota and an 1 µg.ml^{-1} or 10 µg.ml^{-1} antibiotic-treated microbiota.

Discussion

Using a fermentation screening platform, a straightforward comparison of the impact of GOS on the gut microbiota treated with four antibiotics at two doses was possible. The antibiotics belong to the most widely-used classes of antibiotics used in Europe. The doses selected (1 μg.ml^{-1} and 10 μg.ml^{-1}) are in the range of MIC-values of currently used antibiotics.[19] The changes in the microbiota composition upon antibiotic treatment (Figure 1) were in line with previous results [16], except for the increase of bifidobacteria after 1 μg.ml^{-1} DOX treatment. The impact of tetracycline treatment, such as doxycycline, on bifidobacteria has been reported to be variable due to the presence or absence of a resistance gene depending on the species.[20] Overall, the changes resulted in changes of metabolic activity, even upon the use of a sub-lethal dose as 1 μg.ml^{-1}.[21]

Antibiotic-treated microbiota changes upon GOS addition

In AB/GOS-MB, changes in the microbiota composition were mainly detected for the *Bifidobacterium* spp. and *Lactobacillus spp.*, which was expected as GOS is a prebiotic known to stimulate specifically these bacteria.[8] The growth of other bacteria which may utilise the partly degraded substrate present was below the detection level and co-dependence that exists amongst members of the microbiota[21, 22] was not observed. The changes in levels of bifidobacteria and lactobacilli were concomitant with the degradation of GOS and were antibiotic dependant (Table 3). The level of bifidobacteria did not increase in CLI/GOS-MB at both doses within 32h of fermentation, which is in line with the observed low degradation of GOS (about 40% remaining). Hence, partial degradation of GOS is likely due to the activity of lactobacilli that are known to grow on GOS, but to a lesser degree than bifidobacteria[23] On the contrary, levels of bifidobacteria increased upon GOS addition in CIP/GOS-MB and in DOX/GOS-MB at low dose (1 μg.ml^{-1}). The growth of these bacteria was facilitated as they were not affected by CIP and DOX (Table 2) and could utilise GOS (Figure 3). Furthermore, the competition for GOS intermediate degradation products was reduced, e.g. lower levels of *Bacteroides* in DOX/GOS-MB.[24] At high dose, the degradation of GOS occurred in both DOX/GOS-MB and CIP/GOS-MB, likely due to *Lactobacillus gasseri* action that were highly abundant (Table 3). Finally, levels of bifidobacteria, specifically *B. longum,* increased upon GOS in AMX/GOS-MB at both doses. This outcome is of high interest as the levels in the controls were low due to AMX action. The recovery of *B. longum,* therefore, reveals a potential beneficial effect of GOS addition in AMX-disturbed microbiota.

Table 3: Overview of bacteria growth, GOS degradation and ratio of SCFA and intermediate organic acids in non-treated and antibiotic-treated microbiota after 32h or 48h *in vitro* fermentation

Treatment		Bacteria growth		GOS degradation		Ratio SCFA : intermediate OA
		Bifido-bacterium[a]	*Lacto-bacillus*[b]	Remaining GOS (%)	Last remaining DPs	
Non-treated		Log_{10} 9.6 ±0.1	+	0%	3<DP<5	91 : 9
AMX/GOS-MB	1 µg.ml^{-1} 32h	Log_{10} 9.2 ±0.2[#] *B. longum*	--	4%	DP<3	56 : 44
CIP/GOS-MB		Log_{10} 9.6 ±0.3[#] *B. longum* *B. animalis*	++ *L. brevis*	2%	DP<3	100 : 0
CLI/GOS-MB		Log_{10} 4.9 ±0.3	+ *L. brevis*	35%	Most structures	100 : 0
DOX/GOS-MB		Log_{10} 9.7 ±0.2[#] *B. ruminatium* *B. adolescentis*	+ *L. brevis*	9%	3<DP<5	93 : 7
AMX/GOS-MB	10 µg.ml^{-1} 48h	Log_{10} 9.6 ±0.2[#] *B. longum* *B. thermophilum*	--	3%	DP<3	32 : 68
CIP/GOS-MB		Log_{10} 6.3 ±0.2[#]	++ *L. gasseri*	13%	DP<3	38 : 62
CLI/GOS-MB		Log_{10} 5.6 ±0.2[#]	--	42%	Most structures	80 : 20
DOX/GOS-MB		Log_{10} 5.3 ±0.3	++ *L. gasseri*	9%	DP<3	0 : 100

[a] as determined by qPCR (n=3). Values are expressed in Log_{10} (copies.g^{-1} faeces).

[b] as determined with I-chip (++: high abundance, +: medium abundance , --: below detectable level)

[#] significant difference between GOS and no GOS addition ($p<0.01$)

DP: Degree of Polymerisation

Degradation of GOS in antibiotic-treated microbiota

An almost complete degradation of GOS was observed in three antibiotic-treated microbiota using a low dose. The rate of degradation of oligosaccharides with different DPs varied depending on the antibiotic used. This preferential degradation was linked to the specific growth of bifidobacteria. (Table 3). The degradation of oligosaccharides of DP>5 was quick for both AMX/GOS-MB and CIP/GOS-MB, and correlated with the stimulation of mainly *B. longum.* This species is known to produce a membrane-bound extracellular endogalactanase that liberates galacto-trisaccharides from β(1,4) galacto-

oligosaccharides.[25] It is probably due to the endo-activity of this enzyme that the oligosaccharides of DP>5 are readily degraded. On the contrary, for DOX/GOS-MB, a fast degradation of small DP followed by a subsequent degradation of larger DPs was observed, which is characteristic for *B. adolescentis*[25, 26] that was stimulated. The correlation between GOS degradation and recovery of specific bifidobacteria species for specific antibiotic used indicates that preference of certain oligosaccharides by the microbiota is important to be considered when aiming at the recovery of this microbiota after an antibiotic treatment. Investigating in more detail which specific structure of GOS are preferentially degraded upon a specific antibiotic treatment could, for instance, ease the choice for an adequate synbiotic to be added during the antibiotic treatment to limit risk for AAD.[27] Another option could be to adjust the oligosaccharides composition of a prebiotic mixture depending on the antibiotic treatment used in order to stimulate specific bifidobacteria species present in the host gut.

Metabolic activity of the microbiota after antibiotic treatment

The metabolic functions of the microbiota include the degradation of the substrate and production of organic acids, which are most often SCFA.[28] In non-treated microbiota, the degradation of GOS resulted in high amount of SCFA and a low amount of intermediate acids as described before.[13, 29] At low dose, for CIP/GOS-MB and DOX/GOS-MB, the profile of organic acids was similar to that of GOS-MB, indicating that the metabolic activity of the microbiota might be as good as in non-treated microbiota. In strongly disrupted microbiota, e.g. 10 $\mu g.ml^{-1}$ AB-MB or 1 $\mu g.ml^{-1}$ CLI/GOS-MB, monosaccharides accumulated and a low total content of organic acids was observed, which is consistent with literature.[6] The presence of monosaccharides that would usually be quickly utilised by the microbiota might be a reason for pathogen colonisation, e.g. *Clostridium difficile* during antibiotic treatment. Furthermore, a downward shift in the ratio SCFA:intermediate acids was also observed in our study upon antibiotic treatment (Table 3), indicating that the ability to convert intermediate acids to SCFA was reduced. The presence of lactate and acetate is in line with the degradation of GOS by bifidobacteria and lactobacilli.[30] However, the absence of propionate and butyrate indicates that key groups of bacteria, such as *Bacteroides* and butyrate-producing bacteria, are lacking. Accumulation of succinate in 1 $\mu g.ml^{-1}$ treated samples revealed that the activity of mainly *Bacteroides* was affected, while accumulation of lactate in 10 $\mu g.ml^{-1}$ treated samples suggest that the lactate-utilising bacteria were also affected. The altered concentrations of SCFA and intermediate organic acids are unusual in the colon. The impact on the differences in metabolites produced might have consequences on colon health.

Conclusion

Using a fermentation screening-platform, our results clearly revealed that the recovery of an antibiotic-treated microbiota upon GOS addition is antibiotic dependant and, thereby, explains previous discrepancy in literature. The combination of GOS-AMX appeared to be of high interest as a recovery of mainly *Bifidobacterium longum* was observed and could be correlated to specific degradation patterns of GOS. Furthermore, the study emphasised that despite the specific growth of beneficial bacteria and the concomitant degradation of GOS, the metabolic activity of the antibiotic-treated microbiota may still be disturbed as compared to the non-treated microbiota. Further *in vivo* research should focus on the impact of specific structure of GOS to prevent intestinal diseases and on the consequences of the altered concentrations of SCFA and intermediate organic acids on colon health.

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Chapter 4

Impact of galacto-oligosaccharides and its specific size-fractions on non-treated and amoxicillin-treated human inoculum

Abstract

The impact of galacto-oligosaccharides (GOS) and of its specific size-fractions on non-treated microbiota (NT-MB) and on amoxicillin-treated microbiota (AMX-MB) were revealed using a recently described fermentation screening-platform. The *Bifidobacterium* species were highly impacted by amoxicillin and both GOS and GOS size-fractions stimulated their recovery. Interestingly, the level of *Bifidobacterium* spp. tended to be higher upon the addition of original GOS and low size-fractions (dimers-trimers) for NT-MB and of large size-fractions (tetramers to hexamers) for AMX-MB. Chromatographic analyses corroborated the finding that these size-fractions were preferentially degraded by the respective microbiota. Furthermore, new insights were obtained on the microbiota metabolism for AMX-MB: the amount of lactate was higher upon fermentation of low size-fractions, while more butyrate was present upon fermentation of large size-fractions. In conclusion, GOS can be used in advantage to support butyrate production and the recovery of the beneficial bifidobacteria from amoxicillin treatment, especially the large size-fractions.

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Introduction

Galacto-oligosaccharides (GOS) are well accepted as a prebiotic and contribute to human health.[1] GOS are produced by trans-galactosylation of lactose by β-galactosidases from yeast, fungi or bacteria, resulting in complex mixtures of oligosaccharides with different degrees of polymerisation (DP) and glycosidic linkages.[2] The DP varies from DP2 to DP8. Possible linkages are β(1-1), β(1-2), β(1-3), β(1-4) and β(1-6), the latter two being the most common ones.[3] Due to the diversity of possible glycosidic linkages, various isomeric oligosaccharides are present in each size-fraction.[3] The β-glycosidic linkages between the saccharide units can specifically be hydrolysed in the colon by dedicated enzymes from *Bifidobacterium* and *Lactobacillus* species. In pure cultures, different *Bifidobacterium* species have shown to have preferential utilisation of selected DP of GOS. For instance, *Bifidobacterium longum* subsp. *infantis* ATCC 15697 preferred GOS of DP4, whereas *Bifidobacterium adolescentis* ATCC 15703 utilised preferentially GOS of DP3.[4] In the same study, *Bifidobacterium longum* subsp. *longum* DJ010A was reported to partly utilise larger GOS oligosaccharide. Furthermore, the type of glycosidic linkage can also influence the stimulation of specific species. For instance, *Bifidobacterium breve* 26M2 and *Bifidobacterium lactis* BB12 were shown to have preference for β(1-6) and β(1-1) linkages over β(1-4) linkages.[5]

Prebiotics, such as GOS, have been suggested to help balancing the microbiota composition during antibiotic treatment and, thus, to limit antibiotic-associated disease, such as diarrhoea.[6, 7] Antibiotic treatments are known to disturb the gut microbiota and, therefore, the colonic fermentation. Amoxicillin is one of the mostly used antibiotic in Europe. It also has one of the highest incidence (5-35%) of antibiotic-associated diarrhoea.[8] This bactericidal antibiotic belongs to the penicillin class of antibiotics and has a broad spectrum of action, including an impact on bifidobacteria. In mice, it has been reported that the fermentation of dietary fibre fermentation as well as the subsequent short chain fatty acid (SCFA) production were reduced upon antibiotic treatment.[9] Similarly, the gut microbiota composition and metabolic activity were affected in an *in vitro* fermentation using human inoculum treated with different antibiotics.[10] In the latter study, growth of specific bifidobacteria (mainly *Bifidobacteria longum*) was observed upon addition of GOS to an amoxicillin-treated microbiota. The specific growth of this species seemed to be correlated with a preferential degradation of the large oligosaccharides present in the GOS mixture.

In the present study, the effects of GOS and the individual size-fractions of GOS on the microbiota composition and metabolic activity were determined for a non-treated microbiota (NT-MB) and subsequently for an amoxicillin-treated microbiota (AMX-MB).

The degradation rate and speed of the individual oligosaccharides were monitored as well as SCFA production and changes in microbiota composition, with an emphasis on *Bifidobacterium* spp.

Material and methods

Antibiotic

Amoxicillin (AMX) (≥90%) was obtained from Sigma-Aldrich (St Louis, MO, USA).

Prebiotic

Vivinal® GOS (FrieslandCampina Domo, Borculo, The Netherlands) was fractionated to obtain a Galacto-oligosaccharides (GOS) preparation with <3% (w/w dry matter) monomers and lactose. For the fractionation, Vivinal® GOS was treated with a lactase to hydrolyse the lactose into glucose and galactose, after which the monosaccharides were removed by nanofiltration. The degree of polymerisation (DP) of the oligosaccharides present in the GOS preparation ranged from 2 to 8.

Fractionation of GOS

The oligosaccharides present in the purified GOS were fractionated by size exclusion chromatography (SEC). An Äkta purifier (GE Healthcare, Uppsala, Sweden) was equipped with three serially connected HiLoad 26/60 Superdex 30 prep-grade columns (GE Healthcare). The columns were maintained at 35ºC. GOS (180 mg dissolved in 1.5 mL Milli-Q water) was applied onto the column and was eluted with 0.5% (v/v) EtOH in Milli-Q water at a flow rate of 1 mL/min. A refractive index RI-detector (RI-72, Showa Denko, Tokyo, Japan) was used to monitor the eluate. The system was controlled using Unicorn software. Fractions (1.9 ml) were collected, pooled according to the SEC-RI profile (data not shown) and subsequently freeze-dried.

Experimental set up

Using a recently developed fermentation screening-platform (96 wells of 1.5ml), the antibiotic amoxicillin (1 $\mu g.ml^{-1}$) and the purified GOS or size-fractions of GOS (4,2 $mg.ml^{-1}$) were simultaneously added to the Standard Ileal Efflux Medium (SIEM) and *in vitro*

fermented (37°C ; pH 5.8) using faecal inoculum (0.1% v/v) of healthy adults under anaerobic conditions.[11] Selection of volunteers (n=8) and methodology to obtain and store the faecal samples were performed as described previously.[11] To investigate the microbiota metabolic activity, samples (70 µl) were collected at 6 time points during fermentation. On basis of test experiment that determined the course of GOS degradation, the time range was chosen was 8h and 24h fermentation for the non-treated microbiota and, between 16h and 32h for the fermentations using 1 $\mu g.ml^{-1}$ amoxicillin (data not shown). The samples collected were boiled (5min) and stored at -20°C. To investigate the microbiota composition, additional samples (70µl) were collected from the same well at 24h and 48h, and directly stored at -20°C.

Monitoring GOS degradation

High performance Anion Exchange Chromatography (HPAEC) was used to quantify GOS degradation. The samples collected were 10× diluted with Millipore water (final concentration: 0.42 $mg.ml^{-1}$) and analysed using an ICS5000 HPLC system (Dionex, Sunnyvale, CA, USA), equipped with a CarboPac PA-1 column (2 mm ID × 250 mm; Dionex) in combination with a CarboPac PA guard column (2 mm ID × 25 mm) and a ISC5000 ED detector (Dionex) in the PAD mode. A flow rate of 0.3 $mL.min^{-1}$ was used with the following gradient of 0.1 M sodium hydroxide (solution A) and 1 M sodium acetate in 0.1 M sodium hydroxide (solution B): 0–20 min, 0–20% B; 20–26 min washing step with 100% B; 26–41 min, equilibration with 100% A. Ten µl of sample was injected each time.

Organic acids analysis

A GC analysis was performed on a TRACE™ GC Ultra Gas Chromatograph system coupled to a FID detector (Interscience, Breda, The Netherlands) to quantify the SCFA production during fermentation:[12] 50µl of diluted sample (0.42 $mg.ml^{-1}$) or standard (1 $mg.ml^{-1}$ to 0.125 $mg.ml^{-1}$) mixed with 50µl of 0.15 M oxalic acid stood for 30 min before addition of 150 µl of water. Next, samples (1 µl) were injected to a CP-FFAP CB column (25m x 0.53mm x 1.00 µm, Agilent, Santa Clara, CA, USA). The temperature profile was as follow: start at 100°C, increase to 155°C at 5°$C.min^{-1}$, and held at this temperature 1 min. GC data was processed using the Xcalibur® software (Thermo Scientific).

The intermediate organic acids (lactate and succinate) were quantified on a Dionex Ultimate 3000 HPLC (Dionex) equipped with an ion-exclusion Aminex HPX – 87H column (7.8 x 300mm) combined with a guard column (Bio-Rad, Hercules, CA, USA), and an RI-101

refractive index detector (Shodex, Kawasaki, Japan). The mobile phase was 5mM H_2SO_4, the flow rate was 0.6 $mL.min^{-1}$ and the elution temperature was 65°C. Samples (10µl - 0.42 $mg.mL^{-1}$) were injected onto the column. The concentrations of organic acids were expressed as $\mu mol.mg^{-1}$ of GOS.

Microbiota composition analysis

The Intestinal (I)- Chip, developed at TNO (Zeist, The Netherlands), was used to investigate the composition of the microbiota. This DNA based microarray enabled the detection of over 400 bacterial targets from the human large intestinal microbiota. Total faecal DNA from samples collected was isolated, amplified, purified and hybridized as described previously.[11] The hybridization took place on a microarray constructed and validated as described before,[13] using intestinal bacteria primers instead of oral primers. Imagene 5.6 software (BioDiscovery, Marina del Rey, CA, USA) was used to analyse the results. Genes with a signal intensity higher than 3 (>10^5 bacteria) in more than 10 samples were used to describe the bacterial fingerprint.

Total bacteria and Bifidobacterium spp. quantification using quantitative PCR

Quantitative PCR (qPCR) was performed to investigate the variation in the number of total bacteria and of *Bifidobacterium* spp. during fermentation. Primers used to measure the number of total bacteria were the universal primers 16S-uni-II-R [10 pmol.µl-1] (GGA CTA CCA GGG TAT CTA ATC CTG TT) and 16S-uni-II-F [10 pmol.µl-1] (TCC TAC GGG AGG CAG CAG T), and probe 16S-uni-II [5µM] (6FAM-CGTATTACCGCGGCTGCTGGCAC-TAMRA). The primers to measure the number of *Bifidobacterium* spp. were 16S-Bif-F [10 pmol.µl-1] (GGA GCA TGC GGA TTA ATT CG), 16S-Bif-R [10 pmol.µl-1] (GAC CAT GCA CCA CCT GTG AAC), 16S-Bifspec (6FAM-CTG GGC TTG ACA TGT T) (Applied Biosystems, Bleiswijk, The Netherlands). The amplification was performed with 5 µl DNA sample and 25 µl q-PCR mixture.10 Total microbial faecal DNA was diluted 1:10 to quantify the number of total bacteria and 1:100 to quantify the number of *Bifidobacterium* spp. The experiment was performed using the 7500 Fast Real Time PCR system (Applied Biosystems) at settings previously described.[11]

DNA of the microbiota from the control and *Bifidobacterium longum* was used as quantitative standards for total bacteria and *Bifidobacterium* spp., respectively (1 $fg.\mu l^{-1}$ to 1 $ng.\mu l^{-1}$).

Results

Fractionation of GOS and oligosaccharide identification

GOS was fractionated based on de degree of polymerisation (DP) using SEC. Each pool consists majorly of oligosaccharides of one DP, with minor contamination of the preceding or following DP, as determined by MALDI-TOF-MS (data not shown). The composition of GOS (weight percentage) was 3% DP1, 12% DP2, 49% DP3, 20% DP4, 11% DP5, 4% DP6, <1% DP>6. Monosaccharides and oligosaccharides of DP>6 were excluded as monosaccharides are absorbed in the upper part of the gastro-intestinal tract and are not relevant for the gut fermentation, while the abundance of oligosaccharides of DP>6 was too low to obtain a sufficient amount needed for the fermentation experiments. The fractions DP2 to DP6 were further analysed with HPAEC (Figure 1A). As it can be seen, the complexity of the elution pattern increases with increasing DP, which confirms the presence of high numbers of isomeric structures present in GOS.[3] The pattern of peaks obtained per DP was comparable to previous data[3] (Figure 1B).

