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Modulation of the murine microbiome with a concomitant anti-obesity effect by *Lactobacillus rhamnosus* GG and *Lactobacillus sakei* NR28

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Abstract

The microbiota of the gastrointestinal tract (GIT) constitutes the major part of the total human microbiome and is considered to be an important regulator of human health and host metabolism. Numerous investigations in recent years have focused on the connection between the human microbiota and metabolic diseases such as obesity, type II diabetes and atherosclerosis. Yet, little is known about the impact of probiotic consumption on the GIT microbial population and the potential effect on chronic diseases. In this study, the modulation of the microbial community in the murine small intestine resulting from probiotic feeding was investigated and was found to be associated with an anti-obesity effect. Changes in the microbiota of the mouse faeces and small intestine were monitored using quantitative real-time PCR and by following the mRNA expression levels of various obesity-related biomarkers following probiotic feeding in a mouse model. *Lactobacillus rhamnosus* GG and *Lactobacillus sakei* NR28 (a putative probiotic strain isolated from kimchi) were administered at a daily level of approximately 1×10^8 viable bacteria per mouse (C57BL/6J mice) for up to three weeks. Feeding these strains resulted in a significant reduction of epididymal fat mass, as well as obesity-related biomarkers like acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase-1 in the liver. The total number and ratio of the microbial groups, i.e. *Firmicutes, Bacteroidetes, Clostridium* cluster I and XIVab, and *Lactobacillus* spp. were modulated in the small intestine, and the *Firmicutes:Bacteroidetes* ratio was decreased. In contrast, no noticeable effect of probiotic feeding could be detected on the faecal microbiota, neither quantitatively, nor with regard to the bacterial groups (*Firmicutes, Bacteroidetes, Clostridium* cluster I and XIVab, and *Lactobacillus* spp.) studied.

Keywords: LGG, *Lactobacillus sakei,* probiotic feeding, murine small intestine, anti-obesity effect

1. Introduction

Obesity disorders are a growing epidemic around the world, especially in humans from developed countries who enjoy a so-called 'Western' lifestyle. The simplest explanation of obesity can be excessive fat accumulation by more calorie input than expenditure, thereby causing a health risk with various consequences that include cardiovascular disease, type II diabetes mellitus and colon cancer (Armougom *et al.*, 2009; DiBaise *et al.*, 2008; Larsen *et al.*, 2010; Raoult *et al.*, 2008). Recent reports suggest an important role of the gut microbiota in obesity and other chronic diseases as a result of abnormal energy equilibrium (DiBaise *et al.*, 2008; Gionchetti *et al.*, 2000). The gut microbiota has been shown to impact energy efficiency of the host, like energy intake, transport, conversion, and storage, all of which can contribute to development of diet-induced obesity in mouse models as well as (obese) humans (Ley *et al.*, 2005, 2006; Samuel and Gordon, 2006; Turnbaugh *et al.*, 2006, 2008).

Colonisation of germ-free mice with gut microbiota harvested from conventionally housed mice caused rapid increase in fat mass in spite of decreased food consumption (Bäckhed *et al.*, 2004). Not only the mouse model but also mechanisms related to human obesity indicate an altered number of microbiota of the 2 dominant phyla, the *Bacteroidetes* and the *Firmicutes*, in the gastrointestinal tract (GIT) (Ley *et al.*, 2006; Turnbaugh *et al.*, 2006). According to DiBaise *et al.* (2009) the major *Firmicutes* in human faeces comprised *Ruminococcus, Clostridium, Peptostreptococcus, Lactobacillus* and *Enterococcus*, while *Bacteroidetes* was mainly represented by the genus *Bacteroides.* Zhang *et al.* (2009) used pyrosequencing and showed strong domination of *Clostridium* in the *Firmicutes* in patients with gastric bypass, while Mariat *et al.* (2009), using quantitative PCR, found that *Clostridium leptum*, and *Clostridium coccoides* were highly represented in the microbiota of infant faeces. Zhao *et al.* (2011) showed that the region affected the numbers of *Bacteroides prevotella* and the *Clostridium perfringens* subgroup in the faecal microbiota of elderly subjects in China. It should be emphasised that the data mentioned here are mainly based on analysis of faecal samples; reports on human and murine small intestinal microbiota are extremely sparse.

