Modulation of gut microbiota by pre/probiotics and the potential health promoting outcomes for the host

Modulering av tarmmikrobiota av pre/probiotika og de potensielt helsefremmende utfallene for verten

Philosophiae Doctor (PhD) Thesis

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Özgün

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Summary

Gut microbiota is considered an organ that co-develops with the host throughout its life. The composition and metabolic activities of gut microbiota are subjected to a complex interplay of the host genetics and environmental factors, such as life-style, diet, stress and antimicrobials. A deeper understanding of these factors is required for optimizing the strategies for manipulation of gut microbiota in order to fight disease and improve the host health. However, despite the wealth of knowledge that exists on the effects of environmental factors on gut microbiota, there are still many unanswered questions. Therefore, the studies in this thesis aimed to evaluate the impact of different environmental factors, prebiotics and bacteriocin-producing probiotics, on the composition and the activity of gut microbiota.

We used pig models to examine alginate and resistant starch, which are dietary fibers with contrasting physicochemical properties, and mouse models to assess a variety of class II bacteriocin-producing LAB strains: *Lactobacillus sake* Lb 706, *Pediococcus acidilactici* 347, *Enterococcus faecium* L50, *Lactobacillus plantarum* C11B and *Lactococcus garvieae* DCC43, producing sakacin A, pediocin PA-1, enterocins P, Q and L50, plantaricins EF and JK and garvicin ML respectively. In these *in vivo* studies, 16S rRNA gene sequencing of fecal samples was performed for the comparative analysis of gut microbiota.

Gut microbiota was modified by the interventions at diverse levels correlating with the physicochemical properties of the prebiotics, and the different antimicrobial spectra of the bacteriocins. Although the modifications varied among interventions and the treatments, in general, they were seemingly beneficial for the host with the enhancement of metabolically reputable bacterial lineages and the reduction in pathogen-associated phylotypes. Prebiotic treatments in growing pigs (particularly with resistant starch) resulted in significant alterations of the gut microbiota and their imputed functions; however, redundancy was observed in key functions of the microbiome that likely contribute to the normal gut activity. On the other hand, bacteriocin-producing probiotics did not change the overall composition of the gut microbiota in mice. Nevertheless, alterations in the abundance of the bacteria were displayed at deeper taxonomic levels (i.e. genus); however, these changes usually disappeared when the treatments were over. These indicate the relative resilience of the murine gut to the intervention of the bacteriocin-producing probiotics.

Overall, our results propose that the manipulation of the gut microbiota by the prebiotics allow significant beneficial alteration of the gut microbiota, and the use of the class II bacteriocins (via their probiotic producers) emerges as an attractive therapeutic strategy with a target specificity without disrupting other inhabitants of the gut.

Sammendrag

Tarmmikrobiotaen kan ansees som et organ som utvikler seg i samråd med verten gjennom hele dets liv. Tarmmikrobiotaen blir utsatt for et komplekst samspill av vertens genetikk og vertens miljø, herunder miljøfaktorer som livsstil, kosthold, stress, og antimikrobielle midler. Slike påvirkninger kan ha en effekt på tarmmikrobiotaens sammensetning og funksjoner. Til tross for en mye kunnskap om effekten av miljøfaktorer på tarmmikrobiota, er det fortsatt mange ubesvarte spørsmål. En dypere forståelse av effekten av disse faktorene er derfor nødvendig for å forbedre strategiene for manipulering av tarmmikrobiotaen, i bekjempelse av sykdommer og i jakten på bedre helse. Studiene i denne avhandlingen tar derfor sikte på å evaluere effekten av ulike miljøfaktorer, prebiotika og bakteriosin-produserende probiotika, på sammensetningen og funksjonene til tarmmikrobiotaen.

Vi brukte grisemodeller for å undersøke alginat og resistent stivelse, som er kostfibre med kontraste fysisk-kjemiske egenskaper, og musemodeller for å vurdere en rekke klasse II bakteriosin produserende LAB stammer: *Lactobacillus sake* Lb 706, *Pediococcus acidilactici* 347, *Enterococcus faecium* L50, *Lactobacillus plantarum* C11B og *Lactococcus garvieae* DCC43, henholdsvis produserende sakacin A, pediocin PA-1, enterocins P, Q and L50, plantaricins EF og JK og garvicin ML. I disse *in vivo* studier, ble avføringsprøver benyttet for 16S rRNA-gen sekvensering i den komparative analysen av tarmmikrobiota.

Tarmmikrobiotaen endret seg i tråd med de fysisk-kjemiske egenskapene til prebiotika og de forskjellige antimikrobielle spektra av bakteriosinene. Endringene varierte med type inngrep og behandling, men var generelt gunstige for verten, med en forbedring av de gode metabolske bakterielinjer og en reduksjon av patogen-assosierte fyla. Behandling av voksende griser med prebiotika (spesielt med resistent stivelse) resulterte i betydelige endringer i tarmmikrobiotaen og deres antatte funksjoner, men redundans av viktige funksjoner ble observert, noe som trolig bidrar å opprettholde normal tarmaktivitet. Forsøk med bakteriosin-produserende probiotika i mus viste ingen endring i den totale sammensetningen av tarmmikrobiotaen. Derimot, ble endringer i komposisjonen observert ved dypere taxonomiske nivå (dvs. genus-nivå) under behandlingen, men disse endringene forvant ofte når behandlingen var over. Dette er en indikasjon på tarmmikrobiotaens generelle motstand mot påvirkninger forårsaket av bakteriosin-produserende probiotika.

Samlet sett, foreslår resultatene våre at manipulering av tarmmikrobiota med prebiotika har en positiv effekt på tarmens bakterioflora, og bruk av klasse II bakteriosiner via bakteriosin-produserende probiotika fremkommer som en attraktiv terapeutisk strategi der behandlingen er rettet mot bestemte målgrupper uten å forstyrre de andre innbyggerne i tarmen.

List of papers

List of papers included in this thesis:

PAPER 1

Umu ÖC, Oostindjer M, Pope PB, Svihus B, Egelandsdal B, Nes IF, Diep DB (2013). Potential applications of gut microbiota to control human physiology. *Antonie Van Leeuwenhoek* 104(5):609-18. doi: 10.1007/s10482-013-0008-0

PAPER 2

Umu ÖC, Frank JA, Fangel JU, Oostindjer M, da Silva CS, Bolhuis EJ, Bosch G, Willats WG, Pope PB, Diep DB (2015). Resistant starch diet induces change in the swine microbiome and a predominance of beneficial bacterial populations. *Microbiome* 16;3:16. doi: 10.1186/s40168-015-0078-5

PAPER 3

Umu ÖC, Bäuerl C, Oostindjer M, Pope PB, Hernández PE, Pérez-Martínez G, Diep DB (2016). The potential of class II bacteriocins to modify gut microbiota to improve host health. (Submitted manuscript)

Additional papers:

Porcellato D, Frantzen C, Rangberg A, Umu ÖC, Gabrielsen C, Nes IF, Amdam GV, Diep DB (2015). Draft genome sequence of *Lactobacillus kunkeei* ar114 isolated from honey bee gut. *Genome Announc* 19;3(2). pii: e00144-15. doi: 10.1128/genomeA.00144-15

Umu ÖC, Salazar N, Oostindjer M, Pope PB, Martínez B, Gueimonde M, Diep DB (2016). In vitro characterization of bacteriocins for their impact on gut bacterial populations of infants. (Manuscript)

Abbreviations

16S rRNA: Prokaryotic small subunit ribosomal ribonucleic acid

OTU: Operational taxonomic unit

RS: Resistant starch

ALG: Alginate

LAB: Lactic acid bacteria

SCFA: Short chain fatty acids

1. Introduction

1.1. Gut microbiota

The development of the microbiota in the gut is an evolutionary progression for animals that entails gathering microbes necessary for their survival. The symbiosis between the gut microbes and the host is predominantly due to the acquirement of nutrition [1]. Animals provide food for microbes and microbes ferment what animals cannot digest providing maximum gain of energy from food, helping homeostasis and keeping pathogens away. There exists synergy between gut microbiota and the host, controlled via signals that are received and responded by the immune system of the host [1].

The gut microbiota is considered a separate organ due to the great number of microbes and their diversity and impact on host physiology [2]. It consists of up to 100 trillion cells and approximately 1,000 different species encoding 100-fold more unique genes than human genome [3, 4]. The inhabitants of gut microbiota include methanogenic archaea (mainly *Methanobrevibacter smithii*), eukaryotes (mainly yeasts) and viruses (mainly phages) [5]. However, it is dominated by bacteria, most abundantly by Firmicutes and Bacteroidetes, and other phyla including Actinobacteria, Proteobacteria and Verrucomicrobia and Fusobacteria in human [6, 7].

Defining the healthy microbiota forms the baseline to understand the microbiota-host interactions, as well as the associations with disease and disorders. Human gut microbiota have been designated to be clustered into specific groups [8], resulting with three clusters (enterotypes) named with respect to the genera that have variation in the levels: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3). These enterotypes are driven by species composition, and are not nation or continent correlated [8]. However, it is currently challenged that the enterotypes are likely to be more of gradient than this discrete grouping [7]. Moreover, projects in a large scale, such as the US Human Microbiome Project (HMP) [4] and the European Metagenomics of the Human Intestinal Tract (MetaHIT) [9], and also many others [10], aimed to identify healthy (normal) microbiota and have made considerable progress. However, in spite of the common bacterial inhabitants, it is difficult to define the composition of the normal or healthy microbiota due to the complexity of the microbiota and its variation between and within individuals [5].

Inter-individual variations are a commonly observed phenomenon in gut microbiota studies [5]. Although the cause of the inter-individual variations are not well known, diet, environment, host genetics and early microbial exposure are likely to play a role [4]. The greater variations are usually encountered at deeper taxonomic levels rather than at phylum level [11].

In addition to the inter-individual variations, the gut microbiota diversity and composition varies within individuals through life. In early stages of life, the microbiota has low diversity and low complexity. The initial colonizers in neonates include facultative anaerobes, such as *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Enterobacter* spp. that gain the suitable environment from the first days for the colonization of obligate anaerobes, such as *Bifidobacterium*, *Bacteroides*, *Clostridium* and *Eubacterium* spp. [12, 13]. Subsequently, the microbiota slowly develops to an adult-like, more diverse and stable state at around the 3 years of age [14, 15]. Microbial colonization of the intestine in early stage of life is an important factor for the health later in life and it is affected by factors, such as genetics, mother microbiota, delivery type, antibiotics and diet (i.e. breast- or formula-feeding) [16]. The composition and diversity of gut bacteria are shaped through life largely based on what is present at the beginning, which is relevant to the development of proper immune system and reduction of the risk of diseases later in life [12]. The modification of gut microbiota diversity and composition through life is substantially correlated with the change of diet at different stages of life, especially in the transition period between infancy and childhood when the diet is shifting from breast milk to table foods. This shift in diet also leads to the enrichment of genes associated with carbohydrate utilization, vitamin biosynthesis, and xenobiotic degradation [17]. In the adulthood of healthy humans, microbiota is more stable. The ecosystem is in a homeostatic equilibrium with temporal balance between different microbial groups, the epithelial tissue of the intestine and the immune system of the host [14, 18]. However, after approximately 65 years of age, the composition of gut microbiota alters with high inter-individual variability, most likely due to the physiological changes in the intestines that affect food digestion and absorption, and immune function [19].

1.1.1. Response to environmental factors

Environmental factors such as host genetics, aging, health, general lifestyle, early colonization, use of antibiotics and diet are important factors affecting the gut microbiota diversity and

composition [11, 20]. The gut microbiota generally have remarkable stability against such exposures and disturbances, known as resilience, and the community is usually drawn back to its original state before the disturbance [5, 21]. Resilience is presumably a mechanism to suppress the blooms of subpopulations and/or to promote the abundance of the desired bacteria [22]. An interactive network plays a role in this, where different groups of bacteria rely on each other and the signals from the host to survive and to persist within the host. However, the resilience of the communities varies and the recovery or disruption of the stable state may depend on the community, the disturbance and the exposure time [5]. For example, long-term dietary interventions associate strongly with enterotype clustering in gut overcoming resilience, while short-term interventions do not thoroughly change the gut microbiota composition and may not overcome inter-individual variations [20, 23].

In addition to the resilience of gut microbiota, the gut microbiome exhibits functional redundancy, which guarantees that the key functions are maintained for normal gut functioning [22]. The functions of microbiota are conserved among individuals as a core microbiome and have impact on the host [1, 24]. A variety of gut bacteria share the functional traits and ensure that crucial functions are present in gut (e.g. the bacterial housekeeping functions involved in metabolic pathways and the putative gut-specific functions involved in adhesion to host protein, etc.), which sustains robustness in the gut ecosystem [25].

1.1.2. Characterization of gut microbiota

The development of culture-independent methods has contributed with studies of non-culturable microorganisms that constitute the majority of gut microbiota (~75%). Further developments and advantages on the community analysis of gut microbiota have also been provided by the metagenomics approach. Metagenomics is the study of genomic material (i.e. genomic DNA) isolated directly from the environment that can be performed targeted towards specific genes (e.g. 16S ribosomal RNA) or untargeted (e.g. shotgun sequencing) [7]. In metagenomic studies designated for microbial community analyses, taxonomic marker genes are used to characterize the populations in the ecosystem. The most commonly used marker for this purpose is 16S ribosomal RNA (16S rRNA) gene due to its favorable features, i.e. this region varies proportionally to the evolutionary distance between distinct genomes and contains both highly

conserved and ubiquitous sequences [26]. However, the genetic resolution of 16S rRNA is insufficient for the reliable classification of microbes into discrete species units [27]. Therefore, operational taxonomic unit (OTU), which is a cluster of the sequences with a certain similarity cutoff, is used and the species level is usually defined with an identity of 97% [26, 28].

The analysis of 16S rRNA gene amplicon sequencing data for microbial community structure consists of three main workflows (Figure 1) although some small differences can be observed based on the sequencing platform and the bioinformatics tools used [29].

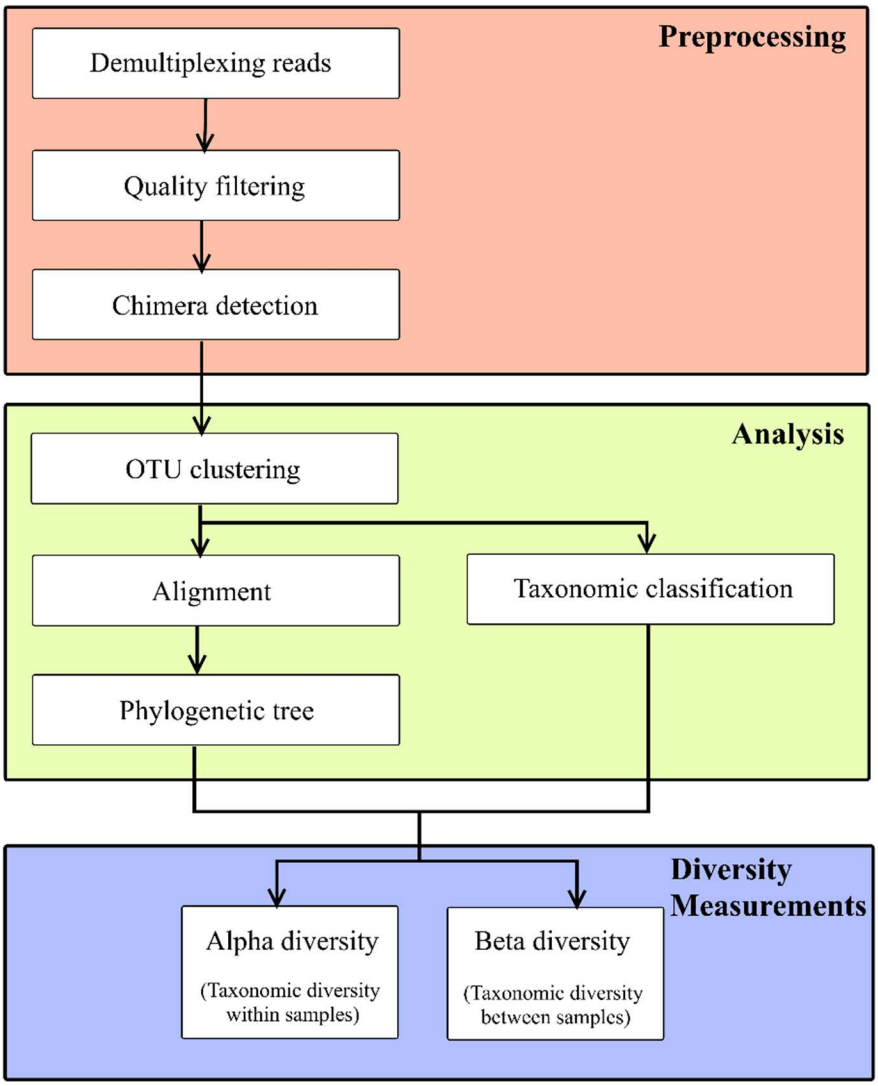

Figure 1. The main steps of 16S rRNA gene amplicon sequencing data analysis for microbial community structure.

There are several tools to analyze 16S rRNA gene sequencing data: some are included in analysis pipelines as a set of multiple algorithms for the analysis steps, while they can also be used alone for only specific step/s of analysis. The most commonly used bioinformatics pipelines for 16S rRNA gene sequencing data are Quantitative Insights into Microbial Ecology (QIIME) [30], mothur [31] and Meta Genome Rapid Annotation using Subsystem Technology (MG-RAST) [32], which produce comparable and reliable results of the community composition [29]. QIIME and mothur offer multiple tools for most of the steps, while MG-RAST has limited choices (Table 1).

Table 1. Comparison of features and implemented tools of QIIME, mother and MG-RAST (Adapted from Plummer et al., 2015).

	QIIME	**mothur**	**MG-RAST**
Licence	Open-source	Open-source	Open-source
Programming languages	Phyton	C++	Perl
Primary usage	Command line	Command line	GUI (website)
Sequencing technology compatibility	Illumina, 454, Sanger, Ion Torrent, PacBio	Illumina, 454, Sanger, Ion Torrent, PacBio	Illumina, 454, Sanger, Ion Torrent, PacBio
Quality control	YES	YES	YES
16S rRNA gene databases searched	RDP, SILVA, Greengenes and custom databases	RDP, SILVA, Greengenes and custom databases	M5RNA, RDP, SILVA and Greengenes
Alignment method	PyNAST, MUSCLE, INFERNAL	Needleman-Wunsch, blastn, gotoh	BLAT
Taxonomic analysis/assignment	UCLUST, RDP, BLAST, mothur	Wang/RDP approach	BLAT
Clustering algorithm	UCLUST, CD-HIT, mothur, BLAST	mothur, adapts DOTUR and CD-HIT	UCLUST
Diversity analysis	alpha and beta	alpha and beta	alpha
Phylogenetic tree	FastTree	Clearcut algorithm	YES
Chimera detection	UCHIME, chimera slayer, BLAST	UCHIME, chimera slayer, and more	No

GUI: Graphical User Interface; RDP: Ribosomal Database Project; M5RNA: Non-redundant multisource ribosomal RNA annotation; PyNAST: PythonNAST; MUSCLE: MUltiple Sequence Comparison by Log-Expectation; INFERNAL: INFERence of RNA Alignment; BLAST: Basic Local Alignment Search Tool; BLAT: BLAST-Like Alignment Tool; CD-HIT: Cluster Database at High Identity with Tolerance.

1.2. Diet and gut microbiota

Diet, prebiotics, probiotics, antimicrobial agents and fecal transplantation are strategies that have potential to modify and manipulate the gut microbiota [24]. Diet is one of the most important environmental factors that affect the gut microbiota since it acts as direct substrate for the microbiota via its indigestible ingredients and some digestion by-products. The significance of diet on the gut microbiota can also be seen in evolutionary development of gut microbiota in mammals such that the bacterial diversity among animals varies depending on diet type, i.e. increase from carnivory to omnivory to herbivory [33].

The main diet components, i.e. protein, fat and carbohydrate, have a remarkable impact on gut microbiota [24]. Enterotypes *Bacteroides* and *Prevotella*, are greatly correlated with the long-term diets of protein and animal fat, and carbohydrates respectively [20, 33]. Moreover, African children with a predominantly vegetarian diet (high fiber, starch and plant polysaccharides and low animal protein and fat) have a significantly higher Bacteroidetes:Firmicutes ratio, with particular increase in *Prevotella* and *Xylanibacter* genera, compared to European children with carbohydrate-rich diet [34]. The high abundance of these dietary fiber-fermenting phylotypes in African children is likely to contribute to the energy extraction in gut, as well as to provide protection against inflammation and non-infectious colonic diseases [24, 34].

Gut microbiota modification is promising to protect the host against diseases and improve some physiological aspects. It has been shown that diet interventions and long-term diet habits can shape the gut microbiota in aged subjects, i.e. mice and human, associating with an improved health [35, 36]. In addition, the modifications may help to recover from the unhealthy state. The balance in the normal gut microbiota can be disrupted (dysbiosis) due to several reasons, and it can lead to many diseases and disorders in host [37]. The cause and effect relation between the microbiota and disease/disorders are not well known; however, interventional studies aiming to develop strategies to modify gut microbiota and restate the normal growth and activity of beneficial phylotypes, are becoming more common [24].

1.2.1. Dietary fibers & Prebiotics

Dietary fibers are important components of the intervention studies for gut microbiota since they cannot be digested or absorbed in the upper part of gastrointestinal tract; however, they can be fermented by gut microbiota in the lower part of gastrointestinal tract (the large intestine) [38]. Many different definitions were suggested for dietary fibers by different organizations including CODEX Alimentarius Commission, European Food Safety Authority (EFSA), Food Standards Australia and New Zealand (FSANZ) and American Association of Cereal Chemists (AACC) [39]. However, all definitions agree on that dietary fibers are non-digested fibrous substances intrinsic in food as part of the fiber complex and most of the definitions also include the condition of having at least one proven health benefit [39]. The health benefits may refer to reduction of risk of some disease and disorders (e.g. coronary heart disease, stroke, hypertension, diabetes, obesity, certain gastrointestinal diseases), enhancement of immune functions or improvement of gut health via physiological effects (e.g. delaying gastric emptying, inducing gastrointestinal hormones, promoting growth of beneficial bacteria in colon) [40].

Dietary fibers can be classified differently depending on their role in the plant, fiber components, polysaccharide type, simulated gastrointestinal solubility, site of digestion, digestion products and physiological classification [41]. One of the most commonly used classifications is based on the solubility, i.e. being soluble or insoluble in water, as shown in Table 2.

Table 2. Classification of dietary fibers based on solubility (Adapted from Staffalo et al., 2012)

Category	Subcategory	Fiber fraction	Main food resource
Soluble fiber	Viscous	β-glucans	Grains
		Pectins	Fruits, vegetables, legumes, sugar beet, potato
		Gums & Mucilages	Leguminous seed plants, seaweed extracts, plant extracts, microbial gums, psyllium
	Non-viscous	Sugars	Lactulose
		Oligosaccharides	Various plants and synthetically produced
		Inulin	Chicory, Jerusalem artichoke, sugar beet, onions
Insoluble fiber		Cellulose	Plants
		Hemicellulose	Cereal grains
		Lignin	Woody plants
		Cutin/suberin/other plant waxes	Plant fibers
		Chitin and chitosan, collagen	Fungi, yeasts, invertebrates
		Resistant starches	Plants
		Curdlan (insoluble β-glucans)	Bacterial fermentation

The beneficial returns of dietary fibers mostly depend on their physicochemical characteristics (e.g. viscosity, solubility, fermentability) [42]. The fermentation of dietary fibers by gut microbiota contributes to the host health mostly due to the produced metabolites such as short chain fatty acids (SCFA) (e.g. butyrate, acetate and propionate), which act as energy source for colorectal tissues, stimulate cellular mechanisms that retain tissue integrity, contribute to immune system, and possibly have anti-inflammatory effects [7, 43, 44]. Furthermore, other organic acids such as formate, lactate and succinate, which are produced via fermentation of fibers, lower the pH in intestines and prevent the growth of pathogenic bacteria [45].

Prebiotics are a subgroup of dietary fibers with a variety of health benefits [46]. A prebiotic is defined as a "selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health." [47]. The targeted bacterial groups by prebiotics are usually lactobacilli

and bifidobacteria and the most extensively studied prebiotics are inulin, oligofructose, and fructooligosaccharides [24, 46].

A food ingredient should fulfill three criteria to be categorized as prebiotic [47]:

1. Resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption;
2. Fermentation by intestinal microflora;
3. Selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being.

1.2.1.1. Resistant starch (RS)

Resistant starch is a dietary fiber and considered as prebiotic since it meets the aspects of prebiotics [46, 48]. It provides prebiotic type fermentation within colon and confers many metabolic benefits such as increasing bile salt turnover and laxation, reducing risk of gastrointestinal tract cancers, lowering postprandial glucose response and blood lipid levels [48, 49]. Moreover, RS (type 3) increases the butyrate concentration via its fermentation by gut microbiota and contributes to epithelial cell growth and proliferation [50]. There are different types of RS that have been defined based on the physicochemical properties [51] (Table 3).

Table 3. Classification of types of resistant starch (RS) in terms of food sources, and factors affecting their resistance to digestion in the colon (Adapted from Nugent et al., 2015)

Type of RS	**Description**	**Food source**
Type 1	Physically protected	Whole- or partly milled grains and seeds, legumes, pasta
Type 2	Ungelatinized resistant granules with B-type crystallinity and are hydrolyzed slowly by amylases	Raw potatoes, green bananas, some legumes, high amylose starches
Type 3	Retrograded starch (i.e. non-granular starch-derived materials)	Cooked and cooled potatoes, bread, cornflakes, food products with prolonged and/or repeated moist heat treatment
Type 4	Chemically modified starches due to cross-bonding with chemical reagents, ethers, esters, etc.	Some fiber-drinks, foods in which modified starches have been used (e.g. certain breads and cakes)

The metabolic benefits of RS and the group of bacteria in gut that respond to RS vary depending on the type, which makes the effects on gut microbiota subtle and intricate [52]. Type 3 RS, which was assayed in this thesis, is considered the most resistant form of RS among the other types [57]. It has been shown to mainly promote the growth of *Ruminococcus bromii*, *Eubacterium rectale* and *Roseburia* spp. populations in gut of different animal models and human [10, 56, 54]. Moreover, *R. bromii* was suggested to be a keystone species in RS degradation, particularly Type 3, which is required for the other bacteria to utilize the products from RS [58]. However, in spite of the promising findings, the absolute role of gut microbiota in the physiological benefits of RS is still unknown.

1.2.1.2. Alginate (ALG)

Alginate is a polyuronic saccharide consisting of 1→4 linked α-L-guluronic acid (G) and β-D-mannuronic acid (M) pyranose residues in an unbranched chain. It is mostly isolated from cell walls of the brown seaweeds and commonly used in food industry as thickener and stabilizing or emulsifying agent [59]. This viscous dietary fiber confers many health benefits due to its gel-forming ability and other physicochemical properties including fermentability by gut microbiota [60]. The gel structure that is formed under acidic conditions of stomach provides the extension of gastric emptying duration and the control of appetite and reduces the rate of intestinal absorption [59, 61]. Moreover, ALG may refine gut barrier function, reduce damaging effects of luminal contents, control Type II diabetes and obesity by enhancing satiety [60]. ALG was shown *in vitro* and *in vivo* to be fermented in low rate by gut microbiota; however, its fermentability increases by time [59]. For example, the intestinal degradation of alginate in pig models was shown to be less than 55% on day 39 and it increased up to around 80% on day 74 [62]. In another study with rats, alginate recovery from fecal samples decreased from 64% to 39% over 4-week feeding period [63]. Moreover, ALG modifies gut flora to a certain extent; therefore, it is considered as prebiotic [63, 64]. However, still very little is known about relationship between the gut bacteria and ALG, e.g. which gut bacteria are affected by ALG and how their activities influence the host.

1.3. Bacteriocins & Probiotics

Competition and cooperation between microbes in the gut are the major shaping forces of the communities in such a complex environment. The competition in the gut generally consists of two main types: Exploitative competition, which entails limiting resources for others, and interference competition, which involves direct harming of other strains via antimicrobial production [65]. Diet is a factor for exploitative competition among gut microbiota providing nutrition for selective group of bacteria as mentioned in the previous section. On the other hand, bacteriocins play role in interference competition among gut microbiota helping the producer to colonize and create the niche environment in the ecosystem [66, 67].

Bacteriocins are "bacterially produced, small, heat-stable peptides that are active against other bacteria and to which the producer has a specific immunity mechanism" [68]. They are produced by a variety of microorganisms, i.e., gram-positive and gram-negative bacteria and some archaea [67]. The bacteriocins produced by gram-positive bacteria, mostly by lactic acid bacteria (LAB), are classified into two major groups; class I (Lanthionine-containing bacteriocins/lantibiotics) and class II (non-lanthionine-containing bacteriocins) bacteriocins. Lantibiotics are post-translationally modified small peptides of 19-38 amino acids in length, which include the best known, broad-antimicrobial spectrum bacteriocin nisin [66, 69]. Class II bacteriocins, which were studied in this thesis, are non-modified or subjected to minor modifications, i.e., disulfide bond formation or circularization. This group of bacteriocins includes a heterogeneous class of small (<10 kDa), heat-stable peptides. Although the classification varies in the literature, they are divided into following four subclasses according to Cotter et al. [68]:

- Class IIa bacteriocins are known as pediocin-like bacteriocins with relatively narrow antimicrobial spectrum. They are typically active against *Listeria*, while the other genera in inhibitory spectra includes *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Clostridium* [70].
- Class IIb bacteriocins are two-peptide bacteriocins that require the combined action of two different peptides with the encoding genes next to each other in the same operon. These bacteriocins have often narrow spectrum activity [71, 72].
- Class IIc bacteriocins are circular bacteriocins with the N- and C-termini covalently linked, which results with a cyclic structure [68].

- Class IId is a miscellaneous group containing all other remaining bacteriocins that do not fit into any of the aforementioned groups [68].

1.3.1. Biosynthesis and regulation

The genes that are required for the production of bacteriocin are organized in the operon clusters. These genes consist of structural gene encoding precursor peptide, immunity gene and the genes involved in regulation and transportation [73]. The biosynthetic pathway for most class II bacteriocins is illustrated in Figure 2.