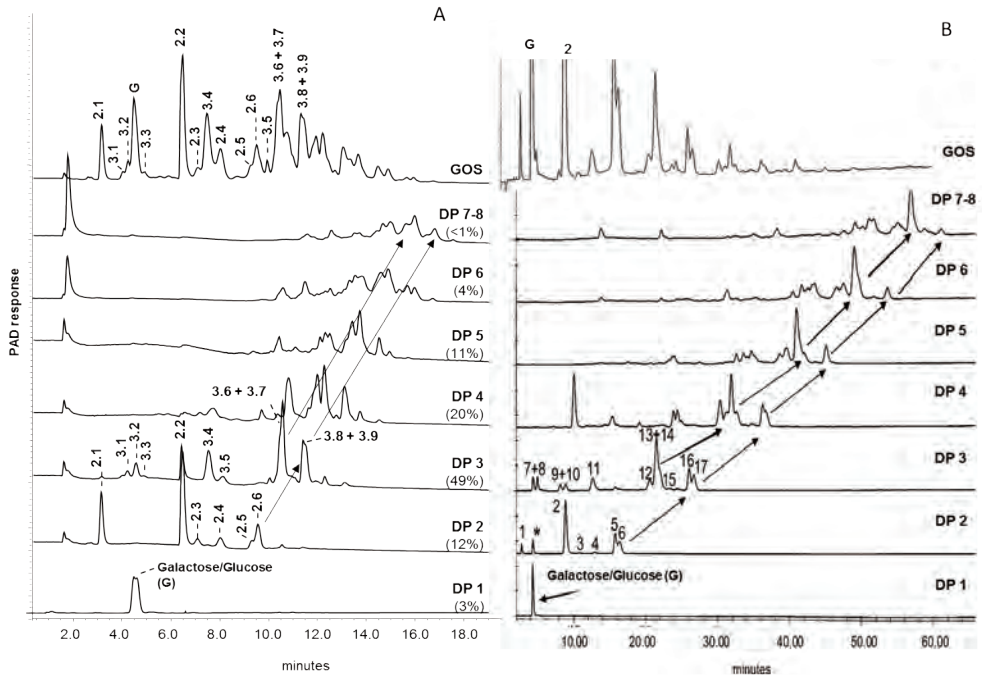

Figure 1: HPAEC-PAD chromatograms of GOS and size-fractions of GOS obtained by SEC from this study (A) and from Coulier *et al*[3] (B). Peak identification are given in Table 1. Ratio of each size-fraction in the GOS complex mixture is indicated in figure A (weight %).

By comparing the two patterns, the oligosaccharides from the DP2 fraction and most oligosaccharides from the DP3 fraction could be identified (Table 1). For the DP2 fraction, β(1-2), β(1-3), β(1-4), β(1-6) as well as α- or β(1-1) linkages were identified. For the DP3 fraction, identified structures were structures present in the DP2 fraction having an additional β-linked Gal, mostly at the O-4 position, added to the non-reducing terminal residue. For the fractions DP4 to DP6, it was more difficult to fully identify individual structures due to the complexity of the pattern. The different size-fractions obtained were now used in an *in vitro* fermentation to study their effect on the composition and metabolic activity of healthy adult microbiota with and without amoxicillin treatment.

Table 1: Identities and levels of DP2 and DP3 oligosaccharides in GOS (adapted from Coulier *et al*).[3]

Peak in this study	Peak from Coulier *et al*[3]	Compound	wt % in DP2
2.1	1	β-D-Gal-(1↔1)-α-D-Glc + β-D-Gal-(1↔1)-β-D-Glc	7
2.2	2	β-D-Gal-(1→4)-D-Glc (lactose)	27
2.2	2a	β-D-Gal-(1→6)-D-Glc (*allo*-lactose)	15
2.2	2b	β-D-Gal-(1→4)-D-Fru (lactulose)	5
2.3	3	β-D-Gal-(1→3)-D-Gal	1
2.4	4	β-D-Gal-(1→4)-D-Gal	3
2.5	5	β-D-Gal-(1→3)-D-Glc	26
2.6	6	β-D-Gal-(1→2)-D-Glc	16
3.1, 3.2, 3.3	7 + 8	?[a]	
-	9 + 10	?[b]	
3.4	11	β-D-Gal-(1→4)-β-D-Gal-(1→6)-D-Glc *or* β-D-Gal-(1→6)-β-D-Gal-(1→4/6)-D-Glc[c]	
3.5	12	β-D-Gal-(1→4)-β-D-Gal-(1→6)-D-Glc *or* β-D-Gal-(1→6)-β-D-Gal-(1→4/6)-D-Glc[d]	
3.6 – 3.7	13 + 14	β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc + β-D-Gal-(1→4)-β-D-Gal-(1→4)-Fru[e]	
-	15	β-D-Gal-(1→6)-β-D-Gal-(1→2)-D-Glc	
3.8	16	β-D-Gal-(1→4)-β-D-Gal-(1→3)-D-Glc	
3.9	17	β-D-Gal-(1→4)-β-D-Gal-(1→2)-D-Glc	

[a] Total of four small peaks, all containing (1↔1) linkages in their structure.
[b] Total of two small peaks, all containing (1↔1) linkages in their structure.
[c] Coelution with one small unknown peak.
[d] Coelution with two small unknown peaks.
[e] Coeluting peaks.

Impact of GOS addition on the microbiota composition

Changes in the microbiota composition during *in vitro* fermentation were studied by quantifying the levels of total bacteria and *Bifidobacterium* spp. and by interpreting the microbiota fingerprints obtained with the I-chip.

Total bacteria and Bifidobacterium spp. quantification

For the non-treated microbiota (NT-MB), the total numbers of bacteria and of *Bifidobacterium* spp. after 24h of fermentation were $10^{9.1}$ and $10^{6.2}$ copies.g^{-1} faeces, respectively (Table 2). Addition of GOS and its sub-fractions resulted in increase of the number of total bacteria after 24h of fermentation (about +0.7 $\log_{10}$). The addition of GOS and of the DP3 fraction tended to induce the highest *Bifidobacterium* spp. increase (about +3.8 $\log_{10}$), while the DP4 and DP5 fractions tended to induce the lowest increase (about +3.0 $\log_{10}$). This bifidogenic effect was still visible after 48h of fermentation.

For amoxicillin-treated microbiota (AMX-MB), the total bacteria was $10^{7.6}$ copies.g^{-1} faeces, so 1.5 $\log_{10}$ lower than in NT-MB due to the action of AMX during 24h of fermentation. A recovery of the total bacteria was observed after 48h of fermentation, reaching $10^{8.1}$ copies.g^{-1} faeces, which was about 1 $\log_{10}$ lower than the total number of bacteria for NT-MB. Addition of GOS and its sub-fractions to the AMX-MB did not influence the total number of bacteria during the fermentation time studied. Furthermore, *Bifidobacterium* spp. were 1.7 $\log_{10}$ lower due to the action of AMX during 24h of fermentation as compared to NT-MB. Addition of GOS and its sub-fractions induced growth of *Bifidobacterium* spp. after 24h of fermentation (about +0.9 $\log_{10}$). No major effect among the sub-fractions was observed within 24h of fermentation. After 48h of fermentation, however, it was clear that *Bifidobacterium* spp. was mostly stimulated upon addition of DP4 and DP5 fractions (about + 2.3 $\log_{10}$), while they were less stimulated upon addition of GOS of DP2 fraction (+1.4 $\log_{10}$).

In conclusion, addition of GOS and GOS fractions stimulated the growth of bifidobacteria for both non-treated and amoxicillin-treated microbiota. For NT-MB, the highest growth was obtained upon addition of GOS and DP3 fraction. In contrast, for AMX-MB, the highest growth was obtained upon addition of DP4 and DP5 fractions.

Table 2: Numbers of total bacteria and *Bifidobacterium spp. (Log10 copies*.g^{-1} faeces) during *in vitro* fermentation using adult inoculum treated with 1 µg.ml^{-1} AMX with and without addition of GOS and size-fractions of GOS as measured with qPCR.

		Total Bacteria		*Bifidobacterium*	
		non-treated	AMX	non-treated	AMX
no GOS	24h	9.1	7.6	6.2	4.5
	48h	9.0	8.1	6.5	5.9
GOS	24h	10.0	7.3	9.9	5.5
	48h	9.1	8.3	9.5	7.5
DP2	24h	10.3	7.4	9.5	5.4
	48h	9.4	8.3	9.2	7.2
DP3	24h	9.8	7.8	9.9	5.5
	48h	9.1	8.5	9.4	7.6
DP4	24h	9.3	7.2	9.2	5.3
	48h	9.4	8.2	8.9	8.3
DP5	24h	9.5	7.2	9.2	5.1
	48h	9.0	8.5	9.0	8.1
DP6	24h	9.5	7.1	9.5	5.5
	48h	9.1	8.3	8.6	7.9

Microbiota fingerprinting using the I-chip

The impact of GOS and its sub-fractions on the overall microbiota composition of NT-MB and AB-MB was determined using the I-chip (Figure 2). The impact was observed mainly within the bifidobacteria population for both NT-MB and AMX-MB. Independently of the addition of GOS and its sub-fractions, several *Bifidobacterium* spp. (*B. adolescentis, B. angulatum, B. longum, B. thermophilum*) were stimulated for NT-MB, whereas for AMX-MB mainly *B. longum* was stimulated. The bifidobacteria growth occurred within 24h of fermentation in NT-MB and within 48h of fermentation for AMX-MB.

For AMX-MB, the impact of the addition of GOS and its sub-fractions was also observed for *Lactobacillus* and *Enterobacteriaceae*. The abundance of *Lactobacillus gasseri* increased upon addition of GOS, DP2 and DP3 fractions for AMX-MB as compared to NT-MB. The abundance of some of the *Enterobacteriaceae,* which were observed to decrease upon AMX treatment, remain equal to the non-treated groups upon addition of DP2 and DP3 fractions of GOS. This observation indicates that high concentrations of small oligosaccharides may enhance the recovery of these potential pathogens up to a level as found in healthy adults, or limit the action of AMX.

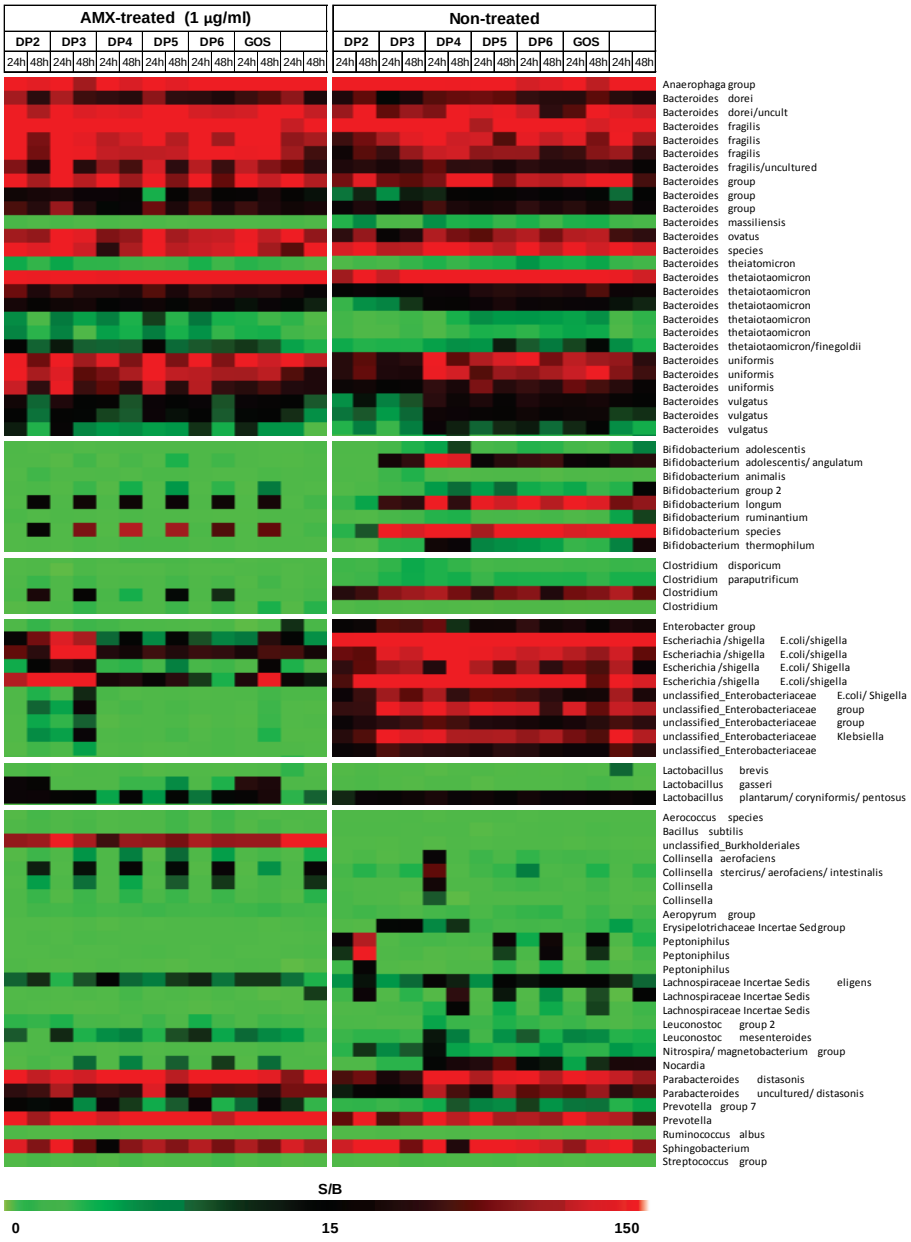

Figure 2: Bacterial fingerprints obtained with the I-chip revealing the impact of addition of GOS and size-fractions of GOS on non-treated and AMX-treated (1 µg.ml^{-1}) microbiota from healthy adults. Signal compared to the background (S/B): Green: below detectable level, Black: medium abundance, Red: high abundance.

Impact of GOS addition on the microbiota metabolic activity

Changes in the microbiota metabolic activity were studied by monitoring the degradation of GOS and its sub size-fractions as well as by monitoring the levels of organic acids during *in vitro* fermentation.

Degradation of GOS and specific size-fractions

The degradation of GOS and its sub-fractions during fermentation with and without AMX treatment were monitored using HPAEC (Figure 3).

The sub-fractions of GOS were mostly degraded (<10% remaining oligosaccharides) within 24h of fermentation, except for the DP3 fraction (>20% remained), for both NT-MB and AMX-MB. The degradation rate of the sub-fractions of GOS was influenced by the amoxicillin treatment. For NT-MB, the oligosaccharides preferentially degraded after 18h of fermentation were oligosaccharides of DP2 followed by oligosaccharides of DP3 and DP6, and at last oligosaccharides of DP4 and DP5. At 24h of fermentation, oligosaccharides of DP3 were still present, most likely because of the degradation of oligosaccharides of DP4 to DP6. For AMX-MB, all oligosaccharides were degraded at a same speed within 24h of fermentation except for the oligosaccharides of DP3 that remained until 32h of fermentation.

The degradation of **GOS** was slower for AMX-MB (20% oligosaccharides remaining) as compared to NT-MB (<5% oligosaccharides remaining) after 24h of fermentation. For AMX-MB, oligosaccharides of DP2 largely accumulated till 24h of fermentation and were then degraded within 32h of fermentation. About 10% of oligosaccharides of DP3 could still be detected after 32h of fermentation. Oligosaccharides of DP4 to DP6 were completely degraded within 24h of fermentation. The differences in the degradation of GOS and its individual sub-fractions emphasise that DP4 to DP6 fractions were preferentially degraded for AMX-MB independently from the presence of smaller structures. Hence, oligosaccharides of DP4 to DP6 appeared to be a potential successful substrate to stimulate a higher recovery of certain *Bifidobacterium* species.

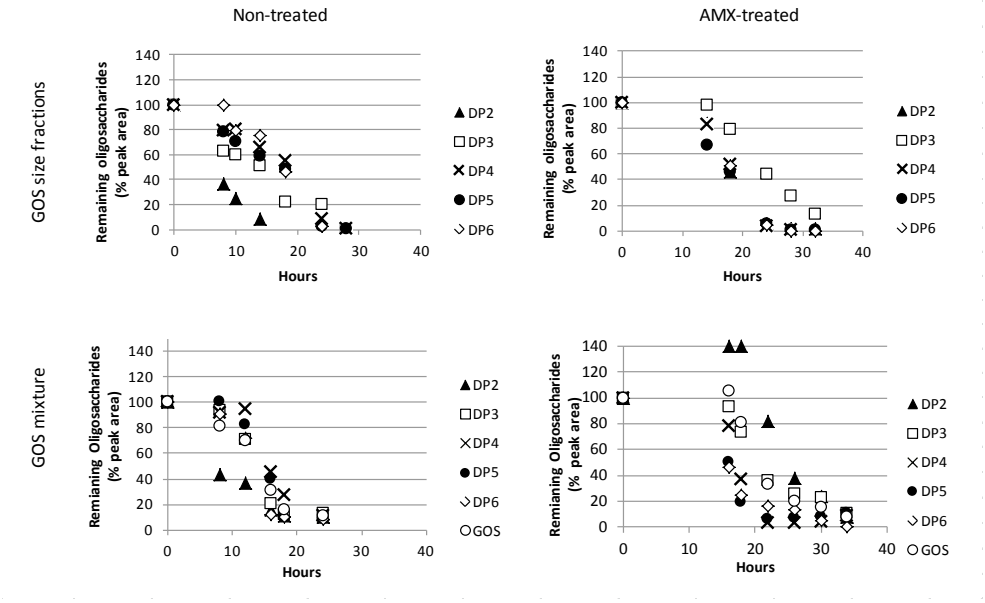

Figure 3: **Proportion (% peak area) of remaining oligosaccharides from GOS and individual size-fractions of GOS during *in vitro* fermentation using a non-treated and AMX-treated (1 µg.ml^{-1}) microbiota.** Concentrations per DP present in the size-fractions or in the GOS mixture were set to 100%.

With respect to the degradation of **isomeric** oligosaccharides present in DP2 fraction of GOS, the first major difference concerns the structures β-D-Gal-(1→4/6)-D-Glc and β-D-Gal-(1→4)-D-Fru that were completely degraded within 8h of fermentation by NT-MB whereas these structures were degraded last by AMX-MB (Figure 4). Another major difference concerned β-D-Gal-(1→4)-D-Gal that remained one of the last structure to be degraded by NT-MB, whereas it was degraded first by AMX-MB. The oligosaccharides with an α- or β[1-1] linkage were difficult to be degraded by both NT-MB and AMX-MB. With respect to the DP3 fraction of GOS, one of the major differences concerned β-D-Gal-(1→4)-β-D-Gal-(1→3)-D-Glc that remained one of the last to be degraded by NT-MB whereas it was degraded first by AMX-MB. Also, the oligosaccharides with α- or β[1-1] linkage were easily degraded by NT-MB, whereas some of them were last to be degraded by AMX-MB.

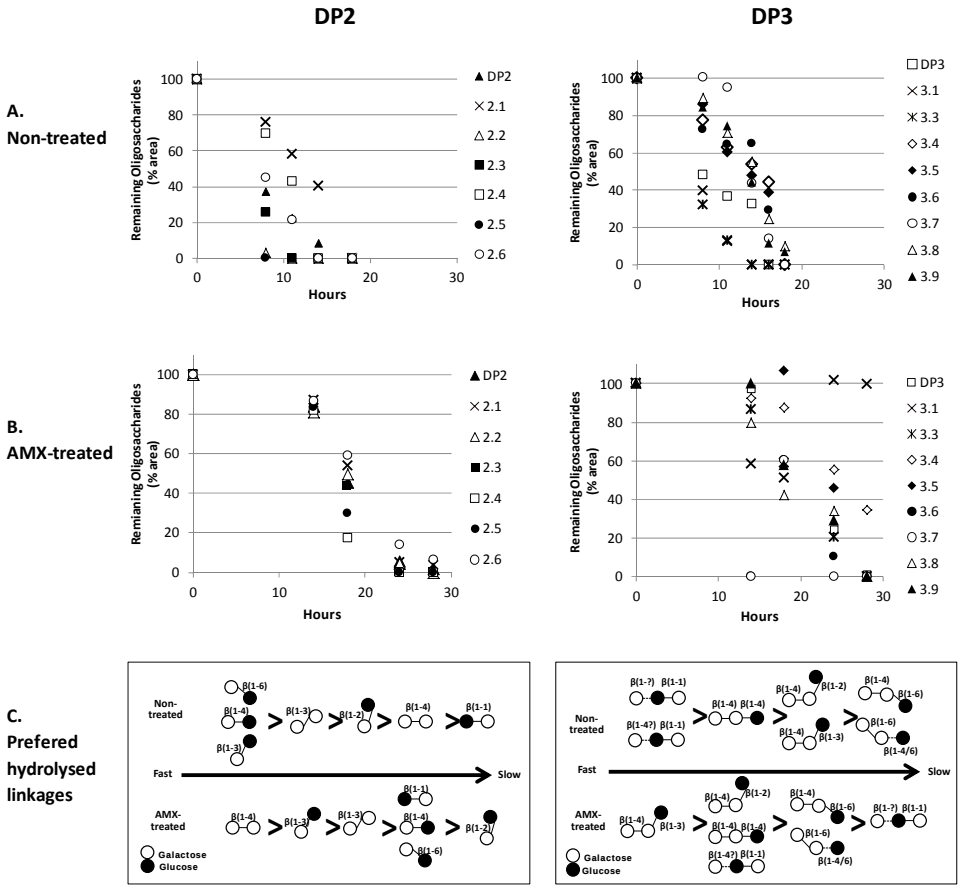

Figure 4: **Proportion (%) of remaining oligosaccharides of DP2 and DP3 during *in vitro* fermentation using a non-treated microbiota (A) and 1 µg.ml^{-1} AMX-treated microbiota (B), and schematic representation of the preferred hydrolysed linkages (C).** Identification of the isomeric structures in DP2 and DP3 fractions are given in Table 1.

Levels of organic acids

The total level of organic acids (OA), being intermediate acids, such as lactate (L) and succinate (S), and SCFA, such as acetate (A), propionate (P) and butyrate (B), was determined to be approximately 13 to 15 µmol.mg substrate^{-1} in most samples (Figure 5). The levels were lower (5 – 10 µmol.mg substrate^{-1}) for NT-MB and AMX-MB supplemented with oligosaccharides of DP2. The proportion of OA for NT-MB was similar for GOS and its sub-fractions (A:P:B:S:L = 75:6:5:11:2). One exception was that, for the DP2 fraction, more acetate and no propionate was detected. The proportion of OA changed upon amoxicillin treatment. Succinic accumulated (about 3.5 times more than for NT-MB) while less acetate was produced (about 1.5 times less than for NT-MB). Also, the proportion of OA for AMX-MB was influenced by the DP: Butyrate was detected (around 5%) upon addition of oligosaccharides of DP4 to DP6 after 36h of fermentation for AMX-MB, while lactate was detected (around 7%) upon addition of oligosaccharides of DP2.