Recent applications of advanced high-throughput sequencing-based techniques allowed better insight into the complexity of gut microbiota, including uncultivable species, under particular conditions such as obesity. A decreased *Bacteroidetes* proportion and a shift in the *Firmicutes:Bacteroidetes* ratio in obese and lean human subjects implied that there may be a microbiome marker by which obese and lean humans may be distinguished (Eckburg *et al.*, 2005; Gill *et al.*, 2006; Ley *et al.*, 2006; Rajilic-Stojanovic *et al.*, 2007; Turnbaugh *et al.*, 2009). Finding a microbiome marker for obese and lean humans or animals is still an ongoing challenge, and controversial explanations are documented in various publications (Duncan *et al.*, 2008; Lyra *et al.*, 2010; Venema 2010). Yet, even when Duncan *et al.* (2008) found no evidence that the ratio of *Bacteroidetes* to *Firmicutes* among faecal bacteria has a function in human obesity, it should be remembered that these studies have been conducted on human faecal microbiota. However, studies conducted on the murine GIT are still inconclusive.

Administration of probiotics constitutes one of the most widely used approaches to modulate intestinal microbiota in a beneficial way (Steer *et al.*, 2000). Numerous probiotic strains have been evaluated for their effects against diarrhoea and inflammatory bowel disease (Gionchetti *et al.*, 2000; Isolauri *et al.*, 1999). Various lactic acid bacterial strains with probiotic properties are widely being used as starter cultures for food fermentation, and also as animal feed supplements they have been shown to be beneficial for weight gain and improved feed conversion (Khan *et al.*, 2007). For humans, no definitive relationships between the use of probiotics and their effect on obesity and microbiome changes have been shown yet (Erhlich, 2009; Raoult, 2009). However, the small intestine and its microbiota constitute a most vulnerable environment in response to probiotic consumption. Moreover, this is the most important compartment of the GIT for digestion, energy absorption and microbial host interactions.

When studying faecal samples of autistic children, Song *et al.* (2004) considered *Clostridium subterminale, C. perfringens,* and *Clostridium paraputrificum* to be typical of *Clostridium* cluster I, while they used a mixed culture of strains of *Clostridium symbiosum, Clostridium bolteae* and *Ruminococcus gnavus* as representatives of *Clostridium* cluster XIVab. Controversial opinions exist regarding the presence of bifidobacteria in the murine intestinal tract. Their survival rate in the murine GIT appears to be low, but may be increased, e.g. by high amylose maize (amylomaize) starch granules (Wang *et al.*, 1999). Moreover, they were not indicated in an analysis of 16S libraries of FvB mouse gastrointestinal microbiota of the small intestine, large intestine and caecum (Salzman *et al.*, 2002). Still, in view of the possible consumption of their own faeces, the presence of bidifobacteria in the small intestine of the experimental mice was taken into consideration in this study.

In this study, we investigated the modulation of the murine microbiome by probiotic feeding, independently of obesity and a high fat diet. We used the 16S rRNA gene targeting probe to differentially detect *Bacteroidetes, Firmicutes, Bifidobacterium, Lactobacillus* and *Clostridium* clusters I and XIVab, and to evaluate the composition of the murine faecal and small intestinal microbiome for three weeks with two probiotic strains. We assessed their relative incidence within the microbiota using a quantitative real-time PCR (qRT-PCR) assay. We also analysed the influence of the microbiota on hepatic *de novo* lipogenesis, by monitoring weight difference (including white adipose tissue) between control and probiotic fed groups. Also, mRNA levels of three well-known obesity biomarker genes of the liver (Horton *et al.*, 2002; Tappy and Lê, 2010), i.e. acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase-1 (SCD-1) were analysed using qRT-PCR.

2. Materials and methods

Animals

The animal study was approved by the ethical committee of the Handong Global University in Korea. Seven-week-old, specific pathogen free male C57BL/6J mice were supplied from Hyochang Science, Taegu, Korea. Autoclaved tap water and lab chow (Hyochang Science) were provided *ad libitum*, while the animals were housed at 23 °C, 55±10% humidity, in a 12 h light/dark cycle. All 24 mice

were separated into 3 different groups receiving different treatments as described below.

- Group A: normal chow diet with 10 µl phosphate buffered saline (PBS) (control),
- Group B: normal chow diet with 1×10^8 cfu viable cells of *L. sakei* NR28 suspended in 10 µl PBS and administered by oral gavage, and
- Group C: normal chow diet with 1×10^8 cfu viable cells of *L. rhamnosus* GG suspended in 10 µl PBS and administered by oral gavage.