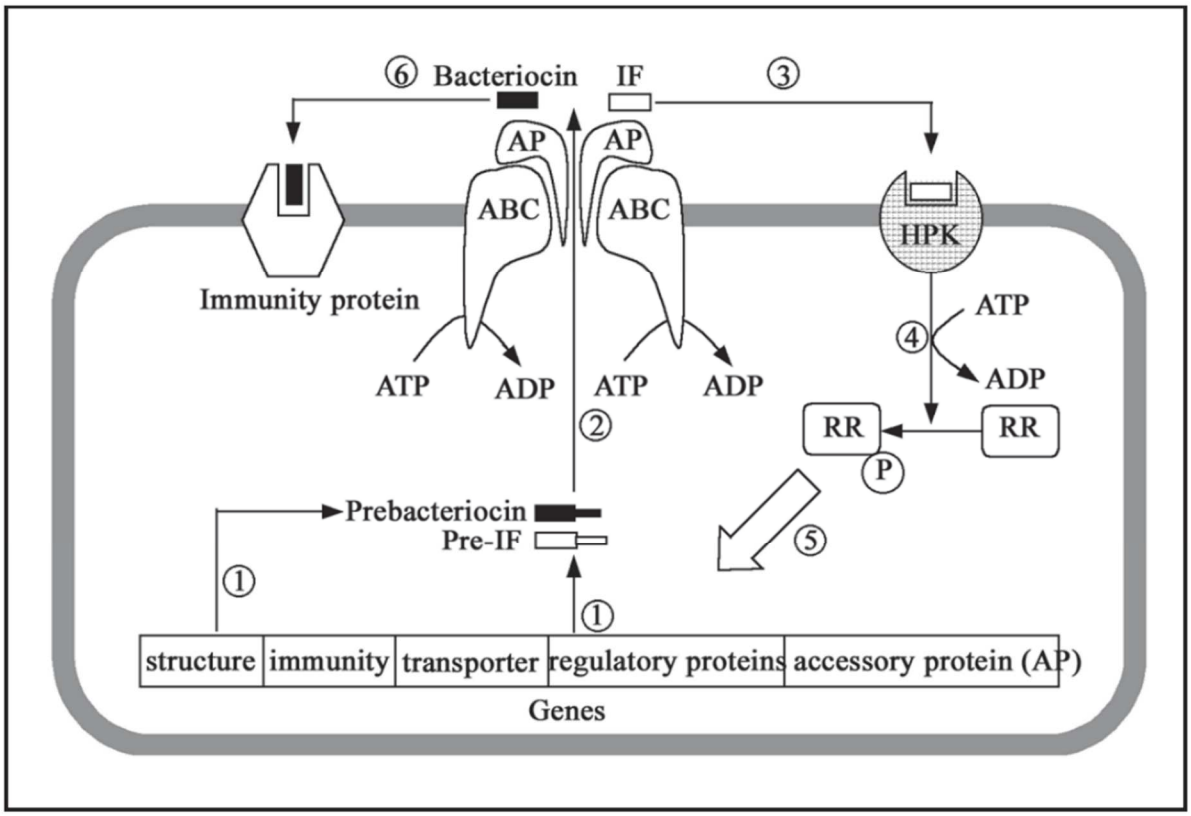

Figure 2. The biosynthesis pathway of class II bacteriocins (Adapted from Chen et al., 2003). (1) Formation of prebacteriocin and prepeptide of induction factor (IF); (2) The prebacteriocin and pre-IF are processed and exported by a dedicated ABC-transporter, resulting in the release of mature bacteriocin and IF; (3) Histidine protein kinase (HPK) senses the presence of IF and autophosphorylates; (4) The phosphoryl group (P) is subsequently transferred to the response regulator (RR); (5) RR activates transcription of the regulated genes; and (6) Immunity is processed.

Class II bacteriocins are usually synthesized as pre-bacteriocins carrying an N-terminal leader peptide that provides intracellular inactivation of the bacteriocin and serves as recognition site for the transportation and exportation processes [74]. Most of the class II bacteriocins contain double-glycine-type leader peptide while some others, such as class IIc bacteriocins, contain *sec*-dependent leader peptide [66]. The double-glycine-type leader peptide is removed during the exportation from the cell and the mature bacteriocin is produced. The processing during the exportation is performed by a dedicated ATP-binding cassette (ABC) transporter and an accessory peptide [75]. In addition, some other bacteriocins, such as enterocin L50 (class IIb) and enterocin Q (class IId) produced by *Enterococcus faecium* L50, do not contain any leader peptide and are exported from the cell via an unknown mechanism [76].

The production of several class II bacteriocins are transcriptionally regulated through a signal transduction system, which is composed of three components: an induction factor (IF), a histidine kinase protein (HK) and a response regulator (RR) [74, 77]. IF, which is a bacteriocin-like peptide with no antimicrobial activity, plays role as an induction factor. A dedicated ABC-transporter cleaves the leader peptide of IF specifically and exports IF as mature peptide concomitantly [73]. The secreted IF serves as an indicator of cell density, is sensed by HK and causes activation of RR, which eventually activates the expression of all operons necessary for the synthesis, transport and regulation of the bacteriocin. This regulatory network is normally referred to as quorum sensing mechanism because it is regulated by cell density. Thus, this mechanism will ensure that bacteriocin production is finely tuned to a certain critical mass (threshold) of bacteria at which bacteriocins are used to fight against competitors for the limited nutrients [66]. The bacteriocins sakacin A (class IIa) produced by *Lactobacillus sakei* Lb706 and plantaricins EF and JK (class IIb) produced by *Lactobacillus plantarum* C11 are examples of the bacteriocins regulated by a three-component signal transduction system [78, 79].

Bacteriocin producers have self-protection mechanism against their bacteriocins referred as immunity. The immunity protein is specific to and co-expressed with the bacteriocin. The immunity genes are located next to the structural genes in the operons [74].

1.3.2. Mode of action

Bacteriocins perform many distinct modes of action. Some causes disruption of target membrane integrity leading to cellular leakage and cell death while others act as enzyme inhibitors in biosynthetic pathways (e.g. DNA, RNA or protein synthesis) [80]. Bacteriocins of LAB mostly act by pore formation in the cell membrane of the target bacteria [73]. The interaction between the bacteriocin and the cell membrane of the target occurs initially via the electrostatic interactions of the positively charged peptide and the anionic lipids in the membrane of Gram-positive bacteria [66, 73]. In the subsequent events, the pores are formed through some mechanisms (see below) leading to detrimental effects such as leakage of molecules and loss of the membrane potential. The attribute of the pores varies from bacteriocin to bacteriocin in terms of size, stability and conductivity [66].

It has been discussed whether the pore formation mediated by bacteriocins occurs by targeting a specific receptor in the target cell envelope. Some mechanisms with specific target molecules has been discovered (Figure 3). Nisin and some other class I bacteriocins specifically target Lipid II, which plays role in peptidoglycan biosynthesis machinery within the cell envelope of bacteria. They may act by inhibiting the peptidoglycan biosynthesis in the target cell or by using this interaction for the pore formation in the cell membrane [80]. On the other hand, other bacteriocins such as class IIa and class IIc bacteriocins target the cell envelope-associated mannose phosphotransferase system (Man-PTS), which play role in transportation of sugars e.g. mannose as well as glucose, and eventually cause pore formation [80–82].

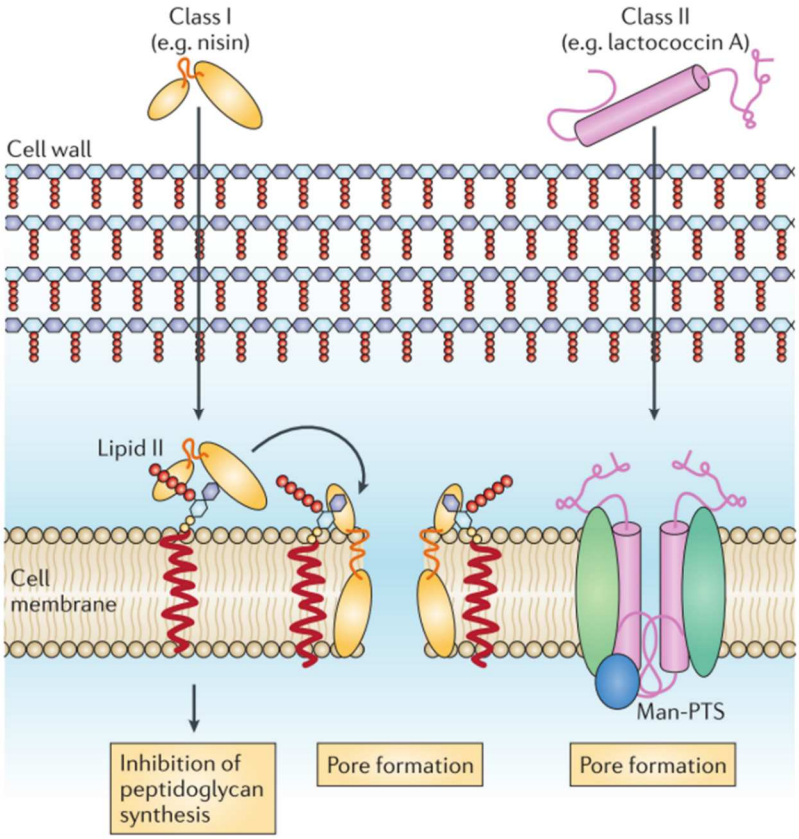

Figure 3. Action mechanisms of representative bacteriocins against Gram-positive targets (Adapted from Cotter et al., 2013).

1.3.3. Bacteriocins of lactic acid bacteria (LAB) in gut

The bacteriocins produced by LAB have received special attention since these bacteria are commonly found in food and feed fermented products, and are therefore generally regarded as safe (GRAS) for human consumption [69]. LAB also survive in gastrointestinal tract via the buffered environment they create as a consequence of their metabolic activities (i.e. with the balance between sugar fermentation and decarboxylation/deimination), and colonize gut resulting with complex molecular cross-talk with host and other bacteria [83]. Bacteriocins are used by LAB as weapons with a variety of inhibition spectra to compete with other bacteria that are likely to share the same niche. Most of the bacteriocins target species or genera closely related to the producers, while some can have much broader spectra [69, 84]. LAB can compensate for

the variety of the targets and the relatively narrow spectra of the bacteriocins by the production of multiple bacteriocins that belong to different classes [66].

In the gut, bacteriocins may act as colonizing agents, killing peptides or signaling peptides (Figure 4). They may help the producer survive and colonize in the gut, and inhibit the closely related competitive strains or pathogens. Bacteriocins may also have impact on microbial populations in the gut and host immune system [84, 85]. These activities of bacteriocins may contribute to the probiotic functionality of the bacteriocin-producing LAB.

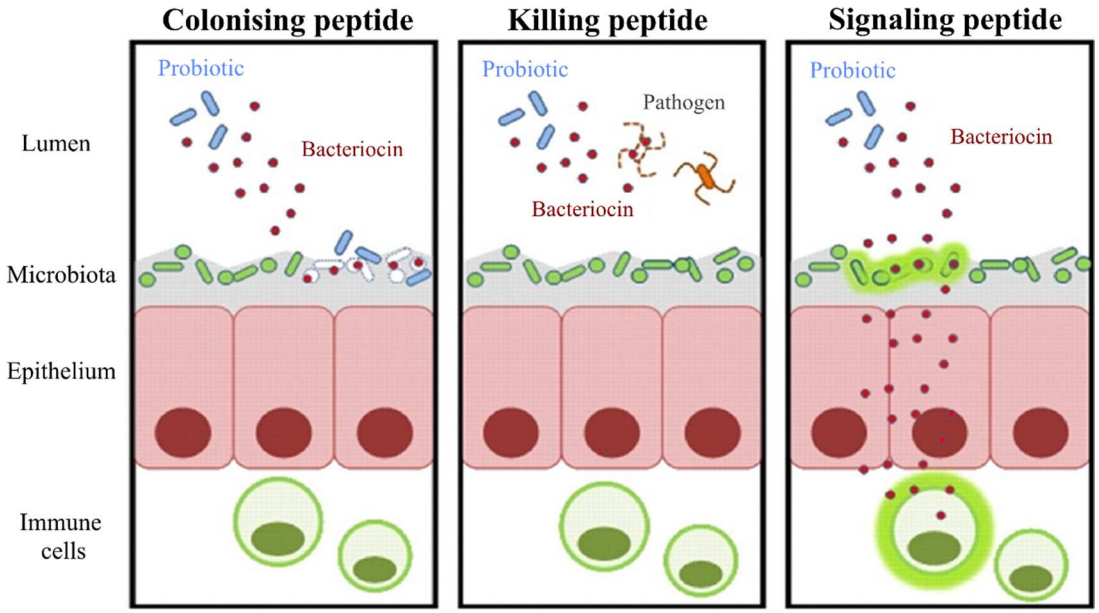

Figure 4. Actions of bacteriocins that could contribute to probiotic traits of the producer in gut (Adapted from Dobson et al., 2012).

Use of probiotics or antimicrobials to manipulate the gut microbiota have been an attractive therapeutic strategy. However, regarding antimicrobials, the inhibition spectrum (target specificity) is an important factor since the antimicrobials with broad spectra, such as antibiotics, may cause dysbiosis perturbing the gut microbiota [24]. The occurrence of dysbiosis depends on the gut microbiota composition, the resistance genes among the gut community and the mode of action of the antimicrobial [24, 86]. The disturbed microbiota may lead to the overgrowth of

pathogens or to adverse health effects to host [24]. In this context, bacteriocins exhibit remarkable advantages over antibiotics due to their relatively narrow spectra. Moreover, bacteriocins have other conveniences such as the variety of target specificity, non-toxicity to the host, antagonistic activity against important pathogens and the possibility of *in situ* production by probiotics [80].

1.4. Animal models in gut microbiota studies

It would be ideal to involve humans as research subject in the studies; however, it is challenging, especially in the diet-related gut microbiota studies, due to the difficulties in controlling their diets and life-styles during the experimental period. Therefore, model organisms are good alternatives with the ease of the experimental control and the opportunity of engineering [15].

Pigs are a commonly used model for humans because of many functional and structural similarities. For instance pigs and humans have similar genome, body size, anatomy and physiology of the digestive system, diet type (being omnivorous), propensity to obesity and social behaviours [87–89]. They also have a particular advantage to be used as model in gut microbiota studies due to their similarity to human in predominant gut phyla, i.e. Bacteroidetes and Firmicutes, and many genera such as *Prevotella*, *Streptococcus, Lactobacillus, Coprococcus, Blautia*, *Oscillibacter*, *Faecalibacterium* and *Roseburia* [88, 90, 91]. However, there are also some important differences in gut microbiota of pigs compared to human microbiota, i.e., pigs have lower amounts of *Bifidobacterium* and *Bacteroides* populations, while the abundance of streptococci is higher; moreover, the most abundant phylotype is lactobacilli although its abundance varies a lot with age [88, 91].

Mice are another animal models that have been used frequently in the studies to investigate the role of the gut microbiota in many physiological aspects that are also present in humans [15]. They are advantageous as model animals since they are well characterized, small and maintained easily, their genome has been sequenced completely, and they have quick reproduction and similar digestive tracts to humans [15, 92]. The main composition of murine gut microbiota at phylum level is similar to human gut microbiota with high abundances of Firmicutes, Bacteroidetes and Proteobacteria [90]. Moreover, most of the commonly encountered genera in the gut microbiota of mice are similar to the humans' [90, 92]. However, the abundances may

vary, e.g., the abundances of *Prevotella*, *Faecalibacterium* and *Ruminococcus* are higher in human gut microbiota, as compared to murine gut microbiota, while vice versa for the *Lactobacillus*, *Alistipes* and *Turicibacter* populations. In spite of the differences, dominant bacterial families were found to be similar in mice and human guts with the similar driving forces, i.e. enterotypes [93].

In addition to animal models with normally colonized gut microbiota, germ-free animals that are sterile and not exposed to any microorganisms, or gnotobiotic animals that are colonized with known bacteria, are good alternatives for the proof of principle to direct and interpret human studies, including diet alteration and probiotic intake studies [7, 25].

1.5. Aim of the thesis

The importance of the gut microbiota composition and activity for the host has been better understood recently, with increasing evidence on their associations with the host physiology and health status. The associations may be in both ways, e.g. leading to diseases and disorders, or improving health, and are usually influenced by environmental factors. Diet and antimicrobials are important factors in shaping the composition and activity of the complex populations in the gut. There is a body of knowledge that exists on the beneficial impacts of dietary fibers and probiotics via modulation of the gut microbiota; however, there are still many unanswered questions on their impacts on gut microbiota composition, interactions and activity. Therefore, the overall aim of this thesis was to identify the changes in gut microbiota populations and activities by the pre/probiotics interventions and their possible benefits on host physiology.

The current studies focused on the modifications of gut microbiota by the interventions of dietary fibers ALG and RS that meet the aspects of prebiotics, and the producers of class II bacteriocins as probiotics, with the following tasks:

- A brief overview on the factors affecting gut microbial composition and metabolites that influence host physiology. This is in order to reflect the recent status of the field before starting our studies (Paper 1)
- Identification of changes/responses in the gut microbiota of growing pigs fed with diets containing gel-forming ALG and fermentable RS, and the prediction of important interactions and functional changes within the microbiota (Paper 2)
- Evaluation of LAB strains, which produce different class II bacteriocins with various target specificities and inhibitory spectra, for their impact on the gut microbiota structure of healthy mice and some host-related aspects. (Paper 3)

2. Main results

Paper 1

Potential applications of gut microbiota to control human physiology.

Umu OC, Oostindjer M, Pope PB, Svihus B, Egelandsdal B, Nes IF, Diep DB (2013). *Antonie Van Leeuwenhoek* 104(5):609-18

The current knowledge regarding gut microbiota composition and metabolites was discussed in this mini-review, with a focus on their modulations due to environmental factors. Moreover, the possible effects of these modifications on the host physiology and metabolism, including obesity and satiety control via the gut-brain axis, were reviewed.

There is increasing evidence supporting that the physiology and health of the host are influenced by the gut microbiota composition and metabolic activities. Gut microbiota is mostly regulated by environmental factors such as diet (e.g. non-digestible dietary fibers). Manipulation of the gut microbiota populations and their metabolism is promising in terms of e.g., triggering specific host responses and treating gut microbiota-associated disease and disorders. However, the gut is a complex ecosystem due to the multi-directional interactions (e.g. diet-microbe, microbe-microbe, microbe-host), as well as the metabolites produced by microbiota (e.g. SCFA that may serve as signaling mediators in diverse activities in the host). These indicate that the interplay between gut microbiota and the host is rather complex and intricate. Therefore, further intervention studies will help more to explain the alterations in gut and their consequences, and they will help design biotechnological and therapeutic applications in the future.

Paper 2

Resistant starch diet induces change in the swine microbiome and a predominance of beneficial bacterial populations.

Umu ÖC, Frank JA, Fangel JU, Oostindjer M, da Silva CS, Bolhuis EJ, Bosch G, Willats WG, Pope PB, Diep DB (2015). *Microbiome* 16;3:16

Pig models were used for the comparative analysis of gut microbiome to evaluate their response to diets containing ALG or RS. 3-month-old growing pigs were fed with diets containing either ALG, RS or digestible starch (control diet). The fecal samples were collected over 12 weeks and processed for 16S rRNA gene amplicon sequencing.

The greatest alteration in gut microbiota structure was detected in pigs fed with RS, which exhibited decrease in alpha diversity, and a notable predominance of metabolically reputable bacterial populations of *Prevotella*, *Ruminococcus,* Lachnospiraceae, as well as others such as Veillonellaceae, *Bulleidia* and *Dialister*. ALG-containing diet did not change the diversity and the composition of gut microbiota of the pigs significantly; however, some populations such as *Roseburia*, *Ruminococcus*, and *Lachnospira* shifted to have significantly higher relative abundance compared to control (CON) pigs.

The feed degradation was determined using comprehensive microarray polymer profiling (CoMPP) of digested material. The change in relative abundances of plant cell wall polysaccharides and proteins (basal diet components) over time was similar in pigs fed with different diets, while the correlated bacterial phylotypes differed notably depending on the diet.

Moreover, the predictions of imputed function from 16S rRNA gene sequencing data showed that the imputed functions varied remarkably in pigs fed with RS, but up to a certain level in pigs fed with ALG compared to control pigs. Despite the variations in imputed functions, some key pathways such as degradation of starch and other plant polysaccharides were predicted to be kept unchanged among the different diets.

Paper 3

The potential of class II bacteriocins to modify gut microbiota to improve host health.

Umu ÖC, Bäuerl C, Oostindjer M, Pope PB, Hernández PE, Pérez-Martínez G, Diep DB (2016). (Submitted manuscript)

The impact of bacteriocin-producing LAB on the gut microbiota of mice was analyzed using 16S rRNA gene amplicon sequencing approach. Mice were fed with the bacteriocin-producing strains or their isogenic strains that do not produce any bacteriocin (or produce fewer) for 14 days of total 28-day experimental period. The bacteriocins assessed were sakacin A (produced by *Lactobacillus sake* Lb 706), pediocin PA-1 (*Pediococcus acidilactici* 347), enterocins P, Q and L50 (*Enterococcus faecium* L50), plantaricins EF and JK (*Lactobacillus plantarum* C11B) and garvicin ML (*Lactococcus garvieae* DCC43). These bacteriocins are classified in different subclasses of class II bacteriocins with different target specificities and antimicrobial spectra.

Overall, the bacterial composition and diversity in treated samples were similar to the control. However, some significant changes were observed at deeper taxonomic levels and these changes varied depending on the antimicrobial spectra of bacteriocins. Sakacin A and pediocin PA-1 (class IIa) and plantaricins (class IIb) with narrow antimicrobial spectrum exhibited less modifications, whereas the producers of garvicin ML (class IIc) and enterocins (class IId and IIb) were the most effective in causing changes in bacterial populations. Many of the modifications/changes disappeared after bacteria treatment ended, which indicates the resilience of the microbiota. Some genera, which include potential pathogenic strains, were negatively affected, such as *Staphylococcus* by enterocins, Enterococcaceae by garvicin ML and *Clostridium* by plantaricins. In addition to possible targets of the bacteriocins, other bacterial groups were affected, which were likely to be indirect effects, e.g. Prevotallaceae Ruminococcaceae and Rikenellaceae populations increased by the producer of garvicin ML.

The production of garvicin ML and enterocins, which enhanced the size of the producer-affiliated populations, likely contributed to the colonization or longer stay of the producers in gut. The producers of sakacin A, garvicin ML and plantaricins increased the count of total LAB that are mostly known for their beneficial effects on the host. These activities of the bacteriocin producers

indicate the contribution of the bacteriocins to the probiotic properties of their producers. Moreover, garvicin ML caused changes on the blood serum levels of lipid components, some of which are normally regarded as beneficial to host health.

3. Discussion

The gut microbiota is densely populated by microorganisms with a vast array of genes and displays numerous effects on host's health. It has complex and heritable traits such as being shaped by the host's genetic components [94]; however, environmental factors that the host is exposed to throughout life are important factors to modulate composition and metabolic activity of gut microbiota [95]. In spite of the excessive increase in the studies that investigate both microbe-microbe and host-microbe interactions, and the descriptions of many factors that may influence gut microbiota, the dynamics of gut microbiota are still poorly understood. Therefore, in this thesis, we include a brief overview of the field reflecting the recent status before starting our studies (Paper 1), and studies on the important forces that drive microbial and functional variations in gut among individuals: prebiotics as diet components (Paper 2) and probiotics that produce bacteriocins as antimicrobial agents (Paper 3). In addition, the possible consequences of these interventions are discussed in terms of the impact on the host physiology.

3.1. Modulation of gut microbiota

The gut microbiota has a symbiotic relationship with the host, where it plays a key role in gut functioning (reviewed in Paper 1). Environmental factors can affect this relationship via modifying gut microbiota composition, resulting in either improved health or an unhealthy state of the host. Intervention studies can increase our understanding on how gut microbiota affects the host or vice versa.

Dietary interventions have strong influence on gut microbiota composition and metabolism, likely due to the exploitative competition, since the diet is mostly a limiting resource in gut resulting with competitive exclusion among the microorganisms [3, 65]. The competitiveness of gut microbiota associates with the exploitation of the supply of the limited nutrients such as carbohydrates. Non-digested dietary components, e.g. dietary fibers (including prebiotics), may lead to metabolic regulation, bacterial population shifts and metabolic cross-feeding among gut microbiota [96]. The impact of dietary fibers on host varies based on their physicochemical properties such as solubility, water holding capacity, viscosity and fermentability that may also associate with appetite, energy intake and body weight of the host [97]. In our study (Paper 2),

we observed the different levels of modifications on gut microbiota of growing pigs by diet intervention using dietary fibers with different physiochemical properties. ALG is a gel-forming soluble fiber and partially digested by digestive enzymes of the host, while RS is a fermentable and insoluble fiber being resistant to digestion. The composition and diversity alterations in gut microbiota was greatest in pigs fed with RS (Type 3). The bacterial diversity was reduced, most likely due to the selection of particular genera among the Firmicutes phylum, and many bacterial phylotypes displayed shifts in relative abundance. Concordantly to the other studies that evaluated RS (type 3) [10, 56, 54], the *Ruminococcus* genus (including *R. bromii*) increased in relative abundance. Moreover, a broad variety of genera was increased in relative abundance, including *Bulleidia*, *Megasphaera*, *Dialister*, an unclassified Lachnospiraceae genus, and *Prevotella*. The phylotypes affiliated to *Prevotella*, Lachnospiraceae and *Ruminococcus* are considered beneficial since they are reputable for production of SCFA that are metabolites of polysaccharide degradation, which may contribute to the host via different ways (described in Paper 1). On the other hand, ALG also altered the relative abundances of some populations although the diversity did not change compared to the control diet pigs. The metabolically favored phylotypes, i.e., *Ruminococcus*, *Lachnospira* and *Roseburia* increased in relative abundance. The number of Bifidobacteria increased in human subjects by ALG previously [98]; however, we could not detect *Bifidobacterium* in any pigs regardless of diet because this group of bacteria is minor in pigs [99, 100].

Contrary to the dietary interventions that cause exploitative competition in gut environment, antimicrobials produced by bacteria, which harm other bacteria directly, contribute to the interference competition [65]. The interventions of antimicrobials themselves or their producers (e.g. probiotics) in the gut may result in the modifications of the gut microbiota populations, depending on the target specificity [24]. In our study (Paper 3), the class II bacteriocins produced by LAB strains did not cause any significant change in the overall structure of the gut microbiota in mice. However, bacterial populations were found affected at deeper taxonomic levels (i.e. genus), especially by the producers of the bacteriocins with relatively broad-inhibitory spectrum compared to the ones with narrow-inhibitory spectrum. The target specificity of the bacteriocins is a very favorable property especially when compared to antibiotics that normally disrupt or provoke large changes in the gut microbiota with their very broad antimicrobial spectrum. This disruption (or the changes) potentially cause some opportunists to take over in the gut (e.g.

Clostridium difficile) [101] or lead to many diseases and disorders [102, 103]. The tested bacteriocins have diverse target bacteria *in vitro* including some pathogenic and problematic strains. Some of the populations that were decreased by the treatments were the taxa that involve important pathogenic species. These traits of the LAB-produced class II bacteriocins offer the opportunity of manipulation of the specific populations by bacteriocin producers without disturbing the symbiotic inhabitants of the gut. Moreover, the administration of the bacteriocins via the gut- or fermented food-associated producer strains that are endurable in acidic conditions provide the advantages of bacteriocin production *in situ* as well as preservation of the bacteriocins from proteolysis during gastric transit.

The different levels of modulation of gut microbiota by these interventions have potential to be used for therapeutic purposes, for example, switching from unhealthy state to healthy state by dietary fibers or inhibiting bacterial phylotypes with target specificity administrating bacteriocin producers without major disturbances on the main structure. Moreover, the modifications such as the enhancement of growth of SCFA producers may be promising for the use of interventions (especially prebiotics) in order to improve the gut health and satiety of the host via different mechanisms, e.g. brain-gut axis (Paper 1).

3.2. Resilience and functional redundancy

The disruption of the stable state of the gut microbiota or the degree of modulation varies depending on the modulating factor/treatment and the resilience of the community [5]. The disruption of the balance in gut microbiota may result in diseases or disorders, or may improve host health. In our studies, the dietary fiber supplementation in the diets of growing pigs for 12 weeks led to significant changes in gut microbiota (especially for RS) with a predominance of beneficial bacterial populations, overcoming the resilience of gut microbiota during the experimental period (Paper 2). This seemingly led to positive consequences, such as enrichment of bacteria with reputable metabolic activities (e.g. SCFA production). On the other hand, the bacteriocin treatments exhibited only fine modifications on mice gut microbiota, which were usually reversible, indicating the relative resilience of the microbiota to the bacteriocin treatments (Paper 3).

The presence of functional core in gut ecosystem has been highlighted for humans and the important functions were common among subjects despite of the inter-individual variations on microbiota compositions [9]. In a concordant manner to humans, we observed the maintenance of key functions in growing pigs even when the gut microbiota composition has been shifted by diet (Paper 2). Principally, the relative abundance of starch and sucrose metabolism KEGG pathway that includes not only starch, but also cellulose, xylan, betaglucan, and pectin conversion was consistent among the pigs treated with different diets. The degradation of these polysaccharides by the gut bacteria can be considered as an important function of microbiota because it serves as fermentation substrate and energy source for them, which results in production of various metabolites, and increases the digestion efficiency of the host. The functional core is provided by a set of non-redundant genes shared among different bacterial species bringing along the hypothesis of functional redundancy that contribute to the normal functioning of the gut [7, 25]. The functional redundancy hypothesis was supported by our study; namely, the gut microbiota of pigs with different feeding regimes had similar capacities for polysaccharide degradation, but different phylotypes correlated with the relative abundance of the polysaccharides. Despite the microbiota composition shift, the important gene functions were mostly conserved; this suggests the replacement of the bacteria that are involved in similar activities in the gut.

3.3. Host-related aspects of the interventions

It is obvious that the composition and activity of the gut microbiota are involved in regulation of the host's metabolism and are associated with the physiology and health of the host through their lifespan [37, 104]. Therefore, the modulation of gut microbiota by external factors including the diet (e.g. involving prebiotics in diet), probiotics and antimicrobial-based intervention may lead to physiological changes in the host [24].