Overall, the proportion of OA was more influenced by the antibiotic treatment than by the size-fractions of GOS present. Nevertheless, it was clear for AMX-MB that lactate tended to be produced upon addition of small size-fractions and butyrate tended to be produced upon addition of larger size-fractions.

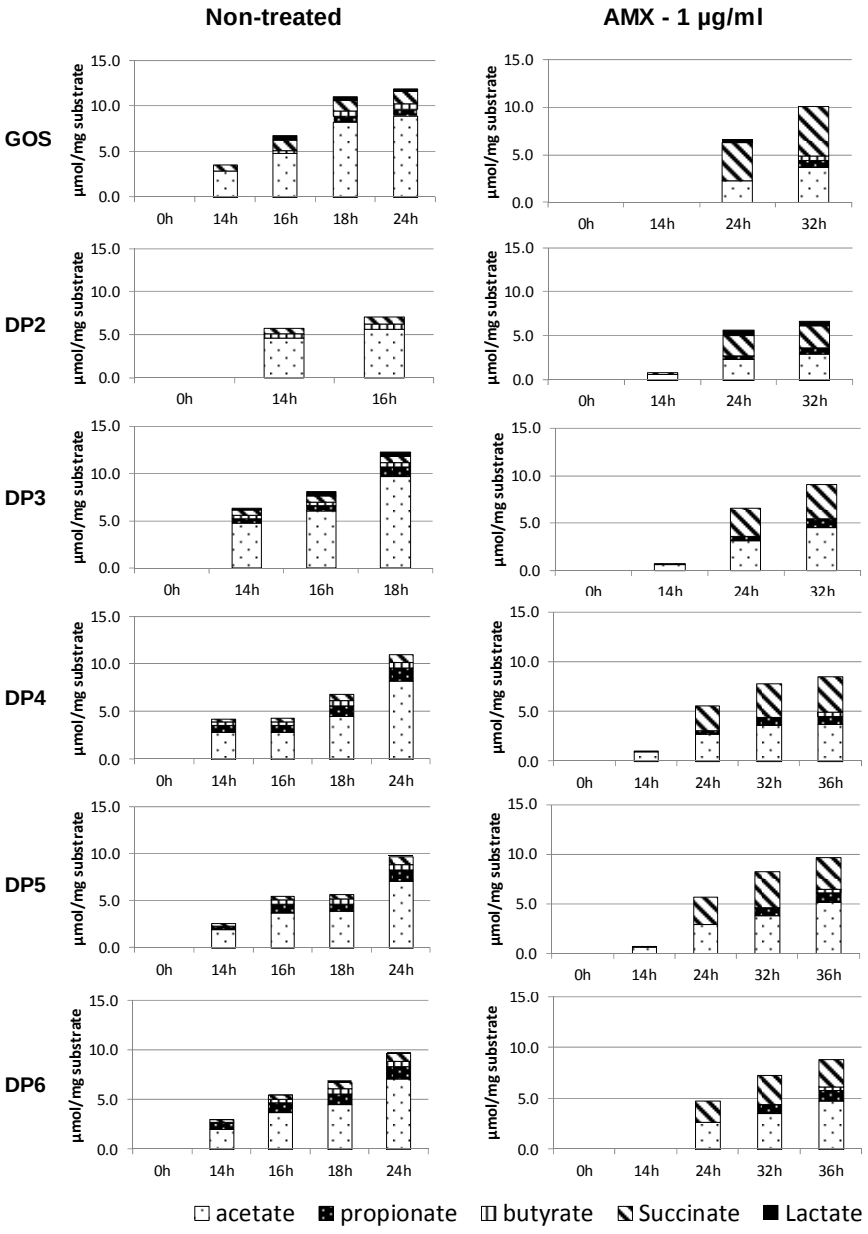

Figure 5: SCFA and intermediate organic acid levels during fermentation of GOS and size-fractions of GOS using a non-treated and AMX-treated microbiota (1 µg.ml^{-1}).

Discussion

Fermentation of GOS and size-fractions of GOS by non-treated microbiota

The complex mixture of GOS was completely fermented by the non-treated human microbiota. The bifidogenic effect of GOS as observed in many studies[14, 15] was, again, confirmed. Several *Bifidobacterium* spp. were stimulated, mainly *B. longum* and *B. adolescentis/angulatum.* These species are quite common in the healthy adult microbiota.[16] The same *Bifidobacterium* spp. were stimulated by the different GOS size-fractions. This contradicts previous studies reporting that specific size-fractions are preferentially degraded by specific *Bifidobacterium* spp.[4] However, those results were based on pure *Bifidobacterium* cultures. In a complex ecosystem as used in the present study, the different *Bifidobacterium* spp. present degrade synergically and simultaneously the substrate. Hence, no specific species is stimulated by a certain size-fraction. Nevertheless, it was clear in our study that the DP2 fraction was degraded the fastest and, together with DP3, tended to stimulate *Bifidobacterium* spp. more as compared to the DP4, DP5 and DP6 fractions. A preferred use of small DP oligosaccharides has also been seen for fructans, with FOS being faster degraded than Inulin.[17] Our observations suggest that small oligosaccharides were efficiently used by the *Bifidobacterium* spp. present. The reported carbohydrate uptake strategy of bifidobacteria[18] and the large amount of *intra-cellular* enzymes produced by these bacteria[18] corroborate this conclusion. The degradation rate of GOS and the DP3 fraction were similar. Since the highest recovery of bifidobacteria was also found upon the addition of this fraction, the current mixture of GOS, mostly consists of oligosaccharides of DP3 (49% w/w), is, therefore, well adapted to support bifidobacteria growth in non-treated microbiota.

Beside a preference for certain size-fractions, it appeared from this study that, within a specific size-fraction, some types of linkages are preferentially hydrolysed. The α- or β(1-1) linkages in DP2 fractions were hydrolysed the slowest. This result indicates that enzymes able to hydrolyse β(1-1) linkages were produced by certain *Bifidobacterium* spp.[19] These enzymes were, however, present in a low amount[18] or had a low activity. In contrast with a slow hydrolysation of α- or β(1-1) linkages in DP2 fractions, oligosaccharides of DP3 having an α- or β(1-1) linkage were preferentially hydrolysed (Figure 4). Previous literature reported that this specific feature might help the action of other β-galactosidases.[5] An accumulation of the oligosaccharides of DP2 with α- or β(1-1) linkage was, however, observed in our study (data not shown). This observation suggests that only the β(1-4) linkage between the two galactoses next to the β(1-1) linkage was easily cleaved. Furthermore, the β(1-4) and β(1-6) linkages were hydrolysed the fastest in DP2 individual

fraction. For GOS of DP3, it seems that β(1-6) branched GOS were more difficult to hydrolyse. This observation is in contradiction with previous results for pure *Bifidobacterium* cultures.[5] These authors reported a preference for oligosaccharides of DP3 with β(1-6) linkages over that of with β(1-4) linkages. The discrepancy is most likely due to the fact that the two *Bifidobacterium* spp. studied by these authors (*B. lactis* and *B. breve)* differ from the ones stimulated in our study (*B. longum* and *B. adolescentis)*.

The fermentation of GOS resulted in the production of SCFA.[14, 15] *Bifidobacterium* spp. are reported to produce lactate and acetate.[20] The diversity of the SCFA observed is illustrative for the synergic action of the microbiota to degrade and use intermediate degradation products. Only a small amount of lactate was detected, indicating that it was probably converted to SCFA, such as butyrate.[2μ] The SCFA profiles were similar upon the fermentation of GOS and of most GOS size-fractions, indicating the good functioning of the gut microbiota. In conclusion, GOS that mostly consists of oligosaccharides of DP3 is suitable to support *Bifidobacterium* spp. growth and to induce butyrate production in human microbiota.

Fermentation of GOS and size-fractions of GOS by AMX-treated microbiota

The AMX treatment clearly disrupted the microbiota composition. The main bacterial groups that were affected constituted of *Enterobacteriaceae, Clostridium, Bifidobacterium* and *Lactobacillus*, as reported before.[11] Addition of GOS and specific size-fractions mainly stimulated the recovery of *Bifidobacterium* spp. The recovery of *Bifidobacterium* spp. within 48h of fermentation was mainly due to the increase of *B. longum.* This species was shown not to be affected by an amoxicillin treatment in infants.[22] *B. longum*, therefore, had a potential to use available substrate to grow in the absence of competitors. The growth of this species tended to be higher upon addition of DP4-DP6 fractions than upon addition of GOS. Furthermore, oligosaccharides of DP4-DP6 were shown to be preferentially degraded by the amoxicillin-treated microbiota. As *B. longum* has already been reported to grow on DP5 and DP6 in a monoculture,[4] it indicates that *B. longum* is the main bifidobacteria species active in the microbiota after amoxicillin treatment and has most potential to grow. *B. longum* is one of the few bifidobacteria species that has a membrane bound endo-galactanase.[18] The fast degradation of the large fractions of GOS for AMX-MB and the fast degradation of the specific type of linkages β-D-Gal-(1→4)-D-Gal suggest that this enzyme was active. As the highest growth of *B. longum* was observed upon addition of these large fractions, it is likely that mainly this micro-organism benefits

from the degradation products. This support the hypothesis that *B. longum* has a mechanism to incorporate galacto-oligosaccharides.[18] Hence, we hypothesize that a faster recovery of *B. longum* upon AMX treatment would be possible upon addition of a GOS mixture enriched in oligosaccharides of DP4-DP5 and DP6 with a linear structure.

Other groups of bacteria were also stimulated in AMX-MB depending on the size of GOS present. The smaller size-fractions (DP2 and DP3) stimulated *Lactobacillus gasseri* and limited the decrease of *Enterobacteriaceae* usually observed due to the action of AMX. The growth of *Lactobacillus* could be beneficial for the host as it is a reported beneficial group of bacteria.[23] However, limiting the decrease of *Enterobacteriaceae* by AMX could be a risk for pathogen development and gut diseases.[24] Limiting the decrease of *Enterobacteriaceae* upon addition of small size-fractions might be an indication that 1) these bacteria can utilise the substrate themselves, 2) they can cross-feed on the degradation products released by the bifidobacteria, 3) oligosaccharides that bind to the bacterial cell wall[24] may limit AMX action. In contrast there are indications that GOS may have anti-adhesive activity and directly inhibit the adherence of *E. Coli* to the host epithelial cell surface.[25] In future research, it would be of interest to investigate whether and which specific GOS oligosaccharides can bind to pathogens in more details.

Amoxicillin treatment also disrupted the organic acid production as higher concentrations of lactic and succinic acid were observed. An increase of succinate upon antibiotic treatment was previously reported.[26] The changes in organic acids levels was influenced more by the antibiotic treatment than by the different size-fractions. It was striking though that lactate was higher upon low DP addition and butyrate tended to be higher upon larger DP addition. Oligosaccharides of DP2 are easily incorporated into bifidobacteria cell via specific transporters,[18] resulting in a high lactate and acetate production. The accumulation of lactate indicates the disruption of the lactate-utilising bacteria microbiota. The production of butyrate during fermentation whereas bifidobacteria do not produce this SCFA is an indication that addition of large size-fractions stimulated the recovery of butyrate-producing bacteria, most likely through cross-feeding.

Overall, in a non-treated microbiota the level of bifidobacteria tended to be higher upon addition of original GOS and low size-fractions, than upon addition of the large size-fractions. In an amoxicillin-treated microbiota the addition of GOS stimulated the recovery of bifidobacteria, mainly *B. longum* and induced butyrate production. The addition of large size-fractions of GOS resulted in a higher recovery of bifidobacteria and higher butyrate level than the low-size fractions. Hence, we conclude that in particular the presence of large oligosaccharides within GOS is responsible for their beneficial effect to counter the

negative impact of amoxicillin on the microbiota. It is, therefore, suggested to investigate the effect of a GOS enriched with large oligosaccharides on an amoxicillin treated microbiota.

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Chapter 5

Galacto-oligosaccharides positively impact the gut microbiota of healthy adults receiving amoxicillin treatment

Abstract

In a double-blind randomized parallel intervention study, the effects of the intake of galacto-oligosaccharides (GOS) on the gut microbiota of 12 healthy adults receiving amoxicillin (AMX), aged 18-45 years with a normal BMI (18-25 $kg.m^{-2}$), were determined. All subjects received AMX (375 mg; 3x per day) for 5 days and either GOS (n=6) or placebo (maltodextrin, n=6) (2.5 g; 3x per day) during and 7 days after the AMX treatment. Faecal samples were collected twice before starting the treatment and at days 2, 5, 8, 12, 19 and 26. Due to AMX treatment, a decrease of *Bifidobacterium* spp., an overgrowth of *Enterobacteriaceae* and a disruption of the metabolic activity of the microbiota (increase of succinate and mono- and oligosaccharides in faecal samples) were observed for both groups. Positive effects of GOS intake were observed on the total bacteria population and bifidobacteria levels. The total bacteria level was significantly higher ($p<0.05$) directly after AMX treatment upon GOS compared to placebo intake. Furthermore, the bifidobacteria level tended to be higher ($p<0.15$) during and was significantly higher ($p<0.05$) directly after the AMX treatment upon GOS compared to placebo intake. The bifidobacteria activity and subsequent cross-feeding of other microbiota species after the AMX treatment was reflected through the significant increase of butyrate ($p<0.05$) when GOS was consumed as compared to placebo. Despite the low number of subjects, our findings confirm previous results obtained *in vitro,* namely that GOS intake supports the production of butyrate and the recovery of the beneficial bifidobacteria from amoxicillin treatment.

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Introduction

In Europe, the median consumption of antibiotics was 18.3 Defined Daily Doses per 1000 inhabitants per day in 2010.[1] The main side effect of these medicines is antibiotic-associated diarrhoea (AAD) that occurs in 5-10% of outpatient cases and 10-35% of inpatient cases. Among those, the antibiotic amoxicillin (AMX) has one of the highest incidences.[2] Prebiotics, defined as "*selectively fermented ingredients that allow specific changes, both in the composition and/or activity of the gastrointestinal microflora that confer benefits upon host well-being and health*",[3] have been suggested to prevent this common side effect of antibiotics. However, literature data concerning prevention of AAD is not consistent,[4, 5] probably due to the variability in parameters among the studies. Parameters, such as prebiotics, antibiotics, dosages and age of subjects are known to have a high impact on the microbiota composition.[6]

Recently, an *in vitro* study using a fermentation screening-platform allowed the straightforward comparison of the impact of four often used antibiotics on healthy adult microbiota with and without the addition of galacto-oligosaccharides (GOS), a known prebiotic.[7] This study revealed that the recovery of bifidobacteria upon GOS addition was antibiotic and dose dependant. For instance, GOS did not impact the recovery of bifidobacteria after a clindamycin treatment, whereas GOS positively impacted the recovery of bifidobacteria after an AMX treatment. Further understanding of the impact of GOS on the gut microbiota *in vivo* is essential as the microbiota is considered as a key factor in human health.[8] Besides the action of antibiotics on the microbiota composition, antibiotics are known to negatively impact the metabolic activity of the microbiota as well.[9] An accumulation of monosaccharides and a high level of lactate and succinate was observed during *in vitro* fermentation using an AMX-treated microbiota supplemented with GOS.[7] However, the metabolic activity of an antibiotic-treated microbiota upon GOS addition has never been addressed *in vivo*. Most *in vivo* studies investigated the effect of fructo-oligosaccharides (FOS) and focused on the diarrhoea frequency and prevention of *Clostridium difficile* infection.

In this intervention study, we determined the effects of GOS intake on the microbiota of healthy adults during and after receiving an AMX treatment in order to investigate whether trends from previous *in-vitro* study[7] represents the situation in healthy humans as well. The impact of GOS on the microbiota composition was assessed by quantifying changes in total bacteria and bifidobacteria levels and by interpreting the microbiota fingerprints obtained with a phylogenetic microarray. The impact of GOS on the microbiota metabolic activity was assessed by monitoring the concentration of short-chain fatty acids, mono- and oligosaccharides in the faecal samples.

Material and methods

Subjects

Twelve subjects were recruited in Wageningen (The Netherlands) and surroundings. The subjects aged 18 to 40 years old, had a normal BMI (18.5 – 25.0 kg/m^2) and a western diet.

Due to the exploratory nature of this trial, no statistical examination of the number of participants was taken into account. Based on other studies investigating the microbiota functioning,[10-13] the selected number of 12 volunteers is considered sufficient to validate whether trends from previous *in-vitro* results can be detected in healthy humans as well. This number allows a first evaluation of the response of the treatment considering individual variation in the microbiota.

Subjects were excluded if they smoked, used drugs, had gastro-intestinal diseases themselves or in their families, travelled to Asian, African or Latin American countries in the last 6 months, had hypersensitivity or allergies to the products used in the study (amoxicillin, lactose), had history of allergies, or had hepatic disease or renal failure. Subjects were also excluded if they used other medications than N-acetyl-p-aminophenol (paracetamol) or acetylsalicylic acid (aspirin), used antibiotics in the last 3 months, had more than 3 antibiotic treatments in the last 2 years or consumed prebiotics or probiotics (a list of products was provided) in the last month before the study. Female subjects were also excluded if they used the contraceptive pill, were pregnant (including planning to be), gave birth in the last 6 months or were lactating. The study was conducted according to the guidelines laid down in the declaration of Helsinki and all procedures involving human subjects were approved by the Medical Ethics Committee (METC) of Wageningen University (registration number NL 42438.018.12). Written informed consent was obtained from all subjects. The study was registered in the U.S. national Institute of health clinical trial database (ClinicalTrials.gov no NCT01848535).

Supplements

The **antibiotic** Amoxicilline disper tablet 375 (Sandoz BV, Almere, The Netherlands) was provided by a local pharmacy. Amoxicillin (AMX) was investigated in this study because of its risk for diarrhoea and its frequent use.[14] A low dose of AMX (375 mg; 3x per day) and the shortest duration (5 days) of treatment were chosen to limit the risks of side effects for the volunteers. The **prebiotic** Vivinal® GOS (FrieslandCampina Domo, Borculo, The Netherlands) was specified by the supplier to have a dry matter of 75% (w/w), of which 59% (w/w) as galacto-oligosaccharides, 21% (w/w) as lactose, 19% (w/w) as glucose, and

1% (w/w) as galactose. The degree of polymerisation (DP) of the oligosaccharides within Vivinal® GOS ranged from 2 to 8. The dose of Vivinal® GOS (2.5 g; 3x per day) was chosen to be sufficient to have a bifidogenic effect and to be low enough to limit gastro-intestinal discomfort, such as flatulence.[15] The **placebo**, Maltodextrin SPG 30 (powder) (AVEBE U.A, Veendam, The Netherlands) was specified by the supplier to comprise 6% (w/w) glucose, 15% (w/w) maltose, 18% (w/w) maltotriose, 8% (w/w) maltotetraose, 12% (w/w) maltopentaose, 17% (w/w) maltohexaose and 24% (w/w) of higher oligosaccharides. The degree of polymerisation was similar to that of GOS. The prepared matodextrin syrup had a dry matter of 75% (w/v) and a density of 1.2. The differences in sweetness and viscosity between the prebiotic and placebo syrups were masked upon solubilisation in 150 ml orange juice.

Experimental design

The study was a double blind randomized parallel intervention study comprising 12 days of intervention and 14 days of follow-up (Figure 1). The 12 subjects were randomly divided into two groups: the first group received AMX and GOS (n=6), while the second group received AMX and placebo (n=6). AMX (375 mg; 3x per day) was given for 5 days. GOS or placebo (2.5 g; 3x per day) solubilised in 150 ml orange juice was given simultaneously to AMX for 5 days and was continued for 7 days after the AMX treatment ended. The intervention products were consumed at breakfast, lunch and dinner. The effects of GOS or placebo intake were monitored up to 14 days after the intake ended. This follow up period will also enable to investigate the resilience of the microbiota after the intervention period.

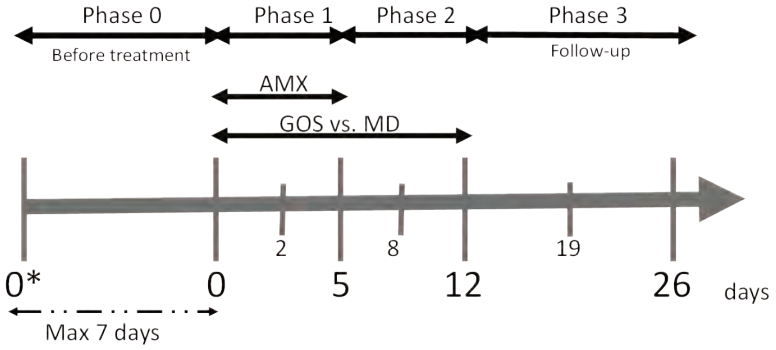

Figure 1: Scheme of the intervention study. AMX: amoxicillin, GOS: galacto-oligosaccharides, MD: Maltodextrin (placebo), 0* refers to samples collected at screening (1 to 7 day(s) before day 0).

Faecal sample collection and storage

Faecal samples were collected at 8 occasions: at screening (day 0*), day 0, day 2, day 5, day 8, day 12 day 19 and day 26. Subjects were free to deliver the faecal sample on the indicated day or the day after, and were free to deliver the faecal sample at home or at work. After delivering the first faecal sample (day 0*), the subjects had the opportunity to stop the study if they found the procedure too invasive. If the subjects wanted to go on with the study, the subjects were free to deliver the faecal sample of day 0 within 7 days after delivering the sample of day 0*. The subjects started the antibiotic treatment and GOS/placebo intake after delivering the faecal sample of day 0. Samples collected at day 0* (1x) and at day 0 (1x) were used as baseline to estimate the individual variability of the microbiota composition and activity.