The experiment comprised one week of adaptation followed by three weeks of bacterial (probiotic) feeding. On the last day of the experiment, faecal samples and other mice organs, i.e. small intestine, liver, and epididymal adipocytes were collected, weighed, and kept at -80 °C subsequently to sacrificing the mice with carbon dioxide overdose. Faeces and homogenised total small intestines were analysed to monitor the modulation of major microbial groups after bacterial (probiotic) feeding, while the liver was homogenised to analyse obesity related biomarkers.

Bacterial strains and culture conditions

The bacterial strains and their culture conditions are listed in Table 1. While the *L. rhamnosus* GG strain (LGG) is a well-known commercial probiotic strain, the *L. sakei* strain NR28 was isolated from Korean kimchi, and its functional properties were studied and reported earlier (Lee *et al.*, 2011). The LGG and *L. sakei* NR28 strains were grown and prepared for feeding daily during the three weeks feeding period. Strains were grown for 8 hours to reach their late log phase and collected and washed 2 times with PBS, and administered to each mouse at a dose of approximately 1×10^8 cfu viable cells per day per animal for three weeks. Each strain was suspended in 10 µl of PBS and administered by oral gavage.

Extraction and purification of DNA and qRT-PCR

DNA was extracted by the repeated bead beating plus column (RBB+C) method from both faeces and total small intestinal samples as described by Yu and Morrison (2004). Briefly, the total faeces or homogenised small intestine was suspended with 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate) containing 0.3 g of sterile zirconia beads, and homogenised for 3 min at maximum speed on a Mini-Beadbeater™ (BioSpec Products, Bartlesville, OK, USA). Protein was removed by using 10 M ammonium acetate, and nucleic acids were precipitated by using 70% ethanol. QIAamp DNA stool Mini Kit (Qiagen, Valencia, CA, USA) was used for the purification column.

For qRT-PCR *Taq*Man probes were used to enumerate major groups of microbiota from faecal and small intestinal samples. The methods used are shown with reference to each primer and probe in Table 2. Briefly, amplification was performed with ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The 25 µl reaction mix contained 0.4 µM of forward and reverse primers, 0.2 µM of florescent probe, 12.5 µl of HotStart-IT Probe qPCR Master Mix (Affymetrix, Santa Clara, CA, USA), 1.25 µl of DNA sample, 0.05 µl of ROX (reference dye), and rest

Table 1. Bacterial strains used in this study and their growth conditions.

Strain	Description/purpose	Medium[1]
Lactobacillus rhamnosus GG ATCC53103	administered microorganism; 'control' probiotic strain; positive control for *Firmicutes* primer and probe	MRS (Difco, Franklin Lakes, NJ, USA)
Lactobacillus sakei NR28	administered microorganism (Lee *et al.*, 2011); putative probiotic strain	MRS (Difco)
Escherichia coli ATCC25922	positive control for universal primer and probe	BHI (Difco)
Bacteroides fragilis ATCC25285	control strain for *Bacteroidetes* primer and probe	Chopped Meat Medium (Holdeman and Moore, 1975)
Bacteroides thetaiotaomicron ATCC29148	control strain for *Bacteroidetes* primer and probe	Chopped Meat Medium
Bifidobacterium animalis ATCC25527	control strain for *Bifidobacterium* primer and probe	*Bifidobacterium* Medium (Sutter *et al.*, 1985)
Clostridium perfringens ATCC13124	control strain for *Clostridium* cluster I primer and probe	Reinforced Clostridial Medium (Hirsch *et al.*, 1954)
Clostridium bolteae WAL16351	control strain for *Clostridium* cluster XIVab primer and probe	Reinforced Clostridial Medium

[1] Growth temperature 37 °C, aerobic conditions.

with nuclease-free water. The thermocycling conditions are programmed as described in the references listed in Table 2. For absolute quantification, qRT-PCR standard curves were made using a representative 'reference' strain for each of the major microbial groups selected, as listed in Table 1. Standard growth curves were made of each strain, and DNA was extracted and purified by using the RBB+C method (Yu and Morrison, 2004), and serially diluted (10-fold) more than 3 to 5 times to measure the Ct value of each diluent by using the corresponding primer and probes. The PCR efficiency and R-squares of standard curves were calculated (Smith and Osborn, 2008) and are listed in Table 3.

qRT-PCR detection of mouse obesity-related mRNA

Obesity-related biomarkers were examined by qRT-PCR and their relative expression was determined using the delta-delta Ct method with β-actin gene as an endogenous control gene as described by Livak and Schmittgen (2001). Total RNA from mouse liver was extracted using homogeniser and RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using the Improm-II Reverse Transcription System (Promega, Madison, WI, USA). The resulting cDNA was examined quantitatively by an ABI 7500 Real-Time PCR System (Applied Biosystems). The 25 µl reaction mix contained 0.4 µM of forward and reverse primers, 12.5 µl of HotStart-IT SYBR Green qPCR Master Mix (Affymetrix), 1.25 µl of cDNA sample, 0.05 µl of ROX, and rest with nuclease-free water. The thermocycling conditions were programmed at 95 °C for 2 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. The melting curves were performed at the end of a cycle to ascertain the specificity of the primers and the purity of the final PCR product. The primers are listed in Table 4.