Prebiotics, in the contexts of their definition, confer beneficial effects on the physiology of the host by their physicochemical properties and by being fermented by intestinal bacteria and stimulating their growth and/or activity (discussed in Paper 1). ALG and RS have been previously studied for their effects on the physiology and feeding patterns of the growing pigs and different outcomes have been displayed [105]. The pigs fed with ALG-containing diet experienced higher

feed intake to compensate for reduced digestible energy compared to the control diet, and had less back-fat thickness and carcass efficiency (i.e. less absorption of nutrients and less conversion to fat and muscle). On the other hand, RS pigs used digestible energy more efficiently and the average daily weight gain was normal in spite of low digestible energy intake; moreover, the colon and total gastrointestinal tract empty weight were increased in RS pigs. These indicate the fermentation of RS in colon resulting in the extra energy supply for the pigs by their intestinal bacteria. We showed that the ALG and the RS exerted different influences on the interactions and functions of the gut microbiome of growing pigs (Paper 2). The other physicochemical properties of the ALG, such as gel forming capability, are seemingly more dominant in affecting the physiology of the host than the fermentability as we observed less alteration in the gut bacterial populations and in the imputed functions of the microbiome compared to RS. However, the function of RS on the host physiology is presumably highly modulated by the altered gut microbiota activities, e.g. encouraging the growth of the phylotypes producing SCFA that are used as energy source, and alters the microbiome functions largely.

Probiotics have also been a focus of many intervention studies showing that the administration of probiotics in sufficient doses will impact the intestinal microbiota of the consumer and impart numerous health benefits [106]. The mechanisms used by probiotics for improving health include production of antimicrobials such as bacteriocins [84]. Bacteriocin production is considered an important trait of probiotics. It provides advantages to the producer; for example, on colonizing the gut while competing with the bacteria that share the same niche, inhibiting the pathogenic strains and triggering host's immune system via various signaling pathways [84, 107]. We proposed that some class II bacteriocins contribute to the probiotic traits of the producer LAB strains promoting health-bringing effects in mice (Paper 3). Among the tested bacteriocins, garvicin ML produced by *Lactococcus garvieae* DCC43 came into prominence with its greater positive effects on both gut bacterial populations and the host-related parameters that may be indicative in health condition, i.e. reduction in triglycerides and increase in high-density lipoprotein levels of blood serum in mice. Garvicin ML enhanced some SCFA-producing phylotypes such as Prevotellaceae, Rikenellaceae and Ruminococcaceae, apparently as a secondary effect due to the interactions between gut populations. We also observed reduction in relative abundances of Enterococcaceae, *Staphylococcus* and *Clostridium* by garvicin ML, enterocins P, Q and L50 and plantaricin EF and JK respectively, which include important

pathogenic strains. Sakacin A, plantaricins EF and JK and garvicin ML increased the count of total LAB, which contributes to host physiology via numerous mechanisms [83]. Moreover, some of the bacteriocin-producing strains (i.e., *Enterococcus faecium* L50 producing enterocins P, Q and L50 and *Lactococcus garvieae* DCC43 producing garvicin ML) enlarged the relative abundance of producer-affiliated bacterial taxa. These findings indicate that these bacteriocins may contribute to the probiotic properties of the producer strains, potentially conferring proposed health benefits to the host. However, the source of the effects could be the alteration of some microbiota populations or due to other pathways included in microbe-host interactions. Moreover, we could analyze limited host-related blood serum parameters for the influence of the bacteriocins. Therefore, a deeper understanding of the host-related aspects is a prerequisite for optimizing therapeutic strategies for the use of bacteriocin-producing probiotics to manipulate the gut microbiota.

4. Concluding remarks and future perspectives

The current work evaluated the impact of interventions of prebiotics and bacteriocin-producing probiotics on the gut microbiota composition and activity using pig and mouse models respectively. Gut microbiota was modulated by these interventions at varying levels correlating with the physicochemical properties of the prebiotics and the antimicrobial spectra of the bacteriocins produced by the probiotics.

The most pronounced variations in composition and imputed functions of gut microbiota were demonstrated by RS in growing pigs. The modulation of the bacterial populations by RS appeared with the predominance of beneficial phylotypes. Moreover, in spite of the significant change on the imputed functionality of predicted metagenomes, especially by RS, the functional redundancy was demonstrated for the key functions, i.e. the capacity to degrade starch and other dietary polysaccharides of the microbiota. On the other hand, the administration of bacteriocin-producing strains resulted in the overall microbiota structure being largely unchanged, although the relative abundance of deeper taxonomic lineages were modified. These modifications included the reduction of some phylotypes to which pathogenic strains are affiliated and the enhancement of the growth of potentially beneficial bacteria (e.g. total LAB). Other positive aspects of bacteriocins, which contribute to the probiotic traits of the producer, included the ability to promote the gut colonization of the producer, and to modulate the host-related parameters conceivably in a health-bringing manner. The relatively stable microbiota composition and the small changes in gut bacterial populations, most of which disappeared after the treatment ceased, highlighted the resilience of murine gut microbiota to the bacteriocin producers. This brings the advantage of these probiotic strains for the therapeutic or health improving purposes without disturbing the commensal inhabitants of the gut.

Based on the findings in this thesis, we propose that prebiotics and bacteriocin-producing probiotics can be used to promote changes beneficial for the host. However, some factors need further detailed investigations, e.g. shotgun metagenomics analysis to figure out the metabolic capabilities of the key populations within the microbiome of the host treated with prebiotics, and further assessment of probiotics in different experimental designs in order to fully recognize their health bringing effects.

The varieties in the design of pre/probiotics studies, e.g. in methods, models, intervention period, as well as the differences between individuals make the evaluation of the results complicated. Therefore, standardization of the experiments using large-scale experimental designs, and further investigation on safety assessments would help to prove the effects and to put these applications into practice in humans.

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REVIEW PAPER

Potential applications of gut microbiota to control human physiology

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Abstract The microorganisms living in our gut have been a black box to us for a long time. However, with the recent advances in high throughput DNA sequencing technologies, it is now possible to assess virtually all microorganisms in our gut including non-culturable ones. With the use of powerful bioinformatics tools to deal with multivariate analyses of huge amounts of data from metagenomics, metatranscriptomics, metabolomics, we now start to gain some important insights into these tiny gut inhabitants. Our knowledge is increasing about who they are, to some extent, what they do and how they affect our health. Gut microbiota have a broad spectrum of possible effects on health, from preventing serious diseases, improving immune system and gut health to stimulating the brain centers responsible for appetite and food intake control. Further, we may be on the verge of being capable of manipulating the gut microbiota by diet control to possibly improve our health. Diets consisting of different components that are fermentable by microbiota are substrates for different kinds of microbes in the gut. Thus, diet control can be used to favor the growth of some selected gut inhabitants. Nowadays, the gut microbiota is taken into account as a separate organ in human body and their activities and metabolites in gut have many physiological and neurological effects. In this mini-review, we discuss the diversity of gut microbiota, the technologies used to assess them, factors that affect microbial composition and metabolites that affect human physiology, and their potential applications in satiety control via the gut-brain axis.

Keywords Gut microbiota · Obesity · Health · Diet · Satiety

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Introduction

Gut microbiota as a whole with its great diversity and quantity is known to have a profound impact on human physiology and health. The most consistently found bacterial phyla in the human gut are Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Actinobacteria and Fusobacteria. In healthy human guts, the most predominant phyla are Bacteroidetes and Firmicutes (92.6 %), as determined by 16S rDNA sequencing applied to fecal samples (Alonso and Guarner 2013; DiBaise et al. 2008; Sanz et al. 2012; Tagliabue and Elli 2012). On species level, the composition of microbiota varies between individuals;

therefore each person has his or her own unique gut microbiota composition (Eckburg et al. 2005). The composition of microbiota also varies between the different regions of the gastrointestinal tract (GIT), from mouth to anus (Latulippe et al. 2013). The density of microbiota and the ratio of anaerobic to aerobic bacteria increase while descending along the digestive tract: the total number of bacteria is 10^2 cfu/mL in the stomach, $10^{1–3}$ cfu/mL in duodenum, $10^{2–4}$ cfu/mL in jejunum, $10^{7–9}$ cfu/mL in ileum and $10^{10–12}$ cfu/mL in the colon (DiBaise et al. 2008; Sartor 2008; Shigwedha and Jia 2013) (Fig. 1).

The microbial communities in the gut play an important role in the regulation of food intake and the uptake of energy and nutrition from food during gut passage. As a result, the growth and development of the host's body (weight) are regulated, most probably to a great extent, by gut microbiota. Germ-free animal studies provide solid evidence for a symbiotic relationship between gut microbiota and the host, and the health benefits of having gut microbiota (Alonso and Guarner 2013; Backhed 2012). Microbial communities of the gut contribute to increased resistance to infections by preventing the growth of pathogens, activating the host's immune system and stimulating proliferation of epithelial cells along the gut (Alonso and Guarner 2013). Consequently, adverse disturbances in the gut microbiota's composition can contribute to many disorders and diseases (Fig. 1). This includes antibiotic-associated diarrhea, pathogenesis of sepsis, autoimmunity and related disorders, inflammatory bowel disease (IBD) (including two distinct disease patterns of ulcerative colitis (UC) and Crohn's disease (CD)), irritable bowel syndrome (IBS) and metabolic syndrome (Alonso and Guarner 2013; Grenham et al. 2011). Obesity which is one of the most common and major health concerns worldwide is also known to be influenced by gut microbiota. Therefore, in order to exploit these microbes safely and efficiently for our health benefits it is crucial to

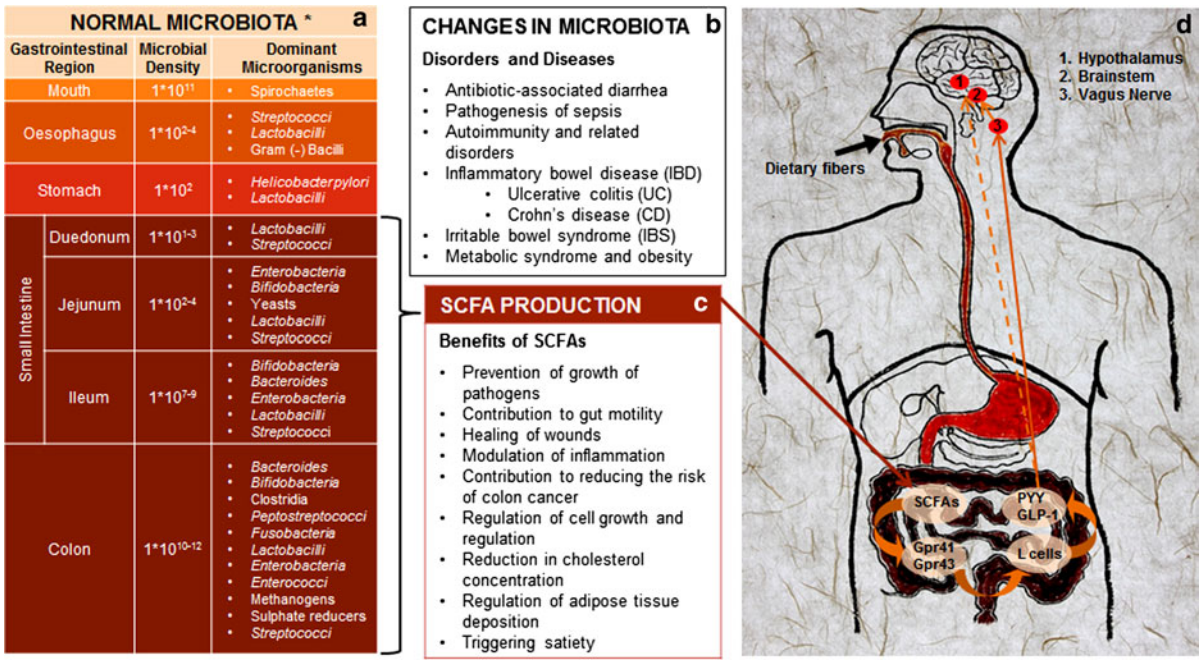

Fig. 1 The gut microbiota and their implications in human health and diseases, and how dietary fibers affect satiety via microbial activities. **a** Common gut bacteria found in the different parts of the GIT (DiBaise et al. 2008; Sartor 2008; Shigwedha and Jia 2013). **b** Changes in the composition of gut microbiota may result in disorders and diseases. Dietary fibers are known to improve the growth of normal gut microbiota. When ingested by host, they are fermented by microbiota in intestines, and short chain fatty acids (SCFAs) are produced. **c** SCFAs enhance satiety beyond many other benefits to health. **d** In the gut, produced SCFAs are ligands for G-protein-coupled receptors (Gpr41 and Gpr43); these receptors are expressed in L-cells and stimulate secretion of satiety-related hormones such as glucagon like peptide-1 (GLP-1) and peptide YY (PYY). The hormone signals follow the vagal pathway from the gut to the brainstem or directly go to the neurons in hypothalamus and influence satiety and food intake

identify and characterize the microbial species living in our gut and how they as individuals and communities affect our health.

In this minireview, we will start with an overview of some technologies commonly being used to study gut microbiota. Then we will focus on some aspects of gut microbiota relevant to obesity, the consequences of change in diet on microbiota and their metabolites, with special focus on the potential to exploit these microorganisms in satiety control via the brain-gut axis.

Characterization of the gut microbiota

Characterization of the gut microbiota can be done using culture-based methods and molecular genetic tools. However, culture-based methods are often inefficient and inadequate because they are time consuming, laborious, costly, and more importantly, 60–80 % of the microorganisms cannot be cultivated (Blaut et al. 2002; Tagliabue and Elli 2012). Several culture-independent methods have been developed to circumvent the shortcomings of culture-based methods. Denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) are examples of culture-independent methods. These technologies are based on 16S rRNA gene and have been used for bacterial community profiling over the past 20 years. However, both methods are dependent on DNA amplification by PCR, an approach which is inevitable to encounter biased amplification of DNA targets from a mixed population, hence potentially giving rise to misinterpretation of the relative abundance of bacteria in an ecosystem (Su et al. 2012). Recently, the most common and preferred approach is called metagenomics, a molecular approach in which all genetic materials are extracted directly from the collected samples without further cultivation. In this approach, the genetic materials obtained will therefore represent all microorganisms (including the non-culturable ones) in the community (Alonso and Guarner 2013). Fluorescent in situ hybridization (FISH) is one of the earliest metagenomic techniques to detect uncultured microbial community (Morgan and Huttenhower 2012). This method is based on 16S rRNA gene targeted oligonucleotide probes for diversity analysis, and community can be detected at phylum level to species level. However, FISH is dependent on probe sequences and cannot detect unknown bacteria. In recent studies, high-throughput DNA sequencing approaches are used for characterization of microbiota (Morgan and Huttenhower 2012). This approach can provide rapid phylogenetic identification in terms of size and diversity, and also unknown bacteria can be identified. Metagenome or mass genome sequencing projects have long been seen as a relatively time-consuming and very expensive approach. Therefore, they have been until recently available to only a few groups with research activities involving bacterial genomes and communities. However, the advent of high throughput nanotechnologies in genome DNA sequencing, and the drastic reduction in costs related the sequencing itself (e.g., less chemicals, more automation) make the technology now a commonplace to many laboratories. Therefore, huge amounts of sequencing data are currently produced in a relatively short time. Further, most metagenome projects are currently carried out and entirely dependent on skilled bioinformaticians or/and metagenomics experts. Future challenge is to design softwares that are more user-friendly and amenable also for non-bioinformatic researchers.

Obesity relating to gut microbiota

The number of people that are classified as obese (Body Mass Index (BMI) > 30) has been more than doubled worldwide since 1980. The most recent data from the Centers for Disease Control and Prevention (CDC) estimates that 35.7 % of U.S. adults and 16.9 % of U.S. children and adolescents were obese in 2009–2010 (Ogden et al. 2012). Obesity is a result of a higher intake of energy-yielding nutrients, mainly starch, fat and sugar, than is being used by the body. Thus, diet is the main cause, as the misbalance between energy intake and energy expenditure occurs due to increased accessibility of high calorie food products and a sedentary life style. The nutrient gain and energy regulation in the host can be affected by gut microbiota (Delzenne et al. 2011; DiBaise et al. 2008). It is now known that the compositions of gut microbiota of obese and lean mice differ, and that when microbiota of obese mice is transplanted to lean mice, the total body fat content of the lean mice increases rapidly (Backhed et al. 2004; Tagliabue and

Elli 2012). It has been reported in many studies that in obese mice and humans, the number of Firmicutes is higher than that of Bacteroidetes (Delzenne et al. 2011; Diamant et al. 2011; DiBaise et al. 2008; Ley et al. 2006; Sanz et al. 2012). However, it has also been claimed that the changes of microbiota at the phylum level are not as significant as those at the smaller taxonomic levels (Cani and Delzenne 2009; Parnell and Reimer 2012b). Obesity is also associated with a decrease in the number of archaea, *Methanobrevibacter smithii* (Parnell and Reimer 2012a).

Moreover, gastric bypass operation in humans which aims to treat obesity often causes weight reduction and change in gut microbiota. In this type of surgery, the stomach is divided into two parts and the small intestine is rearranged to connect both parts of the stomach; therefore, the food intake decreases and the satiety feeling is enhanced (le Roux et al. 2011; Thirlby et al. 2006). A shift in microbiota is commonly observed after gastric bypass, possibly because of the resultant reduced food intake, and in effect, this could have altered the metabolic status in the gut and hence the human health (Tremaroli and Bäckhed 2012). It was shown in a study by Osto et al. (2013) that the gut microbiota of rats changed after Roux-en-Y gastric bypass (RYGB) surgery (one of the most common gastric bypass surgeries). In this study, RYGB operation and sham operation (a placebo surgery) were performed on rats, and certain bacteria were identified using quantitative PCR. The change in microbiota appeared to be similar to the changes occurring after prebiotic treatment and weight loss in humans and rats although this change was independent of weight lost. In another recent study on humans (Kong et al. 2013), the richness of gut microbiota increased after RYGB surgery and changes were observed in seven predominant genera as inferred by 16S-gene sequencing results. It was also observed that some genera of Firmicutes such as *Lactobacillius*, *Dorea* and *Blautia* and *Bifidobacterium* belonging to Actinobacteria phylum, reduced in size. On the other hand, some genera of Bacteroidetes phylum which are *Bacteroides* and *Alistipes* and the genus *Escherichia* belonging to Proteobacteria, increased in number after RYGB.

In sum, the changes in composition of gut microbiota and the presence of certain bacteria have an impact, to a varied extent, on obesity or weight loss, and this is most probably related to energy regulation. Moreover, we can observe in gastric bypass studies aiming at weight loss that this kind of operations often causes a shift in microbiota. Therefore, the weight loss and gut microbiota should be investigated in a two-way manner.

Effects of environmental factors on gut microbiota

Many environmental factors such as maternal gut microbiota, host immunity, antibiotic usage, stress and diet can cause changes in the composition of gut microbiota in humans and animals (Kau et al. 2011; Mujico et al. 2013). The effects of environmental factors on gut microbiota were investigated using human intestinal tract chip (HITChip) microarrays in short and long term in a 12-year study on 5 young adults (Rajilić-Stojanović et al. 2012). It was observed that the presence of some typical gut bacteria was stable in the gut although their size could vary depending on environmental factors. Among the factors, diet has a significant impact. Changing type of the diet (e.g. increased or decreased proportion of proteins, carbohydrates or fat) normally results in significant changes in gut microbiota composition, and it is probably the easiest way to control the composition as the change occurs very quickly- even within a day (Clemente et al. 2012; Tremaroli and Bäckhed 2012). The reason is that diet is the main source of energy for gut microbiota which ferment the food indigestible for the host. Different types of food ingested contribute to the growth of different types of bacteria. (Scott et al. 2012). To illustrate, *Bacteroides* cluster was predominant when food contained high levels of protein and animal fat, while high protein levels alone had a negative impact on *Bifidobacterium*, *Megasphaera* and *Lactobacillus* sp. On the other hand, *Prevotella* became predominant in case of high carbohydrate consumption (Latulippe et al. 2013). Moreover, a study by Mujico et al. (2013) showed in mice that high fat diet had a positive impact on the number of Firmicutes phylum while it affected the count of Bacteroidetes phylum adversely.

Gut microbiota consist of different types of living microorganisms interacting with a physiologically changing host. Therefore, it is expected the development and survival of the species in this community to be affected by many environmental factors and it is confirmed by several studies as discussed above. For a microorganism, one of the major factors that control

its growth and prevalence in a community is food. Further, components of food such as fibers which cannot be digested by host but only fermented by gut microbiota, is one of the most dominant factors on the composition and diversity of microbiota.

Dietary fibers and their effects on gut microbiota and health

Dietary fibers are a large group of carbohydrates that are an intrinsic component in many foods and normally cannot be digested or absorbed in the upper part of GIT of humans. According to the report, European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (2010), dietary fibers comprise non-starch polysaccharides, resistant starch, resistant oligosaccharides with three or more monomeric units, and other non-digestible components which are minor in amount and naturally associated with dietary fiber polysaccharides such as lignin. Some examples of dietary fibers are β-glucans, resistant starch, mannans, fructans, xylans, pectins and marine algae (seaweed) polysaccharides such as alginates, chitosan and fucoidan (Wanders et al. 2011).

Fibers differ in physicochemical properties such as solubility, water holding capacity, viscosity and fermentability (Wanders et al. 2011) that may affect satiety and energy regulation as well as physiology by different pathways. For example, fibers can affect the transit time of food through GIT, change absorption rates and affect signals in the gut-brain axis.

Dietary fibers can to a variable extent be fermented by gut microbiota in the lower part of GIT. They have an important impact on the composition of gut microbiota because different types of fibers are substrates for different types of microbes in the gut, thus certain bacteria can be predominant depending on the type of fibers consumed (Jeffery and O'Toole 2013). Some studies (Flint 2012; Tremaroli and Bäckhed 2012) have shown that a diet high in resistant starch increases the number of *Ruminococcus bromii* (up to a level of 25 % of total gut bacteria), Actinobacteria, specific types of Bifidobacteria sp. (such as *Bifidobacterium adolescentis*), *Oscillibacter* and *Eubacterium rectale* (Flint 2012; Xu et al. 2013). Walker et al. (2011) investigated the effect of resistant starch on 14 overweight men applying different diets. The phylogenetic analysis was done by 16S rDNA sequencing. It was observed that there was a shift in microbiota composition between the diet high in resistant starch and non-starch polysaccharides diet. At the same time, the change in microbiota composition was consistent for each individual. It was concluded that different types of the resistant starch can enhance or reduce the growth of certain bacterial groups depending on the initial gut bacteria composition (Flint 2012; Walker et al. 2011). Also it is claimed that the consumption of xylans increases the number of *Prevotella* and *Xylanibacter* which are the members of Bacteroidetes (Scott et al. 2012; Tremaroli and Bäckhed 2012). Inulin on the other hand increases the levels of *Faecalibacterium prausnitzii* and *Bifidobacterium* sp. (Scott et al. 2012).

Substances that have a positive impact on the growth of certain gut microbiota are normally referred to as prebiotics. Some prebiotics have indeed a remarkable effect on control of body weight in both animal models and humans. It was found that the body fat in rats fed with a diet high in prebiotic fibers was significantly lower than the ones fed with diet high in protein and control diet (Reimer et al. 2012). In similar studies on humans it has also been shown that the increase in fiber intake is associated with weight loss due to decrease in energy intake (Slavin 2013).

Prebiotic fibers have many different beneficial effects on health, physiology and metabolism depending on their structure, physical and chemical properties. For instance, guar gum, β-glucan, oat bran, pectin and resistant dextrins may lower blood lipid and most of them may reduce blood glucose response; inulin and oligofructose may improve the growth of beneficial gut microbiota and fructo-oligosaccharides may enhance calcium absorption (Latulippe et al. 2013). Alginate, a product derived from seaweed, has been exploited to improve gastrointestinal barrier function carried out by intestinal epithelium. Intestinal epithelium acts as a selective filter that prevents harmful microorganisms and substances while facilitating translocation of essential nutrition after digestion. It may also control Type II diabetes by reducing fasting blood glucose and glycated hemoglobin (HbA1c) (Dettmar et al. 2011). Resistant starch, may provide prebiotic type fermentation within the colon and increase bile salt turnover (Dongowski et al. 2005). Type III resistant starch, obtained by cooking and cooling starch-containing food, may also contribute to epithelial cell growth and prevention of colonic

diseases by increasing the butyrate production which is one of the major short chain fatty acids produced by gut bacteria (Lesmes et al. 2008).

It is now known that there are many physiological influences by dietary fiber intake on humans. These influences can be due to the physicochemical properties of fibers as they affect the travelling of food through GIT. Fermentation of fibers by gut microbiota has also a huge impact, probably more than the mentioned properties of fibers, on human physiology. For instance, as discussed below, many metabolites produced during fermentation serve as signaling mediators in diverse activities in the host.

Short chain fatty acids (SCFAs)

There are different types of SCFAs produced by gut bacteria; some of them are formic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and caproic acid, with acetic acid, propionic acid and butyric acid being the predominant ones (Soldavini and Kaunitz 2013). Many methods have been used for the characterization of SCFAs in colonic and fecal samples, including gas chromatography (GC), vacuum ultrafiltration followed by GC, ion chromatography (IC), IC with solid-phase extraction and high performance liquid chromatography (HPLC). Among these, HPLC is mostly used as it is less time-consuming and pre-purification steps are not needed as they are for GC (Huda-Faujan et al. 2010).

Unabsorbed nutrients and viscous fibers may slow gastric emptying and thereby may influence the transit time of food through the intestinal tract (Blackwood et al. 2000; Bosaeus 2004; Scazzina et al. 2013; Slavin 2013). However, a different result was found by Wanders et al. (2013). In this study, control diet (with no fiber addition) and diet with cellulose, guar gum (a viscous fiber) and alginate (a fiber with water holding capacity) were tested for their impacts on gastric emptying and it was found that none of them decreased the gastric emptying rate and it was even increased by alginate. These conflicting results illustrate the complex nature behind gastric emptying and also suggest that other unknown factors might be involved. The unabsorbed macronutrients and fibers are fermented in the lower part of the intestine, mostly in the colon where bacteria density is highest. As a result of fermentation, SCFAs are produced by gut microbiota. They are important energy sources for the host as they are rapidly absorbed by colonic mucosa. These SCFAs may contribute to health benefits (Fig. 1) by increasing acidity in the GIT which in turn prevents the growth of pathogens. The SCFAs may have impact on intestinal motility (Alonso and Guarner 2013; Roberfroid et al. 2010; Tremaroli and Bäckhed 2012), and on healing of wounds and modulation of inflammation (Tremaroli and Bäckhed 2012). Some SCFAs can also affect the host in a defined manner, i.e., butyric acid in reducing the risk of colon cancer (Hamer et al. 2008; Havenaar 2011; Roberfroid et al. 2010) and in cell growth and differentiation (Alonso and Guarner 2013); acetate in the regulation of cholesterol synthesis and adipose tissue deposition (Roberfroid et al. 2010), and propionate also in decreasing the cholesterol concentration in blood and inhibiting fatty acid synthesis (Hosseini et al. 2011). Beyond all these benefits, SCFAs are also believed to play a key role in triggering satiety (Haenen et al. 2013).

The type of SCFAs produced depends on the carbohydrate consumed and the predominant gut microbiota. For example, some bacteria in the Clostridia class of Firmicutes, *Roseburia*, *Eubacterium* and Lachnospiraceae produce butyrate while *Bifidobacteria* produce lactate and acetate and *R. bromii* produces acetate (Tagliabue and Elli 2012).

SCFAs are important metabolites of gut microbiota for the host physiology and different types of SCFAs affect the host differently. Therefore, with detailed knowledge on the specific microorganisms that produce defined sets of SCFAs, one may be able to control host physiology via microbial activities in the gut. One of these physiological features is satiety; SCFAs may be used to control satiety via gut-brain axis as they give rise to the stimulation of endocrine system in the GIT.

The gut-brain axis and satiety

The gastrointestinal epithelium contains many different enteroendocrine cells. These cells form the largest endocrine organ in the body and they respond to neural and physical stimuli as well as chemical stimuli by releasing more than 20 different regulatory peptide hormones (Murphy and Bloom 2006). Peptide YY (PYY), glucagon like peptide-1 (GLP-1),

oxyntomodulin (OXM) and cholecystokinin (CCK) are some of the products secreted by enteroendocrine cells that play an important role in the control of satiety and food intake by influencing appetite regulation centers in brain (Sam et al. 2012).

The regulation of food intake and energy homeostasis is performed by the vagus nerve, brainstem and the center in brain which is the arcuate nucleus (ARC) of the hypothalamus. ARC contains orexigenic neurons stimulating appetite and anorexigenic neurons inhibiting appetite (Sam et al. 2012) (Fig. 1). The neural signals from mechanoreceptors and chemoreceptors in the gut and signaling hormones secreted in the intestine follow the vagal pathway from gut to the brainstem or directly go to neurons in the hypothalamus and influence satiety and food intake (Murphy and Bloom 2006).

Fiber-derived SCFAs produced by gut microbiota are known to have satiety enhancing properties. They are ligands for G-protein-coupled receptors (Gpr41 and Gpr43) expressed in PYY-producing enteroendocrine L-cells in the intestine and activate the satiety center in the brain by stimulating the secretion of satiety-related hormones such as GLP-1 and PYY (da Silva et al. 2012; Diamant et al. 2011; Tagliabue and Elli 2012) (Fig. 1). Parnell and Reimer (2012b) tested on lean and obese rats fed with different doses of inulin-oligofructose diet to investigate the impact of this diet on gut satiety hormones. It was observed that the prebiotics inulin and oligofructose increased the levels of GLP-1 and PYY in rats in a dose-dependent manner.

Through the gut-brain axis, the impact of gut microbiota is not limited only to satiety, food intake and energy harvesting. Studies with germ-free versus conventional-reared mice indicated that gut microbiota have also an impact and control on brain biochemistry and behavior via immune, neural and metabolic mechanisms (Latulippe et al. 2013). It has been claimed that anxiety levels are lower in germ-free mice compared to those colonized with normal gut microbiota (Cryan and O'Mahony 2011; Heijtza et al. 2011). Although germ-free mice studies are not representative for humans in terms of physiology and neurology, this study shows at least that gut microbiota has an impact on animal behavior (Cryan and O'Mahony 2011). Colonization early in life may also affect motor control and influence brain and behavior in later periods of life. There are indications that these effects of gut microbiota on the brain may also have an implication on neurodevelopmental disorders such as autism and schizophrenia (Heijtza et al. 2011).