At each occasion (except day 0), the subjects dropped the faecal sample in a specimen collection container placed on the toilet seat, collected part of the faecal sample using a spoon and gloves, and placed it in 1 plastic cup (120ml). The subjects filled 3 plastic cups using the same faecal sample up to maximum 60 ml. In case of limited faecal material available, the faecal sample was divided equally over the 3 cups. After collecting the faecal samples, the subjects placed the 3 cups in the coolest environment possible (usually 4˚C), phoned immediately the investigator who collected the cups within 1/2h and stored them at -80˚C. The first cup was used for DNA isolation, the second cup was used for measuring mono- and oligosaccharides and SCFA in the faeces and the last cup was used to perform a potential failed analysis again. At day 0, an anaerobic sachet was placed in the specimen collection container, roughly separated from the faecal sample by a plastic layer. The subjects closed the container air-tight, placed it at 4˚C, and phoned immediately the investigator who collected the container within 1/2h. In order to allow further fermentation studies, the faecal sample collected on day 0 was processed in an anaerobic chamber. Part of the faecal sample was divided among 3 cups as described above, and part was suspended in modified standard ileal efflux medium (SIEM)[16] containing 12% (v/v) glycerol. The faecal suspension and the cups were stored at -80˚C.

Stool and gastro-intestinal discomfort

Subjects kept a diary to record compliance to the treatment, stool frequency and consistency, and gastro-intestinal discomfort. Compliance to the treatment was reported by circling ‘yes’ or ‘no’ for the intake of antibiotics and/or the orange juice at breakfast

(7h00-9h00), lunch (12h00-14h00) and dinner (18h00-21h00). A number was indicated to report stool frequency. The Bristol scale was provided and used to score the faecal consistency from type 1 (separate hard lumps to type 7 (watery no solid pieces). Diarrhoea is defined by the world health organization[17] as the condition of having three or more loose or liquid bowel movements per day. Based on this definition, the scores on frequency and consistency were considered serious if subjects had ≥3 stools per day and/or scored ≥ type 6 on the Bristol scale. When subjects reported a serious score, the general practitioner was informed and decided whether the subject was withdrawn from the study or not. If subjects scored a type 6 stool for 3 days in a row, the subject was automatically withdrawn from the study and followed by the general practitioner until symptoms abated. The METC was informed about withdrawal. A 5-point scale from 1 (not at all) to 5 (very much) was used to score markers for discomfort (flatulence, bloating, heartburn, nausea). Free space was left to report other discomforts, medicine used and other remarks.

DNA isolation

The faecal sample collected in 1 cup was thawed overnight at 4˚C and homogenised by manual stirring. Total faecal DNA was isolated as described by Crielaard *et al*[18] with some minor adjustments: The faecal sample (100 mg) was mixed with 250 µl lysis buffer (Agowa, Berlin, Germany) and 250 µl zirconium beads (0.1 mm) and 200 µl phenol, before being introduced to a BeadBeater (BioSpec Products, Bartlesville, OK, USA) for 2 min. Because of a high level of impurities in the faeces, the phenol extraction was performed twice. The DNA isolation was performed in duplicate (from the same cup) for the samples of subjects 11 and 12 at day 0, day 5, day 12 and day 26 to investigate about the homogeneity of the sample collected in 1 cup.

Microbiota composition analysis

The Intestinal (I)- Chip, developed at TNO (Zeist, The Netherlands), was used to investigate the composition of the microbiota. This DNA based microarray enabled the detection of more than 400 bacterial targets from the human large intestinal microbiota. The total faecal DNA isolated was amplified, purified and hybridized as described previously.[16] The hybridization took place on a microarray constructed and validated as described before,[18] using intestinal bacteria primers instead of oral primers. Imagene 5.6 software (BioDiscovery, Marina del Rey, CA, USA) was used to analyse the results. Genes with a

signal intensity higher than 3 (>10^5 bacteria) in more than 10 individual samples were used to describe the bacterial fingerprint.

Total bacteria and Bifidobacterium spp. quantification

Quantification of the number of total bacteria and of *Bifidobacterium* spp. in the faecal samples was performed as previously described using quantitative PCR (qPCR).[7]

Water extractable carbohydrates and organic acids extraction

Extractions of water extractable carbohydrates and organic acids were performed according to Albrecht *et al* [19] and Jonathan *et al*,[20] respectively, with minor modifications. The thawed faecal samples were 20× diluted (w/v) with Millipore water (250 mg in 5 ml Millipore water). The diluted faecal slurry was centrifuged (3500 g, 15 min, T=4˚C). Aliquot of the supernatant (1 ml) was boiled (5 min) to inactivate the enzymes and filtered through a 0.22 µm membrane. The faecal extract obtained was used for mono- and oligosaccharides analysis and organic acids analysis.

Monitoring mono- and oligosaccharides in faeces

High performance Anion Exchange Chromatography (HPAEC) was used to quantify the mono- and oligosaccharides present in the faecal extracts. An ICS5000 HPLC system (Dionex, Sunnyvale, CA, USA), equipped with a CarboPac PA-1 column (2 mm ID × 250 mm; Dionex) in combination with a CarboPac PA guard column (2 mm ID × 25 mm) and a ISC5000 ED detector (Dionex) in the PAD mode was used. A flow rate of 0.3 mL.min^{-1} was used with the following gradient of 0.1 M sodium hydroxide (NaOH), 1 M sodium acetate (NaOAc) in 0.1 M NaOH: 0-3 min, 20-50 mM NaOH; 3-12 min, 50-75 mM NaOH; 12-15 min 100 mM NaOH; 15-35 min 0–200mM NaOAc in 0.1 M NaOH; 35–50 min washing step with 1M NaOAc in 0.1 M NaOH; 50-53 min 100mM NaOH; 53-68 min equilibration with 20 mM NaOH. Ten µl of sample was injected each time. Roughly, the monomer were considered to elute between 0 min and 12 min, while the oligosaccharides (including disaccharides) were considered to elute between 15 min and 35 min. Lactose was used as a quantification reference. The mono- and oligosaccharides levels were, thereby, expressed in mg saccharides (lactose equivalent) per gram of faeces. In the conditions stated above, lactose (0.01 mg.ml^{-1}) had a peak area of 22 nC.min^{-1}.

Organic acids analysis

High Performance Liquid Chromatography was performed to quantify SCFA (acetate, propionate, butyrate, valerate) and intermediate organic acids (lactate and succinate) present in the faecal extracts. A Dionex Ultimate 3000 HPLC (Dionex) equipped with an ion-exclusion Aminex HPX – 87H column (7.8 x 300mm) combined with a guard column (Bio-Rad, Hercules, CA, USA), and an RI-101 refractive index detector (Shodex, Kawasaki, Japan) was used. The mobile phase was 5mM H_2SO_4 and the flow rate was 0.6 $mL.min^{-1}$ at 65°C. Samples (10µl) were injected onto the column. Standards of known concentration (0.25 to 2 $mg.ml^{-1}$) were used for quantification.

Data analysis

The significance of the difference between treatments was assessed by a Student t-test. The significance of the difference in time was also assessed by Student t-test to address the effects of AMX on the microbiota. P-values were calculated assuming equal variance and two-tailed distribution. Correlations were considered significant at a P-value lower than 0.05.

The data matrix of bacterial fingerprints obtained with the I-chip was analysed with Significant Analysis Microarray (SAM) to identify markers significantly different between predefined groups and with a hierarchical clustering based on Euclidian distances (TM4 software, Rockville, MD, USA).[21] Predefined groups tested were “GOS group” vs. “placebo group” to determine the effect of GOS and “phase 0” vs. “phase 1” to determine the effect of AMX in both GOS and placebo groups. The statistical analysis was performed before breaking the treatment codes. Due to the small group studied, we did not only look at the statistical significant differences, but also at individual trends.

Results

Population characteristics

Out of the 12 recruited subjects, faecal samples of 2 subjects were not analysed because these subjects did not comply with the treatment assigned. One subject forgot to 3 times drink the juices and to twice take the antibiotic tablet. The other subject forgot to once take the antibiotic tablet. The studied population, therefore, consisted of 5 men and 5 women that aged 26 ± 4 years and had a BMI of 22.7 ±1.9 kg/m^2.

Effect of GOS on stool and gastro-intestinal discomforts

During phase 0 (before the treatment), the mean stool frequency (1,6 ±0.5) and consistency on the Bristol scale (type 3.4 ±0.5) were similar for both groups. Overall, the frequency of defecation and consistency of faecal samples were constant over 26 days, and hence did not statistically differ. On an individual level, the scores on frequency and consistency tended to increase (up to 3 stools per day and up to a consistency of type 5 on the Bristol scale between days 2 and 5) during phase 1 (AMX+GOS/MD) for 3 subjects (2 from the placebo group, 1 from the GOS group), before returning to initial values during phase 2 (GOS/MD). A change in the stool frequency and consistency (≥3 stools per day and/or ≥ type 6 on the Bristol scale) was observed for 2 subjects from the GOS group during AMX treatment (phase 1): One subject reported 1 loose stool of type 6 on days 2 and 3 and the other subject reported 3 loose stools of type 6 and type 7 on days 4 and 5. Based on the definition of the world health organization, the latter subject had diarrhoea and was withdrawn from the study after day 5. The data set of this subject (days 0 to 5) was included in the study as it might provide insight on why the subject suffered from diarrhoea.

Effects on markers for gastrointestinal discomforts, such as flatulence and bloating feeling, were reported were mild (maximum scores of 2 to 3 on the 5-points scales). No differences were seen between the GOS and placebo group.

Microbiota composition

Effects of GOS on the composition of AMX-treated microbiota were studied by quantifying the levels of total bacteria and *Bifidobacterium* spp. and by interpreting the microbiota fingerprints obtained with the I-chip

Quantification of number of total bacteria and Bifidobacterium spp.

During **phase 0** (before treatment; days 0* and 0), the number of total bacteria and the level of *Bifidobacterium* spp. were similar for both group, around $10^{10.3}$ copies.g^{-1} faeces and $10^{9.5}$ copies.g^{-1} faeces, respectively. Individual variation of numbers of total bacteria and *Bifidobacterium* spp. before treatment can be seen in table 1. One subject in the placebo group presented a much lower proportion of *Bifidobacterium* spp. (<1%) as compared to the other subjects (~9%).

Table 1: Number of total bacteria and *Bifidobacterium* spp. before treatment (Day 0* and Day 0). Values are expressed as mean ± standard deviation of $\log_{10}$ (copies.g^{-1} faeces). *refers to samples collected at screening (1 to 7 day(s) before day 0).

	GOS					**Placebo (MD)**				
Subjects	1	5	6	8	12	2	3	7	9	11
Total bacteria	**10.4** ±0.1	**10.1** ±0.4	**10.2** ±0.1	**10.5** ±0.1	**9.5** ±0.1	**10.4** ±0.1	**10.4** ±0.1	**10.4** ±0.2	**10.4** ±0.1	**10.4** ±0.1
Bifidobacterium	**9.8** ±0.6	**9.4** ±0.1	**10.1** ±0.3	**9.7** ±0.1	**9.5** ±0.2	**10.3** ±0.5	**9.6** ±0.1	**7.5** ±0.8	**10.0** ±0.3	**9.4** ±0.1

Figure 2 shows the variation of the normalised levels of total bacteria and *Bifidobacterium* spp. along the study excluding the data set of subject 7, which was considered not to be representative due the low level of *Bifidobacterium* spp. If the data set was included, the trends were similar, although less significant (Suppl. Data, Figure S1). During **phase 1** (AMX + GOS/MD; days 2 and 5), the level of total bacteria remained similar to the level at day 0 for both GOS and placebo groups. The level of *Bifidobacterium* spp. at days 2 and 5 significantly decreased for both groups as compared to day 0 ($p<0.05$) due to AMX addition, but it tended to decrease less for the GOS group (-0.5 $\log_{10}$) compared to the placebo group (-1 $\log_{10}$) ($p<0.15$). During **phase 2** (GOS/MD; days 8 and 12), the levels of total bacteria and *Bifidobacterium* spp. were significantly higher for the GOS group than for the placebo group ($p<0.05$). For the placebo group the level of *Bifidobacterium* spp. recovered to its initial value at day 12. For the GOS group, the level of *Bifidobacterium* spp. recovered to its initial value already at day 8 and was significantly higher at day 12 than at day 0 (+0.7 $\log_{10}$; $p<0.05$). During **phase 3** (follow up period; days 19 and 26), the increased levels of total bacteria and *Bifidobacterium* spp. for the GOS group returned to their initial levels, while the lowered level of total bacteria for the placebo group also returned to its initial levels.

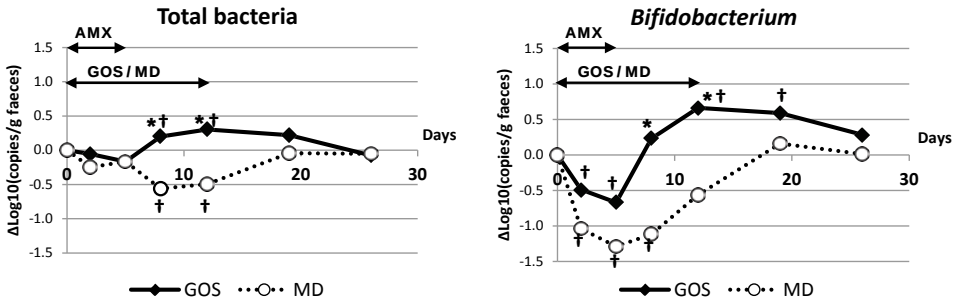

Figure 2: Variation in the number of total bacteria and *Bifidobacterium* spp. during and after treatment for healthy adults receiving AMX with GOS (n=5 until day 5, n=4 after day 5) or with placebo (MD; n=4). Values are expressed as mean of the normalised $\log_{10}$ (copies.g^{-1} faeces). Significant difference between GOS and placebo groups is indicated with * ($p<0.05$). Significant difference in time per treatment is indicated with † ($p<0.05$).

Bacterial fingerprints of the overall microbiota

The bacterial fingerprint of each subject during **phase 1** (before the treatment; days 0* and 0) is given in figure 3. Similar bacterial fingerprints were obtained for the 2 baseline samples for each individual indicating a good stability of the microbiota within 7 days. The main bacterial groups present in the microbiota of each individual were *Bacteroides, Bifidobacterium, Escherichia, Lachnospiraceae, Lactobacillus* and *Peptostreptococcaceae*. On an individual level, differences could be detected for both groups at species level, especially for *Bacteroides, Bifidobacterium*, and *Akkermansia*. Overall, the GOS group had an initial microbiota that was richer in *Bifidobacterium* spp., *Bacteroides uniformis* and *Alistipes putredinis* than the placebo group (Figure 4-A). The difference in bifidobacteria level was solely due to the low level of bifidobacteria for subject 7. If the data set from this subject was excluded from the SAM analysis, no difference in the bifidobacteria level was detected between the two groups.

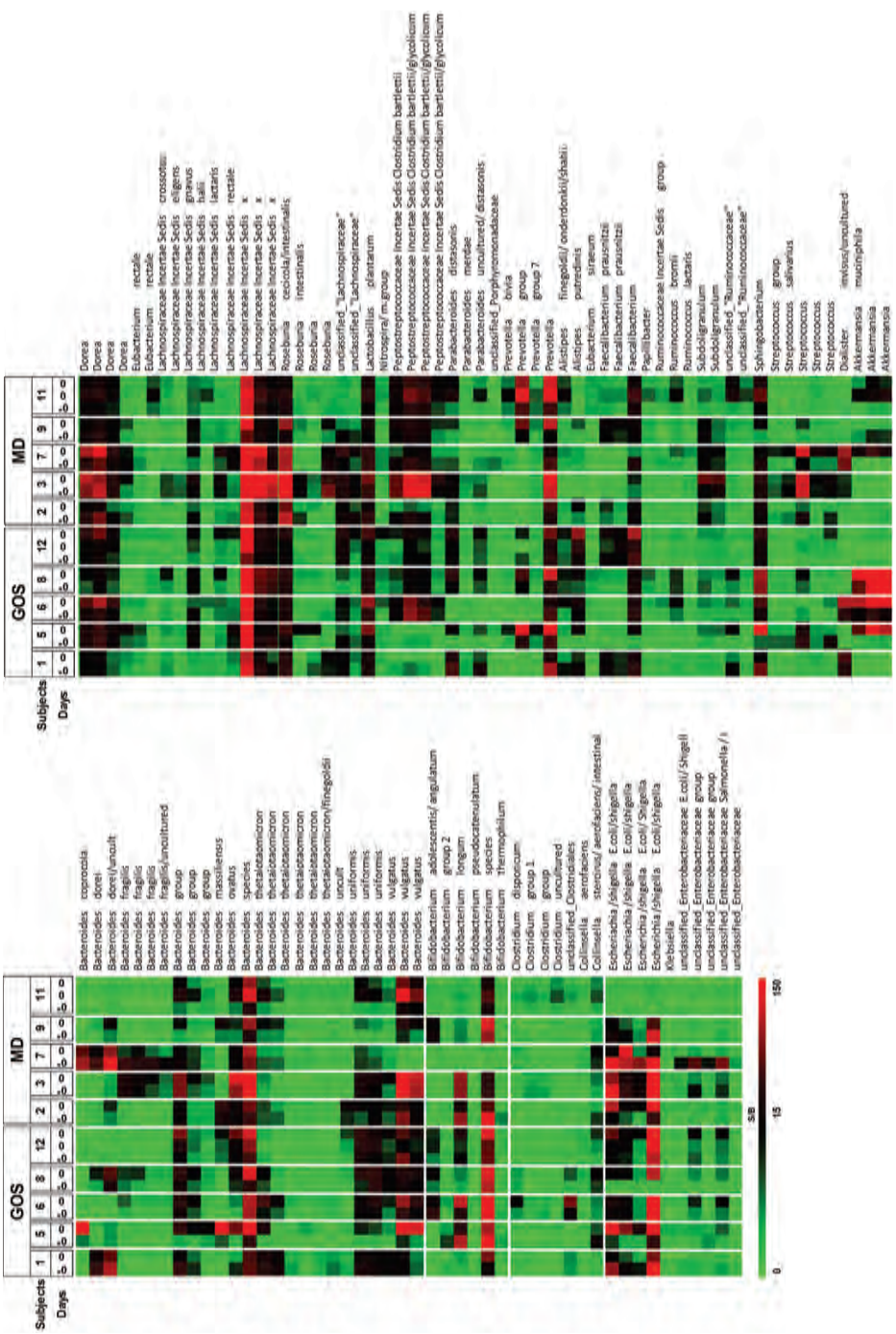

Figure 3: Bacterial fingerprints of the microbiota from healthy adults before treatment (Day 0* and Day 0) obtained with the I-Chip. Signal compared to the background (S/B): Green: below detectable level, Black: medium abundance, Red: high abundance. * refers to samples collected at screening (1 to 7 day(s) before day 0). Analysis of sample Day 0 was done in duplicate for subjects 11 and 12.

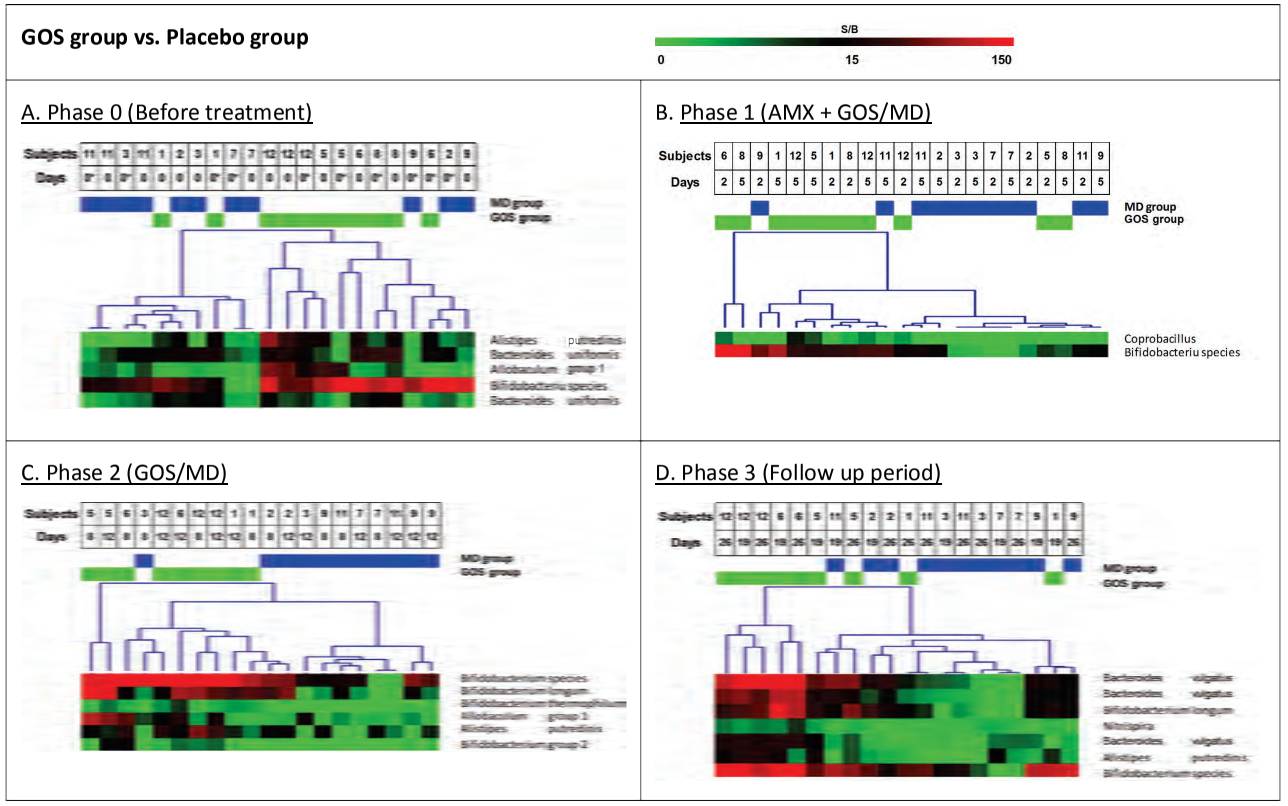

Figure 4: Bacterial groups significantly different between the GOS and placebo (MD) groups at different periods of the treatment as detected by SAM analysis. Four periods were defined: Phase 0 (A; before treatment), Phase 1 (B; AMX + GOS/MD), Phase 2 (C; GOS/MD), Phase 3 (D; follow up period). Signal compared to the background (S/B): Green: below detectable level, Black: medium abundance, Red: high abundance. * refers to samples collected at screening (1 to 7 day(s) before day 0). Analyses of samples Day 0, Day 5, Day 12, Day 26 were done in duplicate for subjects 11 and 12.