Statistical analysis

Parametric *t*-tests was used to compare both mean cfu logarithmic values of major microbial groups per gram of small intestine and faeces between the control and the probiotic feeding groups, and the relative expression level of analysed genes from the liver.

3. Results

Groups of microbiota of the faeces and small intestine were enumerated after administration of the probiotic strains (Figure 1A,B). Also, the relative abundance of each

Table 2. Primers and probes used in this study for detection of major microbial groups.

Target		Sequence	Control strain	Reference
Total bacteria	forward	5'-AGA GTT TGA TCC TGG CTC AG-3'	*Escherichia coli*	Haakensen *et al.* (2008)
	reverse	5'-ATT ACC GCG GCT GCT GG-3'		
	probe [1]	CY3-5'-CTT GCT GCC TCC CGT AG-3'-BHQ2		
Firmicutes	forward	5'-AGA GTT TGA TCC TGG CTC AG-3'	*Lactobacillus rhamnosus* GG	Haakensen *et al.* (2008)
	reverse	5'-ATT ACC GCG GCT GCT GG-3'		
	probe [2]	FAM-5'-CTG ATG GAG CAA CGC CGC GT-3'-BHQ1		
Bacteroidetes	forward	5'-AAC GCT AGC TAC AGG CTT AAC A-3'	*Bacteriodes thetaiotaomi-cron*	Dick and Field (2004)
	reverse	5'-ACG CTA CTT GGC TGG TTC A-3		
	probe [3]	FAM-5'-CAA TAT TCC TCA CTG CTG CCT CCC GTA-3'-TAMRA		
Clostridium cluster I	forward	5'-TAC CHR AGG AGG AAG CCA C-3'	*Clostridium perfringens*	Song *et al.* (2004)
	reverse	5'-GTT CTT CCT AAT CTC TAC GCA T-3'		
	probe [2]	FAM-5'-GTG CCA GCA GCC GCG GTA ATA CG-3'-BHQ1		
Clostridium cluster XIVab	forward	5'-GAW GAA GTA TYT CGG TAT GT-3'	*Clostridium bolteae*	
	reverse	5'-CTA CGC WCC CTT TAC AC-3'		
	probe [2]	FAM-5'-GTG CCA GCA GCC GCG GTA ATA CG-3'-BHQ1		
Lactobacillus spp.	forward	5'-TGG ATG CCT TGG CAC TAG GA-3'	*L. rhamnosus* GG	Haarman and Knol (2006)
	reverse	5'-AAA TCT CCG GAT CAA AGC TTA CTT AT-3'		
	probe [2]	FAM-5'-TAT TAG TTC CGT CCT TCA TC-3'-BHQ1		
Bifidobacteria	forward	5'-CGC GTC YGG TGT GAA AG-3'	*Bifidobacterium animalis*	Delroisse *et al.* (2008)
	reverse	5'-CCC CAC ATC CAG CAT CCA-3'		
	probe [2]	FAM-5'-AAC AGG ATT AGA TAC CC-3'-BHQ1		

[1] CY3: water-soluble fluorescent cyanine dye family; BHQ2: Black Hole Quencher 2 dye.
[2] FAM: fluorescein amidite dye; BHQ1: Black Hole Quencher 1 dye.
[3] TAMRA: tetramethyl-6-carboxyrhodamine dye.

Table 3. PCR efficiency of each standard curve.

Group	Slope	Y-intercept	$E^1 = (10^{-1/slope})$	E^1 (%)	R^2
Total bacteria	-3.457	42.27	1.9467	94.67%	0.9989
Firmicutes	-3.430	41.01	1.9566	95.66%	0.9960
Bacteroidetes	-3.357	44.98	1.9854	98.54%	0.9983
Clostridium cluster I	-3.652	35.88	1.8786	87.86%	0.9909
Clostridium cluster XIVab	-3.327	42.66	1.9981	99.81%	0.9928
Lactobacillus spp.	-3.363	41.12	1.9831	98.31%	0.9975
Bifidobacteria	-4.329	52.81	1.7022	70.22%	0.9925

[1] *E* means amplification efficiency.