While the microbiota can have stimulatory effects on the brain, the brain itself can also influence gut microbiota directly by triggering lumina propria to release signaling molecules into the GIT or indirectly by changing motility, secretion and permeability of intestine, making the gut-brain axis a two-way-street for signals (Rhee et al. 2009).

There are many factors affecting gut-brain interaction and still very little is known despite of many studies presently reported. A detailed understanding of the intricate interaction between gut microbiota and the brain and their effects on physiology needs future studies, especially at molecular level where signaling mechanisms occur, between protein–protein interactions and ligand–protein interactions along the gut to the brain.

Healthy food, healthy gut bacteria, healthy people

Through 1,000 years of experience humans have realized that our health is greatly influenced by the type of foods we consume and that certain foods appear healthier than others. It is now becoming evident that some of these health-inducing effects result from the microbial activities in our gut. These microorganisms, which outnumber the total cells in our body at least ten times, whose genes outnumber ours about 1,000 times, help us to degrade our food, provide us energy, metabolize the vitamins we need, prevent us from getting infections, sharpen our immune system, control our appetite, etc., hence influencing on us both physically and mentally. In the study of Zhao (Hvistendahl 2012), a microbiologist whose health was declining due to a significant increase in body weight, he designed a diet program for himself based on a mixture of whole-grains together with prebiotic ingredients such as Chinese yam and fermented bitter lemon. After 2 years on the diet program, he lost 20 kilos in weight and showed reduced blood pressure and cholesterol levels. This physical and physiological transformation was found to be closely associated with a significant shift in gut microbiota caused by the diet change: one important finding was *F. prausnitzii* (Hvistendahl 2012) which is a bacterium with anti-inflammatory properties

tested in vivo and in vitro (Sokol et al. 2008). This bacterium was found to flourish, from an undetectable level before diet control to 14.5 % of his total gut bacteria after diet control (Hvistendahl 2012). In the same study, tailor-designed diet programs containing prebiotic foods were also applied to 123 clinically obese volunteers. It was observed that most of the volunteers (93 of 123) lost nearly 7 kg of weight, and while beneficial bacteria increased, the toxin-producers decreased. This study illustrates that our health, the food we eat and the composition of gut microbiota are closely connected, and that healthy foods recruit good gut bacteria which are necessary to promote the health-bringing effects. Transplantation of a healthy gut microbiota is now promising therapeutic approach to restore the normal function of our gut after diseases and disorders caused by pathogenic gut microbiota although it is not a common practice because of complexity and little knowledge on the composition and activity of the microbiota (Clemente et al. 2012; Khoruts and Sadowsky 2011). Furthermore, it is possible in the near future that we will be able to selectively program the microbial activity in our gut in order to achieve a certain favorable physiological condition or a mental state for a defined purpose. For example, one such mental state is the feeling of satiety. Inducing satiety will reduce hunger, thus motivation to eat. As a result, health may be improved by creating a better balance between energy intake and energy expenditure.

Concluding remarks

It is now evident that gut microbiota has an important role on human physiology and health ranging from maturation of host immune system, prevention of pathogen invasion, to controlling satiety and obesity, etc. Detailed knowledge on these mechanisms can be used to prevent human diseases, disorders, or to control certain physiological or mental state by regulating the factors that affect the composition of gut microbiota and their metabolic activities. Among these factors, diet appears to be one of most promising approaches. Different types of diet can facilitate different sets of bacteria to become predominant in the gut. Given the right conditions, each set of bacteria can potentially be manipulated to produce a defined set of metabolites which in turn trigger a specific host response (e.g., satiety). Hence, one can in principle use diet to trigger specific host response via gut microbial activities. However, the interaction between gut microbiota and its human host is in most cases multifactorial and complex, and most of the changes along a signaling pathway between microbes and the host are not well known, especially at the molecular level, i.e., how signals (inputs) are produced, transmitted and processed further to eventually result in adaptive responses (outputs). Furthermore, behind the interactions with the host, gut microbiota as a whole is a complex system and it is recently considered as a separate organ. Therefore, it is also worthwhile to study gut microbiota itself in terms of fundamental biology (e.g., intra- and inter-species cell-to-cell communication), population metagenetics, evolution etc. as the gut has many different kinds of dwellers such as bacteria, archaea, yeasts and human cells. To understand the interactions between them and the regulation of many metabolic pathways in the microbial community is equally important as to get knowledge on human health, diseases and disorders. For the identification and genetic characterization of the members of gut microbiota, metagenomics is an important tool to disclose all members within the gut community under different physiological or mental conditions. Sequencing technology is developing rapidly and huge amounts of data can now be readily produced within a relatively short time. However, to analyze these data in an efficient manner and to organize them into meaningful biological networks are much more challenging because it needs to combine advanced knowledge from different research disciplines including microbiology, microbial genetics and physiology, cell biology, human physiology, nutrition, chemistry and maybe most importantly, bioinformatics which glues other disciplines together. When functional networks of these disciplines come into place and become available for different research fields, gut microbiota will provide a tremendous source of opportunities for different research avenues, especially in human medicine and biotechnology.

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Paper 2

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Microbiome

RESEARCH Open Access

Resistant starch diet induces change in the swine microbiome and a predominance of beneficial bacterial populations

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Abstract

Background: Dietary fibers contribute to health and physiology primarily via the fermentative actions of the host's gut microbiome. Physicochemical properties such as solubility, fermentability, viscosity, and gel-forming ability differ among fiber types and are known to affect metabolism. However, few studies have focused on how they influence the gut microbiome and how these interactions influence host health. The aim of this study is to investigate how the gut microbiome of growing pigs responds to diets containing gel-forming alginate and fermentable resistant starch and to predict important interactions and functional changes within the microbiota.

Results: Nine growing pigs (3-month-old), divided into three groups, were fed with either a control, alginate-, or resistant starch-containing diet (CON, ALG, or RS), and fecal samples were collected over a 12-week period. SSU (small subunit) rDNA amplicon sequencing data was annotated to assess the gut microbiome, whereas comprehensive microarray polymer profiling (CoMPP) of digested material was employed to evaluate feed degradation. Gut microbiome structure variation was greatest in pigs fed with resistant starch, where notable changes included the decrease in alpha diversity and increase in relative abundance of Lachnospiraceae- and *Ruminococcus*-affiliated phylotypes. Imputed function was predicted to vary significantly in pigs fed with resistant starch and to a much lesser extent with alginate; however, the key pathways involving degradation of starch and other plant polysaccharides were predicted to be unaffected. The change in relative abundance levels of basal dietary components (plant cell wall polysaccharides and proteins) over time was also consistent irrespective of diet; however, correlations between the dietary components and phylotypes varied considerably in the different diets.

Conclusions: Resistant starch-containing diet exhibited the strongest structural variation compared to the alginate-containing diet. This variation gave rise to a microbiome that contains phylotypes affiliated with metabolically reputable taxonomic lineages. Despite the significant microbiome structural shifts that occurred from resistant starch-containing diet, functional redundancy is seemingly apparent with respect to the microbiome's capacity to degrade starch and other dietary polysaccharides, one of the key stages in digestion.

Keywords: Growing pigs, Resistant starch, Alginate, Gut microbiota, 16S rRNA gene, Bacterial community

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Background

The gut microbiome of animals comprises a broad diversity of bacterial and archaeal phylotypes and is considered a separate organ due to the influence of its metabolic traits on host physiology [1]. Its key roles include modulating food intake, growth and development of the body, energy uptake from food, immune system and proliferation of epithelial cells, and resistance to infections [2]. Diet is one of the most important factors influencing gut microbiome structure and function, which indirectly modulates metabolic activities of the host [3].

Dietary fibers are defined as a large group of carbohydrates that play an important role in the gut microbiome as well as in the physiology of the host [4]. Resistant starch is an example of a dietary fiber that cannot be broken down by digestive enzymes or be absorbed in the small intestine but can be fermented by microbes in the lower gastrointestinal tract [5]. Diets rich in resistant starch have potential health benefits, such as lowering postprandial glycemia and insulinemia, enhancing absorption of minerals including calcium and iron, and prolonging the duration of satiety [6,7]. The fermentation byproducts of resistant starch (that is, short-chain fatty acids) also contribute to host health in many ways [5]. For example, butyric acid is the main energy source for colonic epithelial cells and may play a role in preventing colon cancer [8]. There are four types of resistant starch defined by their physicochemical properties, with each type affecting the gut microbiome structure differently [9]. Type 1 consists of physically inaccessible starch; type 2, granular starch; type 3, retrograded starch obtained by cooking and cooling the starch; and type 4, modified starch. Type 3 is considered the most resistant form and is totally resistant to digestive enzymes [6].

Alginate is a viscous dietary fiber consisting of guluronic acid (G) and mannuronic acid (M) that forms a gel at low pH (such as in the stomach). This gel structure slows down gastric emptying and reduces the rate of intestinal absorption of metabolizable nutrients, subsequently lowering the blood cholesterol and glucose levels [10]. Alginate may assist in the refinement of gastrointestinal barrier function and was previously shown to increase mucus layer thickness and replenishment rate, which are fundamental for the colonic mucus barrier [10]. The gel structure of alginate may also play a role in controlling obesity and type II diabetes [11] as well as limiting the adverse effects of luminal contents adsorbing a number of damaging agents such as mutagens, toxins, and carcinogens [10], thus reducing colonic exposure to these agents. Alginate-containing diets have demonstrated a satiating effect on pigs (short-term satiety) primarily due to the gel forming capability [7,12]. While fermented at a low rate by gut microbiota [10], alginate has been shown to also affect microbiome structure at some level, demonstrating its potential as a prebiotic [13,14]. However, the microbe-alginate relationship has not been evaluated in detail.

Pigs are frequently utilized as models for humans due to their similar body size, genome, digestive tract, diet type as well as other anatomical and physiological features [15,16]. Their gut microbiome also exhibits similar structural features to the extent that their use as model animals in gut microbiota studies is believed to be advantageous [17]. Previously, it has been shown that alginate and resistant starch (type 3) display different effects on the physiology and feeding patterns of growing pigs [12]. The feed intake of ALG pigs was higher than CON pigs to compensate for the reduced digestible energy intake with ALG and to result in an overall similar digestible energy intake to CON pigs. Digestible energy intake is reduced by resistant starch with increase in fermentation and more efficient use of digestible energy.

In this study, it was hypothesized that the diets containing these two contrasting dietary fibers exert different influences on the pig gut microbiome and affect important interactions and functionalities within the microbiota. Feeding trials were conducted on young animals (growing pigs) where the total energy intake should be less variable than in adult animals, as all individuals require high-energy intake for growth. Therefore, any change in microbiome structure and function in response to dietary fibers may be more visible. Microbiome analysis was conducted over a 12-week period encompassing: SSU rDNA amplicon sequencing, functional analysis of predicted metagenomes, and CoMPP analysis of plant cell wall components (PCWCs). Correlation and co-occurrence analysis were additionally conducted between the relative abundances of operational taxonomic units (OTUs) and post-digestion PCWCs.

Results

Feeding trials and microbiome data collection

In order to characterize the effects of ALG and RS on the pigs' gut microbiomes, we assessed the community structure via 16S rRNA gene analysis. Using amplicon pyrosequencing, we obtained 251,522 SSU rRNA gene fragments in total (approximately 524 nt). Quality filtering and clustering analysis resulted in 2,621 total OTUs from 61 samples. Functional capabilities of each microbiome were predicted using KEGG pathway analyses of simulated metagenomes and compared between diet types to identify differences. CoMPP analysis was used to measure relative PCWC levels in the original feed as well as fecal samples in order to monitor changes of the individual polysaccharides and proteins that were available to the microbiome populations for ingestion (Figure 1). As expected, starch levels (detected using the CBM20 probe) were consistent in the original

A

		HG partially methylesterified (mAb LM19)	Backbone of rhamnogalacturonan I (mAb INRA-RU1)	(1→4)-β-D-galactan (mAb LM5)	(1→5)-α-L-arabinan (mAb LM6)	(1→4)-β-D-[galacto](gluco)mannan (mAb LM21)	(1→3)-β-D-glucan (mAb BS-400-2)	(1→3)(1→4)-β-D-glucan (mAb BS-400-3)	Xyloglucan (XXXG motif) (mAb LM15)	(1→4)-β-D-xylan (mAb LM10)	(1→4)-β-D-xylan/arabinoxylan (mAb LM11)	(1→4)-β-D-xylan (mAb LM23)	Cellulose (crystalline) (CBM3a)	Extensin (mAb LM1)	Extensin (mAb JIM20)	AGP (mAb JIM13)	AGP, β-linked GlcA (mAb LM2)	Starch (CBM20)
Control Diet	Feed	63	40	87	45	25	16	52	62	0	45	0	36	0	6	9	0	25
	CON.T1	19	24	8	27	29	26	65	32	39	66	2	18	7	13	28	20	2
	CON.T2	16	22	5	24	0	26	51	28	31	49	2	16	0	8	24	14	0
	CON.T3	13	19	2	20	0	19	45	27	29	38	5	15	0	9	20	9	0
	CON.T4	23	25	4	25	2	21	52	33	31	37	5	18	0	11	22	12	0
	CON.T5	12	19	0	22	2	17	46	30	31	37	9	16	0	10	20	9	0
	CON.T6	21	24	8	24	0	19	47	30	29	40	7	16	0	11	19	5	2
	CON.T7	6	17	0	19	2	15	47	31	27	33	11	16	0	9	17	2	0
Alginate Diet	Feed	65	40	88	46	39	17	71	61	0	60	0	37	0	6	9	0	28
	ALG.T1	25	26	8	30	12	26	62	34	41	69	2	19	7	12	30	19	5
	ALG.T2	25	24	13	27	5	22	46	26	32	47	5	15	2	11	23	10	0
	ALG.T3	37	31	20	29	2	22	46	25	25	42	0	17	0	12	21	3	0
	ALG.T4	10	18	0	21	0	19	38	23	26	30	6	14	0	10	19	0	0
	ALG.T5	10	19	0	22	0	17	43	26	32	38	7	15	0	11	19	0	2
	ALG.T6	4	14	0	17	2	13	37	23	27	31	6	13	0	8	15	0	2
	ALG.T7	0	13	0	16	0	13	42	27	30	35	4	14	0	8	16	0	0
Resistant Starch Diet	Feed	78	47	100	54	44	23	75	69	0	67	0	39	0	8	12	0	24
	RS.T1	25	27	9	31	0	25	59	31	34	62	0	18	8	12	28	16	2
	RS.T2	16	22	7	25	2	23	54	29	37	60	2	16	0	8	26	13	4
	RS.T3	11	19	6	24	10	21	46	28	26	40	3	18	0	11	21	4	0
	RS.T4	10	17	2	21	4	20	41	24	21	30	2	15	0	10	19	3	0
	RS.T5	5	16	2	21	0	20	42	29	23	28	7	16	0	10	19	4	0
	RS.T6	8	19	0	23	2	19	44	29	29	33	10	15	0	13	19	2	0
	RS.T7	11	19	4	23	0	19	47	32	27	33	4	16	0	12	21	9	0

B

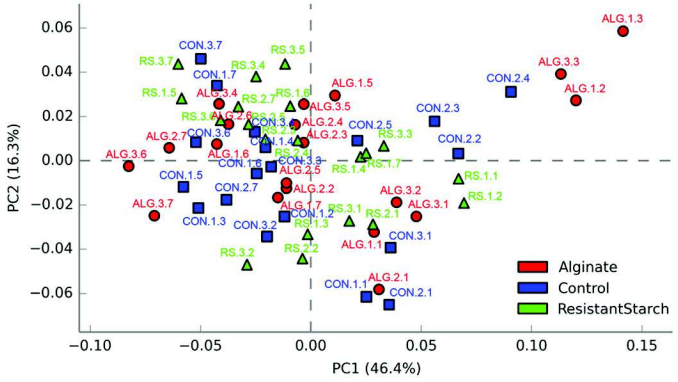

Figure 1 Comprehensive microarray polymer profiling (CoMPP) of plant cell wall components (PCWCs) and principle component analysis (PCA). Heatmap **(A)** shows the relative abundances of PCWCs in each sample. Color intensity is proportional to mean spot signal. T1 to T7 refer to the time-points when samples were collected. PCA plot **(B)** shows the comparison of PCWC composition between diets. Labels contain name of diet type (CON, ALG, RS), pig number for the specific diet with numbers between 1 and 3 and time-point numbers between 1 and 7 in the order (starting from T1 as first time-point). HG, homogalacturonan; AGP, arabinogalactan protein; GlcA, glucuronic acid.

feed samples for all diets (Additional file 1: Table S1). No starch was detected in the fecal samples of any of the pigs, irrespective of the solubility of the starch component in their diet. Overall, a variety of pectic substrates, hemicellulosic substrates (including xyloglucans, xylans, mannans, and betaglucans), and cellulose were detected in all diet groups. The change in relative abundance of these PCWCs (decrease or increase depending on the PCWC) over time was consistent across all samples, and no differences were observed between diets (Figure 1). Alginate levels were unable to be reported via CoMPP analysis due to the lack of a suitable probe; however, previous pig feeding trials using alginate have indicated that this polysaccharide is detectable in fecal material and is not digested completely [18].

Microbiome diversity

Alpha diversity analyses were performed upon all samples to determine how the different diets affected the microbiome of each animal over the 12-week period. Shannon index plot (Figure 2) and rarefaction curves (Additional file 2: Figure S1) were generated for each diet group to compare the species diversity within each microbial community. Each method demonstrated that the diversity of bacterial OTUs at species level significantly ($P < 0.01$) decreased in the microbiomes of RS pigs compared to CON pigs, while there was no obvious difference in diversity

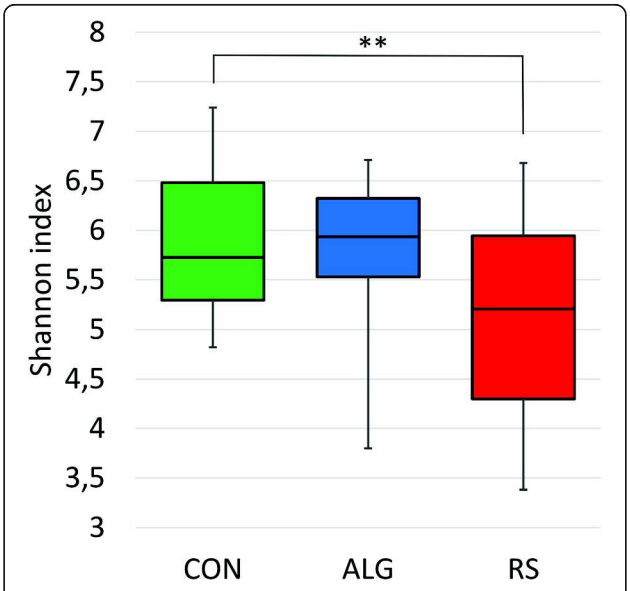

Figure 2 Community diversity represented by Shannon index at an OTU level for samples from each diet. Shannon indexes were calculated based on the average of ten iterations at equal subsampling size of 1,781 for each sample. Each bar represents the samples from the pigs fed with different diets; alginate-containing diet (ALG) blue, control diet (CON) green, and resistant starch-containing diet (RS) red.

between ALG and CON pigs. Moreover, time did not significantly affect bacterial alpha diversity in any of the diet groups (ANCOVA, $P = 0.053$) (Additional file 3: Figure S2).

To explicitly compare the microbiomes of the individual animals used in this study, distance matrices were calculated by unweighted UniFrac [19], visualized via principle coordinate analysis (PCoA) (Figure 3A), and statistical analyses were performed on distance matrices for significance testing (Figure 3B). Pigs fed with the same diet tended to cluster together (Figure 3A), while time did not significantly affect the bacterial community composition of fecal samples within each diet over the 12-week period (Additional file 4: Table S2). CON pigs were shown to cluster in close proximity after samples T2 to T3, indicating that they were acclimatized to their diet within 1 to 3 days after the start of the prebiotic diet. The relatively sporadic clustering between the three pigs fed with the same diet and sampled at the same time was possibly due to the inter-individual variation (Additional file 5: Figures S3 and Additional file 4: Table S2), a commonly observed phenomenon [20,21]. As expected, the first time-point (T1, day −7) samples of all diets had similar microbiome structure since all pigs were fed with the same commercial basal diet at this time. However, from time-point 2 (T2, day 1) when pigs were fed with different diets, their microbiomes started to diverge from each other, with those from RS pigs in one direction while those from ALG and CON pigs jointly in another direction. The structural shift of the microbiome of RS pigs compared to CON and ALG pigs were statistically significant, whereas ALG pigs had similar microbiome composition to CON pigs (Figure 3B).

Taxonomic affiliations

Overall, the microbiomes of the individual pigs were dominated by the phyla Firmicutes (88.2% in CON pigs, 90.1% in ALG pigs, and 88.3% in RS pigs) and Bacteroidetes (9.7% in CON pigs, 8.6% in ALG pigs, and 10.2% in RS pigs). The other phyla present in low abundance (less than 2.1%) were Actinobacteria, Cyanobacteria, Spirochaetes, TM7 (candidate division), Tenericutes, and a number of unclassified bacteria. Although most of these phyla were present in samples across all diets, Spirochaetes were not detected in RS pigs and TM7 was observed only in ALG pigs.

At deeper taxonomic levels, a greater number of significant differences were observed (Additional file 6: Figure S4). At the family level, the following families were more abundant in RS pigs than CON pigs: Erysipelotrichaceae ($P < 0.001$), Veillonellaceae ($P < 0.001$), Lachnospiraceae ($P < 0.01$), an undefined Firmicutes family ($P < 0.001$), and Prevotellaceae ($P < 0.001$). In contrast, the families

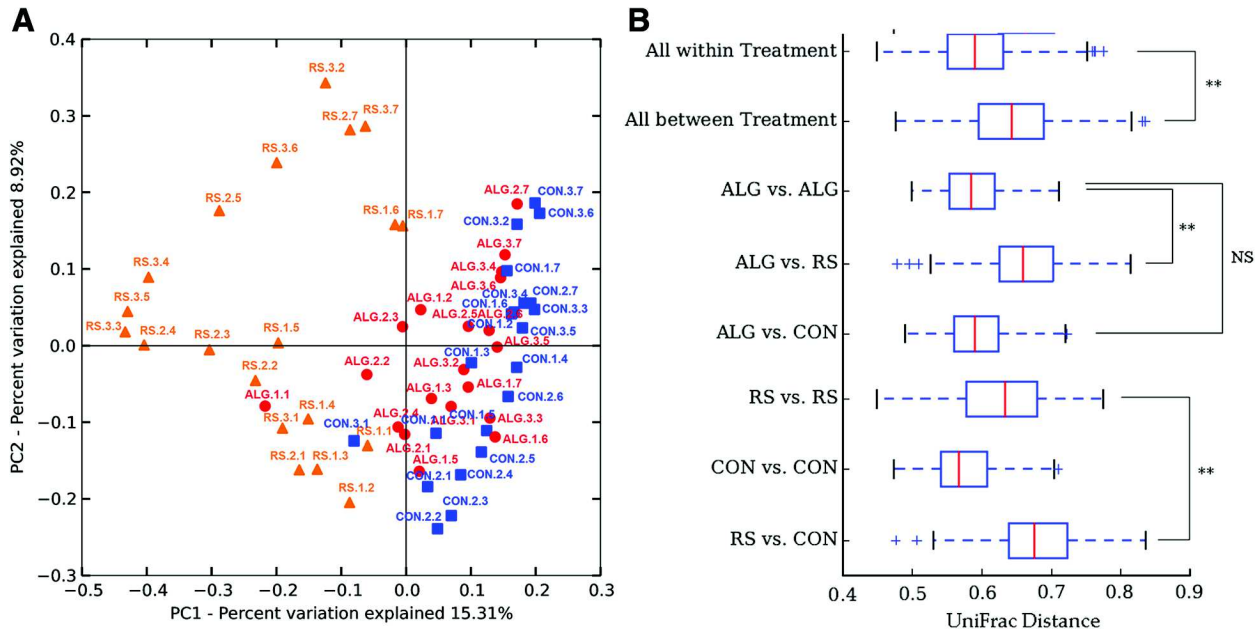

Figure 3 Comparison of the gut community composition. **(A)** Principle coordinate analysis (PCoA) plot generated based on the calculated distances in an unweighted UniFrac matrix. Samples were grouped by color and shape in terms of diet group they belong to; alginate-containing diet (ALG) red (circle), control diet (CON) blue (square), and resistant starch-containing diet (RS) orange (triangle). Labels contain name of diet type (CON, ALG, RS), pig number for the specific diet with numbers between 1 and 3, and time-point numbers between 1 and 7 in the order (starting from T1 as first time-point). **(B)** The statistical significances of differences in unweighted UniFrac distances between diets. Significance degree (calculated using Student's *t*-test with 1,000 Monte Carlo simulations) is represented as no significance ($P > 0.05$) with NS; $P < 0.05$ with one star (*); $P < 0.005$ with two stars (**).

unclassified RF39 (affiliated to Mollicutes) ($P < 0.01$) and Clostridiaceae ($P < 0.001$) appeared significantly less abundant in RS pigs than in CON pigs. The relative abundance of only unclassified F16 family (affiliated to TM7) ($P < 0.01$) was higher in the microbiome of ALG pigs than that of CON pigs, whereas the unclassified RF39 (affiliated to Mollicutes) ($P < 0.05$) and Clostridiaceae ($P < 0.001$) were less abundant. At the genus level, ANCOVA resulted with many genera with significant relative abundance differences in RS and less in ALG compared to CON (Figure 4). *Bulleidia* ($P < 0.001$), *Megasphaera* ($P < 0.001$), *Dialister* ($P < 0.001$), an undefined Veillonellaceae genus ($P < 0.001$), *Ruminococcus* ($P < 0.001$), unclassified Lachnospiraceae genus ($P < 0.001$), an undefined Firmicutes genus ($P < 0.001$), *Prevotella* ($P < 0.01$), and unclassified Prevotellaceae genus ($P < 0.01$) were more abundant in RS pigs compared to CON pigs, while unclassified RF39 genus (affiliated to Mollicutes) ($p < 0.01$), L7A_E11 (affiliated to Erysipelotrichaceae) ($p < 0.05$), Unclassified Ruminococcaceae ($P < 0.001$), *Lachnospira* ($P < 0.05$), *Dorea* ($P < 0.001$), *Blautia* ($P < 0.001$), SMB53 genus (affiliated to Clostridiaceae) ($P < 0.001$), and *Clostridium* ($P < 0.01$) had a significantly lower relative abundance. In the ALG pigs, the most notable observation was the significantly higher relative abundance of unclassified F16 genus (affiliated to TM7) ($P < 0.01$), *Ruminococcus* ($P < 0.05$), *Roseburia* ($P < 0.01$), and *Lachnospira* ($P < 0.05$) compared to CON pigs.

The relative abundances of some of the bacterial families within dietary groups tended to show variations over time (Figure 5). Streptococcaceae and Lactobacillaceae showed an opposing trend in relative abundance variation over time in all diets. Moreover, relative abundance of some families including Lachnospiraceae, Erysipelotrichaceae, and Veillonellaceae varied over time (becoming more abundant and less abundant over time) in an opposing manner to some other families such as Ruminococcaceae,

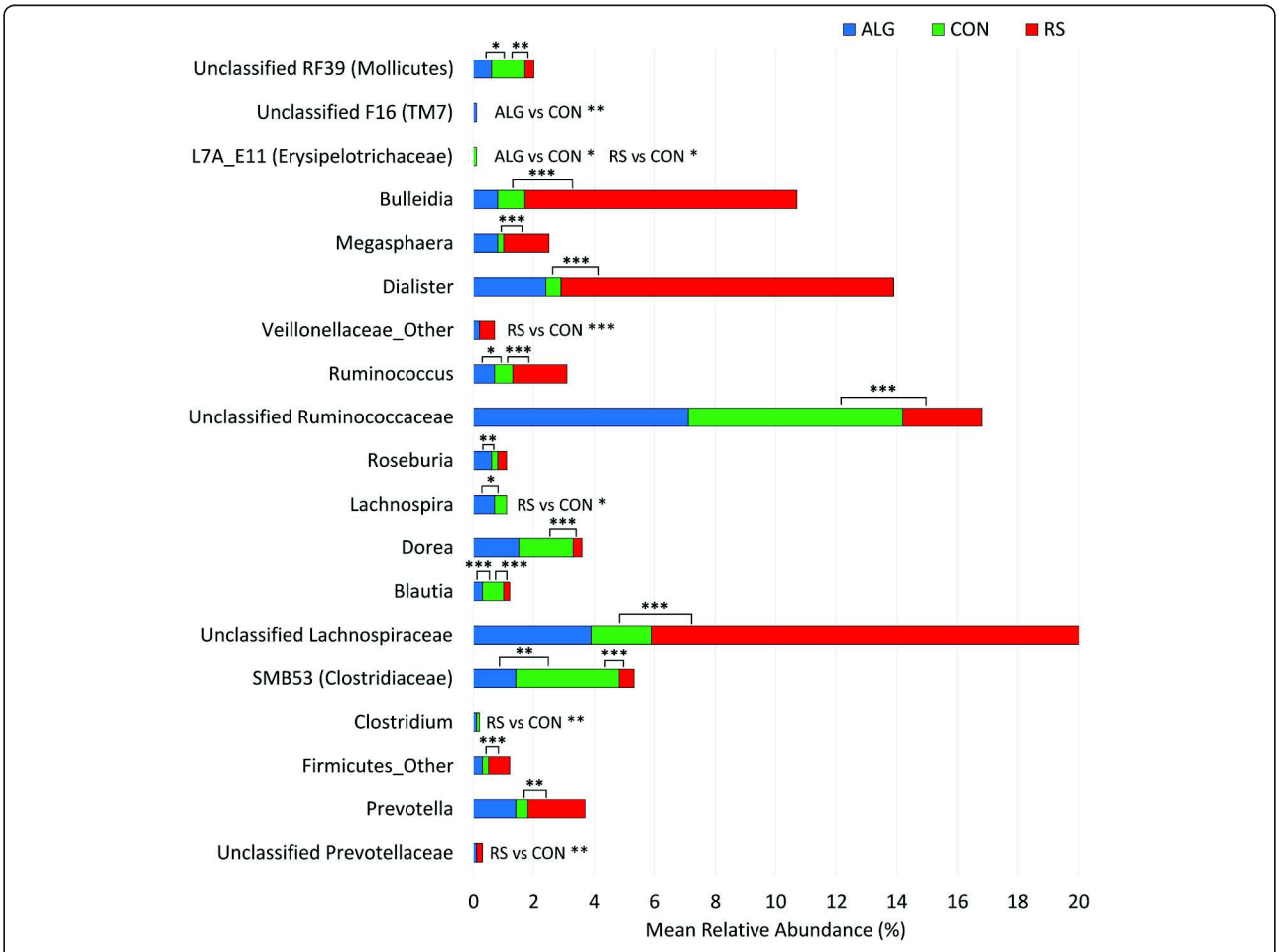

Figure 4 Significantly different bacterial genera in relative abundance between different diets. Genera that have different relative abundances in ALG or RS pigs compared to CON pigs were determined by ANCOVA. The shown mean relative abundance percentages of the taxa were calculated using all samples taken over time within each diet. Significance degree is represented with stars; $P < 0.05$ with one star (*); $P < 0.01$ with two stars (**); $P < 0.001$ with three stars (***). The significance was stated next to the bar together with the abbreviations of compared diets (ALG, CON, and RS) when the bar does not appear for at least one of the diets due to a very low relative abundance percentage.