During the treatment, the impact on the overall microbiota is highly individual dependant. Some trends could be detected by SAM analysis between the GOS and placebo group as well as between the phases of the treatment. During **phase 1** (AMX + GOS/MD; days 2 and 5), the relative abundance of certain *Lachnospiraceae* was lower at days 2 and 5 than at day 0 (Figure 5 A-B) for both groups. The relative abundances of some *Enterobacteriaceae* were also changed: They increased significantly at days 2 and 5 than at day 0 for the GOS group (Figure 4 A) and for 3 out 5 subjects in the placebo group, despite not concluded as significant (Suppl. Data, Figure S2). As the changes of the relative abundances of certain *Enterobacteriaceae* and *Lachnospiraceae* were observed for both groups, they were probably due to the AMX action. The effects of GOS intake were detected on the level of *Bifidobacterium* spp. that were significantly higher for the GOS group than for the placebo group (Figure 4-B). During **phase 2** (GOS/MD; days 8 and 12), the relative abundances of *Bifidobacterium* spp., *B. longum* and *B. thermophilum* were significantly higher for the GOS group than for the placebo group (Figure 4-C). During **phase 3** (follow up period; days 19 and 26), while the microbiota in most subjects returned to its initial composition, a higher level of *Bifidobacterium* spp*., B. longum* and *Bacteroides vulgatus* was detected for the GOS group as compared to the placebo group (Figure 4-D). On an individual level, some bacterial groups (mainly *Bacteroides*) also differed from their initial levels (Suppl. Data, Figure S2).

Metabolic activity of the microbiota

The effect of GOS on the metabolic activity of AMX-treated microbiota was studied by monitoring the levels of SCFA and intermediate organic acids (OA), as well as the mono- and oligosaccharide (including disaccharides) levels in the faecal samples.

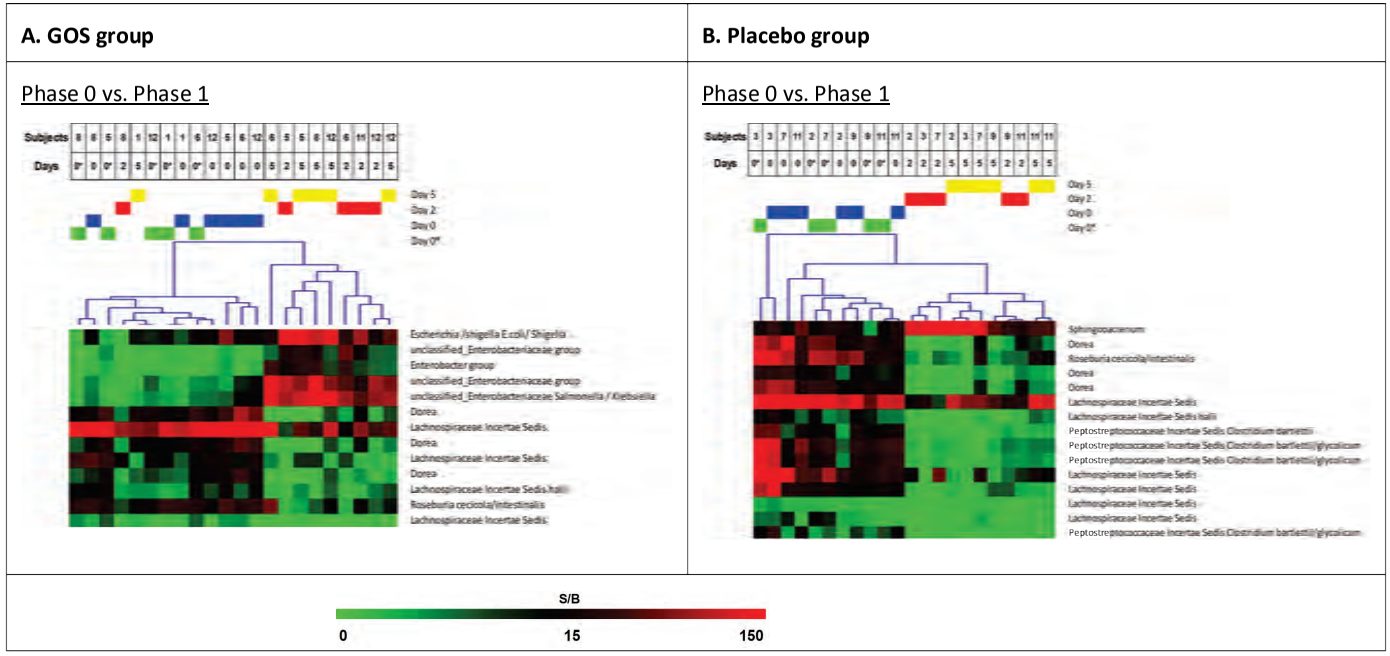

Figure 5: Bacterial groups significantly different between phase 0 and phase 1 for each the GOS and placebo (MD) groups as detected by SAM analysis. Signal compared to the background (S/B): Green: below detectable level, Black: medium abundance, Red: high abundance. * refers to samples collected at screening (1 to 7 day(s) before day 0). Analyses of samples Day 0, Day 5, Day 12 and Day 26 were done in duplicate for subjects 11 and 12.

Levels of OA in faecal samples

The levels of OA, being SCFA and intermediate OA, were measured in the faecal samples of subjects receiving AMX and GOS or placebo (Figure 6).

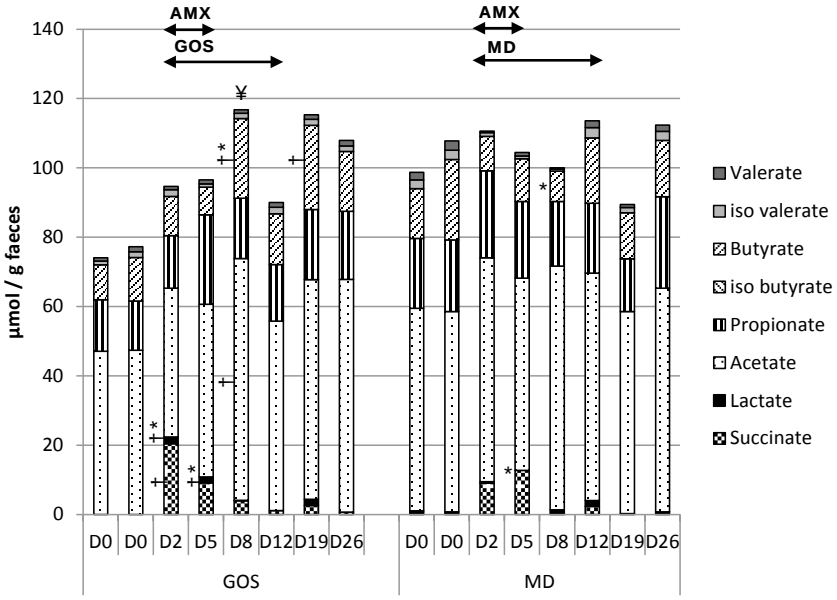

Figure 6: Levels of organic acids in the faecal samples of healthy adults receiving AMX and GOS (n=5 until day 5, n=4 after day 5) or with placebo (MD; n=5). Values are expressed as mean of µmol.g^{-1} faeces. Significant difference between GOS and placebo is indicated with * ($p<0.05$). Significant difference in time per treatment is indicated with † for individual organic acids and with ¥ for total amount of organic acids ($p<0.05$).

During **phase 0** (before the treatment), the total amount of OA in faecal samples was similar for both groups, around 89 ±13 µmol.g^{-1} faeces. The molar proportion (%) of acetate : propionate : butyrate : valerate : succinate : lactate (A:P:B:V:S:L) was around 59:20:16:4:0:0 for both groups. During **phase 1** (AMX + GOS/MD; days 2 and 5), the levels of succinate and lactate tended to increase ($p<0.15$) for the placebo group and was significantly higher for the GOS group as compared to day 0 ($p<0.05$). The proportion of succinate reached up to 22% of the total OA amount. As the succinate and lactate levels seem to increase for both groups, it was due to the AMX action. At day 5, the level of lactate for the GOS group was significantly higher than for the placebo group (2±3 vs. 0±0 µmol. g^{-1} faeces; $p<0.05$). **During phase 2** (GOS/MD; days 8 and 12), the level of butyrate

for the GOS group at day 8 was significantly higher than for the placebo group (26 ±18 vs. 9 ±6 µmol. g^{-1} faeces; ($p<0.05$), although not detected to be different at day 12 anymore. **During phase 3** (follow up period; days 19 and 26), SCFA and intermediate OA levels were similar to their respective initial levels for both GOS and placebo groups.

Mono- and oligosaccharide levels in faecal samples

Mono- and oligosaccharide (including disaccharides) levels were measured in the faecal samples using HPAEC and expressed in mg saccharide (lactose equivalent) per gram of faeces (Figure 7). Before the treatment, the saccharide level in the faecal sample was 23 ±4 mg.g^{-1} faeces, of which 85% monosaccharides and 15% oligosaccharides, for both GOS and placebo group. Overall, the amounts, and thereby the proportion, of mono- and oligosaccharides remained rather stable over the study, except at day 2. At day 2, the levels of saccharides tended to increase for the placebo group (39 ±26 mg.g^{-1} faeces; $p<0,15$) and were significantly higher for the GOS group (57 ±33 mg.g^{-1} faeces; $p<0.05$) as compared to day 0. At day 2, the proportion of monosaccharide increased to 30% and 24% in the GOS and placebo group, respectively. As the level of saccharides increased in both groups, it was due to AMX action. Overall, no differences were detected between the saccharides levels of the GOS groups as compared to the placebo group.

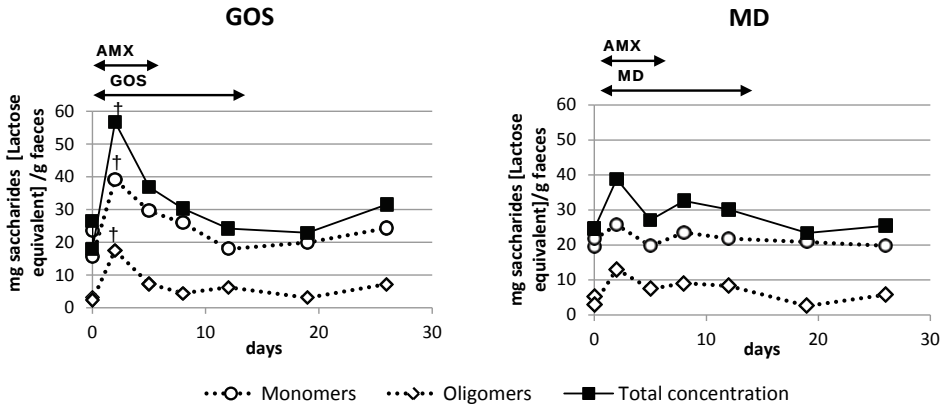

Figure 7: Mono- and oligosaccharide (including disaccharides) levels in the faecal samples of healthy adults receiving AMX treatment and GOS or placebo (MD) intake as measured by HPAEC. Values are expressed as mean of mg saccharide (lactose equivalent).g^{-1} faeces. Significant difference between in time per treatment is indicated with † ($p<0.05$).

Discussion

Starting conditions

Before the treatment (phase 0; days 0 and 0), subjects from both groups had a healthy complex microbiota composition. An individual diversity in the microbiota was observed at species level, as reported previously.[22] The number of total bacteria ($10^{10.3}$ copies.g^{-1} faeces) is in the lower range of what is usually detected (10^{9} to 10^{12} CFU.ml^{-1}),[23, 24] while the number of *Bifidobacterium* spp. ($10^{9.5}$ copies.g^{-1} faeces) is in line with literature.[25] As a result, the proportion of bifidobacteria (17%) is rather high as compared to literature (≤10%).[26] Regarding the activity of the microbiota before the treatment, the total amount of organic acids (102 ±15 µmol.g^{-1} faeces) and their relative concentration (acetate:butyrate:propionate:valerate of 59:20:16:4) in faecal sample were in line with literature, around 100 µmol. g^{-1} faeces total SCFA, [27] a low amount of intermediate organic acids and a molar ratio of acetate:butyrate:propionate of 60:20:20. [28]

Effect of GOS on the microbiota during AMX treatment

Microbiota composition

Although a low dose and a short duration of the AMX treatment were applied, changes in the microbiota composition were expected.[29] For both placebo and GOS groups, the mean of total bacteria remained stable during the AMX treatment, which is in contradiction with previous *in vivo* studies.[30, 31] In our study, a decrease up to 2 $\log_{10}$ was observed for 4 subjects (2 subjects from each group) at different days (day 2, day 5 or day 8 after the treatment ended) (data not shown). This indicates that the response to an antibiotic treatment depends on the individual.[32]

Rather than a decrease of total bacteria numbers, a shift in the bacterial composition was observed during the antibiotic treatment, as indicated before.[33] The decrease of the relative abundances of *Lachnospiraceae* and *Bifidobacterium* and the increase of the relative abundance of *Enterobacteriaceae* observed for 3 out of 5 subjects in the placebo group due to AMX treatment are consistent with literature.[29] Although no significant differences were found between the placebo and GOS groups, the intake of GOS to adults receiving the AMX treatment tended to limit the decrease of *Bifidobacterium,* but did not prevent either the decrease of *Lachnospiraceae* or the growth of *Enterobacteriaceae*. The low level of *Lachnospiraceae* as well as the higher level of *Bifidobacterium* for the GOS group as compared to the placebo group are in line with previous *in vitro* results.[7] The bifidobacteria level measured *in vitro* at 24h of fermentation was already similar or higher

than the level measured at t=0, whereas the bifidobacteria level was still below the initial levels during the AMX treatment in the *in vivo* study. The difference in rate of recovery might be explained by the use of a diluted system *in vitro*. It is also possible that the level of bifidobacteria measured during *in vitro* fermentation was higher because the effect of AMX diminishes in time in an *in vitro* assay. The relative high abundance of *Enterobacteriaceae* is in contradiction with previous *in vitro* data.[7] This discrepancy might be explained by a difference in the initial level of *Enterobacteriaceae in vitro* (low) as compared to *in vivo* (high). Possibly, the difference between the initial levels resides in the variability of the microbiota composition between inocula. It might also be that the pre-culture step of the *in vitro* study induced a preferred growth of *Enterobacteriaceae*.[34] Furthermore, the discrepancy might be due to a different contact between the antibiotic and the bacteria in the batch system (stronger) as compared to in the colon (less severe because of the presence of many vilis). Eventually, the relative high abundance of the *Enterobacteriaceae* should be confirmed by quantification, as microarrays only provide relative changes in the composition of the microbiota.

Overall, it can be concluded that GOS intake did not significantly prevent the changes in the microbiota composition observed *during* the AMX treatment, although GOS tended to limit the decrease of bifidobacteria.

Microbiota metabolic activity

The disruption of the microbiota composition upon AMX was also reflected in observed changes in the metabolic activity. The levels of mono- and oligosaccharides as well as the levels of succinate and lactate tended to increase at day 2 for the placebo group as compared to day 0. Most probably, the bacteria metabolic activity, including enzyme synthesis and fermentation activity, was reduced upon antibiotic treatment, resulting in a lower degradation of the oligosaccharides available,[35,36] an accumulation of monosaccharides, and a lower conversion of the intermediate organic acids into SCFA.[9] The changes in the metabolic activity was also observed upon GOS addition, as reported previously *in vitro*.[7] A significantly higher levels of mono- and oligosaccharides were measured at day 2 as compared to day 0, reflecting the fibre-enriched diet of the GOS group. In addition, the fermentation of GOS by bifidobacteria was reflected by the significant increase of lactate for the GOS group as compared to the placebo group. As the cross-feeding network is disrupted due to AMX action, lactate is accumulated instead of being converted to SCFA, e.g. butyrate.[37] In conclusion, despite an evidence for a partial utilisation of GOS by the microbiota, the overall metabolic activity of the microbiota was still majorly disturbed *during* AMX treatment.

Effect of GOS on the microbiota after AMX treatment

After the AMX treatment, it was clear that the level of bifidobacteria was significantly higher in the GOS group compared to the placebo group. Bifidobacteria species produce lactate and acetate.[37] The high level of butyrate for the GOS group compared to the placebo group suggests that GOS intake also stimulated the recovery of other bacteria of the ecosystem, probably through cross-feeding on lactate and acetate.[37] The significant increase of the level of total bacteria for the GOS group as compared to the placebo group reflects the recovery of the bifidobacteria and of other groups of the microbiota, e.g. butyrate-producing bacteria. Overall, the recovery of the microbiota composition and metabolic activity occurs faster upon GOS addition than placebo after the AMX treatment ended. An increase of bifidobacteria in infants receiving a mixture of FOS and Inulin was also observed *after* AMX treatment.[31] It, therefore, seems that the beneficial effect of GOS on the AMX treated microbiota becomes significant *after* the AMX treatment ended rather than *during* the AMX treatment.

Resilience of the microbiota

In the follow up period (3 weeks after the end of AMX treatment), the levels of total bacteria and of bifidobacteria as well the SCFA profile and the amounts of mono- and oligosaccharides in faeces returned to their initial levels. The resilience of the microbiota composition was reported to occur within a month in a previous study.[10] In the current study we showed that the resilience of the microbiota metabolic activity occurs shortly after the AMX treatment ended, despite the microbiota composition was still altered at that moment. This conclusion illustrates the reported redundancy of the functionality of the microbiota.[26]

Despite a high percentage of resilience observed on the bacterial fingerprints, individual differences at species level were detected, especially for the *Bacteroides* group. These differences at species level might indicate a long term impact of antibiotic on the microbiota.[38] Long term impacts are increasing with increasing exposure events and short resilience time between the subsequent treatments.[32]

Effect of GOS on stools and gastro-intestinal discomfort

In general, no effects on gastro-intestinal discomforts were reported regarding, bloating, flatulence, heartburn and nausea for both placebo and GOS groups. This was expected as

the dose of GOS (7.5g/day) was chosen in the range where GOS has been reported to have bifidogenic effect but limited side effects, such as bloating and flatulence (from 2.5 to 10.0 g/day).[15]

On an individual level, the two subjects that reported one loose stool or one time diarrhoea belonged to the GOS group. Several factors could have caused this effect, among which the disruption of the microbiota by AMX treatment (low number of *Bifidobacterium* spp., increase in *Enterobacteriaceae*), consumption of a particular or spoilt product, or another change in the diet of the subjects. Another speculation could be that the GOS added to the fibre already present in the subjects diet, might have been too much to be handled by the by AMX disturbed microbiota, resulting in an osmotic diarrhoea.

Concluding remarks

This *in vivo* study revealed the potential of GOS to limit the decrease of bifidobacteria during an AMX treatment and to stimulate the bifidobacteria growth after the AMX treatment ended. Considering SCFA profiles and mono- and oligosaccharides levels in faeces, it was also shown that the GOS addition stimulated the recovery of the microbiota activity after the AMX treatment has ended rather than already during the treatment. This research has provided data that confirm previous results obtained in *in vitro* study[7] and, thereby, justify further research involving targeted populations, such as patients, infants or elderly rather than healthy adults.

Acknowledgments

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Supplementary Data

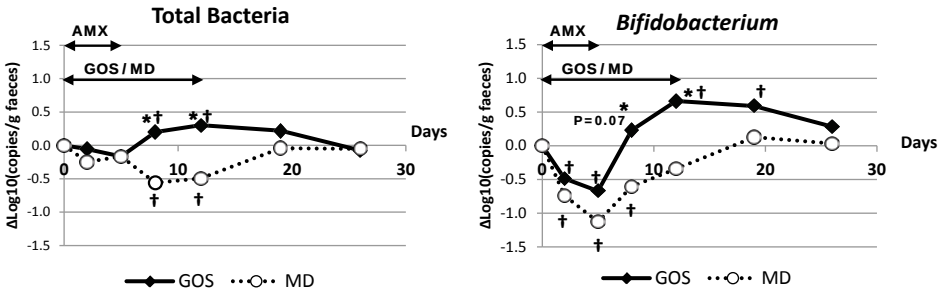

Figure S1: Variation in the number of total bacteria and *Bifidobacterium* spp. during and after treatment for healthy adults receiving AMX with GOS (n=5 until day 5, n=4 after day 5) or with placebo (MD; n=5). Values are expressed as mean of the normalised $\log_{10}$ (copies.g^{-1} faeces). Significant difference between GOS and placebo groups is indicated with * ($p<0.05$ or $p=0.07$ when indicated). Significant difference in time per treatment is indicated with † ($p<0.05$).