Table 4. Obesity related mRNA primers used in this study.

Target		Sequence	Reference
Acetyl-CoA carboxylase	forward	5'-TGACAGACTGATCGCAGAGAAAG-3'	Thomas *et al.* (2007)
	reverse	5'-TGGAGAGCCCCACACACA-3'	
Fatty acid synthase	forward	5'-GCTGCGGAAACTTCAGGAAAT-3'	Yasutomi *et al.* (2008)
	reverse	5'-AGAGACGTGTCACTCCTGGACTT-3'	
Stearoyl-CoA desaturase-1	forward	5'-CCGGAGACCCCTTAGATCGA-3'	Martin *et al.* (2007)
	reverse	5'-TAGCCTGTAAAAGATTTCTGCAAACC-3'	
Beta-actin (housekeeping gene)	forward	5'-GGCGACGAGGCCCAGA-3'	Goidin *et al.* (2001)
	reverse	5'-CGATTTCCCGCTC GGC-3'	

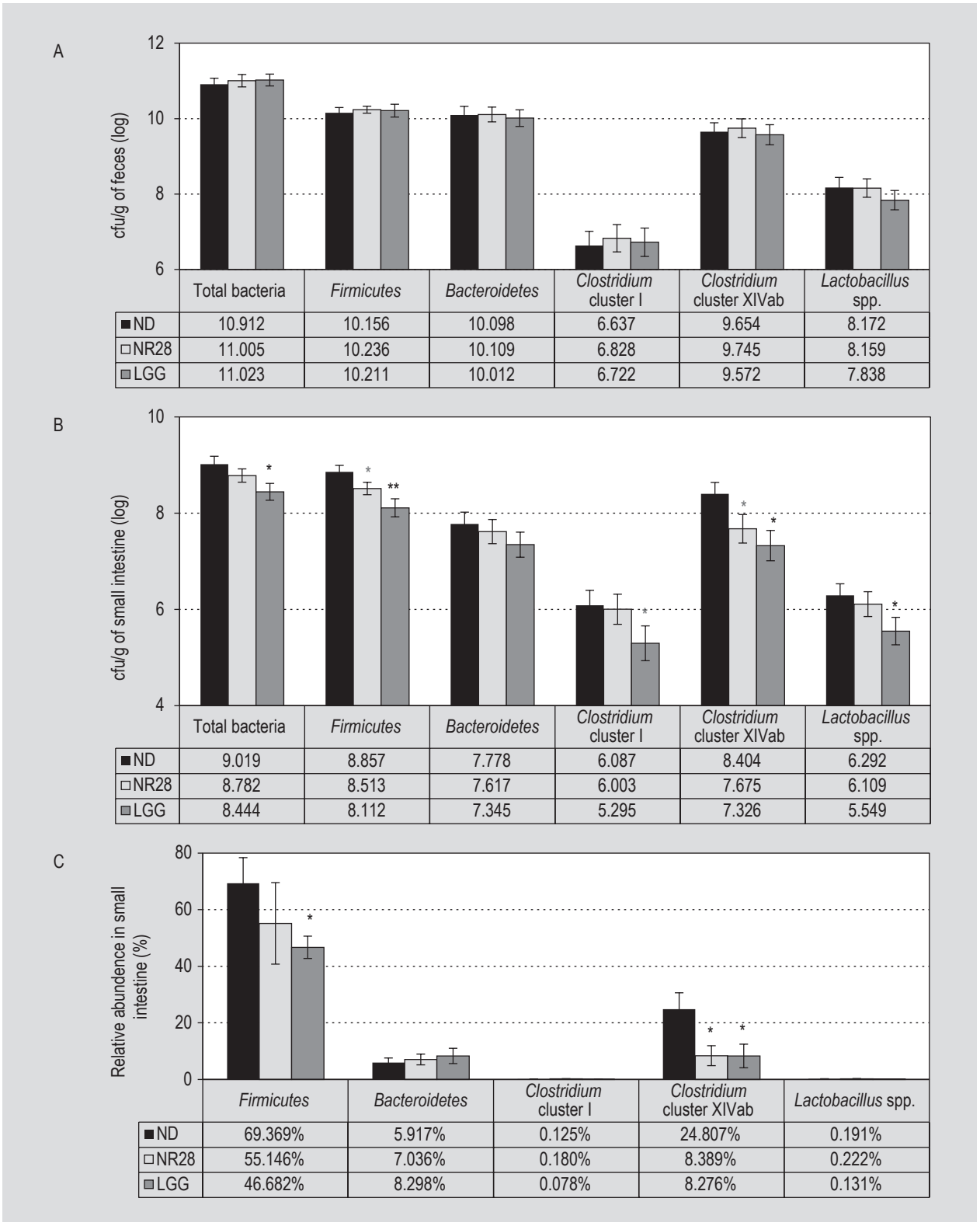
group was calculated in percentage of the total number of microorganisms of the same group in the small intestine (Figure 1C).