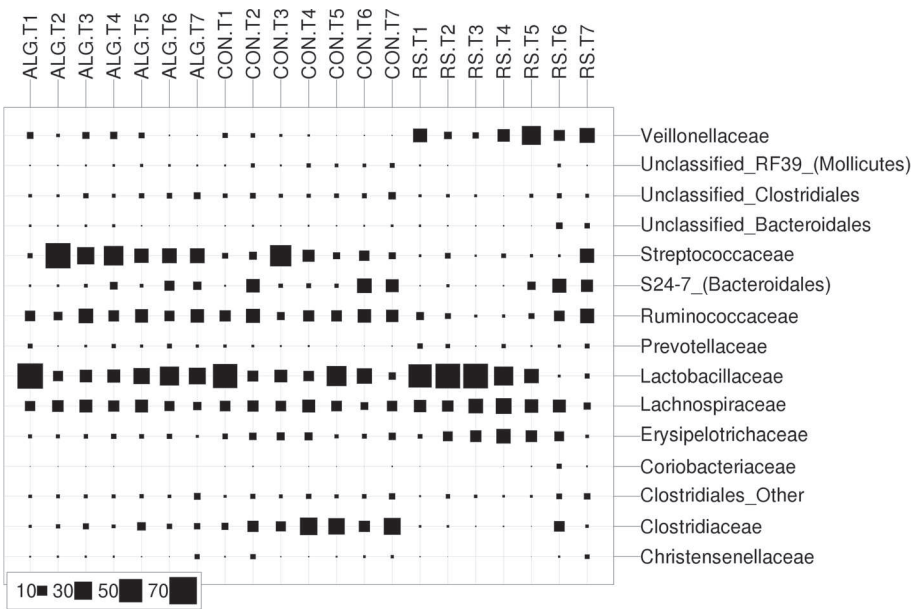

Figure 5 The relative abundances of bacterial families for each fecal sample over time. The size of each square represents the mean relative abundance of bacterial families (%) for the indicated time-point and was determined from fecal samples of three pigs that were fed with the same diet. Samples were ordered in terms of time within each diet and labeled beginning with diet type (ALG, CON, and RS) and time-point from T1 to T7 (T1: day −7, T2: day 1, T3: day 3, T4: day 7, T5: week 3, T6: week 7 and T7: week 12).

S24-7, Clostridiaceae, unclassified Clostridiales, and unclassified Bacteroidales, particularly in RS pigs (Figure 5). The patterns of these contrasting changes between particular families were supported by Pearson's correlations, which were consistent with the different diet types (Additional file 7: Figure S5). For example, specific families that positively correlated with one another and to the RS diet were often negatively correlated to other groups that were positively correlating to the CON diet. ALG correlated positively with Streptococcaceae only, while RS correlated positively with many families such as Veillonellaceae, Lachnospiraceae, Erysipelotrichaceae, and Prevotellaceae that became predominant by RS.

Imputed microbiome function

Given the structural changes within the microbiome of RS and ALG pigs compared to CON pigs, we subsequently examined whether the contrasting diets would also cause functional changes within each microbiome. In the absence of shotgun metagenomic sequencing data, we applied PICRUSt [22] to our 16S rRNA gene survey to predict metagenome functional content. PICRUSt is a computational approach in which evolutionary modeling is used to predict the present gene families from 16S data and a reference genome database [22]. The imputed relative abundances of KEGG pathways in each respective sample were used to predict changes in metabolic function within the microbiomes of ALG and RS pigs compared to CON pigs (Figure 6). The RS diet was predicted to significantly affect ($P < 0.05$) a greater number of KEGG pathways (sevenfold) in the gut microbiome, whereas the ALG diet seemingly had a reduced impact on microbiome function compared to CON diet. The KEGG pathways that exhibited the greatest statistical difference in RS and CON pigs were butanoate, pyruvate, and propanoate metabolism, with all having a higher predicted relative abundance in CON pigs. Interestingly, there were no significant differences in the starch and sucrose metabolism KEGG pathway between RS pigs and ALG pigs compared to CON pigs although a significant difference was observed at one time-point (T3) ($P = 0.046$) between RS and CON pigs (Additional file 8: Figure S6). While this KEGG pathway map encompasses starch conversion, it also includes cellulose, xylan, betaglucan, and pectin conversion (http://www.genome.jp/kegg/kegg2.html, map00500), which are all key PCWCs that were detected using CoMPP analysis.

OTU-PCWC correlations

To investigate correlation/co-occurrence of PCWCs and bacterial taxa, extended local similarity-based networks were applied as they can be used to evaluate correlations between two data types over time. Many different OTUs that were affiliated to various families co-occurred or correlated significantly ($P < 0.001$) with PCWCs in different diet pigs. Although the relative levels of PCWCs did not show any difference between diets (Figure 1), the number of the OTUs varied in the CON, ALG, and RS networks (Figure 7 and Additional file 9: Figure S7).

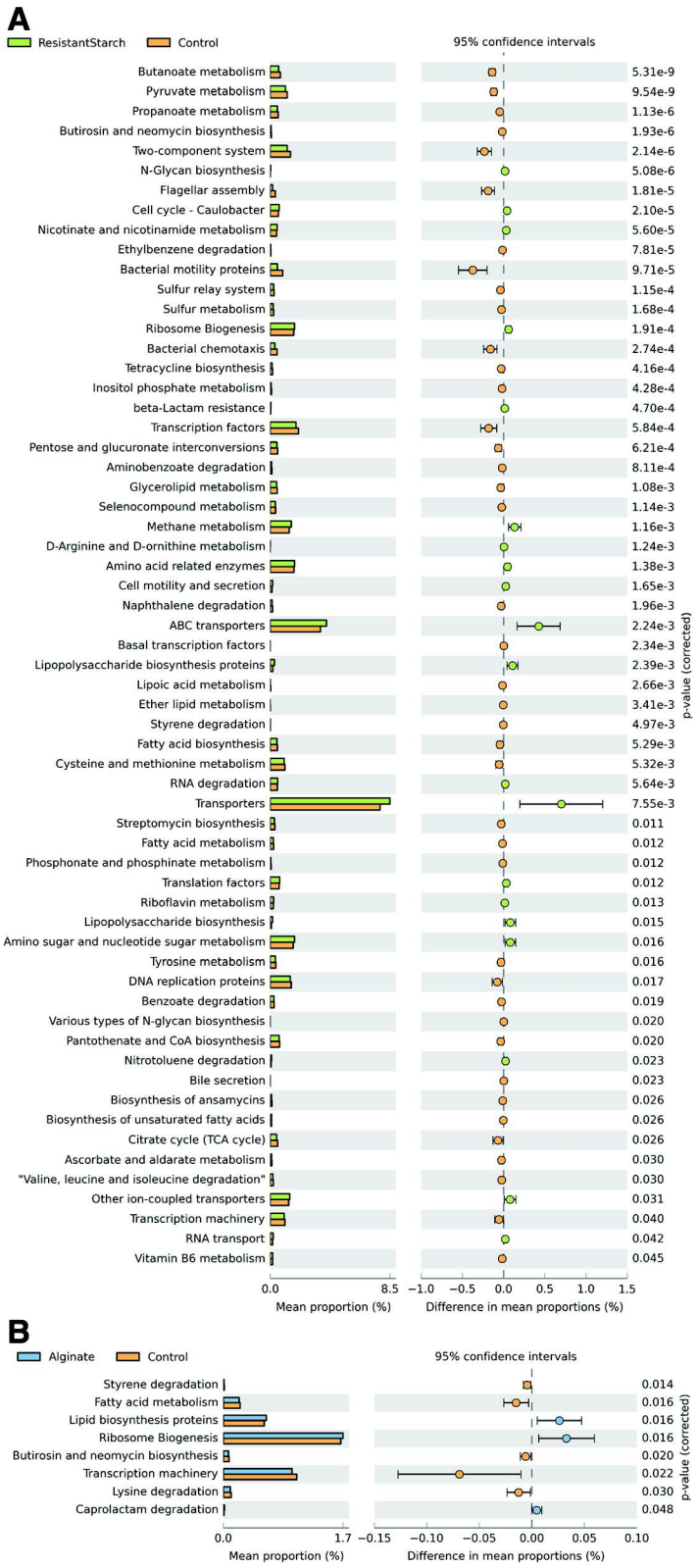

Figure 6 Imputed metagenomic differences between ALG and RS pigs compared to CON pigs. The relative abundance of metabolic pathways encoded in each imputed sample metagenome was analyzed using STAMP [63]. Extended error bars show significantly different KEGG pathway maps in RS **(A)** and ALG **(B)** pigs compared to CON pigs ($P < 0.05$, confidence intervals = 95%).

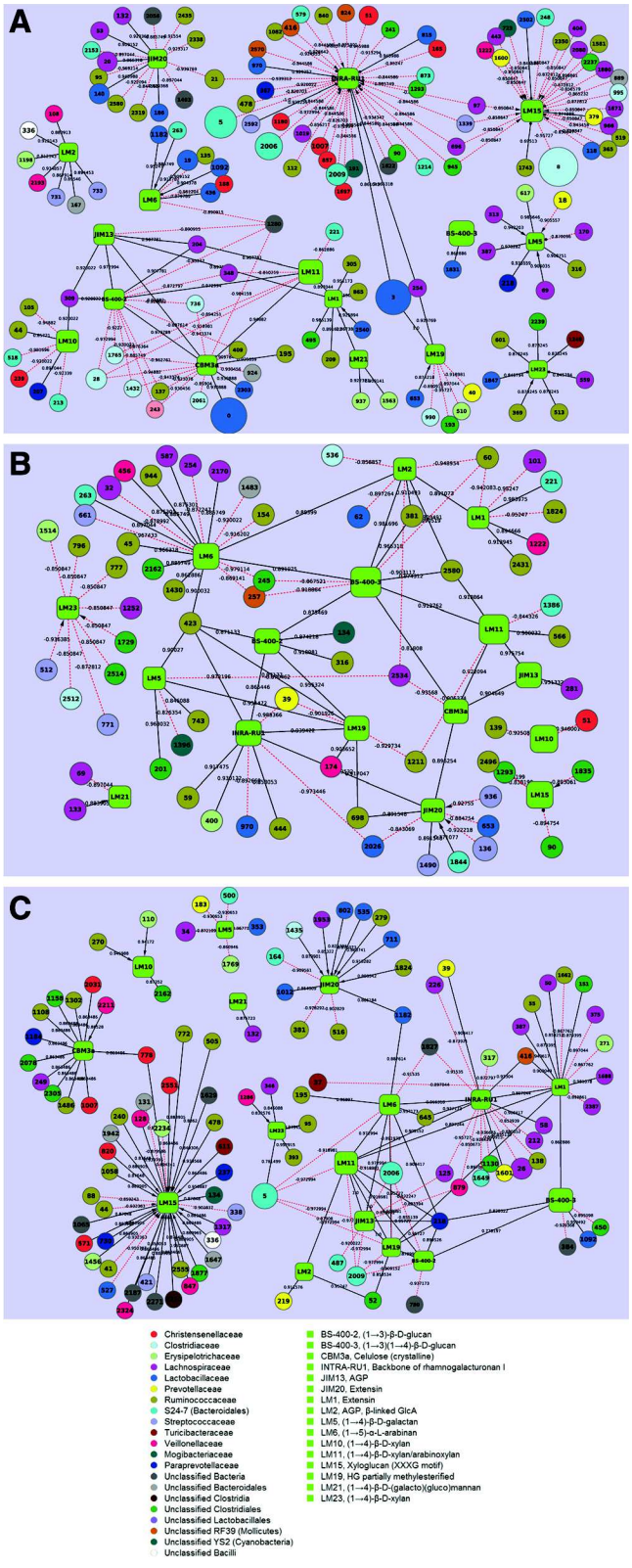

Figure 7 (See legend on next page.)

(See figure on previous page.)
Figure 7 Correlation networks of OTUs and PCWCs in each diet. OTUs were grouped at 97% SSU rRNA gene identity and the networks were plotted based on eLSA with significant local similarity scores ($p < 0.001$). **(A)**, **(B)**, and **(C)** networks represent CON, ALG, and RS, respectively. The numbers on nodes are OTU numbers, and PCWCs are labeled with their targeting monoclonal antibodies. All PCWCs are shown by one color (green) while OTUs belonging to different families are represented by different colors (see legend). The size of each node is proportional to the value of relative abundances. Solid edges (black) are positively associated while dashed edges (red) are negatively associated. Edges without any tip show co-occurrence without time delay; while one, two, and three time-point delays are indicated on the affected feature with an arrow, circle, or diamond tip, respectively. HG, homogalacturonan; AGP, arabinogalactan protein; GlcA, glucuronic acid.

OTUs affiliated to the Ruminococcaceae, Lachnospiraceae, and Lactobacillaceae families were the most abundant taxa that co-occurred/correlated with the PCWCs in all diets.

In the CON network, rhamnogalacturonan I (INTRA-RU1) and xyloglucan (LM15) exhibited the highest number of correlations with different OTUs. These polysaccharides typically had negative correlations (with a one time-point delay such that the shift on OTU relative abundance affects polysaccharide relative abundance with a delay of one time period), suggesting that an increase in the relative abundance of these OTUs was correlated to a decrease in the relative levels of these polysaccharides in CON pigs. Within the RS network, many taxa co-occurred with xyloglucan (LM15), although the OTUs were affiliated to different lineages and the majority of correlations were positive with one time-point delay. Most of the highly abundant S24-7 OTUs (OTU5, OTU2006, and OTU2009) negatively co-occurred in the RS network with more than one PCWC, including arabinan (LM6), arabinogalactan protein (AGP: JIM13), homogalacturonan (HG: LM19), β-(1,3) glucan (BS-400-2), and xylan/arabinoxylan (LM11). However, this varied in the CON network as the same OTUs were only negatively correlated with rhamnogalacturonan 1 (INTRA-RU1). The number of OTUs that exhibited correlations in the ALG network was relatively lower (almost half of CON and RS networks), with the most prominent being negative correlations (with a one time-point delay) between the unclassified Clostridiales and xylan (LM23) and xyloglucan (LM15) as well as Streptococcaceae and xylan (LM23) and glycoproteins (extension: JIM20).

Discussion

SSU rRNA gene amplicon sequence analysis and CoMPP of PCWCs were used to evaluate the effects of dietary fibers (alginate and type 3 resistant starch) on the gut microbiome of growing pigs during a 12-week feeding experiment. The fibers assessed in this study have contrasting properties, the most prominent being the gel-forming capacity of alginate fibers whereas resistant starch is resistant to the host's digestive enzymes but fermentable by gut flora in the lower intestine. Findings by Souza da Silva *et al.* [12,23] demonstrated that these two fibers affected feeding patterns and physiology of growing pigs in different ways. The feeding patterns were affected less by alginate addition in the diet compared to resistant starch addition in a manner that only cumulative and average daily feed intake increased in ALG pigs compared to CON pigs, to achieve similar digestible energy intake. Moreover, both diets increased the relative empty weight of the colon, but only RS increased the weight of the total gastrointestinal tract. This is conceivably the result of an increase in bacterial mass and fermentation end-products [24] or an increase in metabolically active tissue in the colon [12,25]. The gut microbiota plays an important role in host physiology [26], and a different impact on community composition resulting from ingestion of these dietary fibers is therefore expected to occur due to their different physicochemical and metabolic properties. This study showed that resistant starch (type 3) had significant effect on gut community structure of growing pigs while the community composition in ALG pigs was similar to that in CON pigs. Moreover, the demonstrated shift in microbiome structure of RS pigs was specific to diet type in spite of the inter-individual variations.

Alpha diversity within the microbiome was lower in RS pigs compared to CON pigs, which is most likely due to the selection of particular genera among the Firmicutes. Many bacterial lineages exhibited shifts in relative abundances after the commencement of the different diets, with RS pigs being the most pronounced. In some previous studies, performed with varied methods and models, it has been shown that type 2 resistant starch increases *Ruminococcus bromi* and *Eubacterium rectale*, while type 4 resistant starch promotes the growth of *Bifidobacterium adolescentis* and *Parabacteroides distasonis* in human subjects [27], and that *Bifidobacterium*, *Akkermansia*, and *Allobaculum* are increased by type 2 resistant starch in mouse models [28]. Similarly, type 3 resistant starch has led to the increased relative abundances of *E. rectale*, *Roseburia* spp., and *R. bromii* in mouse models [29], *E. rectale*, *Roseburia*, Clos IV *Ruminococci* and *Oscillospira* in obese male humans [30], and *R.bromii* in colonic samples of pig models [31]. In the present study, we observed an insignificant increase in *Roseburia* relative abundance in the microbiome of RS pigs, whereas *Eubacterium* was not detected in any of the pigs irrespective of diet. The *Ruminococcus* genus, including *R. bromii,* which is known for its ability to

degrade resistant starch [32], had a significant increase in RS pigs. In addition, a broad diversity of bacterial genera increased in relative abundance due to RS, including *Bulleidia, Megasphaera, Dialister,* an unclassified Lachnospiraceae genus, and *Prevotella.* The increase in some of these bacterial lineages was also observed previously in growing pigs after 14 days of feeding with type 3 RS compared to CON pigs [23,33]. The increase in relative abundances of *Ruminococcus* (threefold) and *Prevotella* (nearly fivefold) in RS pigs compared to CON pigs is notable due to their ability to use polysaccharides to produce short-chain fatty acids [34] that are known to play a protective role against gut inflammation [35] and be used as an energy source for the host [6]. The predominance of Lachnospiraceae in RS pigs is also noteworthy as previous mouse studies [36] have demonstrated that their presence can lead to a reduction in *Clostridium difficile* colonization, which is an important pathogen for pigs and humans [37-39]. We found an increase in Lachnospiraceae as well as a decrease in Clostridiaceae in RS pig. Although there was no direct correlation between these families, the interaction between specific species affiliated to these families need to be investigated further. *Bifidobacterium,* which is known for its minority in pig intestine [40,41], was not detected in any of pigs regardless of diet type.

Compared to resistant starch, alginate has a low fermentability [10], however, it has been demonstrated to have a positive impact on the total bacterial count in the human fecal microbiome *in vitro* and is believed to have prebiotic effects [13,14]. Its consumption has been shown to result in a significant increase in the number of *Bifidobacteria* and a decrease in the number of Enterobacteriaceae in healthy human subjects [42], whereas the relative abundance of *Bacteroides capillosus* has also been demonstrated in the cecum of rats fed with sodium alginate [43]. In ALG pigs, less variation within the microbiome structure than RS pigs was observed when they both were compared to CON pigs. However, we observed that alginate affects the gut bacterial community via altering the relative abundances of some families and genera. In particular, Clostridiaceae-affiliated phylotypes experienced decreased relative abundance in ALG pigs similar to the RS pigs when compared to the CON animals.

Time did not have a significant influence on alpha and beta diversity metrics within any of the diets. This can be explained by the short experimental period and the maturity (3 to 6 months old) of these growing pigs, which were principally in a child-to-early-adolescent life stage. Diversity levels during this period are typically more comparable to adults and generally more stable than those during the infant period [44-46]. The natural age of completion of weaning in pigs differs from 9 to 20 weeks [47], whereas the onset of puberty in pigs can be as early as 5 months in female pigs [48]. The 3-month-old pigs used in this study were weaned before the commencement of the feeding trials and had only a few months to puberty. Despite the relative stability of diversity metrics, the relative abundances of some families did change over time. These alternating variations between families that were correlated negatively with each other (Additional file 7: Figure S5) may indicate the competitive interactions within the community as a result of substrate change in the community with addition of fibers.

The shifts in microbiome structure of ALG and RS pigs were consistent with imputed functional predictions. ALG had little effect on predicted microbiome function, which was expected since there was little change in the microbiome structure. In contrast, RS pigs experienced greater microbiome structural shifts, subsequently resulting in more predicted changes in the relative abundance of imputed KEGG pathway maps. Many of the significantly altered imputed functions in RS pigs were related to fatty acid metabolism such as butanoate and propanoate. Resistant starch is known to play an important role in fatty acid production in the gut [49,50], therefore it was surprising that imputed butanoate and propanoate metabolisms were associated negatively with RS compared to the CON diet. The KEGG starch and sucrose metabolism pathway map which contains the majority of reactions involving starch, cellulose, xylan, and pectin degradation was not significantly influenced by RS or ALG with all time-points considered. Assessing the individual samples taken over the 12-week time period revealed a similar pattern with the exception of one sample (T3), which demonstrated a higher imputed representation of this KEGG pathway in RS pigs. This result seems to correspond well with CoMPP analysis of PCWCs, which showed polysaccharide degradation consistency between diets over time.

RS and ALG diets were found to influence OTU and PCWC correlations/co-occurrences over time, with the same PCWCs in CON, ALG, and RS pigs often correlated with different OTUs. This was expected given that alginate and resistant starch caused varying changes to microbiome structure, whereas the PCWC availability in the microbiome is believed to be largely unchanged. This was clearly illustrated for the hemicellulose polysaccharide xyloglucan (target of probe LM15), for which the total number and OTU affiliation of correlations varied substantially between CON, ALG, and RS pigs (Figure 7). Many OTUs affiliated to the Ruminococcaceae and Lachnospiraceae families were positively correlated to PCWCs and thus inferred in PCWC metabolism in growing pigs regardless of diet type. Both of these families are well known for degradation of complex plant material (for example, cellulose, hemicellulose) in the mammalian gut environment [51].

Conclusions

In conclusion, RS exhibited the strongest structural variation compared to ALG, which is likely resultant from the contrasting physicochemical properties of these dietary fibers. The increase in relative abundance of Lachnospiraceae-, *Prevotella*- and *Ruminococcus*-affiliated phylotypes in RS pigs can be considered as desirable traits given the reputation of these groups in fiber degradation and production of short chain fatty acids. Moreover, resistant starch and to a lesser extent alginate, influenced the imputed functionality of predicted metagenomes and correlation between bacterial phylotypes and PCWCs. With all data collectively considered, we speculate that despite the microbiome structural differences between diets, functional redundancy exists in the key metabolic stage of polysaccharide degradation. The observed stability in the imputed KEGG starch and sucrose metabolism pathway and consistent PCWC availability between diets supports this hypothesis. Furthermore, the variation in OTU-PCWC correlations between the different diets suggests that different phylotypes possibly drive PCWC utilization within each feeding regime. These hypotheses require further detailed metagenomic investigations to deduce the metabolic capabilities of key uncultured populations within the microbiome of pigs, and form the basis of our ongoing efforts.

Methods

Study design and sampling

Nine pigs (approximately 3 months old) selected for this study were housed, fed, and sampled at the Nutreco Swine Research Centre facilities, Sint Anthonis, The Netherlands [12]. Each group of three pigs was fed with one of three diets: control (CON) containing no prebiotic dietary fiber, alginate-containing (ALG) and retrograded (Type 3) resistant starch-containing (RS). The control diet was formulated to contain 40% digestible starch, and other diets were formulated from control diet by exchanging alginate (sodium alginate in dry form) or resistant starch (retrograded tapioca starch) for digestible starch on a dry matter. (Additional file 1: Table S1, for further diet details refer to [12]). Weight measurements were also performed during the feeding period. There was no significant difference between the weights of the pigs fed with different diets, although all pigs achieved a final weight (99.4 ± 6.7 kg) greater than three times of the initial weight in the experiment (31.7 ± 1.4 kg). The pigs were labeled with respect to diet they were fed with, such as CON.1, CON.2, CON.3, ALG.1, ALG.2, ALG.3, RS.1, RS.2, and RS.3. All pigs originated from the same batch consisting of castrated males with the exception of one female (ALG.2) and were unrelated except for two siblings (ALG.1 and RS.3). Each pig was fed with the aforementioned diet over a 12-week period (T2 to T7), and fecal samples were collected at seven different time-points (T1: day −7; T2: day 1; T3: day 3; T4: day 7; T5: week 3; T6: week 7; T7: week 12). All pigs were fed with a commercial basal diet for 3 weeks before the experiment commenced and the first fecal sample collection (T1). The adaptation to the diets was performed by gradual exchanging of the commercial diet for one of the CON, ALG, and RS during a 7-day period before T2, from which point the complete differentiation in diets started. The 7-day transition period entailed the following stages: 2 days of the animals being fed with the commercial diet (100%); the third day, the commercial diet was supplemented with 20% of the different prebiotic diets; and from days 4 to 7, the percentage of the prebiotic diet was increased in 20% increments until the prebiotic diet reached 100% (T2). A total of 61 fecal samples were used because the rectum of two pigs were empty at the time of collection of fresh fecal samples (pig ALG.1 at T4 and pig RS.3 at T6), and these two samples were subsequently not available. Fresh fecal samples were homogenized and kept at −20°C until analysis.

Cell dissociation and DNA extraction

Bacterial cells were harvested from 0.3 *g* of frozen feces using a cell dissociation protocol as described previously [52]. The samples were suspended in acidic dissociation buffer [53] containing (v/v) 0.1% Tween 80, 1% methanol, and 1% tert-butanol, and cells were harvested from supernatant by quick centrifugation. These steps were repeated five times to increase cell yield. Cell pellets were collected by high-speed centrifugation (14,500 *g* for 5 min) and washed with a wash buffer containing 10 mM TrisHCl and 1 M NaCl. DNA extraction was performed as described in [54] with small modifications. The cells were re-suspended in RBB + C lysis buffer containing 500 mM NaCl, 50 mM TrisHCl, and 50 mM ethylene diamine tetraacetic acid (EDTA) and incubated with lysozyme and mutanolysin enzymes at 37°C for 30 min. Further lysis was carried out by addition of 4% sodium dodecyl sulfate (SDS) and incubation at 70°C for 20 min, mixing the tube by inversion every 5 min. Cetyltrimethyl ammonium bromide (CTAB) buffer was used for DNA precipitation. After repeated treatments with chloroform and phenol/chloroform/isoamyl alcohol, DNA was precipitated by isopropanol, washed once with ethanol, re-suspended in water, and kept at −20°C until further analysis.

Bacterial SSU rRNA gene amplification and 454 pyrosequencing

The SSU rRNA gene fragment hyper variable regions V1 to V3 were amplified from extracted DNA using 8F-515R bacteria-specific primers. The forward primer is a combination of the 454 fusion adapter B sequence and

universal bacterial primer 8F, 5′-*CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG CAA CAG CT*A GAG TTT GAT CCT GG-3′. The reverse primer is a combination of the 454 fusion adapter A sequence including a unique 8 nt multiplex barcode, represented by Ns, and universal bacterial primer 515 R, 5′-*CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG NNN NNN NN*T TAC CGC GGC TGC T-3′. Each PCR reaction consisted of 25 μl iProof High-Fidelity Master Mix (BioRad, Hercules, CA, USA), 0.2 mM forward primer, 0.2 mM reverse primer, 400 ng template DNA, and sterile water to a total volume of 50 uL. The following PCR program was used: denaturation at 98°C for 30 s, 30 cycles of 10 s at 98°C, 30 s at 58°C, and 40 s at 72°C and a final extension at 72°C for 7 min. PCR product concentrations were measured by Qubit® fluorometer using Qubit® dsDNA BR Assay Kit (Invitrogen, Eugene, OR, USA) and checked by gel electrophoresis (1% agarose gel). All PCR products were pooled into one tube in equal amounts and run on a 1% agarose gel. The band containing pooled PCR products was excised and purified using NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany). Pyrosequencing was performed on the 454 GS FLX sequencer (Roche) at the Norwegian Sequencing Center (Oslo, Norway).

Analysis of 16S rRNA gene sequences

The sequencing reads were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0 [55]. Reads of quality lower than 25, lacking a barcode, and/or shorter than 400 or longer than 600 nt were not analyzed further. The remaining reads (93%) were multiplexed to samples based on their nucleotide barcodes. Further error correction was performed using USEARCH version 5.2.236 [56] and UCHIME [57], and the remaining sequences were clustered into OTUs using a 97% sequence identity threshold. A representative sequence set was formed by picking the most abundant sequence from each OTU and aligned against the Greengenes core set database [58] (May 2013 version) by PyNAST [59] with a minimum sequence length of 150 and a minimum identity of 75%. The Ribosomal Database Project (RDP) classifier program [60] was used to assign taxonomy to the aligned sequences with a confidence of 0.8. The alignment was filtered prior to generating a phylogenetic tree using a lanemask to remove highly variable regions and positions that were all gaps. A phylogenetic tree was built using filtered, aligned sequences in FastTree [61] which was subsequently used to generate an unweighted UniFrac distance metric [62]. This metric included the calculated distances between samples based on OTU composition of each sample and visualized by principle coordinate analysis (PCoA).

Functional analysis of metagenomes

Metagenome functional contents of CON, ALG, and RS diet samples were predicted using PICRUSt [22] online Galaxy version. Closed reference OTU table was generated from filtered reads (previously described) in QIIME v1.7.0 [55] using the Greengenes core set database [58] (May 2013 version) and enabling reverse strand matching. A closed reference OTU table was normalized by 16S rDNA copy number, metagenome was predicted, and they were categorized by function based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in PICRUSt online Galaxy version. The obtained biom file was processed by STAMP v2.0.8 [63] for statistical analysis; Welch's *t*-test was applied to compare the KEGG pathways of diet groups pairwise (RS and CON, ALG and CON) with P value <0.05, confidence intervals of 95% and extended error bars were plotted. Boxplots were plotted to further focus on starch and sucrose metabolism pathway.