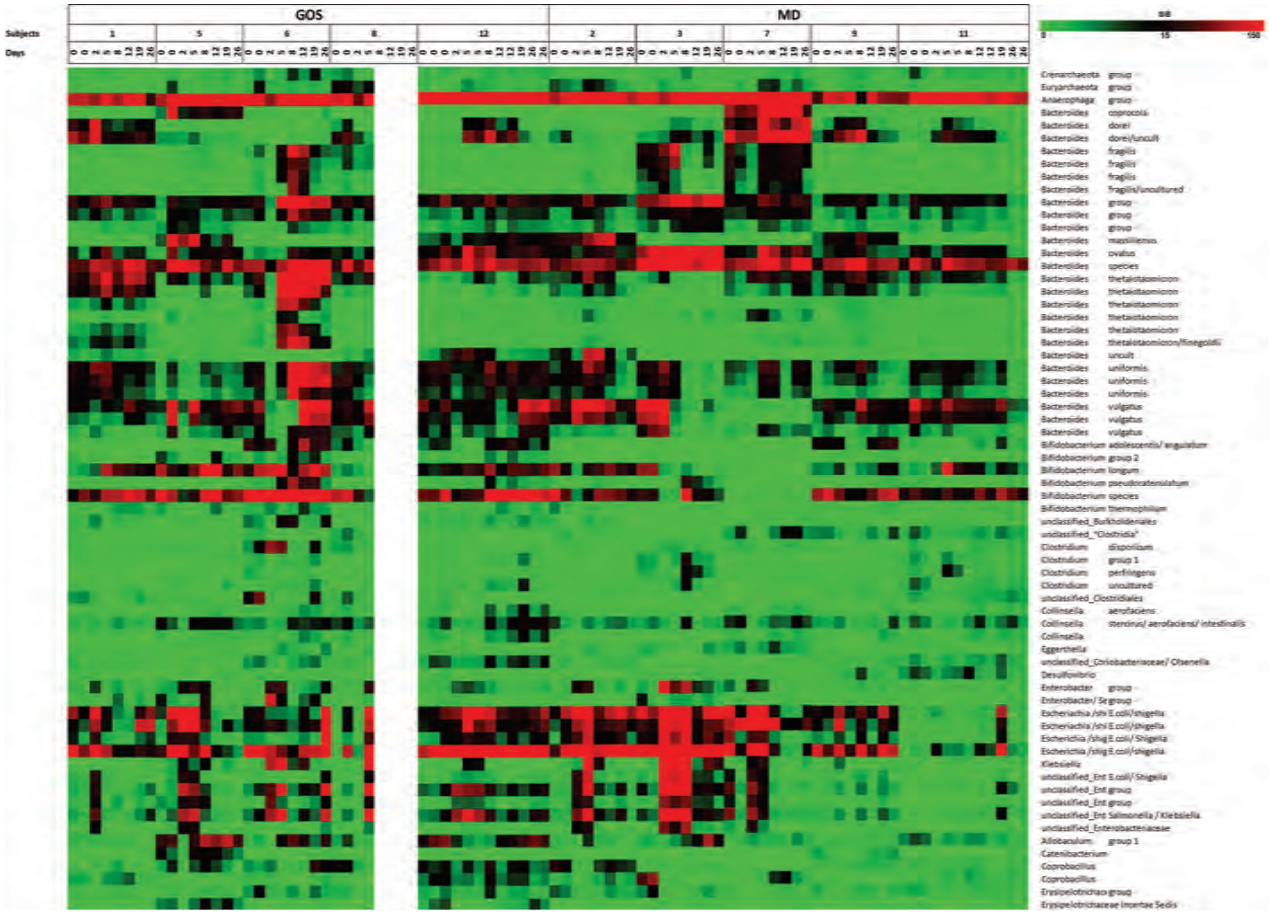

Figure S2: ***Bacterial fingerprints of the microbiota from healthy adults receiving amoxicillin treatment and GOS or MD supplementation obtained with the I-Chip.*** Signal compared to the background (S/B): Green: below detectable level, Black: medium abundance, Red: high abundance. * refers to samples collected at screening (1 to 7 day(s) before day 0). Analysis of sample Day 0 was done in duplicate for subjects 11 and 12.

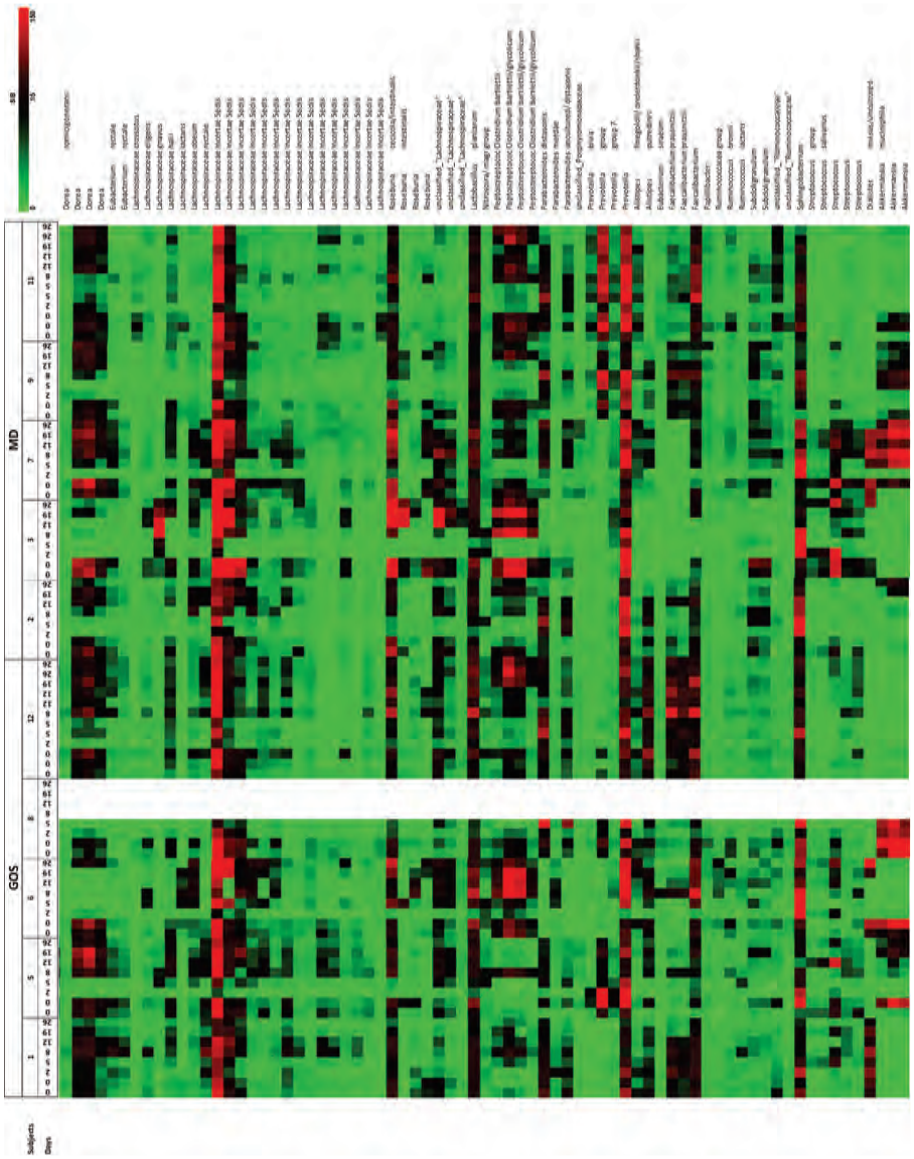

Figure S2 (continued): *Bacterial fingerprints of the microbiota from healthy adults receiving amoxicillin treatment and GOS or MD supplementation obtained with the I-Chip.* Signal compared to the background (S/B): Green: below detectable level, Black: medium abundance, Red: high abundance. * refers to samples collected at screening (1 to 7 day(s) before day 0). Analysis of sample Day 0 was done in duplicate for subjects 11 and 12.

Chapter 6

General discussion

Research aim and approach

Besides treating bacterial infections, antibiotic treatments also impact untargeted bacteria from the microbiota and, therefore, may lead to gut disorders. The side effects of antibiotics on the microbiota have been suggested to be reduced through the addition of prebiotics, which stimulate beneficial bacteria in the microbiota.[1] Nevertheless, results were not consistent in literature and more research was necessary. This thesis aimed to understand whether and by which mechanisms GOS, a known prebiotic, could help to counter the side effects of antibiotics on the human gut microbiota. The approach was to screen for potential positive effects of GOS supplemented to microbiota treated with different antibiotics using a fermentation-screening platform. Next, the specific combination(s) of GOS-antibiotic that showed a positive effects of GOS on the recovery of the antibiotic-treated microbiota were studied in more details. The impact of GOS was studied on the microbiota composition as well as the microbiota metabolic activity disrupted with antibiotic treatments, both *in vitro* and *in vivo*. In this chapter, the main findings of this PhD thesis, the methodology used, and implications for further research are discussed.

Main findings

Effects of GOS on antibiotic-treated microbiota are antibiotic and dose dependant

Part of the orally administrated antibiotics is known to reach the colon and disturbs the microbiota.[2] The impact of antibiotics on the gut microbiota could be successfully monitored using an *in vitro* fermentation screening-platform coupled to a phylogenetic microarray (Chapter 2). Overall, the shift in the bacterial composition increased with an increasing dose, while shifts in bacterial population were also noticed upon sub-Minimal Inhibitory Concentrations (MIC). Next to be dose dependant, the impact on the microbiota was also antibiotic dependant. The antibiotic and dose dependant shifts in the microbiota composition observed *in vitro* were similar to previous *in vivo* results.[3] When GOS was added, the main impact was observed on *Bifidobacterium* spp. level and to a lesser extent on *Lactobacillus* spp. level (Chapter 3). This was expected as GOS has been demonstrated to be specifically degraded by *Bifidobacterium* spp. and *Lactobacillus* spp.[4] Lactobacilli were stimulated upon GOS addition mostly when not initially affected by the antibiotic treatment. It concerned *L. brevis* in 1 $\mu g.ml^{-1}$ treated microbiota and *L. gasserri* in 10 $\mu g.ml^{-1}$ treated microbiota (Chapter 3). Similarly, when the bifidobacteria level was

not decreased upon antibiotic exposure (Ciprofloxacin and Doxycycline), their level obviously increased upon GOS addition. On the contrary, when the bifidobacteria level was dramatically decreased upon antibiotic exposure (Clindamycin), little or no recovery of their level was observed *in vitro* upon GOS addition. A third case was striking as the level of bifidobacteria recovered to the initial level or higher upon GOS addition whereas it was first decreased due to amoxicillin (AMX) action (Chapter 3). The positive effect of GOS on AMX-treated microbiota was confirmed in another *in vitro* experiment (Chapter 4) as well as a human trial involving healthy adults (Chapter 5).

Furthermore, different species of *Bifidobacterium* were observed to grow upon GOS addition depending on the antibiotic treatment, although the same inoculum was used for all conditions (Chapter 3). Clearly, this indicates that antibiotics impact certain bifidobacteria species more than others. Upon AMX treatment, *B. longum* was shown to be specifically stimulated upon GOS addition (Chapters 3 and 4). This species was also still present in faecal samples of infants receiving AMX, whereas in that study the level of other bifidobacteria was largely decreased.[5] In this thesis, the (least affected) *B. longum* could, therefore, benefit from the available GOS substrate and recover beyond its initial level.

Overall, it can be concluded that the recovery of beneficial bacteria as well as which specific species recover upon GOS addition are antibiotic and dose dependant. This suggests that specific combination of prebiotic-antibiotic should be considered to develop strategies countering the sides-effects of antibiotic on the microbiota.

Correlating the microbiota composition to its metabolic activity

Not only the microbiota composition is disrupted by antibiotic treatment, but subsequently the microbiota metabolic activity as well.[2] In this thesis, the metabolic activity was investigated by monitoring both the degradation of the substrate and the production of organic acids.

Oligosaccharide degradation

The degradation rate of GOS was monitored during *in vitro* fermentation using HPAEC (Chapters 3 and 4). A delay in the *in vitro* degradation of GOS was observed upon antibiotic exposure (Chapter 3), which is in line with the increased level of oligosaccharides present in faeces of healthy adults receiving amoxicillin (Chapter 5). The delay in GOS degradation was also observed upon antibiotic treatments that did not

decrease the abundance of living bifidobacteria (Chapter 3). The latter observation indicates that the limited degradation of GOS is not only due to the disruption of the bifidobacteria population but also due to the disruption of enzyme synthesis.[6] Once the degradation of GOS was started, the level of GOS degradation correlated with the level of bifidobacteria (and lactobacilli) growing (Chapter 3). Interestingly, specific size-fractions of GOS were preferentially degraded depending on the antibiotic used (Chapter 3) and within a specific size-fraction, different isomeric structures were preferentially degraded for AMX-treated microbiota as compared to non-treated microbiota (Chapter 4). The preferably degraded GOS structures correlated with the enzymes produced by the specific bifidobacteria species stimulated upon the antibiotic treatments (Chapters 3 and 4).

Overall, the recovery of specific species of *Bifidobacterium* is concomitant to the degradation of particular GOS structure, indicating that the composition of the GOS mixture could be optimized for each antibiotic treatment.

Levels of organic acids

Fermentation of dietary compounds by the microbiota results in the production of organic acids (OA), being intermediate OA, such as lactate and succinate, and end-products such as SCFA. No or low amounts of intermediate OA are usually detected as they are converted to SCFA.[7] Upon antibiotic treatment, the fermentation of GOS by the microbiota was negatively affected as indicated by the accumulation of monosaccharides and the decrease of the amount of SCFA (Chapter 3), which is consistent with literature.[8] The disruption of the SFCA production was in line with the level of disruption of the microbiota induced by the dose and the antibiotic used (Chapter 3). Interestingly, the ratio of SCFA : intermediate OA was shown to decrease from 10:1 in a non-treated microbiota to 0.7:1 in strongly antibiotic-disrupted microbiota (Chapter 3). This indicates that the conversion from lactate/succinate to SCFA was reduced. Different size-fractions of GOS could influence the type of SCFA or intermediate OA produced during *in vitro* fermentation using AMX-treated microbiota (Chapter 4). Nevertheless, the disruption of the SCFA and intermediate OA production occurred despite GOS addition during amoxicillin treatment, both *in vitro* and *in vivo* (Chapters 3-5). The altered concentrations of SCFA and intermediate OA are unusual in the colon and might have consequences on colon health. For instance, a depletion of butyrate might influence the colon epithelial cells as they derive 70% of their energy through the oxidation of this SCFA.[9]

In the human trial, the levels of most SCFA and intermediate OA returned to their initial levels after the AMX treatment ended. Interestingly, after the AMX treatment ended, a higher amount of butyrate was observed for the GOS group as compared to the placebo group, indicating the stimulation of the cross-feeding among bacteria (Chapter 5).

Overall, the SCFA: intermediate OA ratio during the AMX treatment is an indication of the level of disruption of the microbiota. GOS addition did not prevent the disruption of the metabolic activity of the microbiota during the antibiotic treatment in both *in vitro* and *in vivo* experiments, but stimulated the recovery the microbiota metabolic activity after the antibiotic treatment ended *in vivo*.

Cross-feeding activity in antibiotic-treated microbiota upon GOS addition

The degradation of GOS reflects the (recovered) activity of bifidobacteria. However, the accumulation of monosaccharides during the *in vitro* fermentation (Chapters 3-4) and during the AMX treatment in humans (Chapter 5) indicates that the activity of the other bacteria was disrupted despite the GOS addition.

The growth of other bacterial groups was expected upon GOS addition because of cross-feeding.[10] For instance, lactate and acetate produced by bifidobacteria can be utilised by butyrate-producing bacteria such as *Eubacterium*, *Roseburia*.[11] The growth of *Eubacterium* was detected upon GOS addition for ciprofloxacin treatment (Chapter 2). However, in most cases, the growth of butyrate-producing bacteria was not detected in our experiments despite an observed production of butyrate. Most likely, the changes in relative abundance of the targeted species were not detected with the microarray due to the low amount of these species (<1% total bacteria).[11, 12] In the human trial, growth of butyrate-producing bacteria was observed for certain individuals independently from GOS or placebo intake. Quantification by qPCR of these bacteria is recommended to conclude about the indirect effect of GOS on butyrate-producing bacteria.

The production of butyrate was shown to be influenced by the size of GOS added in an AMX-treated microbiota (Chapter 4). Butyrate production is of interest as butyrate is an SCFA known to have beneficial effect of colon health, such as reducing colon cancer.[13] Two other groups of bacteria than the butyrate producing bacterial group were stimulated in AMX-treated microbiota depending on the size of GOS present: *Enterobacteriaceae* and *Lactobacillus*. The relative decreased abundance of *Enterobacteriaceae* observed due to amoxicillin treatment (Chapter 2) was not influenced by the addition of the complex mixture of GOS (Chapter 3), but seem to decrease less upon the addition of small size-

fractions of GOS (Chapter 4). Also, the increase abundance of *Lactobacillus gasseri* was observed upon the addition of small size-fractions of GOS, which is in line with the increase of lactate production observed (Chapter 4). Changes in the abundances of bacterial groups upon the concentration of specific size of GOS might influence the host health. For instance, the growth of *Lactobacillus* could be beneficial for the host as it is a reported beneficial group of bacteria,[14] whereas, limiting the decrease of *Enterobacteriaceae* by AMX could be a risk for pathogen development and gut diseases.[15]

In conclusion, the disruption of the overall metabolic activity is observed despite GOS addition during AMX treatment, but the concentration of specific size of GOS appear to be of importance to stimulate other bacterial groups of the microbiota through cross-feeding.

Methodological considerations

Using in vitro models to predict in vivo responses

Human studies are considered to be superior to study the effect of a compound on the microbiota and subsequent human health. Nevertheless, they are expensive, low throughput and face ethical restrictions.[16] Furthermore, results are not easily comparable as responses are highly individual dependant.[17] Using a fermentation-screening platform with a standardized pool of adult faecal inoculum (n=8) enabled the study of the effects of antibiotics on the human gut microbiota and the effect of GOS on antibiotic-treated microbiota in a high-throughput approach (Chapter 2-4). The pooled inoculum, prepared according to Minekus *et al*,[18] was especially relevant in our study since it limited inter-individual variations and increased the probability to have a larger representation of potential bacterial species in the human colon. Furthermore, despite the fact that the microbiota composition of faecal material might not be representative of the composition of the microbiota in the proximal colon,[19] using a faecal inoculum allowed to investigate the effect of antibiotics and of GOS addition on a complex ecosystem taking cross-feeding interactions into account. Although the high-throughput approach allowed a straightforward comparison of the results, it also had some limitations. The main two limitations were (i) not mimicking the host-microbiota interactions, and (ii) influencing the different growth rates of the bacteria present due to the use of a diluted system. Nevertheless, the controls were similar among different *in vitro* experiments (Chapters 2-4) indicating the reproducibility of the model system. In addition, the beneficial effects of GOS on amoxicillin-treated microbiota observed *in vitro* were evidenced in a human trial (Chapter 5). Despite individual variability, the bifidobacteria level recovered upon GOS intake and the cross-feeding activity of the microbiota upon GOS intake was reflected by

the significant increase of butyrate level in faecal sample after the AMX treatment. It is, therefore, concluded that a batch fermentation model with a pool of faecal inoculum is reliable to predict generic impact on the microbiota of humans.

Study design and outcome of the in vivo study

Study design

A double-blind randomized parallel intervention study was conducted using 12 healthy adults receiving amoxicillin and GOS/placebo (Chapter 5). Healthy adults were recruited because the risk for adverse events was considered to be lower as their microbiota is more mature, diverse and stable as compared to patients, infants or elderly.[20, 21] The risks for diarrhoea do exist for healthy adults receiving amoxicillin treatment but are defined as "nuisance diarrhoea", which is a frequent loose and watery stool with no other complications. Severe adverse such as risk for colitis, which is a potential source of serious progressive disease, is rare (< 0,01%).[22] Furthermore, the dose selected for the *in vivo* trial (375mg 3x per day) was in the low range of commonly prescribed doses,[22] therefore limiting the risk for side effects. An option for future research is to recruit subjects that need antibiotic treatments for medical reason, although the lack of a baseline observation, the diversity of treatment regarding dose and duration as prescribed by the practitioner and the rush in getting started (no real time to inform the volunteer properly) should be considered to have a representative and volunteer-friendly study.

Outcome measured

The outcomes of the impact of GOS on antibiotic-treated microbiota measured were the changes in the microbiota composition of the faecal samples, especially the changes in bifidobacteria level, and the changes in the microbiota metabolic activity, being oligosaccharides and SCFA levels in the faecal sample. Since the microbiota composition is known to change along the colon,[19] and the SCFA produced are absorbed through the portal vein,[13] the measurements performed using the faecal sample might not be representative of the microbiota as present in the proximal colon. Nevertheless, the faecal sample is the only accessible material that can be measured in healthy humans considering ethical issues.

A high standard deviation was observed for SCFA and oligosaccharide levels in the faecal samples. This could be due to the fact that the diet of the subjects was not controlled. Due to a possible difference in the type of fibre and the fibre level in the diet of the two groups

the levels of oligosaccharides and SCFA measured in the faecal sample might have been influenced.[13] Further studies should control the diet of the subjects (at least the fibre intake) and have a non-digestible marker to better understand the effects of GOS on the metabolic activity. Nevertheless, a major strength of this study is that the results can be easily extrapolated to the general population as the real-life situation is represented. Another strength of the study resides in its double blind character. The effect of GOS on bifidobacteria level was significantly different between the two groups despite the limited number of people and the uncontrolled diet. This outcome gives strong evidence that prebiotic can help to recover the microbiota composition after amoxicillin treatment. Overall, this *in vivo* study had a strong experimental set up and, therefore, provided data to justify further large studies involving targeted population.