Probiotic feeding, both of *L. sakei* NR28 (group B) and LGG (group C), resulted in a noticeable reduction in the total number of microorganisms in the small intestine compared to the control (group A) (Figure 1B,C). By contrast, there were no significant differences in the total faecal microbial population in group B and C as a result of probiotic feeding, when compared to the control (group A).

The population groups in the small intestine clearly responded on administration of the respective probiotic strains (groups B and C). This was associated with a significant reduction in the *Firmicutes* and the *Clostridium* cluster XIVab relative to the control (group A) at the end of the feeding period (Figure 1B). The reducing effect of LGG on *Clostridium* cluster XIVab and *Firmicutes* population in the small intestine was stronger than that of *L. sakei* NR28. Compared to the control, the *Bacteroidetes* population decreased insignificantly in the small intestine, although the calculated relative abundance according to the total microbial numbers was slightly increased (Figure 1B,C). Both the total number of microorganisms and the relative abundance of both *Firmicutes* and *Clostridium* cluster XIVab were reduced in the small intestine (Figure 1B,C). There was no significant change in the faecal microbiota, but a slight increase in *Clostridium* cluster I and XIVab resulting from probiotic feeding (Figure 1A). No dramatic changes could be detected in the faeces for any of the major population groups studied. The presence of *Bifidobacterium* spp. in the faeces and small intestinal samples was also investigated. However, due to poor PCR efficiency (Table 3), in addition to small populations, practically no clear modulating effect on the microbiota could be detected in either of the feeding groups (data not shown).

The small intestinal changes in the major microbial population groups studied were underlined by a noticeable reduction in the *Firmicutes:Bacteroides* ratio as a result of probiotic feeding, as compared to the control (Figure 2B). By contrast, the ratio determined for the faeces did not indicate any noticeable pattern or reduction (Figure 2A).

Significantly lower weight gain and total weight were detected in group B *(L. sakei* NR28) as compared to the control (Figure 3A,B), while there was no significant difference in feed consumption between the 3 groups (Figure 3C). There was a hardly noticeable (insignificant) reduction in feed consumption of group B compared to the control. Yet the total weight gain of group B was significantly lower (around 50%) than that of the control. A significant reduction in the epididymal fat mass over



A	Total bacteria	*Firmicutes*	*Bacteroidetes*	*Clostridium* cluster I	*Clostridium* cluster XIVab	*Lactobacillus* spp.
ND	10.912	10.156	10.098	6.637	9.654	8.172
NR28	11.005	10.236	10.109	6.828	9.745	8.159
LGG	11.023	10.211	10.012	6.722	9.572	7.838

B	Total bacteria	*Firmicutes*	*Bacteroidetes*	*Clostridium* cluster I	*Clostridium* cluster XIVab	*Lactobacillus* spp.
ND	9.019	8.857	7.778	6.087	8.404	6.292
NR28	8.782	8.513	7.617	6.003	7.675	6.109
LGG	8.444	8.112	7.345	5.295	7.326	5.549

C	*Firmicutes*	*Bacteroidetes*	*Clostridium* cluster I	*Clostridium* cluster XIVab	*Lactobacillus* spp.
ND	69.369%	5.917%	0.125%	24.807%	0.191%
NR28	55.146%	7.036%	0.180%	8.389%	0.222%
LGG	46.682%	8.298%	0.078%	8.276%	0.131%

Figure 1. Numbers of microorganisms in various groups of murine microbiota after three weeks of probiotic bacterial treatment. (A) Numbers of each group in 1 g of faeces after probiotic treatment. (B) Numbers of each group in 1 g of small intestine after probiotic treatment. (C) Each group's relative abundance out of total number of bacteria in small intestine was calculated on a percentage basis. The relative abundance of each group was calculated from results in (B). ND: group fed 10 µl of phosphate buffered saline (PBS) only; NR28: group fed 1×10^8 cfu of *Lactobacillus sakei* NR28 suspended in 10 µl of PBS; LGG: group fed 1×10^8 cfu of *Lactobacillus rhamnosus* GG suspended in 10 µl of PBS. Statistical significance (parametric *t* test) compared to the ND value for the same group is indicated: * = *P*< 0.05; ** = *P*<0.001.