Plant cell wall component (PCWCs) analysis

CoMPP was used to detect PCWCs in feed and fecal samples as described previously [64]. Two of the samples (CON.2, T6 and CON3, T5) did not contain enough material after the other analyses to be analyzed for PCWC content and were not assessed. Freeze-dried fecal samples (each of 10 mg) were homogenized by mortar and pestle. Alcohol-insoluble residues were obtained by sequential extraction using three solvents: 70% ethanol, methanol/chloroform (1:1), and acetone. Each extraction was followed by vortexing for 30 s and centrifugation at 14,500 *g* for 10 min to remove supernatant. Following this, the acetone was removed using a pipette and the samples were air dried. PCWCs were extracted from the alcohol-insoluble residues using 50 mM diamino-cyclohexane-tetra-acetic acid (CDTA), pH 7.5, and 4 M NaOH with 1% v/v NaBH4, which are known to solubilize pectins and noncellulosic polysaccharides, respectively. For each extraction, 300 μl of solvent was added to each tube and incubated at room temperature with shaking for 2 h. After centrifugation at 2,500 *g* for 10 min, supernatants were retained, diluted (neat, 5-, 25-, and 125-fold) in Arrayjet buffer (50% water, 50% glycerol, and 0.05% Triton X100) and the three dilutions printed in quadruplets onto nitrocellulose membranes. Every replicate was therefore represented by a 16-spot sub-array (four concentrations and four printing replicates). Arrays were probed with monoclonal antibodies (mAbs) or carbohydrate-binding modules (CBMs) (Table 1) and scanned (CanoScan 8800 F, Canon, Søborg, Denmark) and quantified using Array-Pro Analyzer 6.3 (Media Cybernetics, Rockville, MD, USA). The maximal mean spot signal was set to 100%, and all other values within that data set adjusted accordingly. A mean spot signal minimum was set as 5%.

Table 1 The probes used in comprehensive microarray polymer profiling (CoMPP) and the target plant cell wall components (PCWCs)

Monoclonal antibody (mAb) and carbohydrate-binding module (CBM) probes	Target PCWCs
LM19	Homogalacturonan (HG) partially methylesterified
INRA-RU1	Backbone of rhamnogalacturonan I
LM5	(1 → 4)-β-D-galactan
LM6	(1 → 5)-α-L-arabinan
LM21	(1 → 4)-β-D-(galacto)(gluco)mannan
BS-400-2	(1 → 3)-β-D-glucan
BS-400-3	(1 → 3)(1 → 4)-β-D-glucan
LM15	Xyloglucan (XXXG motif)
LM10	(1 → 4)-β-D-xylan
LM11	(1 → 4)-β-D-xylan/arabinoxylan
LM23	(1 → 4)-β-D-xylan
CBM3a	Cellulose (crystalline)
LM1	Extensin
JIM20	Extensin
JIM13	Arabinogalactan protein (AGP)
LM2	AGP, β-linked glucuronic acid (GlcA)
CBM20	Starch

Statistics

The statistical significant test was applied on unweighted UniFrac distance matrices in QIIME v.1.7.0. The parametric *P* values were calculated performing two-sample *t*-tests for the pairs of the groups while nonparametric *P* values were calculated using Monte Carlo permutation ($n = 1{,}000$). The bacterial diversity was calculated at an OTU level using Shannon index that based on the average of ten iterations at equal subsampling size of 1,781. Analysis of covariance (ANCOVA) was run using R (version 3.1.0) package lme4 to identify the effects of time and diets on diversity of bacterial communities (based on Shannon indexes) and the relative abundances of taxa in genus and family levels. In this analysis, ALG and RS samples were compared to CON samples and the taxa with *P* value smaller than 0.01 were included in the plots. Calypso version 3.4 (http://bioinfo.qimr.edu.au/calypso/) was used to generate bubble plot to observe time-dependent changes. Each data point on bubble plot shows the mean relative abundance of bacterial families for the indicated time-point and was determined from fecal samples of three pigs that were fed with the same diet. Pearson's correlations between the bacterial families were calculated and plotted using Calypso Version 3.4. To evaluate the interactions between gut bacteria and PCWCs over time, extended local similarity analysis (eLSA) [65,66] was performed. Cytoscape 2.7.0 [67] was used to process eLSA outputs and generate correlation networks. eLSA output was filtered by local similarity score (LS) and *P* value ($P < 0.001$) to reduce the number of nodes.

Ethical aspects

The housing, feeding, and sampling of the animals were performed at the Nutreco Swine Research Centre facilities (Sint Anthonis, The Netherlands), and all experimental protocols describing the management, animal care, and sampling procedures were reviewed and approved by The Animal Care and Use Committee of Wageningen University (Wageningen, The Netherlands, DEC nr. 2011088.c).

Supporting data

The sff file has been deposited in the SRA (Bioproject ID: PRJNA262976 and Accession number: SRP048624).

Additional files

Additional file 1: Table S1. The diet ingredients and their inclusion percentages.

Additional file 2: Figure S1. Rarefaction curves calculated for each diet group. Curves were calculated for observed species with standard deviation.

Additional file 3: Figure S2. Shannon index variation over time. Shannon indexes were calculated to be the average of ten iterations at equal subsampling size of 1,781 for each sample. Samples were grouped by color in terms of diet group they belong to; control diet (CON) green, alginate-containing diet (ALG) blue, and resistant starch-containing diet (RS) red.

Additional file 4: Table S2. Inter-individual variations and bacterial composition over time.

Additional file 5: Figure S3. Bacterial family relative abundances in every sample. Different colored bars represent different families with size showing abundance of this family. Labels contain name of diet type (CON, ALG, RS), pig number for the specific diet with numbers between 1 and 3, and time point numbers between from 1 to7 in the order (starting from T1 as first time point).

Additional file 6: Figure S4. Bacterial families with significantly different relative abundances between different diets. Families that have different abundances in ALG or RS pigs compared to CON pigs were determined by ANCOVA. The shown mean relative abundance percentages of the taxa were calculated using all samples taken over time within each diet. Significance degree is represented with stars; $P < 0.05$ with one star (*); $P < 0.01$ with two stars (**); $P < 0.001$ with three stars (***). The significance was stated next to the bar together with the abbreviations of compared diets (ALG, CON, and RS) when the bar does not appear for at least one of the diets due to very low relative abundance percentage.

Additional file 7: Figure S5. Correlations between bacterial communities in family level. The correlations were calculated using Pearson's correlation. Positive correlations are displayed with yellow edges and negative correlations with blue edges. The minimum similarity between the edges is 0.25. The blue nodes represent bacterial families and size of each node is proportional to the value of relative abundances. The diets (ALG, CON, RS) are shown with red nodes.

Additional file 8: Figure S6. Starch and sucrose metabolism comparison of RS and CON pigs and ALG and CON pigs over time. The relative abundance of starch and sucrose metabolism pathways encoded in each imputed sample metagenome was analyzed using STAMP [54].

Time points were represented by T1 to T7 (T1: day 0, T2: day 1, T3: day 3, T4: day 7, T5: week 3, T6: week 7 and T7: week 12). Significant difference was considered only when $P < 0.05$.

Additional file 9: Figure S7. Original versions of network plots in Figure 7. The networks are ordered as CON, ALG, and RS.

Abbreviations

AGP: arabinogalactan protein; ALG: alginate-containing diet; CBM: carbohydrate-binding module; CoMPP: comprehensive microarray polymer profiling; CON: control diet; CTAB: cetyltrimethyl ammonium bromide; EDTA: ethylene diamine tetraacetic acid; eLSA: extended local similarity analysis; HG: homogalacturonan; KEGG: Kyoto Encyclopedia of Genes and Genomes; LS: local similarity score; mAb: monoclonal antibody; OTU: operational taxonomic unit; PCR: polymerase chain reaction; PCWCs: plant cell wall components; RDP: Ribosomal Database Project; RS: retrograded (type 3) resistant starch-containing diet; SDS: sodium dodecyl sulfate; SSU: small subunit.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The study was designed by OCOU, MO, PBP, JEB, and DBD. CSS and GB did the sample collection. OCOU performed the sequence analysis and annotation. CoMPP analysis was performed by JF and WGTW. OCOU wrote the manuscript. MO, JAF, PBP, and DBD edited the manuscript. All authors read and approved the final manuscript.

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Paper 3

The potential of class II bacteriocins to modify gut microbiota to improve host health

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Running title: Impact of class II bacteriocins on gut microbiota

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Abstract

In this comparative study, five bacteriocin-producing strains of gut-associated lactic acid bacteria (LAB) and their isogenic non-producing mutants were investigated in mice for their probiotic properties. The bacteriocin producers belong to the following groups: *Lactobacillus sake*, *Pediococcus acidilactici*, *Enterococcus faecium*, *Lactobacillus plantarum*, and *Lactococcus garvieae*, and their bacteriocins, sakacin A (SakA), pediocin PA-1 (PedPA-1), enterocins P, Q and L50 (enterocins), plantaricins EF and JK (plantaricins), and garvicin ML (GarML), respectively, are all classified as class II bacteriocins. These strains were given to mice through drinking water and changes in microbial communities of fecal samples were analyzed using metagenomics approach. In general, we observed that the overall structure of the bacterial composition was not much affected. However, some changes were found in certain bacterial groups at deeper taxonomic levels. For instance, *Staphylococcus* was found inhibited by enterocins, Enterococcaceae by GarML, and *Clostridium* by plantaricins. SakA, plantaricins and GarML increased the proportion of LAB, which are often considered as beneficial gut inhabitants. Moreover, some bacteriocin-producing strains were also associated with serum parameters interpreted as favorable for the host, such as GarML decreasing triglycerides and increasing high-density lipoprotein levels. Taken together, our results indicate that bacteriocin production contributes to the probiotic value of the producer as they can be used to promote health-bringing effects in animals.

Keywords: lactic acid bacteria, class II bacteriocins, *in vivo*, mice, gut microbiota, sakacin A, pediocin PA-1, enterocins, plantaricins, garvicin ML

Introduction

The gut microbiota has recently been considered a separate organ in mammals since it contributes with many benefits to the host, including immunomodulation/stimulation, production of vitamins and inhibition of the growth of pathogens as well as to help extract energy and other nutrients from the ingested food (1, 2). With more than 1,000 bacterial species lining along the gastrointestinal tract (3), these microbial inhabitants are expected to have developed means to compete with each other for common resources and strategies to cope with different insults from the host (4). Survivors are selected on the basis of several aspects, including the ability to deal with host diet, colonization resistance, inhibitory agents (e.g. bile salt, defensins) and other host-mediated effects like improved barrier function and altered immune response (5). Bacteria, the most predominant members of the gut microbiota, use different mechanisms to colonize and persist in the gut. One of these is the production of bacteriocins, which are ribosomally synthesized antimicrobial peptides produced by numerous Gram-negative and Gram-positive bacteria (6). Most bacteriocins have relatively narrow spectra, normally targeting species or genera closely related to the producers. Others, such as the lactococcal bacteriocin nisin can have a much wider spectrum, including important pathogenic or problematic species of *Staphylococcus*, *Listeria*, *Enterococcus* and *Streptococcus* (6, 7). A large number of bacteriocins are produced by LAB, which is a diverse group of bacteria that are frequently found in food and feed, as well as being common inhabitants in the gut environment of a great number of animals including humans. LAB are therefore generally regarded as safe (GRAS) for human consumption and the production of bacteriocins as one of the important probiotic properties. It has been shown that bacteriocins can modulate the host immune system, as well as being able to antagonize opportunists and potential pathogens (8).

Bacteriocins produced by Gram-positive bacteria are classified into two main classes: class I, containing heavily modified (lanthionine-containing) peptides called lantibiotics; and class II, containing non-modified peptides or peptides with minor modifications (such as disulfide bond formation or circularization) (9). Class II bacteriocins can be divided further into subclasses: class IIa, pediocin-like bacteriocins, which are typically very active against *Listeria* and have a relatively narrow spectrum; class IIb, two-peptide bacteriocins, whose activity is dependent on the synergy between two different peptides; class IIc, circular bacteriocins; and class IId, the miscellaneous group which include all other bacteriocins that do not fit into any of the aforementioned groups (9).

Mice have been successfully used as a model to unravel the connection between gut microbiota and a variety of health issues or environmental factors, such as obesity (10), diet (11, 12) and antibiotics (13). Also in humans, different external and internal factors can cause changes in the composition of gut microbiota. For instance, it is well known that diet composition can affect distinct human enterotypes (15–17) and that the administration of antibiotics causes drastic changes in the gut microbiota (18). In addition, the microbiota is altered in certain health conditions such as obesity (19–21), a variety of diseases (22) and stress (4). However, many changes are transient or can be reverted to normal (healthy) conditions soon or later dependent on the type of treatments (3).

A number of bacteriocins have been studied for their ability to inhibit pathogens in the gut, such as *Salmonella enteritidis* (23), *Listeria monocytogenes* (24), *Clostridium difficile* (25), *Staphylococcus aureus* (26), to eliminate multidrug- or vancomycin-resistant enterococci (27, 28) or to deal with some bacteria-related disorders such as obesity (29). Bacteriocins have several advantages over antibiotics in infection treatments because they are more target-specific (thus

avoiding the killing of commensal and beneficial cells), have low or no toxicity toward eukaryotic cells and are active against both pathogens and their derived antibiotic-resistant strains (30). However, most studies lack a detailed assessment on how bacteriocins affect the general composition of the gut microbiota, especially with regard to the probiotic properties of the producers in healthy individuals. In this study, we performed a comparative study to examine the probiotic effects of five different bacteriocin producers on the gut microbiota and other host parameters (including blood serum, weight) in healthy mice. The chosen bacteriocins show great differences in terms of target specificity and width of inhibitory spectrum, which will allow us to affect the gut at different levels and in different directions.

MATERIAL AND METHODS

Animals and housing conditions

Six to eight weeks old BALB/C female mice were grouped into 11 different cages (1 control cage with n=10 and 10 treated cages with n=9) and mice were ear-labelled for individual tracking. Before treatments, mice were left in cages for about 10 days for adaptation to the environment after they were brought to the facility. All mice had *ad libitum* access to water and feed and their health status was carefully observed during the entire experimental process. All animal work and procedures were approved by the institutional Ethics Committee of the CSIC and University of Valencia and performed following the principles of laboratory animal care (as mandatory by European Union Law and 2010/63/EU and Spanish Government RD 53/2013on the protection of animals used for scientific purposes**)**.

Experimental design and sampling scheme

Bacteriocin-producing and non-producing (isogenic mutants) strains of LAB were administrated to mice via drinking water (Table 1). For each bacteriocin system, a pair of bacterial strains were

used: a wildtype producer and an isogenic non-producing mutant, except for one isogenic mutant producing fewer bacteriocins compared to the wildtype strain (see below). The isogenic mutant strains were used as negative controls for bacteriocin production. The wildtype bacteriocin producers were: *Lactobacillus sake* Lb 706 producing SakA (31, 32), *Pediococcus acidilactici* 347 producing PedPA-1 (33), *Enterococcus faecium* L50 producing enterocins P, Q and L50 (34), *Lactobacillus plantarum* C11B producing plantaricins EF and JK (35) and *Lactococcus garvieae* DCC43 producing GarML (36).

Bacterial strains were grown overnight in brain-heart infusion (BHI) medium. Cells were harvested by centrifugation and washed twice with phosphate buffered saline (PBS) before being frozen as stock cultures in 15% glycerol in PBS at -80°C. To prepare bacteria-containing drinking water, each frozen was thawed and diluted to give 100 ml water containing about of 10^9 cells/mL which was then given to each cage (Table 1). The drinking water (with or without bacteria) was renewed on daily basis. The bacterial administration was carried out for 15 days followed by another two more weeks with bacteria-free water. For survival assessment, bacterial cells in drinking water were counted by plating just after dilution and after 24h; this was done once during first week and once during the second week of the bacterial administration regime. All mice from the same cage shared the same water bottle, and water intake was measured daily for each cage. Fecal samples were collected from each mouse once a week during the four-week experiment. The first sample was taken on day 0 (time zero) just before exposure of mice to bacteria-containing drinking water, thus these samples served as base line. The following fecal samples were collected on day 7, day 14, day 21 and day 28 and kept at -80°C until further analysis. Mice were weighed every week on fecal collection day. Blood samples were collected from the facial vein, without anticoagulants, from 4 to 5 randomized mice from each cage on day 15, which was the last day of bacteria

administration. Samples were kept on ice after collection and centrifuged at 1,500 g for 10 min, at 4ºC, to separate serum which was collected and kept at -80ºC prior to analysis. The analyses of triglycerides, total cholesterol, HDL and LDL contents were performed in Laboratory of Analysis, Hospital Clinico Veterinario, Universidad de Murcia, Spain.

LAB counting and bacteriocin activity

Total LAB cells in fecal samples at day 14 were counted for three randomly selected mice per cage. LAB counting and bacteriocin plate assay were performed as follows: Each fecal pellet was dissolved and serially diluted in 0.9% NaCl. Cells (100µL) from each dilution were mixed with 4 mL of MRS soft agar (0.8%), poured onto an MRS agar plate and then covered by another 4 ml of cell-free soft agar (to prevent cells growing on the surface). Plates were incubated anaerobically at 30°C overnight before being covered with another layer of soft agar containing 100-fold diluted overnight culture of a suitable indicator. The plates were again incubated overnight, and total colony forming units (CFUs) and CFUs of bacteriocin producers that formed inhibition zones were scored. The following indicator strains were used: *E. faecium* P21 (LMG 2783) for SakA and PedPA-1, *P. damnosus* (LMG 3397) for enterocins, *L. plantarum* 965 (LMG 2003) for plantaricins, and *L. lactis* IL1403 (LMG2705) for GarML.

DNA extraction

A total of 495 (99 mice x 5 time points) fecal samples were collected during the course of the experiment. DNA from each fecal sample was extracted using Realpure SSS kit (Real Life-Science Solutions, Durviz, Spain) with addition of a bead-beating step. The DNA was quantified using Qubit® fluorometer with Qubit® dsDNA HS Assay Kit (Invitrogen, Eugene, OR, USA). The DNA samples at each time point from the mice sharing same cage were normalized and pooled prior to the amplicon sequencing, giving rise to 55 pooled samples. DNA samples from three

randomly selected mice from the control cage at day 0, day 14 and day 28 were also sequenced to observe the individual variation on fecal microbiota over time.

16S rRNA gene amplification and sequencing

Library preparation for 16S rRNA gene amplicon sequencing was performed as described in the Illumina 16S metagenomic sequencing library preparation protocol (37). Briefly, the V3-V4 region of bacterial 16S rRNA gene (38) was amplified using forward and reverse primers with Illumina overhang adaptors, 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3' and 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3', respectively. PCR products were cleaned up using AMPure XP beads (Beckman Coulter Genomics, USA). A second PCR was carried out for sample specific dual indexing using the Nextera XT Index kit (Illumina, San Diego, California, USA) that contains index primers with 8-base indices adjacent to the P5 or P7. Cleaning-up of indexing PCR products was performed again using AMPure XP beads (Beckman Coulter Genomics, USA). Purified product concentrations were measured by Qubit using Qubit® dsDNA HS Assay Kit (Invitrogen, Eugene, OR, USA) and quality was checked by gel electrophoresis (1% agarose gel). Libraries were normalized and pooled. The pool was quantified with rt-PCR using PerfeCta NGS library quantification kit for Illumina Sequencing platforms (Quanta BioSciences, Maryland, USA). The quantified pool was denaturated prior to loading of samples into the MiSeq machine. Loading of libraries to the sequencer was performed using MiSeq v3 reagent kit (Illumina, San Diego, California, USA).

Analysis of sequencing data

The raw Illumina reads were filtered and de-multiplexed using the Illumina MiSeq Reporter system software version 2. The paired-end MiSeq reads were processed using UPARSE pipeline

(39) implemented in USEARCH (40) (version 7.0.1090). Paired-ends were merged and quality filtering was applied using maximum expected error (maxee) value of 1.0 and discarding the sequences less than 150nt. Sequences were dereplicated, singletons were discarded. Sequences were clustered into OTUs using 97% sequence identity threshold, chimeric sequences were filtered from clustered OTUs using UCHIME (41) and OTU table was created. OTUs were processed further using Quantitative Insights Into Microbial Ecology (QIIME) version 1.8.0. The representative OTUs were picked and aligned against the Greengenes core set database (42) using PyNAST (43) with a minimum identity of 75%. Taxonomy was assigned to aligned sequences using The Ribosomal Database Project (RDP) classifier program (44) with a confidence of 0.8. OTU table was subsampled to normalize the sequence number among samples based on the sample with lowest number of sequences. A phylogenic tree was built using Fast Tree (45) from aligned sequences after the filtration step in order to remove highly variable regions and positions that were all gaps. This tree was used to calculate alpha and beta diversities. Rarefaction curves and Shannon indexes were calculated. Unweighted UniFrac distance metrics (46) were generated and principle coordinate analysis (PCoA) was used to visualize the metrics.

Statistics

The comparisons of Shannon indexes between the treatments were performed in R using an ANCOVA that considered time as a continuous dependent variable with significance at $P < 0.05$. The distances between treatments in PCoA plots were compared in QIIME using a two-sided Student's *t*-test, and the nonparametric p-values were calculated with 1,000 Monte Carlo permutations using Bonferroni correction. ANOVA was performed in R for the comparison of weights of mice over time within each treatment and the change in relative abundance of taxa between treatments. Relative abundance at day 0 was taken as basis within each treatment and the

change corresponding to day 0-relative abundances were compared between treatments. The changes on day 7 and day 14 were together considered to represent the bacteria administration (treatment) period, while day 21 and day 28 represented the post-treatment period. P-values smaller than 0.1 were considered as significant for the relative abundance comparisons when the effect size of testing was high ($R^2 > 0.84$). Serum levels were compared pairwise between the cages treated with bacteriocin-producing and non-producing strains, using two-sided t-tests and plots that were generated in STAMP (47). Pearson correlations ($P < 0.05$) between serum levels and the relative abundance of OTUs in treatments were calculated using CoNet (48) and visualized using Cytocsape 3.1.1 (49).

RESULTS

Water consumption and weight gain of mice

The drinking water was refreshed every day and live bacterial cells were kept between 10^8 to 10^9 cells per ml for bacteria administration. The water intake of all cages was similar to the control cage (CON, without bacteria); i.e., mice consuming daily on average 29 ml/cage or 3.2 ml/mouse. In general, most of the mice gained weight in a relatively normal fashion during the course of the 4 week experiment, with an average initial weight at 18.6 g and final weight at 20 g. However, the increase appeared to differ to some extent for some cages. The weight of mice in the CON cage increased at later time points (day 21 and day 28), while weight of mice had increased significantly by the end of treatment period (day 14) in SakA(+), enterocins(+), GarML(+) cages and by day 7 in GarML(-) cage (Fig. 1).

Determination of LAB and LAB bacteriocin-producers in fecal samples

LAB normally constitute a sizable group in the gastrointestinal tract. We enumerated this group of bacteria in fecal samples to examine how the bacteriocin treatments affect this group, compared to

the bacteriocin-negative (isogenic) cages. Using conditions selective for LAB (MRS plates and anaerobic condition), the counts of total LAB were around 10^8 cfu/g feces for the selected mice from the most of the cages (n= 3 from each cage). However, the number of LAB in fecal samples was significantly higher in bacteriocin positive cages of SakA (2-fold, $P < 0.05$), plantaricins (3-fold, $P < 0.01$) and GarML (2-fold, $P < 0.001$) compared to their bacteriocin negative cages (Fig. S1).

In addition to total LAB enumeration, bacteriocin-producing colonies were counted in the same assay using indicator bacteria that are sensitive to administrated bacteriocins. The portions of bacteriocin producers (tested against the specified indicator strains) among all LAB in bacteriocin positive cages were 25% in SakA(+), 5% in PedPA-1(+), 89% in enterocins(+), 89% in plantaricins(+) and 18% in GarML(+) cages. Interestingly, there were no bacteriocin-producing colonies in samples from the bacteriocin negative cages, except the cage treated with the enterocins isogenic mutant strain (produced enterocin P, 71% bacteriocin producing colonies).

Moreover, sequencing data of the fecal samples showed that the relative abundances of OTUs that were assigned to *Pediococcus* (*Pediococcus acidilactici* particularly), *Lactococcus* (*Lactococcus garvieae* particularly), and unclassified Enterococcaceae, comprising LAB bacteriocin producers, were significantly higher in the cages treated with PedPA-1(+) and (-), GarML(+) and enterocins(+) respectively (Fig. S2). However, *Lactobacillus* had many OTUs assigned; therefore, this effect could not be observed for SakA and plantaricins cages. These results imply that bacteriocin production makes the producers more capable to establish growth in the gut environment.

Community analysis of fecal bacteria

The fecal bacterial community structure was assessed using 16S rRNA gene analysis. Nearly 4.8 million merged and quality filtered sequences were obtained. In total 1,168 OTUs were acquired for all samples after chimera filtering. The community of each sample was subsampled, giving about 15,800 sequences/sample. The inter-individual variations within the cages were converged by the pooling of all feces from each cage at each time point. Rarefaction curves indicated that the diversity of bacterial communities was not significantly affected by any of the treatments (Fig. S3, Table S1).

The distance matrices were calculated by unweighted UniFrac and visualized by PCoA plots to compare the communities of bacteriocin-positive, negative and CON cages. In addition to the pooled samples, we also analyzed fecal samples from three randomly selected mice at the same time points (day 0, day 14 and day 28) to examine whether there were major individual differences compared to the pooled communities. No significant differences were found between the individual communities and the pooled communities (Fig. S4). The OTU compositions of all the treatment samples were similar to CON at control time point day 0 (i.e, before treatments) as expected (Fig. 2). There was a divergence in the community composition over time within the different treatments including CON. However, OTU compositions of samples were not statistically significantly affected by the different treatments (Fig. 2).

Taxa at phylum level

Similar to the OTU composition, the relative abundance of bacteria at phylum level was not strongly affected by bacteriocin producers/non-producers. Overall, the microbiota of mice were dominated by the phyla Bacteroidetes (average of 57% in different treatment groups), Firmicutes (average of 29%) and Verrucomicrobia (average of 10%) (Fig. 3). Phyla with a relative abundance

of less than 2% were Actinobacteria, Proteobacteria, TM7 (candidate division), Tenericutes and a number of unclassified bacteria. Only a few of the low abundant taxa were affected by bacteriocin producers/non-producers. For instance, TM7 (candidate division) decreased by the SakA(+), SakA(-) and GarML(-) strains, and Proteobacteria by the SakA(+) strain. On the other hand, the Actinobacteria population increased by SakA(-) treatment compared to CON.

Modifications at deeper taxonomic levels

When analyzing at deeper taxonomic levels we found a number of changes that were triggered by bacteriocin producers but not by their corresponding non-producers (Fig. 4, Fig. S5 and Fig. S6), thus these changes were likely due to bacteriocin effects. The producers of SakA and GarML increased the Leuconostocaceae population during the treatment period. The producers of PedPA-1 and plantaricins increased and decreased Clostridiaceae population respectively, particularly *Clostridium* genus, with persistent effect both during treatment and post-treatment periods. The Enterococcaceae family population was remarkably increased by the producer of enterocins during the treatment period, but this effect disappeared during the post-treatment period. Unlike enterocins, the producer of GarML inhibited the Enterococcaceae family over the entire course of the experiment. The population of Streptococcaceae (particularly *Lactococcus*) was increased by the GarML producer during the treatment period, on the contrary, this family (particularly *Streptococcus*) became less abundant when treated with the enterocins producer. Moreover, the enterocins producer reduced the Staphylococcaceae family, particularly the *Staphylococcus* genus (Fig. 4).

In some cases, we also observed similar changes caused by both bacteriocin producers and the isogenic mutants. For instance, *P. acidilactici* 347 and the isogenic bacteriocin non-producing mutant reduced Enterococcaceae (during the treatment period) and *Streptococcus* (during the

entire course of experiment). Both strains also significantly increased the *Pediococcus* population but decreased *Lactobacillus* during the treatment period, while the family they are affiliated to, Lactobacillaceae, was not significantly affected. Similarly, *E. faecium* L50 and its isogenic mutant L50-142 reduced *Lactobacillus*. Moreover, *L. plantarum* C11B (bacteriocin producer) during the course of the post-treatment, and its isogenic mutant strain *L. plantarum* C11D3 during the whole experiment, showed a significant adverse effect on the Staphylococcaceae population (Fig. S6).

It is well known that Gram-negative bacteria as well as Gram-positive bacteria that are more distantly related to the bacteriocin producers are generally not sensitive to LAB bacteriocins. We observed that some populations of these bacterial groups were affected, e.g. Firmicutes affiliated-populations: Erysipelotrichaceae, Lachnospiraceae and Ruminococcaceae. However, most of these changes were likely not due to bacteriocin production as both the bacteriocin producers and their isogenic mutants gave similar effects (Table S2). Only the GarML producer, but not the isogenic strain, increased the Ruminococcaceae population in the post-treatment period (Table S2).

Surprisingly, some bacteriocin producers and their isogenic mutants behaved differently toward the Gram-negative bacteria. The Bacteroidaceae population was increased by the SakA producing strain during the treatment period but the isogenic mutant (bacteriocin negative) during the post-treatment period. The Prevotellaceae and Rikenellaceae populations were significantly more abundant in the samples treated with the producer GarML than in those treated with the isogenic one. Nevertheless, there were also cases where the same changes were caused by both the bacteriocin producers and their isogenic mutants. F16 (affiliated to TM7 candidate division) was reduced by both the producer and non-producer strains of plantaricins, and Desulfovibrionaceae,

which is a subgroup of Proteobacteria, was increased in mice fed by both the producers and non-producers of SakA and PedPA-1.

Analysis of blood serum components

Here we focused on the blood serum components at the end of the treatment period, i.e., the levels of triglycerides, total cholesterol, HDL and LDL, which can be used to estimate the risk of some health disorders, such as heart diseases and obesity. A significant decrease in the level of triglycerides, and an increase in the levels of HDL and total cholesterol were triggered by GarML(+), while plantaricins(+) decreased the level of LDL compared to bacteriocin negative treatments (Fig. 5). Correlations between OTUs and the level of the blood serum components were also investigated. Significant positive and negative correlations were found for some OTUs of the families Rikenellaceae, S24-7 (Bacteroidales subgroup), Ruminococcaceae, Lachnospiraceae, Coriobacteriaceae, Dehalobacteriaceae, Unclassified RF39 (Tenericutes subgroup), Bacteroidaceae, Clostridiaceae and Erysipelotrichaceae ($P < 0.05$) (Fig. 6). The most remarkable correlations were that OTUs affiliated to the S24-7 were mostly negatively correlated with serum LDL and triglycerides levels, while OTUs of Erysipelotrichaceae family were positively correlated with the levels of triglycerides. The Bacteroidaceae population were also positively correlated with the level of triglycerides, while one OTU belonging to Rikenellaceae showed a negative correlation. Moreover, the correlation of Ruminococcaceae with the measured serum levels was more OTU specific.