New perspectives for GOS addition to antibiotic-treated microbiota

In chapters 2-4, *in vitro* experiments were performed using adult inoculum. In addition, GOS was always added simultaneously to the antibiotic treatment. Since the microbiota composition is changing with the host age[20] and since *in vivo* data (Chapter 5) indicated that the time of addition of GOS might be of importance, supplementary studies were performed to investigate these issues. The results are described below.

Time of GOS addition

Upon amoxicillin treatment, the level of *Bifidobacterium* spp. recovered upon GOS addition in both *in vitro* and *in vivo* experiments (Chapters 2 to 5). The recovery was, however, observed after the treatment has ended in the *in vivo* trial, suggesting that supplementation GOS after an antibiotic treatment could be more beneficial than during the treatment (Chapter 5). In addition, it was suggested that the diarrhoea developed by one subject belonging to the GOS group was due to the high level of oligosaccharides in the colon as reflected in the faeces during the antibiotic treatment (Chapter 5). This observation reinforces the suggestion to supplement GOS after the AMX treatment.

In order to investigate whether the time of addition of the prebiotic influences the recovery of the microbiota, an *in vitro* fermentation was performed in which GOS (4,2 $mg.ml^{-1}$) was added during or 24h after a 10 $\mu g.ml^{-1}$ amoxicillin treatment (unpublished results). The antibiotic effect was expected to be sufficiently diminished after 24h of fermentation.[23]

In table 1, it is clear that the level of *Bifidobacterium* spp. was significantly higher when GOS was added 24h after treating the microbiota with AMX (9.2 ±0.8 log_{10} copies.ml^{-1}) than when GOS was added simultaneously to the AMX treatment (7.5 ±0.5 log_{10} copies.ml^{-1}), although the level of bifidobacteria was similar after 24h exposure to AMX (6.2 and 6.5 log_{10} copies.ml^{-1}, respectively). These results confirm that the bifidobacteria activity is limited during the AMX treatment both with and without GOS but also suggest that the bifidobacteria were in a "better state" to utilise the GOS when GOS was added after the AMX activity diminished. The rate of degradation of GOS (Table 1) corroborates this suggestion as the microbiota was observed to degrade GOS faster after the AMX activity diminished than when GOS is added simultaneously. In addition, it was observed that the final concentration of SCFA was proportional to the rate of GOS degradation. The highest concentration of SCFA, comprising butyrate, was obtained when GOS was added after AMX activity diminished, while the lowest concentration of SCFA, without butyrate found, was observed when GOS was added simultaneously to AMX (Table 1).

Table 1: Effect of the moment of GOS addition on the level of *Bifidobacterium* spp., percentage of GOS degraded and concentration of SCFA during *in vitro* fermentation using healthy adult microbiota treated with AMX (n=2).

	Time of fermentation	Moment of GOS addition to AMX-treated microbiota	
		During	**After**
***Bifidobacterium* spp. level**	0h	6.9 ±0.2	7.0 ±0.3
log_{10} (copies.ml^{-1})	24h	6.5 ±0.1	6.2 ±0.2
	48h	7.5 ±0.5	9.2 ±0.8
Degradation of GOS (%)	0h*	0 ±2	0 ±0
(based on peak area)	24h*	9 ±4	80 ±8
	33h*	65	nd
	48h*	87 ±4	nd
Concentration of SCFA (μmol.mg^{-1} GOS)	48h		
acetate		4.5 ±0.1	15.5 ±3.0
propionate		2.5 ±0.1	3.8 ±1
butyrate		NF	0.3 ±0.0
lactate		1.1 ±0.1	0.6 ±0.1
Total		8.1	20.2

* time after GOS addition, nd: not-determined, NF: not found

Amoxicillin has a bactericidal mode of action. It inhibits the cell wall synthesis *during* bacterial multiplication. Due to an incomplete cell wall, water is absorbed into the bacterial cell by osmosis, leading to the lysis of the cell. As gram positive and gram

negative bacteria differ in their intracellular osmotic pressure as compared to external environment (10-30 and 3-5 times more, respectively), AMX is more effective against gram positive than gram negative microorganisms.[24] This mechanism of action might explain why GOS added simultaneously to AMX is not ideal. Since GOS stimulates bifidobacteria growth, AMX is more likely to inhibit these growing bacteria. When GOS is supplemented after AMX has lost its activity (by inhibiting other growing bacterial groups), bifidobacteria are free of inhibitors and, hence, are able to multiply and grow. Since the antibiotic action reduced competitors for intermediate substrates, the growth rate of bifidobacteria in a AMX-treated microbiota is higher than in a non-treated microbiota. In conclusion, supplementing GOS to an AMX-treated microbiota helps the recovery of the microbiota, especially if, considering the mode of action of amoxicillin, GOS is supplemented after the AMX activity diminished.

Age of the microbiota host

Healthy adults were recruited because the risk for adverse events was considered to be lower as their microbiota is more mature, diverse and stable as compared to infant or elderly. [20, 21] However, amoxicillin is often used for infants[25] and antibiotics in general are most often prescribed for elderly.[26] It is, therefore, interesting to know how the age of the microbiota host influences the microbiota response to prebiotic upon amoxicillin treatment. For this purpose, *in vitro* fermentations were performed using faecal samples from babies (≤3 months old), adults (25-65 years) and elderly (≥65 years) treated with 10 $\mu g.ml^{-1}$ amoxicillin and supplemented with GOS (4.2 mg/ml).

The initial composition of the microbiota differed for each age-group (Figure 1). The levels of *Bifidobacterium* spp. measured with qPCR decreased with age (40% *Bifidobacterium* spp. in babies- 13% in adults and 1,2% in elderly; data not shown), while the abundance of *Bacteroides* and *Clostridium* measured with the I-chip is higher for the adult and elderly (Figure 1), as also previously reported.[27] The main impact of GOS addition to antibiotic-treated microbiota was observed on *Bifidobacterium* spp. independently from the age of the microbiota host (Figure 1). The bifidobacteria level recovered within 24h of fermentation for amoxicillin-treated baby microbiota, while it recovered within 48h of fermentation for adult and elderly microbiota. Clearly, amoxicillin-treated baby microbiota benefited the most from the GOS addition. Furthermore, specific species of *Bifidobacterium* were stimulated depending on the age of the microbiota host, being mostly *B. breve* and *B. indicatum* in baby microbiota and *B. longum* in adult microbiota. These results are in line with the most abundant bifidobacteria species usually detected in faeces of different age groups.[28] Interestingly, for the elderly microbiota, the abundance

of *Clostridium disporicum* and *Klebsiella*, which are known to be occasional or frequent human pathogens, respectively,[29, 30] increased upon amoxicillin, independently of the GOS addition (Figure 1). The increase in *Clostridium difficile* is quite common for elderly subjects after an antibiotic treatment.[20, 27] The higher susceptibility of elderly people to AAD[21] seems not only to relate to the low levels of bifidobacteria, but also to the overall state and complex interactions among bacteria within the gut ecosystem.

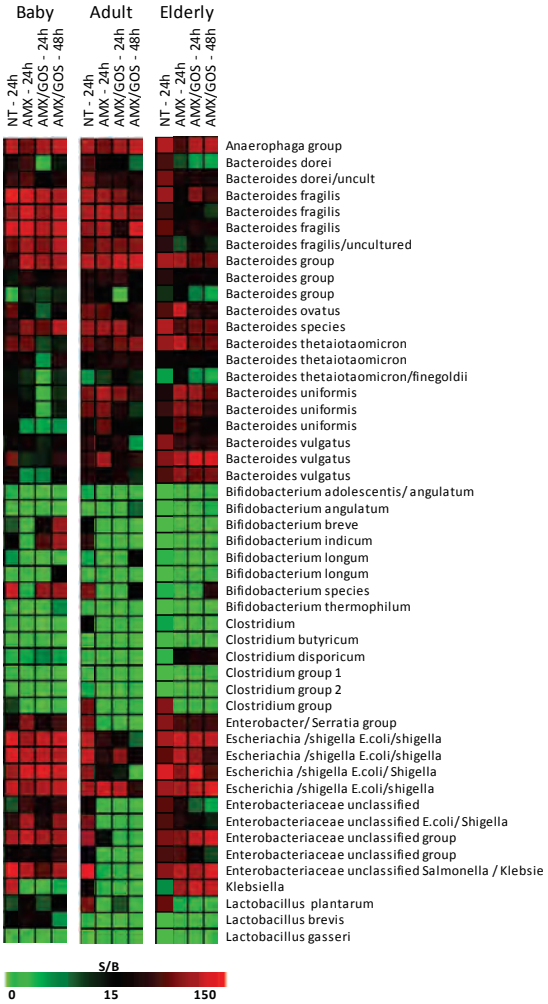

Figure 1: Bacterial fingerprints of the non-treated (NT) and amoxicillin-treated (AMX) microbiota from baby, adults and elderly and effect GOS on AMX-treated microbiota during *in vitro* fermentation. Signal compared to the background (S/B): Green: below detectable level, Black: medium abundance, Red: high abundance.

The metabolic activity of the microbiota (Table 2) was proportional to the level of disruption of the microbiota composition. For baby microbiota (the least disrupted by AMX), the degradation of GOS was the fastest and the SCFA produced were mostly acetate after 24h of fermentation. For adults and elderly microbiota, the degradation of GOS was slower (within 48h fermentation) and up to 8% succinate was produced. The adult and elderly microbiota were richer in *Bacteroides* than the baby microbiota before the AMX treatment (Figure 1). Despite the levels of *Bacteroides* for adult and elderly microbiota were not dramatically affected by AMX, it is likely that the *Bacteroides* activity was reduced, resulting in a limited conversion of succinate to propionate.[31]

In conclusion, supplementing GOS to an antibiotic-treated microbiota, no matter of the age group, seems to help to increase the bifidobacteria levels *in vitro*. Nevertheless, a higher level of bifidobacteria in elderly microbiota did not limit the growth of potential pathogen species, indicating that more complex interaction within the microbiota seem to predispose elderly population to AAD.

Table 2: Influence of the age of the AMX-treated microbiota (baby, adult, elderly) on the percentage of GOS degraded and concentration of SCFA produced during *in vitro* fermentation.

		GOS addition to AMX-treated microbiota of different age		
	Time of fermentation	**Baby**	**Adult**	**Elderly**
Degradation of GOS (% based on peak area)	0h	0	0	0
	12h	44	2	4
	24h	100	33	24
	48h	100	52	82
Concentration of SCFA (µmol.mg^{-1} GOS)				
acetate	48h	9.8	11.4	6.2
propionate	48h	2.1	4.0	3.7
butyrate	48h	1.1	0.0	1.1
lactate	48h	0.0	0.4	0.0
succinate	48h	0.0	1.4	0.8
Total	48h	13.0	17.2	11.8

Future perspectives

The aim of this thesis was to determine when and how GOS could counter the side effects of antibiotics. Overall, the results of this thesis confirmed the hypotheses: 1) The recovery of the microbiota upon GOS addition was antibiotic dependant and 2) the addition of GOS stimulated the growth of bifidobacteria and, subsequently, the functioning of the microbiota through cross-feeding. The detailed results provide insights on how to develop new strategies to counter the side effects of antibiotics.

First, specific bifidobacteria and/or lactobacilli species were shown to recover depending on the antibiotic treatment and dose used. Identifying the best (highly resistant) species for a specific antibiotic treatment could lead to potential new probiotics effective against AAD. For amoxicillin treatment, it is suggested to consider the probiotic *B. longum* to decrease the rate of AAD. This probiotic, in combination with two other probiotics (*L. rhamnosus* and *L. plantarum*) has already been used in a clinical trial.[32] Although no significant decrease of rate of diarrhoea was measured, the outcome of that study should be interpreted with caution as the rate of diarrhoea was surprisingly low, also in the placebo group.

Secondly, adding GOS to antibiotic-treated microbiota had a neutral or a positive impact on the microbiota, independently of the age of the host. Hence, the intake of GOS should be considered upon receiving an antibiotic treatment. The best moment of administration of GOS to stimulate the growth of bifidobacteria upon treatment with a bacteriolytic antibiotic, such as amoxicillin, was suggested to be after the antibiotic treatment ended. This outcome was obtained *in vitro* and should, therefore, be confirmed *in vivo* before drawing strong conclusions. Although, the use of the current GOS mixture is already effective to recover the microbiota composition and metabolic activity upon antibiotic treatment, the results of this study also indicated that specific structures of GOS could influence the rate of recovery of the bifidobacteria. This observation provides new perspectives for the food industry to develop adapted mixtures of GOS or other prebiotics depending on the antibiotic treatment. The new mixture of GOS could be enriched in certain DPs, for instance enriched in large GOS for amoxicillin treatment as suggested in this thesis. Furthermore, new GOS structures could be developed. It is clear that breastfed baby have a microbiota composition that is richer in bifidobacteria and has a lower numbers of *C. difficile* and *E. coli* than formula fed baby,[33] probably due to the composition of the human milk oligosaccharides. These human milk oligosaccharides have been shown to inhibit the adhesion of pathogenic bacteria to the epithelial surface.[34] Mimicking these structure by decorating GOS with sialic or fucosyl group could be of interest to limit the risks for AAD.

Another strategy would be to make use of synbiotics. The specific bifidobacteria species recovering upon a specific antibiotic treatment could be combined with the GOS mixture comprising the ideal composition of oligosaccharides that the species grow preferably on. This strategy would be especially of interest when little or no beneficial bacteria survive the antibiotic treatment (e.g. clindamycin). The intake of a synbiotic (FOS + *B. longum* or *L. acidophilus*) during a treatment of cefpodoxime proxetil successfully limited the decrease of lactobacilli and prevented the growth of *C. difficile*.[35]

Finally, measuring the oligosaccharide degradation during *in vitro* fermentation revealed that some structures of GOS remain longer in time. Similarly as human milk oligosaccharides may limit the adhesion have of pathogenic bacteria to the epithelial surface,[34] it could be of interest to study the anti-adhesive properties of these least degraded GOS in antibiotic-treated microbiota to further investigate how to reduce pathogen colonisation, especially *C. difficile* during antibiotic treatment.

Overall, this thesis showed the potential of GOS to stimulate the recovery of the microbiota after certain antibiotic treatments. For amoxicillin-treated microbiota, the addition of GOS, especially the large oligosaccharides, allowed the recovery of *B. longum* and, subsequently, stimulated the activity of the microbiota through cross-feeding after an AMX treatment.

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Summary

Samenvatting

Résumé

Summary

The bacteria inhabiting the human gut, also called gut microbiota, have been recognised to play a key role in human health. The **first chapter** of this thesis describes the composition and metabolic activity of the gut microbiota and how it contributes to the health of the host. Then, the mostly used antibiotics in Europe and the known prebiotic galacto-oligosaccharides (GOS) are introduced. Both their impacts on the microbiota are addressed as well as how prebiotics have been considered to help preventing the negative side-effects of the antibiotics on the microbiota because of their bifidogenic effect. Finally, the chapter describes fermentation models and diagnostic tools to measure the microbiota composition. The discrepancies in literature regarding the effect of prebiotics in preventing the side-effects of antibiotics lead to the **aim** of this PhD thesis which is to investigate when and how the prebiotic GOS may counter the side-effects of antibiotics on the microbiota. The strategy of the thesis was to address the impact of GOS on both the microbiota composition and the microbiota metabolic activity disrupted with antibiotic treatments.

First, the impact of seven mostly used antibiotics in Europe on adult intestinal microbiota was determined using a new high-throughput *in vitro* approach (**Chapter 2**). The *in vitro* fermentations were simultaneously performed using a screening-platform and the antibiotic-dependant shifts in the microbiota composition were investigated using a phylogenetic microarray. The method was shown to be reliable despite the variations in the number of total bacteria and the presence of dead and/or inactive cells induced by the antibiotic treatments. An overview of the impact of the seven antibiotics on the microbiota was obtained. Subsequently, 4 antibiotics were selected for further experiments based on their mode of action, classification and specific impact on the microbiota.

In **Chapter 3**, the impact of the addition of the prebiotic GOS on an adult gut microbiota treated with the 4 selected antibiotics was investigated using the same high-throughput approach. In addition to the changes in the microbiota composition, the metabolic activity of the microbiota supplemented with GOS was investigated. The metabolic activity was investigated by monitoring the degradation of individual GOS components and the production of short chain fatty acids produced during fermentation using anion exchange chromatographic methods. The comparison of the results revealed unambiguously that the impact of GOS addition on antibiotic-treated microbiota was antibiotic dependant and that the recovery of bifidobacteria was concomitant to the degradation of GOS. The addition of GOS to amoxicillin (AMX)-treated microbiota was especially of interest as, after a decrease of the level of bifidobacteria due to AMX action, a the level of *Bifidobacterium* spp., mainly *Bifidobacterium longum,* recovered to the initial level and beyond. The results

also pointed out that, despite a recovery of bifidobacteria and a degradation of GOS, the functionality of the microbiota was not fully recovered yet as the conversion of succinate to short chain fatty acids (SCFA) was limited.

Chapter 4 focuses on the positive impact of GOS on AMX-treated microbiota described in chapter 3. In order to elaborate on which structures of GOS may stimulate the recovery of specific bifidobacteria species, GOS was fractionated based on size (molecular weight) using size-exclusion chromatography and the different size-fractions were *in vitro* fermented using non-treated and AMX-treated inoculum. The increase of bifidobacteria, especially *B. longum,* upon the addition of GOS after being decreased due to AMX action was evidenced. Interestingly, the growth of bifidobacteria and production of butyrate tended to be higher upon addition of large oligosaccharides (tetramers to hexamers) than small oligosaccharides (dimers-trimers) for AMX-treated microbiota. On the contrary, the growth of bifidobacteria and production of butyrate tended be higher upon addition of small oligosaccharides than large oligosaccharides for non-treated microbiota. Also, within a specific size-fraction, isomeric structures with certain types of linkages were preferentially utilised. β-D-Gal-(1→4)-D-Gal and β-D-Gal-(1→4)-β-D-Gal-(1→3)-D-Glc were the structures preferably degraded by AMX-treated microbiota whereas these structures remained among of the last ones to be degraded by non-treated microbiota. For the non-treated microbiota, the linear structure β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc was preferably degraded. Overall, the results suggest that different size-fractions of GOS contribute to the growth of bifidobacteria species and butyrate production through cross-feeding depending on the treatment applied, and within a size-fraction, specific isomers have a preferential impact.

In **chapter 5**, the effects of GOS on AMX-treated microbiota were investigated in a double-blind randomized parallel intervention study involving 12 healthy adults. The subjects received AMX for 5 days and either GOS (n=6) or placebo (maltodextrin; n=6) during and subsequently 7 days after the AMX treatment. Due to AMX treatment, a decrease of the number of bifidobacteria and a disruption of the metabolic activity, shown through an increase of succinate and oligosaccharides in faecal samples, were observed for both groups. A positive impact of the intake of GOS as compared to placebo was observed on the levels of bifidobacteria and of butyrate. The level of bifidobacteria tended to decrease less ($p<0.15$) during the AMX treatment and was significantly higher ($p<0.05$) after the AMX treatment when GOS was added as compared to placebo. The level of butyrate significantly increased as well ($p<0.05$) after the AMX treatment when GOS was added as compared to placebo. The increased level of butyrate reflected the recovery of the microbiota activity through cross-feeding. Overall, results confirm that the positive effect

of GOS on AMX-treated microbiota observed *in vitro* (Chapters 3 and 4) could be observed *in vivo* as well despite individual variability.

In **chapter 6**, the *in vitro* results (chapters 3 and 4) related to the impact of antibiotic on the microbiota and the effect of prebiotics on antibiotic-treated microbiota were discussed in relation to the results of the *in vivo* study (chapter 5). After discussing some methodological considerations, the results obtained are put into perspective using results from additional *in vitro* experiments on the moment of administration of the prebiotic to the antibiotic-treated microbiota and on the age of the subjects. It is suggested that the positive effect of GOS on the composition and metabolic activity of the microbiota might be even higher if supplemented after the AMX treatment instead of during the AMX treatment. Furthermore, addition of GOS to AMX-treated microbiota positively influenced the level of bifidobacteria in the microbiota of a baby, adult and elderly person. The AMX treated microbiota of the baby benefited the most from GOS addition, while the AMX-treated microbiota of the elderly person still contained a high level of potential pathogens despite the recovery of bifidobacteria upon GOS addition. Finally, the possible impact of this study on new probiotics, prebiotics and synbiotics development to prevent the negative side-effects of antibiotic is discussed.

Summary

Samenvatting

Résumé

De bacteriën die leven in de darmen van de mens, ook wel de microbiota genoemd speelt een belangrijke rol in de menselijke gezondheid. Het eerste hoofdstuk van dit onderzoek beschrijft de samenstelling en stofwisseling van de microbiota in de darmen. Ook is beschreven hoe de microbiota bijdraagt aan de gezondheid van de mens. Vervolgens zijn de veelgebruikte antibiotica, de bekende prebiotische galacto-oligosachariden (GOS) en hun impact op de microbiota beschreven. Hierna wordt ingegaan op gangbare fermentatie modellen en diagnose technieken, welke gebruikt worden om de samenstelling van de microbiota te bepalen. Tenslotte is beschreven hoe prebiotica met een bifidogeen effect de negatieve bijwerkingen van antibiotica op de microbiota helpen voorkomen. De verschillen in de literatuur hierover leiden tot het doel van dit onderzoek, namelijk onderzoeken wanneer en hoe de prebiotica GOS de negatieve bijwerkingen van antibiotica kan voorkomen. De aanpak van dit onderzoek richt zich op de impact van de prebiotica GOS op zowel de samenstelling van de microbiota als het bijbehorende metabolisch proces na verstoring door het gebruik van antibiotica.