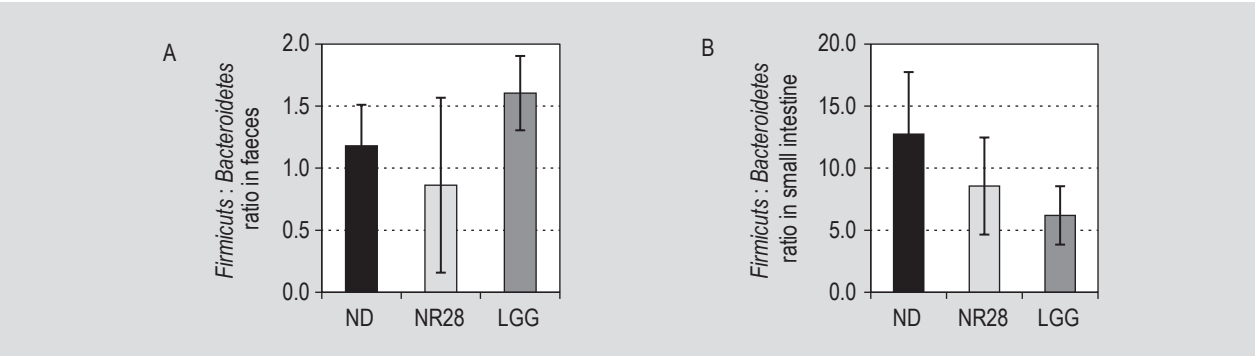


Figure 2. The ratio *Firmicutes:Bacteroidetes* in (A) murine faeces and (B) small intestine following three weeks of probiotic bacteria treatment. ND: group fed 10 µl of phosphate buffered saline (PBS) only; NR28 = group fed 1×10^8 cfu of *Lactobacillus sakei* NR28 suspended in 10 µl of PBS; LGG = group fed 1×10^8 cfu of *Lactobacillus rhamnosus* GG suspended in 10 µl of PBS.

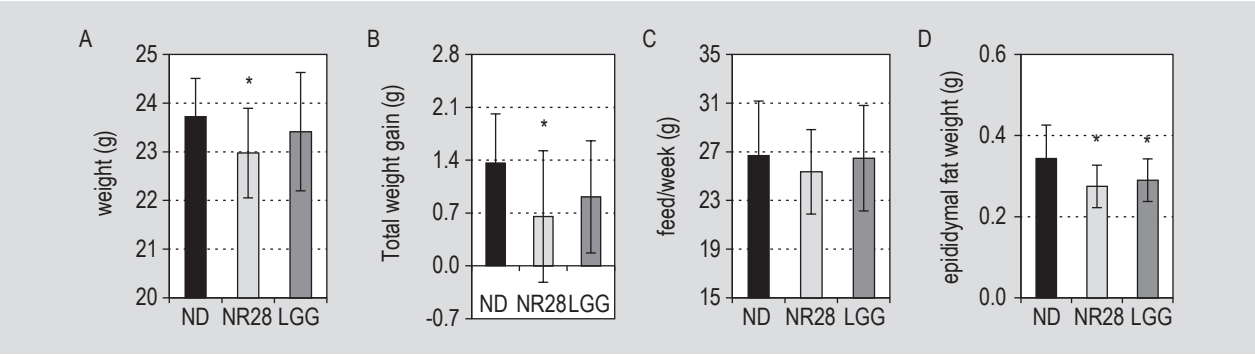


Figure 3. Changes in weight following three weeks probiotic feeding from each group of mice: (A) weight gain, (B) calculated weight gain (final weight – initial weight), (C) calculated feed consumption (total feed consumption / 3 weeks), (D) weight of epididymal adipose tissue.
ND: group fed 10 µl of phosphate buffered saline (PBS) only; NR28: group fed 1×10^8 cfu of *Lactobacillus sakei* NR28 suspended in 10 µl of PBS; LGG: group fed 1×10^8 cfu of *Lactobacillus rhamnosus* GG suspended in 10 µl of PBS. Statistical significance (parametric *t* test) compare to the ND value for the same group is indicated: * = *P*<0.05.

that of the control was detected, both for groups B (*L. sakei* NR28), and C (LGG) (Figure 3D). Within white adipose tissue only the epididymal fat mass was reduced while no significant reduction was observed in visceral fat mass in both groups (data not shown). The relative mRNA levels of obesity related biomarkers from the mouse liver (Figure 4) showed a significant reduction for both treatment groups, compared to the control.