DISCUSSION

Production of bacteriocins by LAB has generally been considered a probiotic trait. However, still only few studies have assessed their probiotic effects in live animals, especially on their impacts on the normal gut microbiota (29, 50, 51). This study therefore aimed to investigate the effects of

LAB producers of the class II bacteriocins SakA, PedPA-1, enterocins (Q and L50), plantaricins (EF and JK) and GarML, on the composition of gut microbiota of healthy mice. These five bacteriocins were selected due to their different physicochemical properties, structures and antimicrobial spectra (Table S3) (52). Another important aspect in the present study was the use of isogenic mutants of the producers to distinguish whether a change observed in the microbiota was due to bacteriocin itself or to other properties of the strain (i.e., non-bacteriocin effects).

In general, our results indicate that the main structure of the gut bacterial composition in mice was relatively resilient to the administration of LAB producers or non-producers. However, at deeper taxonomic levels we could observe some modifications and these changes varied in a manner related to the *in vitro* antimicrobial activities of the bacteriocins, i.e., the narrow-spectrum bacteriocins SakA, PedPA-1 and plantaricins showing less impact on bacterial communities compared to the wider-spectrum bacteriocins enterocins, and GarML.

The populations of Gram-positive bacteria, particularly LAB such as lactobacilli, lactococci, enterococci, streptococci, leuconostoc, and pediococci as well as some other niche competing bacteria like staphylococci, listeria and clostridia are often the targets of LAB-produced bacteriocins (53). These populations were altered mostly when mice were treated with bacteriocin producers. Some of these changes are definitely favorable to the host, such as the reduction in population size of streptococci, staphylococci, and clostridia because these microoganisms are often associated with diverse infections as well as being regarded as opportunists. Some populations of Gram-positive bacteria are, however, also affected negatively by the isogenic non-producers, (e.g. *Staphylococcus* by plantaricins(-), *Streptococcus* by PedPA-1(-)). These changes are likely due to other unknown non-bacteriocin activities.

In addition to modification of specific taxa, some bacteriocin producers (SakA, plantaricins and GarML) significantly increased the counts of total LAB. This is interesting since LAB can have probiotic value via the production of various molecules (e.g. short chain fatty acids, conjugated linoleic acids, exopolysaccharides, fructooligosaccharides and selenoproteins) and favor the production of butyrate and propionate of other mutualistic bacteria (54). LAB are also involved in regulating host metabolism and immune system, controlling infections and modulating inflammation (55).

In spite of several direct correlations, some of the *in vitro* antimicrobial activities of bacteriocins could not be observed in the gut communities in mice. For example, the class IIa bacteriocins, SakA and PedPA-1, are effective against *Enterococcus* strains and GarML against *Lactobacillus* strains *in vitro*; however, these bacteriocins did not inhibit the growth of these populations *in vivo*. One possible explanation is that bacteriocin production could be exposed to innate gene regulation. It is well known that many bacteriocin producers involve the classical two-component regulatory networks to respond to cues from the environments (56, 57). This is highly relevant for bacteriocin production in the gut environment where chemical cues are expected to be greatly complex. Among our selected bacteriocins, the biosynthesis of sakA and plantaricins are in fact regulated by such regulatory networks (58, 59). Similarly, production of enterocins by *E. faecium* L50, is regulated in a temperature-dependent manner (60). Moreover, we cannot rule out the possibility that certain bacteriocins or their producers were weakened by other factors, e.g. the native LAB in gut (55, 61–63).

The fecal populations of *Enterococcus* and *Lactococcus* increased in cages treated with the enterococcal enterocins and the lactococcal GarML, respectively. It is likely that a significant proportion of the reads corresponding to these bacterial groups would correspond to the bacteriocin

producers themselves and that their colonization in gut was facilitated by bacteriocin production. This later notion is supported by the fact that the corresponding isogenic mutants did not result in an increase of these populations. However, the *Pediococcus* population was enhanced by both the PedPA-1 producer and its isogenic mutant; therefore, this enhancement could not be linked only to the bacteriocin production. Moreover, there was no obvious increase in the *Lactobacillus* population in cages treated with SakA or plantaricins (bacteriocins produced by lactobacilli). The quantitative detection of changes of this population was challenging due to the large number of affiliated OTUs. However, it is likely that the growth of native *Lactobacillus* strains was triggered or they were replaced by the administrated bacteriocin-producing *Lactobacillus* strains as suggested before (28).

Bacteriocins may also help the producer to invade new niche by competitive exclusion of other inhabitants, which usually are closely related bacteria, such as LAB in this study. This competition can lead to modifications of other bacterial populations connected in the microbial network (28). This might explain the increase of Prevotellaceae by plantaricins and GarML and the effect of GarML on Ruminococcaceae and other Bacteroidetes affiliated bacteria (e.g., Rikenellaceae). Members of these Gram-negative families are known to produce short chain fatty acids beneficial for host (64–66). Moreover, we also observed that Bacteroidetes phylotypes S24-7, Bacteroidaceae, Rikenellaceae, Ruminococcaceae, Erysipelotrichaceae, Coriobacteriaceae, Lachnospiraceae and *Clostridium* were correlated to serum parameters tested usually in an OTU-specific manner.

Weight gain is one of the parameters to measure host health. The treated mice gained weight in a manner comparable to or better than the CON cage, indicating that the bacteriocin/bacteria treatments did not have a negative impact on the normal growth of mice. The producers of SakA

and enterocins significantly increased the body weight of mice more than their corresponding isogenic mutants. On the other hand, both GarML producer and its isogenic non-producer have a positive impact on weight, implying that the effect was due to the strain *L. garvieae* DCC43, independently of the bacteriocin phenotype. Variation in weight gain in response to administration of bacteriocins or certain strains has been observed before (51, 67). The blood serum levels were significantly changed by the producers of GarML and plantaricins in a potentially positive manner for the host. Triglycerides and LDL, which are physiological indicators of the risk of some health disorders such as heart diseases and obesity, decreased and HDL known as good cholesterol increased. However, it is of future interest to correlate the effects of bacteriocins on host physiology to gut microbiota modifications.

Gut environment is a complex niche where numerous and diverse bacteria are thriving and competing fiercely for common resources. The successful survivors must therefore have developed strategies to coexist with other gut inhabitants and with the host in an interactive network. This network is presumably quite resilient as well as dynamic in order to deal with many different chemical challenges during the daily traffic along the intestinal tract (e.g., ingested food or medicines) and to maintain the diverse functions the gut play (processing the ingested food, producing nutrients, vitamins, immune-stimulation, gut emptying, etc). Such a resilient and dynamic nature of the healthy gut can be seen in our present study. Firstly, the overall structure of microbiota remained largely unaffected by the administration of bacteriocin producers and non-producers of different genera. Secondly, many changes which were observed at deeper taxonomic levels in treated mice, disappeared or were disappearing at the end of the 4-week course of experiment. In terms of probiotic use, these properties are highly appreciated because probiotics are meant to transiently affect the gut microbiota to promote health-bringing conditions for host

(inhibition of potential pathogens, enhanced growth of beneficial bacteria, increase of beneficial blood serum parameters, etc.) without disturbing the gut's main microbial structure and function. Nevertheless, although probiotics have been much studied worldwide in recent years, and that there are numerous studies showing different probiotic effects from LAB and other bacteria, still this research field is, at best, in its infancy, especially with regard to the limited understanding at the molecular and cellular levels. Further, it is important to underline that the use of probiotics to achieve favorable values in animals is a rather complex and unpredictable process as many unknown hurdles along the GIT can deteriorate or repress the probiotic properties. Some probiotic properties can even vary dependently on a number of host parameters, including animals tested, genetic background, age and gender (68). Thus, to fully and safely appreciate their health bringing values, probiotics must be critically and carefully assessed in the relevant models and settings.

SUPPORTING DATA

The fastq files have been deposited in the SRA (Bioproject ID: PRJNA310414 and Accession number: SRP069889).

ABBREVIATIONS

LAB: lactic acid bacteria HDL: high-density lipoprotein, LDL: low-density lipoprotein, CON: control with no treatment, SakA: sakacin A, PedPA-1: pediocin PA-1, enterocins: enterocins Q and L50, plantaricins: plantaricins EF and JK, GarML: garvicin ML, maxee: maximum expected error, OTU: operational taxonomic unit, RDP: Ribosomal Database Project, PCoA: Principle coordinate analysis.

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TABLE 1 Administrated bacterial strains and bacteriocins

Bacterial strain	Bacteriocins in treatments	Class of bacteriocins	Stock Culture (cfu/mL)	References/Source
Lactobacillus sake Lb 706	Sakacin A (+)	Class IIa	$0.16x10^{11}$	LMG Collection
Lactobacillus sake Lb 706B	Sakacin A (-)		$0.2x10^{11}$	
Pediococcus acidilactici 347	Pediocin PA-1 (+)	Class IIa	$2.6x10^{11}$	Martínez et al., 1998
Pediococcus acidilactici 347	Pediocin PA-1 (-)		$2.5x10^{11}$	
Enterococcus faecium L50	Enterocins P, Q and L50 (+)	Class IIb (EntL50) Class IId (EntQ)	$0.6x10^{11}$	Cintas et al., 1998
Enterococcus faecium L50-142	Enterocin P (+) but Q and L50 (-)		$0.5x10^{11}$	
Lactobacillus plantarum C11B	Plantaricins EF and JK (+)	Class IIb	$2.6x10^{11}$	LMG Collection
Lactobacillus plantarum C11D3	Plantaricins EF and JK (-)		$2.3x10^{11}$	
Lactococcus garvieae DCC43	Garvicin ML (+)	Class IIc	$1.7x10^{11}$	LMG Collection
Lactococcus garvieae DCC43	Garvicin ML (-)		$1.7x10^{11}$	

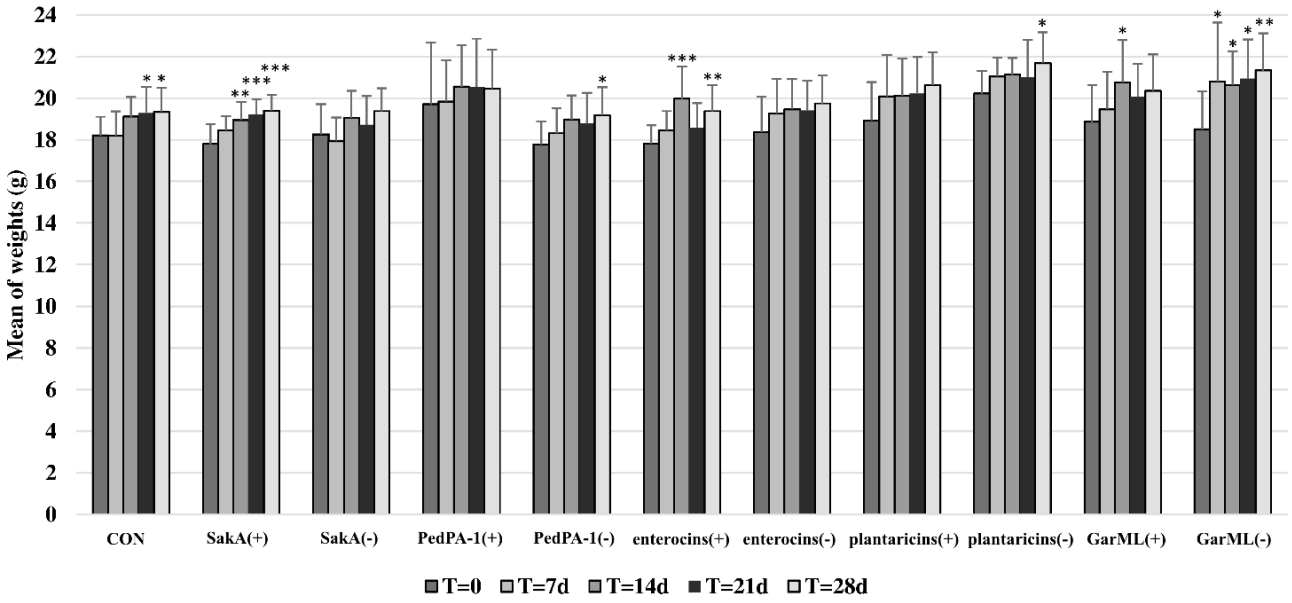

FIG 1 Average weights of mice in the same treatment cage over time. '(+)' represents bacteriocin producer cage while '(-)' represents bacteriocin non-producer cage. ANOVA was performed for the comparison of weights of mice at each time point to day 0 within each treatment. Significance degree is represented with stars; $p<0.05$ with one star (*); $p<0.01$ with two stars (**); $p<0.001$ with three stars (***).

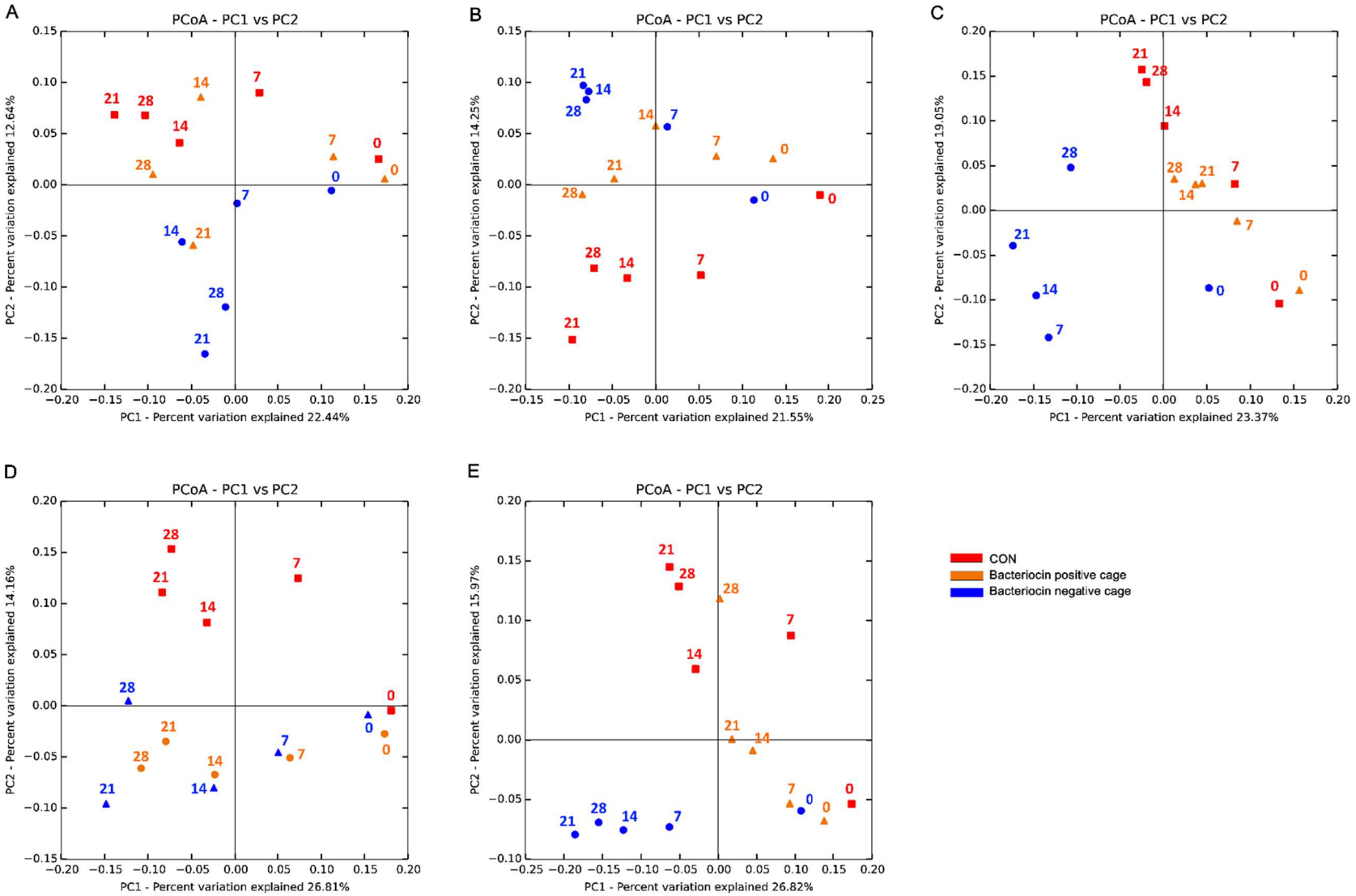

FIG 2 Comparison of bacteria composition of treatments. Principle coordinate analysis (PCoA) plot generated based on the calculated distances in an unweighted UniFrac matrix. The plots labelled with different letters represent different bacteriocin treatments: Sakacin A (A), Pediocin PA-1 (B), Enterocin Q and Enterocin L50 (C) Plantaricins EF and JK (D), and Garvicin ML (E). Samples were grouped by color in terms of treatment group they belong to; CON (red), bacteriocin positive samples (orange) and bacteriocin negative samples (blue). Labels indicate time with day numbers: 0 (day 0), 7 (day 7), 14 (day 14), 21 (day 21) and 28 (day 28).

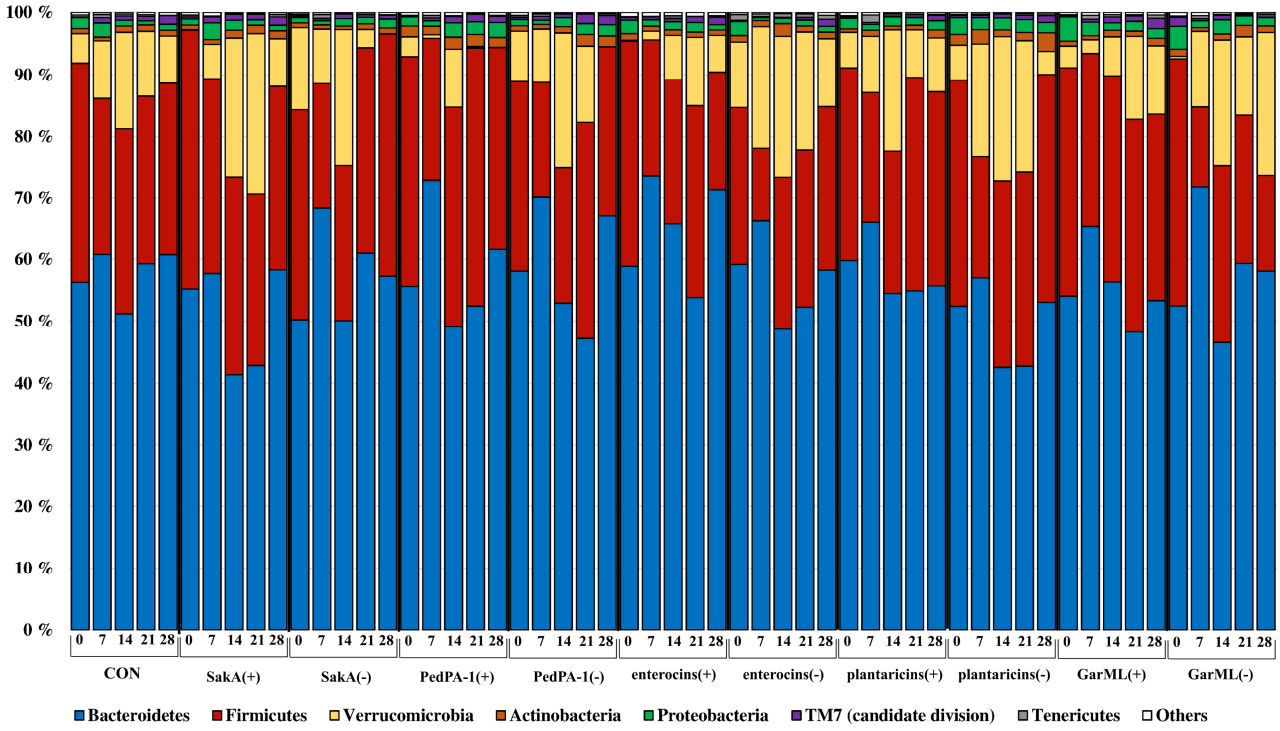

FIG 3 Relative abundances of bacterial phyla in every sample. Different colored bars represent different phyla with size showing relative abundance of this phylum. Labels contain name of treatments and time with day numbers: 0 (day 0), 7 (day 7), 14 (day 14), 21 (day 21) and 28 (day 28).

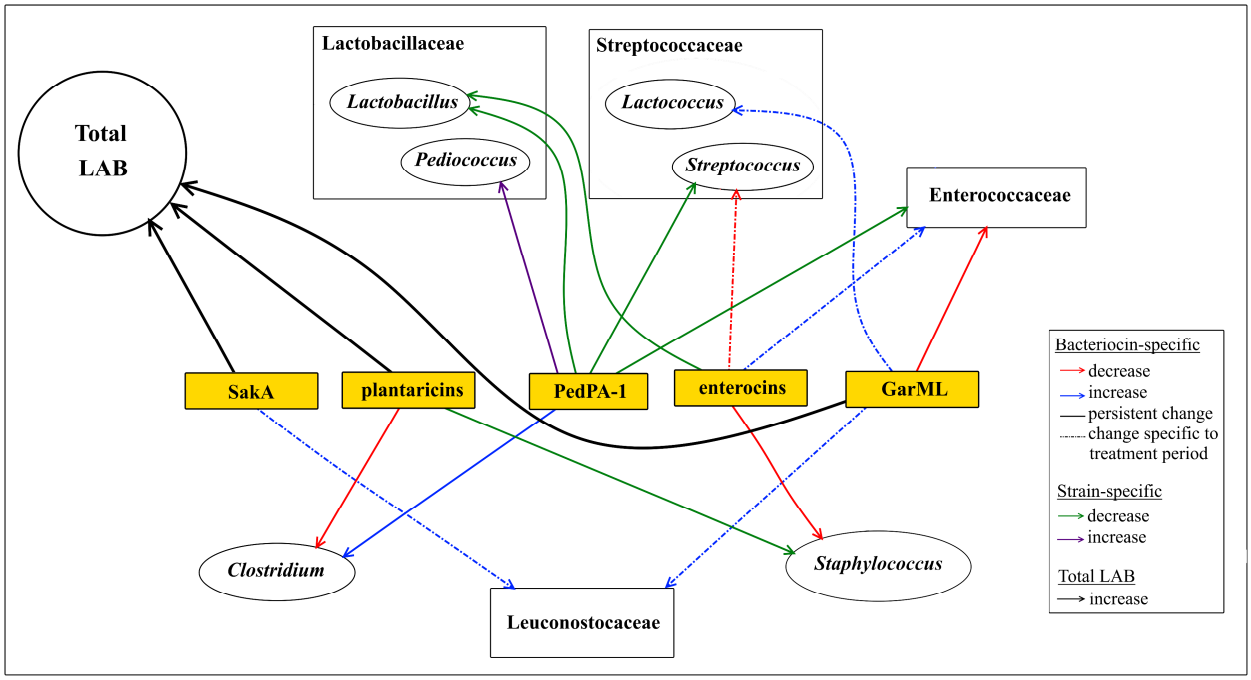

FIG 4 Significant modifications of the relative abundance of LAB and other target bacterial groups of bacteriocins in response to treated bacteriocins or strains. Bacteriocin-specific mofidications, which were observed in the cages of bacteriocin producing strains only, and strain-specific modifications, which were exhibited by both bacteriocin producing and non-producing strains, are represented. Bacteriocin-specific modifications which were recovered in post-treatment period are shown as specific to the treatment period, while the modifications persistent throughout the experimental period are shown as persistent changes. Total LAB count enhancing bacteriocins, based on a colony counting study, are also shown, using black arrows. Bacteriocins are indicated with the following abbreviations: SakA: Sakacin A, plantaricins: Plantaricins EF and JK, PedPA-1: Pediocin PA-1, enterocins: Enterocin Q and Enterocin L50 and GarML: Garvicin ML.

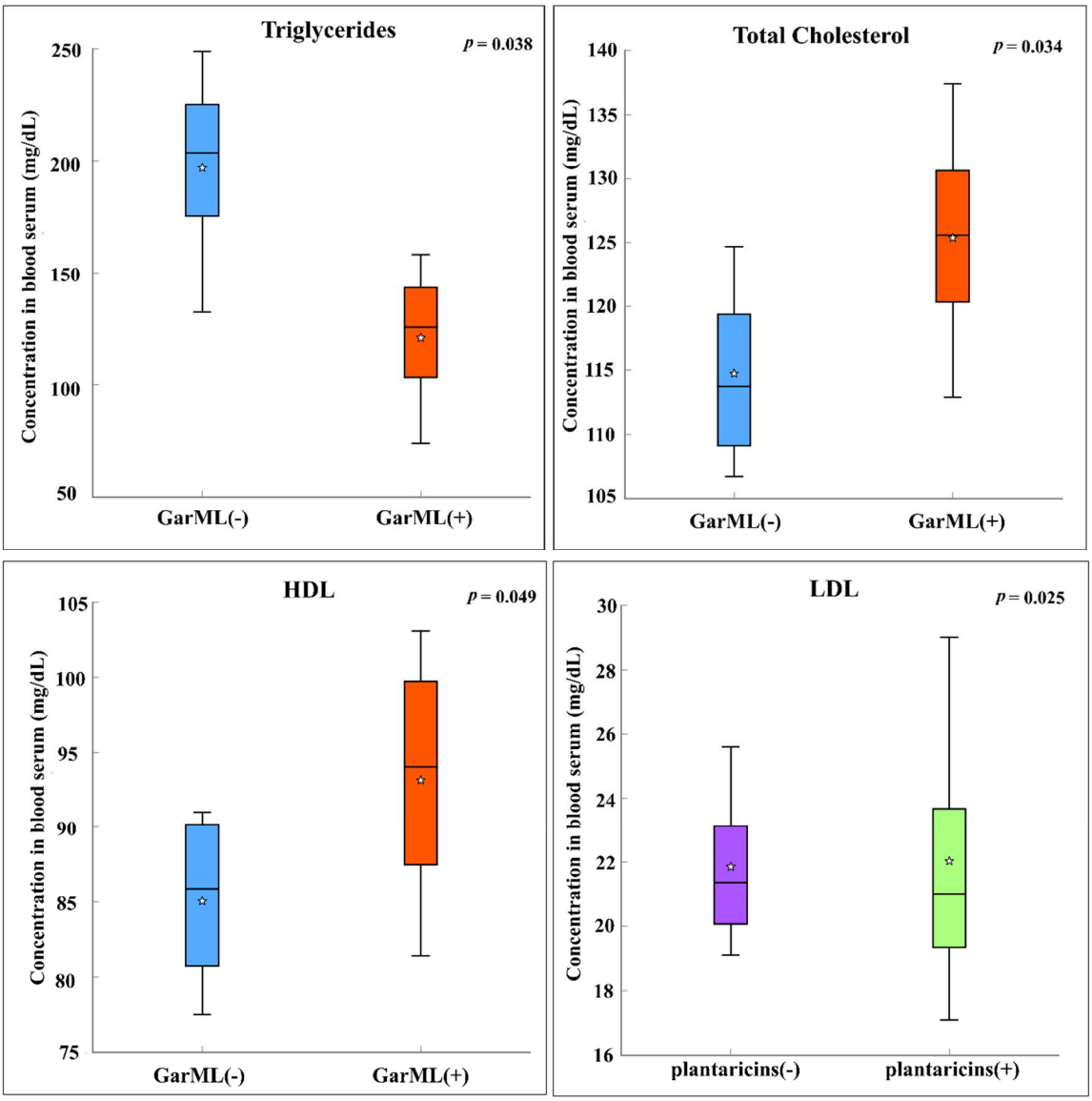

FIG 5 Significant serum level modifications by bacteriocin. The pairwise comparisons between bacteriocin positive and negative treatments were performed using Student's t-test. The boxplots include comparisons with $P < 0.05$ and p-values were indicated in each plot. plantaricins: Plantaricins EF and JK, GarML: Garvicin ML, (+): bacteriocin producing strain, (-): bacteriocin non-producing isogenic strain.