Allereerst is in **hoofdstuk 2** de invloed van zeven veelgebruikte antibiotica in Europa op de darmmicrobiota van een volwassene bepaald. Hiervoor is gebruik gemaakt van een nieuwe *in vitro* aanpak met een hoge verwerkingscapaciteit. Deze *in vitro* fermentaties zijn gelijktijdig uitgevoerd middels een screening platform. Vervolgens zijn de veranderingen in de microbiota die zijn veroorzaakt door de antibiotica onderzocht middels een fylogenetische microarray. Deze methodiek is bewezen betrouwbaar voor het meten van het aantal bacteriën ondanks de aanwezigheid van dode en/of inactieve cellen die zijn veroorzaakt door de antibiotica behandeling. Met behulp van een overzicht van de invloed van de zeven antibiotica op de darmmicrobiota zijn vier antibiotica geselecteerd voor een vervolgexperimenten waarbij met name gelet werd op hun werking, classificatie en invloed op de darmmicrobiota.

In **hoofdstuk 3** wordt de impact van het toevoegen van de prebiotica GOS op de antibiotica-behandelde darmmicrobiota van een volwassene beschreven. Voor deze analyse zijn de 4 geselecteerde antibiotica gebruikt samen met dezelfde *in vitro* aanpak met een hoge verwerkingscapaciteit. Naast de veranderingen in de samenstelling van de microbiota is ook het metabolische proces in de microbiota in aanwezigheid van GOS nader onderzocht. Het metabolisch proces is onderzocht door het meten van de afbraak van specifieke GOS componenten en de vorming van korte keten vetzuren (KKVZ) als gevolg van de fermentatie. Hiervoor is gebruik gemaakt van ionenuitwisselingschromatografie. Uit de resultaten is het duidelijk dat de impact van de toevoeging van GOS op de microbiota afhankelijk is van de gebruikte antibiotica. Tevens blijkt de afbraak van GOS gerelateerd te zijn aan het herstel van de populatie

bifidobacteriën. Een interessante waarneming is dat bij toevoeging van GOS aan de amoxicilline (AMX) behandelde microbiota de hoeveelheid bifidobacteriën in eerste instantie afneemt om vervolgens toe te nemen tot voorbij het oorspronkelijke niveau, waarbij vooral *Bifidobacterium longum* domineert. De resultaten bewijzen ook dat ondanks de toename van bifidobacteriën en de afbraak van GOS, de microbiota nog niet volledig hersteld is gezien het feit dat de omzetting van barnsteenzuur naar kortketenige vetzuren (KKVZ) nog beperkt is.

Hoofdstuk 4 verdiept zich meer op de positieve impact van GOS op de met AMX behandelde microbiota zoals beschreven in hoofdstuk 3. Om in te kunnen gaan op welke componenten van GOS het herstel van specifieke bifidobacteriën specifiek stimuleren zijn eerst alle componenten van GOS onderverdeeld op basis van grootte (moleculair gewicht). Hiervoor is gebruik gemaakt van gelfiltratie chromatografie. De verschillende componenten zijn vervolgens *in vitro* gefermenteerd met niet behandelde en AMX behandelde microbiota. Na de toevoeging van GOS is een toename van het aantal bifidobacteriën zichtbaar, vooral *B. longum*, nadat deze eerst was gedaald door de AMX behandeling. Interessant is dat de groei van bifidobacteriën en de productie van butyraat in een AMX-behandelde microbiota hoger is bij het toevoegen van grote oligosachariden (tetrameren tot hexameren) dan bij kleine oligosachariden (dimeren en trimeren). In tegenstelling hiermee is de groei van bifidobacteriën en de productie van butyraat bij een niet-behandelde microbiota juist hoger bij het toevoegen is van kleine oligosachariden. Binnen een gekozen grootte-fractie worden structuren met bepaalde type glycosidebindingen met voorkeur benut. β-D-Gal-(1→4)-D-Gal en β-D-Gal-(1→4)-β-D-Gal-(1→3)-D-Glc zijn componenten die als voorkeur worden benut door een met AMX-behandelde microbiota. Voor de niet-behandelde microbiota wordt juist vooral de lineaire component β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc benut. De resultaten suggereren dat de verschillende GOS grootte-fracties bijdragen aan de toename van bifidobacteriën en indirect ook de butyraat productie verhogen voor zowel AMX-behandelde als niet-behandelde microbiota.

In **hoofdstuk 5**, is de invloed van GOS op met AMX behandelde microbiota beschreven zoals bepaald in een dubbelblinde, willekeurige en parallelle interventieonderzoek met twaalf gezonde volwassenen. De proefpersonen kregen GOS (n=6) of met een placebo (maltodextrine, n=6) gedurende (5 dagen) en na (7 dagen) een AMX-behandeling. Door de AMX-behandeling is in beide groepen een afname van het aantal bifidobacteriën aangetoond en wijzen een toename van succinaat en oligosachariden in de ontlasting monsters op een verstoring van het metabolisch proces. Een positieve invloed van de inname van GOS in vergelijking met de placebo werd waargenomen middels de aantallen

van bifidobacteriën en het niveau van butyraat. Het aantal bifidobacteriën blijkt af te nemen ($p<0.15$) gedurende de AMX behandeling en is significant hoger ($p<0.05$) na de AMX-behandeling door de inname van GOS in plaats van de placebo. Het niveau van butyraat is na de AMX-behandeling ook significant hoger ($p<0.05$) door de inname van GOS in plaats van de placebo. De stijging van het niveau van butyraat geeft indirect het herstel van de microbiota weer. Ondanks de aanwezigheid van individuele variaties bevestigen de resultaten dat de positieve invloeden van GOS op een met AMX-behandelde microbiota zoals *in vitro* gevonden (hoofdstuk 3 en 4) bevestigd worden middels *in vivo* onderzoek.

In **hoofdstuk 6**, zijn de *in vitro* resultaten (hoofdstuk 3 en 4) naar de invloeden van antibiotica op de microbiota en de invloeden van prebiotica op een met antibiotica behandelde microbiota en vergeleken met de resultaten van het *in vivo* onderzoek (hoofdstuk 5). Na het bespreken van enkele overwegingen aangaande de gebruikte analysemethoden zijn de verkregen resultaten in perspectief geplaatst door gebruik te maken van aanvullende *in vitro* experimenten omtrent het moment van toedienen van prebiotica aan een met antibiotica-behandelde microbiota en met de leeftijd van de microbiota-donoren. De onderzoeksresultaten suggereren dat de positieve invloed van GOS op de samenstelling en metabolische activiteit van de microbiota nog hoger kan zijn als deze wordt toegediend na de AMX-behandeling in plaats van tijdens de AMX-behandeling. Daarnaast heeft de toevoeging van GOS op een met AMX-behandelde microbiota ook positieve invloeden op het aantal bifidobacteriën in de microbiota van een baby, volwassene of een oudere persoon. De met AMX-behandelde microbiota van een baby ervaart de meeste voordelen van GOS, terwijl de AMX-behandelde microbiota van een oudere na een GOS toevoeging naast een hersteld niveau van bifidobacteriën nog steeds een hoog gehalte van potentiele ziekteverwekkers bevat. Tenslotte zijn de mogelijke gevolgen van dit onderzoek voor de ontwikkeling van nieuwe probiotica, prebiotica en synbiotica om de negatieve bijwerkingen van antibiotica te voorkomen besproken.

Summary

Samenvatting

Résumé

L'ensemble des bactéries présentes le long de l'intestin humain, aussi appelé microbiote intestinal, joue un rôle important pour la santé humaine. Le premier chapitre de cette thèse décrit la composition et l'activité métabolique du microbiote intestinal et explique en quoi il contribue à la santé de l'hôte. Les antibiotiques les plus utilisés en Europe et le prébiotique galacto-oligosaccharide (GOS) sont ensuite présentés. Chacun de leur impact sur le microbiote intestinal est décrit, puis un état de l'art du potentiel des prébiotiques à prévenir les effets secondaires des antibiotiques sur le microbiote grâce à leur effet bifinogénique est fourni. Enfin, ce chapitre décrit les modèles de fermentation et les outils de diagnostic de la composition du microbiote. Les divergences dans la littérature sur l'effet des prébiotiques à prévenir des effets secondaires des antibiotiques ont conduit au sujet de cette thèse: étudier dans quelle mesure et de quelle manière le prébiotique GOS peut contrer les effets secondaires des antibiotiques sur le microbiote intestinal. L'étude a été conduite en évaluant les effets du GOS sur la composition et sur l'activité métabolique du microbiote toutes deux perturbées par des traitements antibiotiques.

Tout d'abord, l'impact de sept antibiotiques souvent utilisés en Europe a été déterminé sur le microbiote intestinal adulte en utilisant une nouvelle approche *in vitro* à haut rendement **(Chapitre 2)**. Les fermentations *in vitro* ont été réalisées en utilisant une plateforme de criblage tandis que les changements de la composition du microbiote en fonction de l'antibiotique utilisé ont été déterminés en utilisant une puce à ADN. La méthode à haut rendement s'est révélée fiable malgré les variations du nombre de bactéries totales et la présence de cellules mortes et/ou inactives induites par les traitements antibiotiques. Grâce à cette méthode, un aperçu de l'impact de sept antibiotiques sur le microbiote intestinal a tout d'abord été obtenu. Quatre antibiotiques ont ensuite été sélectionnés en fonction de leur mode d'action, leur classification et leur impact spécifique sur le microbiote intestinal pour poursuivre l'étude.

Dans le **chapitre 3**, l'impact de l'ajout du prébiotique GOS au microbiote intestinal adulte traité par les quatre antibiotiques sélectionnés a été étudié en utilisant la même méthode à haut rendement. L'impact du GOS a été étudié en évaluant les changements dans la composition et l'activité métabolique du microbiote intestinal. L'activité métabolique a été évaluée par des méthodes de chromatographie d'échange d'ions en déterminant le pourcentage de dégradation des composants individuels du GOS et le taux d'acides gras à chaîne courte produits pendant la fermentation. Les résultats ont clairement montré que l'impact du GOS sur le microbiote sous antibiotiques était fonction du type d'antibiotiques et que la dégradation du GOS était simultanée au rétablissement du niveau des bifidobactéries. L'ajout du GOS au microbiote traité par amoxicilline (AMX) s'est révèlé particulièrement performant car, après une diminution du niveau de bifidobactéries due à

l'action de l'AMX, le niveau de *Bifidobacterium longum* est revenu à son niveau initial ou l'a même dépassé. Les résultats ont également souligné que, malgré le retour des bifidobactéries à leur niveau initial et la dégradation du GOS, la fonctionnalité du microbiote n'était pas encore pleinement récupérée puisque la conversion du succinate en acides gras à chaîne courte restait limitée.

Le **chapitre 4** se concentre sur l'impact positif du GOS sur le microbiote traité par AMX tel que décrit au chapitre 3. Afin de comprendre quels composés du GOS stimulent le rétablissement de certaines espèces de bifidobactéries, le GOS a été fractionné en fonction de sa taille (poids moléculaire) en utilisant la méthode de chromatographie par exclusion de tailles. Les fractions de tailles différentes ont été fermentées *in vitro* en utilisant un inoculum non traité et un inoculum traité par AMX. Lors de l'ajout du GOS, le rétablissement du niveau de bifidobactéries, en particulier celui du *B. longum,* a été de nouveau observé. Pour le microbiote traité par AMX, la croissance des bifidobactéries et la production du butyrate avaient tendance à être plus élevées lors de l'ajout de grands GOS (tétramères à hexamères) que lors de l'ajout de petits GOS (dimères-trimères). Au contraire, pour le microbiote non traité, la croissance des bifidobactéries et la production de butyrate avaient tendance à être plus élevées lors de l'ajout de petits GOS que lors de l'ajout de grands GOS. Pour chaque taille de GOS, les isomères de structures préférentiellement dégradées par le microbiote traité par AMX étaient β-D-Gal-(1→4)-D-Gal et β-D-Gal-(1→4)-β-D-Gal-(1→3)-D-Glc. Pour le microbiote non traité, l'isomère préférentiellement dégradée était la structure linéaire β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc. Dans l'ensemble, ces résultats suggèrent que ce sont différentes tailles de GOS qui contribuent à la croissance des bifidobactéries et la production du butyrate selon le traitement appliqué au microbiote et pour une même fraction certains isomères ont un impact préférentiel.

Dans le **chapitre 5**, les effets du GOS sur le microbiote traité par AMX ont été déterminés au cours d'une étude d'intervention, randomisée, réalisée en parallèle, en double aveugle, contrôlée au moyen d'un placebo, sur 12 sujets adultes sains. Les sujets ont reçu de l'AMX pendant 5 jours et soit du GOS (n=6) soit un placebo (maltodextrine, n=6) en simultané et 7 jours après le traitement antibiotique. Une diminution du nombre de bifidobactéries et une perturbation de l'activité métabolique ont été observées pour les deux groupes en raison de l'action d'AMX. La perturbation de l'activité métabolique a été montrée par une augmentation des niveaux de succinate et d'oligosaccharides dans des échantillons de matière fécale. L'effet positif de la prise de GOS par rapport au placebo a bien été observé sur les niveaux de bifidobactéries et de butyrate. En effet, lorsque le GOS a été ajouté, le niveau de bifidobactéries avait tendance à moins diminuer au cours du traitement d'AMX

(p<0,15) et était significativement plus élevée après le traitement d'AMX (p<0,05). Le niveau du butyrate a augmenté de manière significative après le traitement AMX lorsque GOS a été ajouté (p<0,05). Cette augmentation du niveau de butyrate reflète la reprise d'activité du microbiote au moyen d'une alimentation croisée. D'un point de vue général, les résultats confirment que les effets positifs du GOS sur un microbiote traité par AMX observés dans les essais *in vitro* (Chapitres 3 et 4) sont aussi observés dans le test *in vivo*.

Dans le **chapitre 6,** les résultats des essais *in vitro* (chapitres 3 et 4) et de l'étude *in vivo* (Chapitre 5) sont discutés. Après avoir pris en considération des aspects méthodologiques, de nouvelles perspectives ont été dégagées en fonction de résultats d'expériences *in vitro* complémentaires. Ces expériences complémentaires ont montré que l'impact du GOS sur le microbiote sous traitement antibiotiques pouvait être influencé par le moment d'administration du prébiotique au microbiote et par l'âge des hôtes du microbiote inoculé. En effet, les effets positifs du GOS sur la composition et l'activité métabolique du microbiote étaient supérieures lorsqu'il été ajouté *après* le traitement d'AMX et non pas *pendant* le traitement d'AMX. D'autre part, l'ajout du GOS au microbiote traité par l'AMX a stimulé le niveau des bifidobactéries aussi bien pour les microbiotes d'un bébé, d'un adulte et d'une personne âgée, mais à des niveaux différents. Le microbiote du bébé s'est entièrement rétabli du traitement d'AMX avec du GOS alors que le microbiote d'une personne âgée présentait un niveau de bactéries potentiellement pathogènes élevé malgré le rétablissement des bifidobactéries dû à l'ajout du GOS. Enfin, ce chapitre présente les implications possibles de cette étude sur le développement de nouveaux probiotiques, prébiotiques et symbiotiques pour contrer les effets secondaires négatifs des antibiotiques.

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Four years already, so many memories, one thesis (!) and soon new adventures. I am looking forward to it already but for now, let's have a sit and take the time to appreciate the moments that have passed and thank the people that helped me to complete this thesis.

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Not only Henk and Harry guided me during my PhD, but also Frank from TNO, Margriet and Arjen from FrieslandCampina, Jean-Marc from Kalys and Monica from the division of human nutrition of Wageningen University. Thank you for your expert and critical views on the data at the start of the project (Jean-Marc), during the *in vivo* study (Monica), and throughout the whole project (Frank, Margriet and Arjen). Among many things, I will remember the importance of STATISTICS (!) and keeping in mind what's in for the consumer.

Next, I would like to thank all the great colleagues that I worked with. Hakim, if one person was involved in the analyses of the samples as much as I was, it is you! Thank you for teaching me the different microbiological techniques and for analysing a load of samples at the end of the project! Also a special thank you to the technicians at FCH who trained me on the equipment. Without your assistance, research would take even longer! Jolanda, you have already helped and will continue to help so many PhDs for non-scientific related topics. Thank you, you are irreplaceable! Anne W., the human study would not have happened without your precious help for writing the ethical proposal. A special MET-thank you to you! Talking about the human study, this study would also not have happened without the "contribution" of the 12 (anonymous :D) volunteers. In the "name of science", thank you to each of you! During my PhD, I had the opportunity to supervise 5 students. Harshit, Carla, Marcella, Chao and Simone, thank you for choosing and working on my topic during your MSc or BSc theses. Your work definitively helped me to set-up my own experiments and to reflect on my own data. I enjoyed and learned a lot from supervising you. Carla, you got hooked by research; good luck finishing your PhD thesis!

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My thesis was about to recover the balance of the bacteria in the human colon. The following paragraphs are about people who helped me to keep the balance between my professional and personal life. Of course, I would like to thank all the Lobbers. On court, it was great to learn badminton and then to be part of a competition team. A special thank you to my teammates of the last two years: Niels, Ramon, Paul, Patrick, Chen, Nic, Timo (who said there were not enough guys at the Lobbers? ;p), Maartje and my dear double partner Sabina. We had in my view the most cheering, competing and fun team! Off court, starting at the Vlaam's to end at the International club, thank you to all the party animal Lobbers !! Here is no space enough to tell all the stories...

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Once again, thank you all.

Stéphanie

About the author

Curriculum vitae

Stéphanie Émilie Ladirat was born on August 6th, 1985 in Avignon, France. After graduating from high school (Lycée Saint Joseph, Avignon, France) in 2003, she was admitted at the Engineering school of Agriculture, Food science and Environmental studies (ISARA) in Lyon, France, and studied Food Technology and Industrial Management. As part of her curriculum, she left to the Netherlands in 2007 to follow a double diploma programme in Food Technology at Wageningen University. She completed her MSc thesis entitled "*In vitro* fermentation of Konjac Glucomannan using an antibiotic-treated microbiota" at the Laboratory of Food Chemistry under the supervision of Simone Albrecht and Dr. Henk Schols. In 2009, she completed an internship as junior product developer at Pepsico International, Maarsen, The Netherlands. The same year, she obtained her MSc double degree in Food Technology with a specialisation in Food Ingredient and Functionality. After completing her master study, she was offered the opportunity to work as a PhD student at the Laboratory of Food Chemistry under the supervision of Dr. Henk Schols and Prof. dr. ir. Harry Gruppen. The results of her PhD research are presented in this thesis. Currently Stéphanie is working temporarily at the same department as researcher.

Contact e-mail: stephanie.ladirat85@gmail.com

List of publications

Ladirat, S. E.; Schols, H. A.; Nauta, A.; Schoterman, M. H. C.; Keijser, B. J. F.; Montijn, R. C.; Gruppen, H.; Schuren, F. H. J., High-throughput analysis of the impact of antibiotics on the human intestinal microbiota composition. *J Microbiol Methods* **2013,** *92*, 387-397.

Ladirat, S. E.; Schuren, F. H. J.; Schoterman, M. H. C.; Nauta, A.; Gruppen, H.; Schols, H. A., Impact of galacto-oligosaccharides on the gut microbiota composition and metabolic activity upon antibiotic treatment during *in vitro* fermentation. *FEMS Microbiol Ecol* **2013**. DOI 10.1111/1574-6941.12187.

Ladirat, S. E.; Schols, H. A.; Nauta, A.; Schoterman, M. H. C.; Schuren, F. H. J.; Gruppen, H., Impact of galacto-oligosaccharides and its specific size-fractions on non-treated and amoxicillin-treated human inoculum. – *submitted for publication to J Agric Food Chem.*

Ladirat, S. E.; Schoterman, M. H. C.; Rahaoui H.; Mars M.; Schuren, F. H. J.; Gruppen, H.; Nauta, A.; Schols, H. A., Galacto-oligosaccharides positively impact the gut microbiota of healthy adults receiving amoxicillin treatment. – *Submitted for publication to Br J Nutr.*

Overview of completed training activities

Discipline specific activities

Courses

- Summer course Glycosciences[†] (VLAG), Wageningen, The Netherlands, 2010
- Advanced food analysis[†] (VLAG), Wageningen, The Netherlands, 2013

Conferences and meetings

- CCC scientific days on carbohydrates and (gut) health[¥], Groningen, The Netherlands, 2011
- International scientific conference on Probiotics and Prebiotics[¥], Kosice, Slovakia, 2011
- Symposium on Gastro-intestinal models, Kosice, Slovakia, 2011
- CCC scientific days on carbohydrate structures[¥], Groningen, The Netherlands, 2012
- INRA-Rowett symposium on Gut Microbiology[†], Clermont-Ferrand, France, 2012

General courses

- Information literacy including EndNote, WGS, 2009
- VLAG PhD week, 2010
- Mobilizing your scientific network, WGS, 2011
- Scientific writing, WU, 2011
- Techniques for writing and presenting scientific papers, WGS, 2011
- PhD competence assessment, WGS, 2011
- Career perspectives, WGS, 2013

Optionals

- Preparation PhD research proposal
- Study trip to Ghent, Belgium, WU (FCH), 2009
- PhD trip to Switzerland and Italy[†¥], WU (FCH), 2010
- PhD trip to Singapore and Malaysia[†¥], WU (FCH), 2012
- Organization PhD trip Singapore/Malaysia, WU (FCH), 2011-2012
- BSc/MSc thesis student presentations and colloquia, WU (FCH), 2009-2013
- PhD presentations, WU (FCH), 2009-2013
- Project meetings – consortium, 2009-2013

[†] poster presentation, [¥] oral presentation

Abbreviations used:

VLAG: Graduate School for nutrition, Food Technology, Agrobiotechnology and Health Science
CCC: Carbohydrate Competence Centre
WGS: Wageningen Graduate School
WU: Wageningen University
FCH: Laboratory of Food Chemistry

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