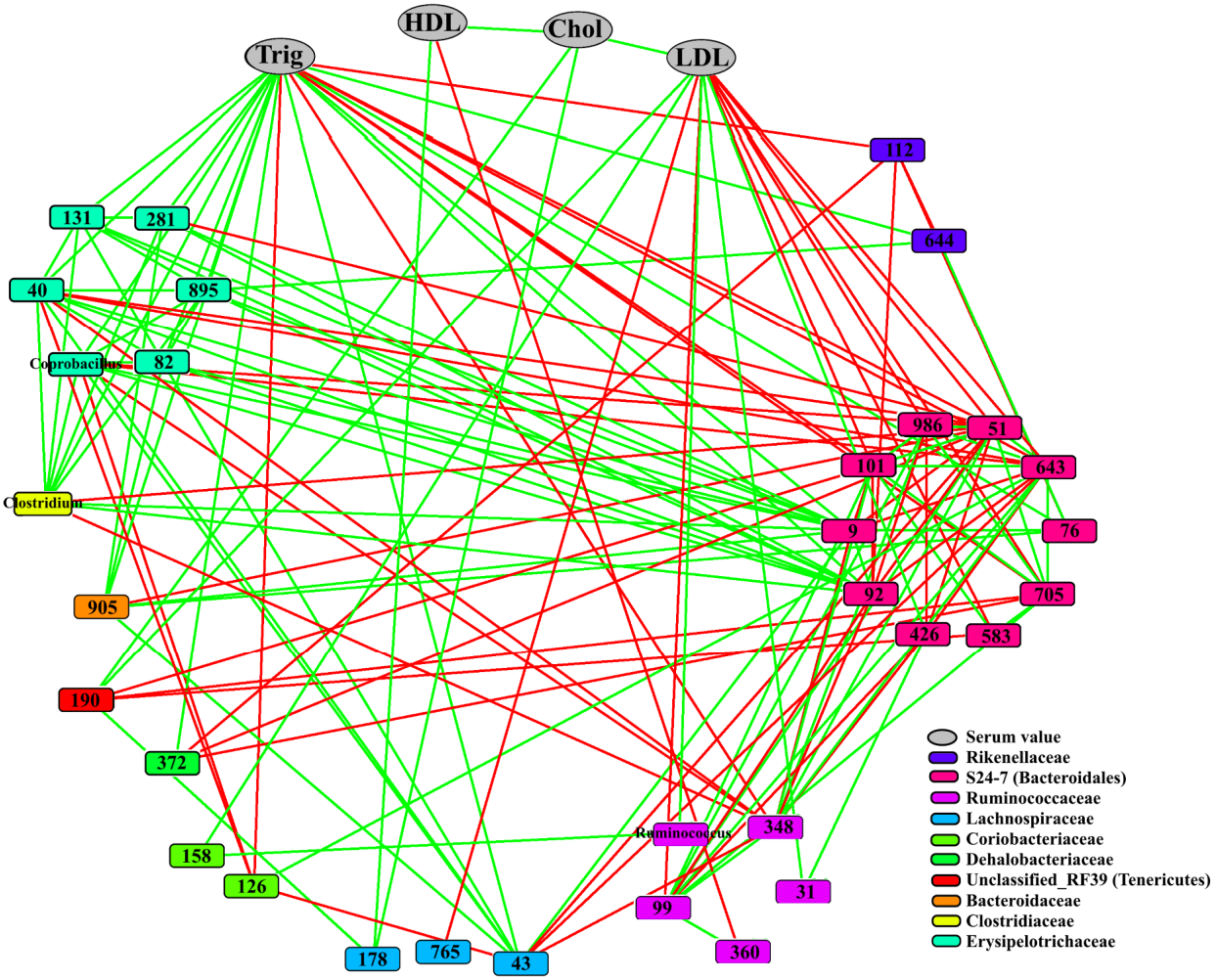

FIG 6 Correlation network of relative abundances of OTUs at day 14 and serum levels. The correlations were calculated using Pearson's correlation in CoNet and the significant ones ($P < 0.05$) were shown on the network. All serum values are shown by one color (grey) while OTUs belonging to different families are represented by different colors (see legend). Positive correlations are displayed with green edges and negative correlations with red edges. OTUs on the nodes were represented with OTU numbers or genus names they belong to and serum values were labelled as Trig: triglycerides, HDL: high-density lipoprotein, Chol: total cholesterol and LDL: low-density lipoprotein.

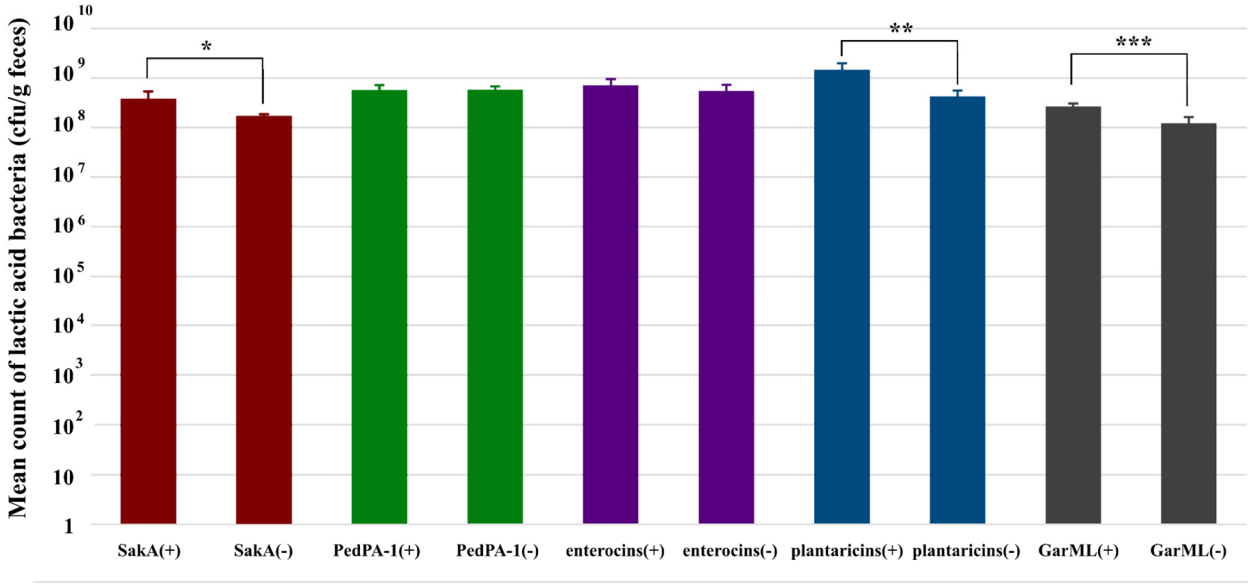

FIG S1 Number of total lactic acid bacteria in treatment samples. Counting of lactic acid bacteria was performed for day 14 samples for fecal samples of three randomly selected mice from each treatment cage. Labels contain name of treatments, significance degree is represented as followings: $P < 0.05$ with one star (*); $P < 0.01$ with two stars (**); $P < 0.001$ with three stars (***).

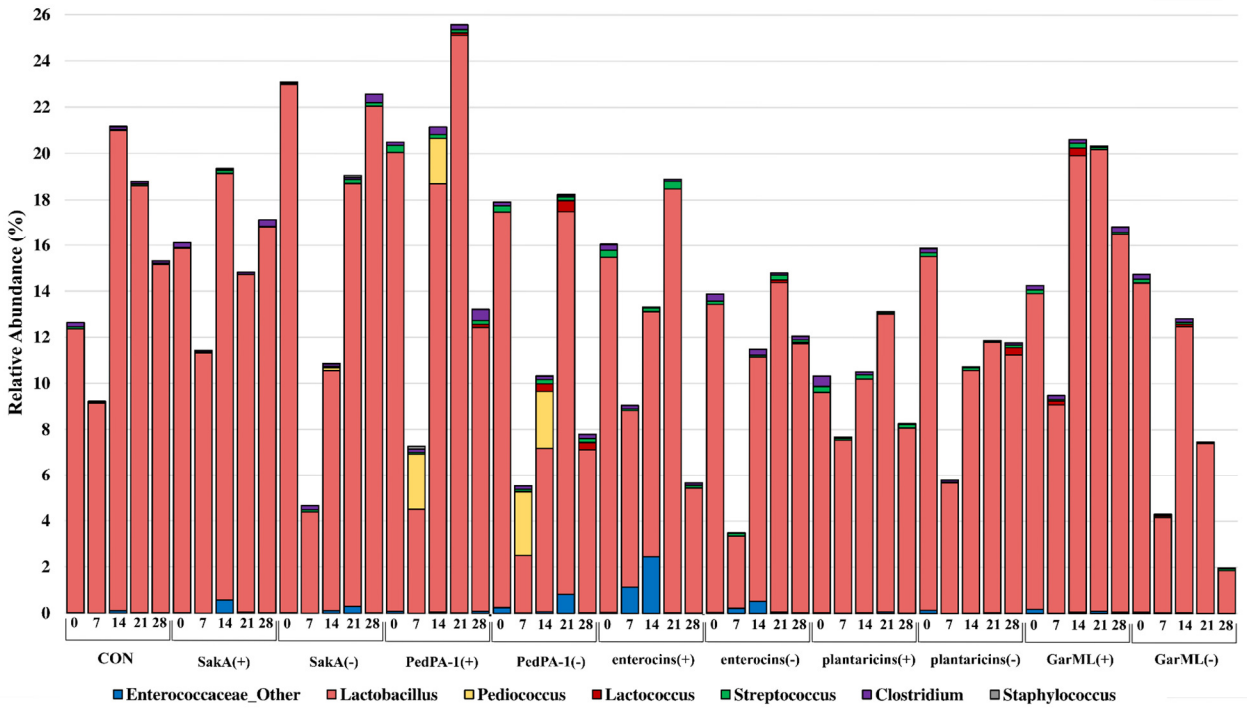

FIG S2 Relative abundances of OTUs at genus level among other OTUs. Different colored bars represent different genera with size showing relative abundance of this genus. Labels contain name of treatments and time with day numbers: 0 (day 0), 7 (day 7), 14 (day 14), 21 (day 21) and 28 (day 28).

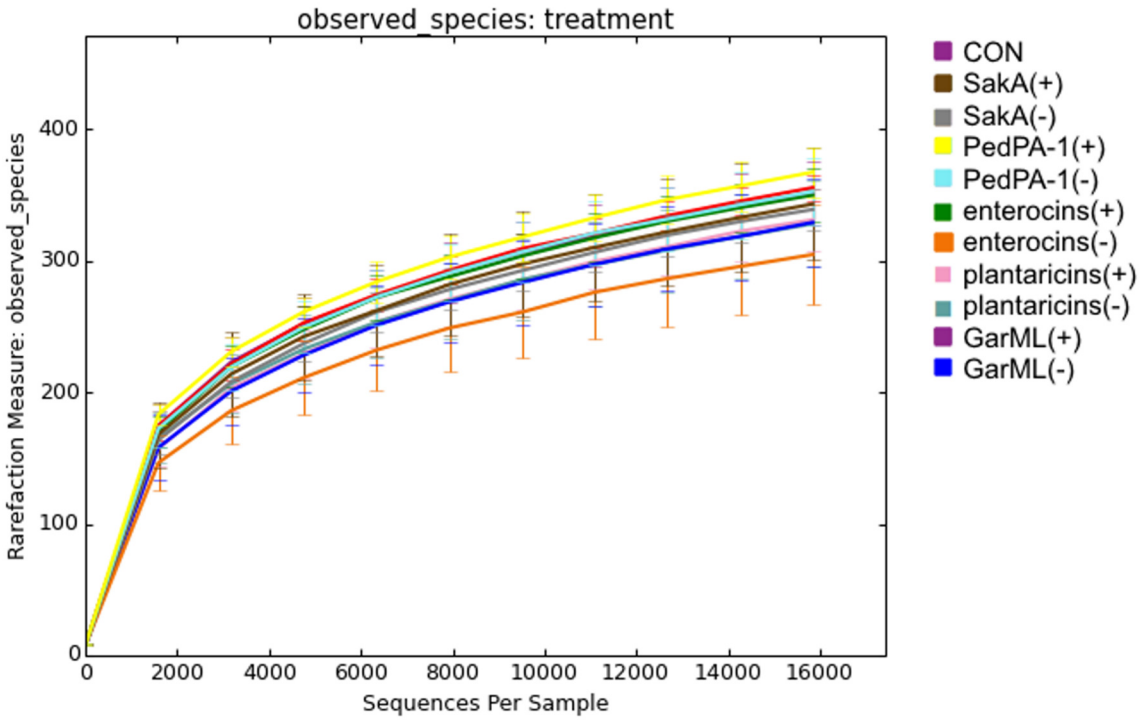

FIG S3 Rarefaction curves calculated for each treatment cage. Curves were calculated for observed species with standard deviation.

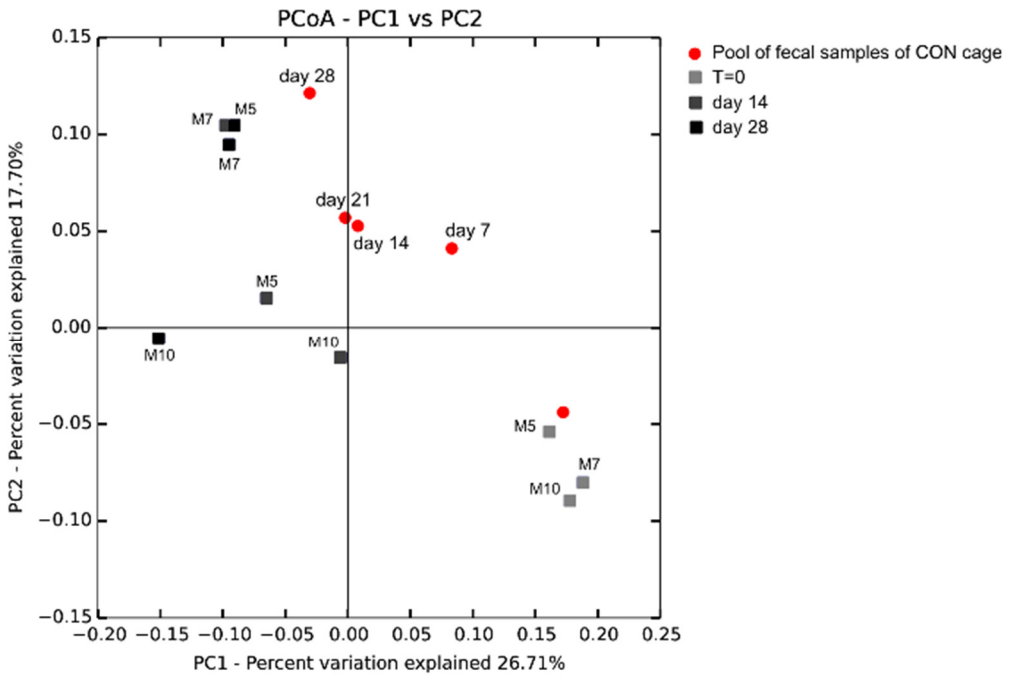

FIG S4 Comparison of bacteria composition of pooled fecal samples and fecal samples from individual mice in CON cage. Principle coordinate analysis (PCoA) plot generated based on the calculated distances in an unweighted UniFrac matrix. Samples were grouped by color and shape such that pool of fecal samples at indicated time point (red circle), day 0 individual mice samples (light grey square), day 14 individual mice samples (grey square) and day 28 individual mice samples (dark grey square). Individual mice were indicated with numbers: M5, M7 and M10.

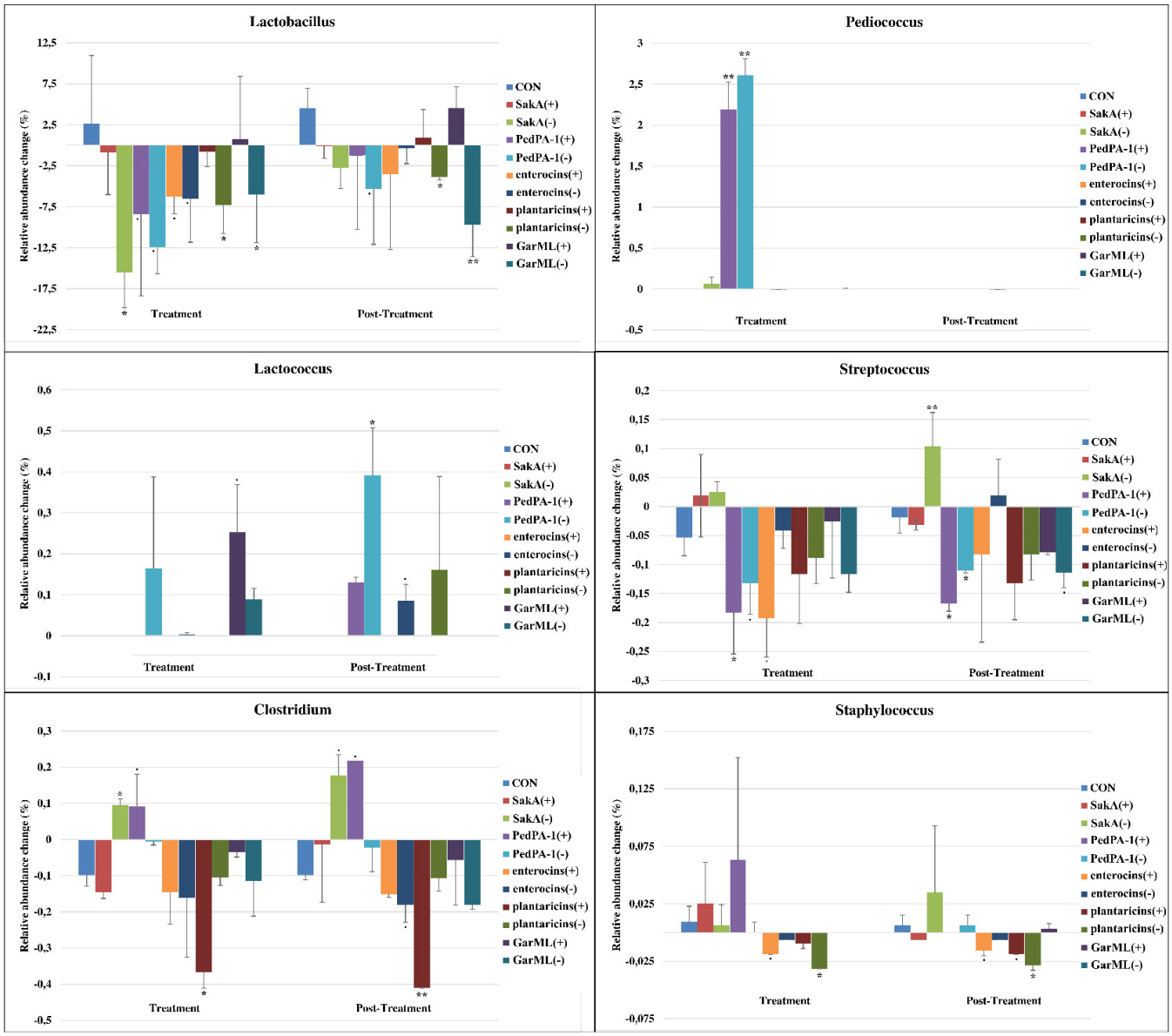

FIG S5 Relative abundance change of LAB and other target bacterial groups of bacteriocins at genus level in treatment and post-treatment periods. Change in relative abundances of genera corresponding to day 0 of treatments were compared to CON using ANOVA. Significance degree is represented as followings: $P < 0.1$ with dot (.); $P < 0.05$ with one star (*); $P < 0.01$ with two stars (**).

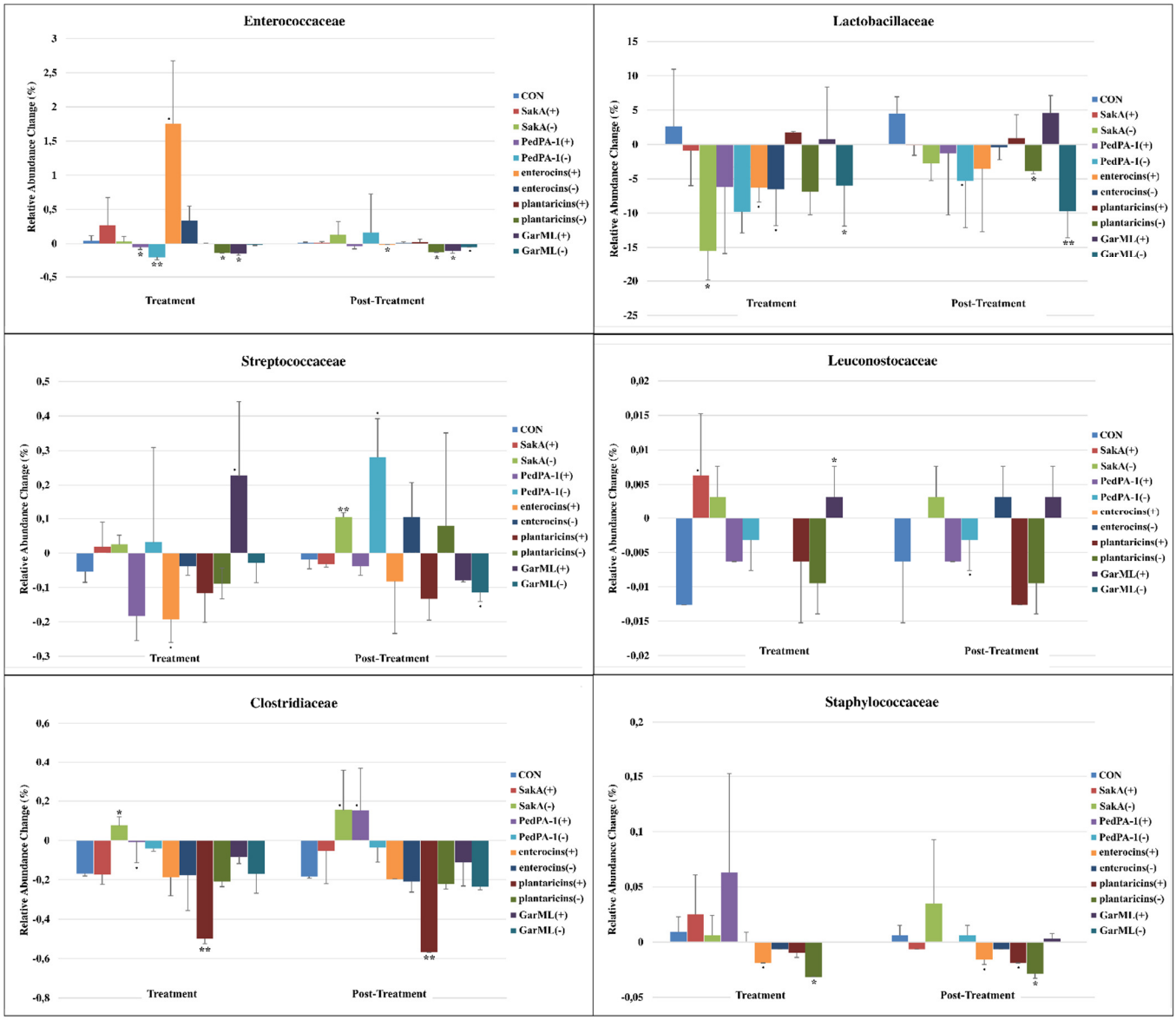

FIG S6 Relative abundance change of LAB and other target bacterial groups of bacteriocins at family level in treatment and post-treatment periods. Change in relative abundances of families corresponding to time 0 of treatments were compared to CON using ANOVA. Significance degree is represented as followings: $P < 0.1$ with dot (.); $P < 0.05$ with one star (*); $P < 0.01$ with two stars (**).

TABLE S1 Shannon indexes calculated as mean of ten iterations at equal subsampling size of 15,840

Treatment	Time (day)	Shannon index
CON	0	6.26
CON	7	6.16
CON	14	5.43
CON	21	5.47
CON	28	5.90
enterocins(+)	0	6.18
enterocins(+)	7	6.08
enterocins(+)	14	5.80
enterocins(+)	21	5.71
enterocins(+)	28	6.09
enterocins(-)	0	5.76
enterocins(-)	7	4.81
enterocins(-)	14	5.11
enterocins(-)	21	5.22
enterocins(-)	28	5.90
GarML(+)	0	6.24
GarML(+)	7	6.30
GarML(+)	14	5.71
GarML(+)	21	5.72
GarML(+)	28	5.80
GarML(-)	0	6.42
GarML(-)	7	5.59
GarML(-)	14	5.39
GarML(-)	21	5.26
GarML(-)	28	5.16
PedPA-1(+)	0	6.10
PedPA-1(+)	7	6.14
PedPA-1(+)	14	6.02
PedPA-1(+)	21	5.84
PedPA-1(+)	28	6.25
PedPA-1(-)	0	5.80
PedPA-1(-)	7	5.83
PedPA-1(-)	14	5.22
PedPA-1(-)	21	6.00
PedPA-1(-)	28	6.40
plantaricins(+)	0	6.29
plantaricins(+)	7	5.70
plantaricins(+)	14	5.40
plantaricins(+)	21	5.41
plantaricins(+)	28	5.70
plantaricins(-)	0	6.20
plantaricins(-)	7	5.76
plantaricins(-)	14	5.26
plantaricins(-)	21	5.14
plantaricins(-)	28	6.08
SakA(+)	0	6.25
SakA(+)	7	6.35
SakA(+)	14	5.04
SakA(+)	21	5.09
SakA(+)	28	5.95
SakA(-)	0	5.39
SakA(-)	7	5.72
SakA(-)	14	5.26
SakA(-)	21	5.82
SakA(-)	28	6.11

TABLE S2 Comparison of relative abundance change of families by administrated strains to CON in treatment (T) and post-treatment (Post-T) periods

	SakA(+)		SakA(-)		PedPA-1(+)		PedPA-1(-)		enterocins(+)		enterocins(-)		plantaricins(+)		plantaricins(-)		GarML(+)		GarML(-)	
FAMILY	**T**	**Post-T**	**T**	**Post-T**	**T**	**Post-T**	**T**	**Post-T**	**T**	**Post-T**	**T**	**Post-T**	**T**	**Post-T**	**T**	**Post-T**	**T**	**Post-T**	**T**	**Post-T**
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.Other						(-) .		(-) .						(-) .						(-) .
k__Bacteria.p__Bacteroidetes.Other.Other.Other								(-) .												
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__						(-) *								(-) *			(+) .			
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae	(+) .			(+)*		(+) .	(-) *	(-) *					(-) .	(-) **		(-) **			(+) .	(+) *
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae			(+)**																	
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae													(+) *			(-) .	(+) *			
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae				(+) .			(-) **	(-) *									(+) .	(+) **		
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7	(-) *	(-).	(-) .	(-) .		(-) .						(-) .		(-) .	(-) *	(-) .		(-) *		(-) .
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae]	(+) .	(+) .	(+) .	(+) **	(+) *	(+) .	(+) *	(+) *	(+)*		(+) **	(+) **	(+) .		(+) *	(+) .	(+) *	(+) *	(+) .	
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae]						(+) *			(+) .				(+) *		(+) .					
k__Bacteria;p__Firmicutes;Other;Other;Other	(-) .	(-) **	(-) .	(-) *						(-) *			(-) .	(-) *		(-) *		(-) *		(-) *
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae									(-) .	(-) .				(-) .	(-) *	(-) *				
k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__Gemellaceae			(+) .	(+) *			(+) *	(+) *	(-) .	(-) *										
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae					(-) *		(-) **		(+) .	(-) *					(-) *	(-) *	(-) *	(-) *		(-) .
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae			(-) *					(-) .	(-) .		(-) .					(-) *			(-) *	(-) **
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae	(+) .							(+) .									(+) *		(+) *	
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae				(+) **				(+) .	(-) .								(+) .			(-) .
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other	(-) .	(-) *		(+) *		(+) .	(+) .	(+) *				(+) *		(+) *		(+) **				
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__				(+) *	(+) .	(+)*		(+) **				(+) .		(+) *		(+) *				
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae														(-) *		(-) *				
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae			(+) *	(+) .	(+) .	(+) .							(-) **	(-) **						
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae			(+) .		(+) *	(+) *														
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae																			(-) .	(-).
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae		(-) .		(+) *	(+) *	(+) **	(+) .	(+) **			(+) .	(+) *								
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae	(-) .						(-) .													
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae		(+) *	(+) *	(+) **		(+) *	(+) .	(+) **		(+) .		(+) *				(+) *		(+) .		
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae]	(-) *							(+) .							(+) .					
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae						(+) .		(+) .			(+) .			(+) *		(+) *				
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae	(+) .		(-) **	(-) *																
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae					(-) *			(+) .												
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae						(+)*														(-) .
k__Bacteria;p__Cyanobacteria;c__4C0d-2;o__YS2;f__				(-) .									(-) *			(+) *		(+) *		
k__Bacteria;p__Cyanobacteria.c__Chloroplast.o__Streptophyta.f__				(+) .															(+) .	
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae		(-) *		(-) .							(-) **	(-) **					(-) *		(+) .	
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae	(+) .	(+) *		(+) **		(+) .		(+) .							(-) .	(-) *			(-) *	(-) *
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae		(-) .				(+) *				(-) .		(-) .								
k__Bacteria;p__TM7;c__TM7-3;o__CW040;f__F16				(-) .					(-) *		(-) *		(-) .	(-) *		(-) *			(-) *	(-) **
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae											(-) .				(+) .		(-) *		(+) *	

TABLE S3 In vitro antimicrobial activity assay of bacteriocins against the indicator strains listed

Indicator Bacteria	LMG collection #	SakA(+)	SakA(-)	PedPA-1(+)	PedPA-1(-)	enterocins(+)	enterocins(-)	plantaricins(+)	plantaricins(-)	GarML(+)	GarML(-)
***Enterococcus avium* 208**	LMG 3465					+				+++	
***Enterococcus faecalis* UI 50**	LMG 2333	+++		+++		+	+				
***Enterococcus faecium* L50**	LMG 2763	+		+							
***Enteroccous faecium* P21**	LMG 2783	+++		+++		++	++				
***Enterococcus* AL41**	LMG 2876			+++							
***Lactobacillus curvatus* 89**	LMG 2355					+					
***Lactobacillus curvatus* CTC 435**	LMG 2371	++		+		+	+			+	
***Lactobacillus sakei* 706**	LMG 2334			+		++				+	
***Lactobacillus sake* LS3**	LMG 2356					(+)					
***Lactocbacillus sakei* 148**	LMG 2361					++				++	
Lactobacillus sakei	LMG 2380	+		+++							
***Lactobacillus sakei* 791**	LMG 2799	+		+		+	+	+		+	
***Lactobacillus salivarius* UCC118**	LMG 2787	+		+++		++				+	
***Lactobacillus curvatus* NCFB 2739 B**	LMG 2715			+++		+		+		+	
***Lactobacillus* ssp 965**	LMG 2003					+		+		+	+
***Lactobacillus plantarum* R**	LMG 2352			+		+				++	
***Lactobacillus plantarum* C11**	LMG 2358			+						+	
***Pediococcus pentosaceus* FBB 63 B**	LMG 2722	+		+++		+	+	+		+	
***Pediococcus acidilactici* Pac1.0**	LMG 2002					+++	(+)	+		+++	
***Pediococcus pentosaceus* FBB61.1**	LMG 2001			+++		+++	+	+		++	
***Pediococcus pentosaceus* PPE 1-2 M**	LMG 2366			+			+			++	
***Lactococcus* F4-13**	LMG 2070			(+)						(+)	
***Lactococcus lactis* IL1403**	LMG 2705					+				+++	
***Lactococcus garvieae* DCC43**	LMG 3390					+					
***Lactococcus* F14**	LMG 2081					+				+++	
***Lactococcus lactis* 1403**	LMG 2130					+				+++	
***Lactococcus lactis* QU5**	LMG 3419					+				+++	
Listeria innocua	LMG 2710	+++		+++		+++	+++				
Listeria innocua	LMG 2785	+		+++		+					
***Listeria ivanovii* Li4**	LMG 2813	++		+++							
***Listeria monocytogenes* 279 serotype 4**	LMG 2650	+++		+++		+++	+++				
Listeria monocytogenes	LMG 2652	+++		+++		+++	+++				
***Listeria monocytogenes* 223 serotype 1**	LMG 2653	+++		+++		++	++				
Staphylococcus aureus	LMG 3242					+				+	+