4. Discussion

The possible correlation between microbiota and obesity has been studied by several workers, suggesting that a particular phylum or species can be a regulator of energy metabolism in the body (Bäckhed *et al.*, 2004; DiBaise *et al.*, 2008; Larsen *et al.*, 2010). *Clostridium* cluster XIVab has been proposed as a phylum with an impact on energy metabolism. This group is known to promote the degradation (fermentation) of indigestible complex polysaccharides and to increase the efficiency of energy metabolism in food (Ley *et al.*, 2005). No significant changes were observed for the *Bacteroidetes* population in the small intestine, and the significance of their role in the small intestine may require further investigation. The Gram-negative anaerobe *Bacteroides thetaiotaomicron* was shown to be a dominant member of the normal distal intestinal human microbiota, and, as part of its 4,779-member proteome, has the enzyme capacity to hydrolyse otherwise indigestible dietary polysaccharides (Xu *et al.*, 2003), a function which may have major significance only in the colon.

Other gene expressions have also been reported to be regulated by gut microbiota. One of the most well known factors is fasting-induced adipose factor (Fiaf). Fiaf is a lipoprotein lipase inhibitor which is strongly expressed during fasting. However, a study by Goodman *et al.* (2011) showed that germ-free mice were resistant to Fiaf expression in the intestine, leading to less fat accumulation, especially epididymal fat. Using Fiaf knock-out mouse, they also confirmed that Fiaf is one key factor of fat accumulation (Bäckhed *et al.*, 2004). The animal and human studies dealing with obesity and microbiota, thus far only provided minor indication of a correlation between

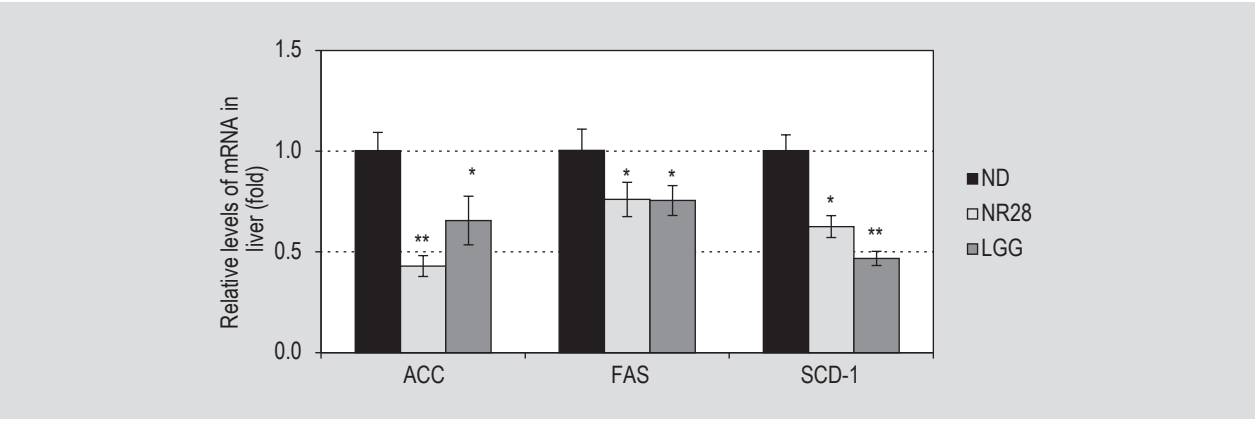


Figure 4. Obesity related mRNA levels of different groups of mice from liver. Relative level of mRNA was calculated by delta-delta Ct method explained by Livak *et al.* (2001). β-actin was used as endogenous control. ACC: acetyl-CoA carboxylase; FAS: fatty acid synthase; SCD-1: stearoyl-CoA desaturasem-1; ND: group fed 10 µl of phosphate buffered saline (PBS) only; NR28: group fed 1×10^8 cfu of *Lactobacillus sakei* suspended in 10 µl of PBS; LGG: group fed 1×10^8 cfu of *Lactobacillus rhamnosus* GG suspended in 10 µl of PBS. Statistical significance (parametric *t* test) compared to the ND value for the same group is indicated: * = *P*< 0.05; ** = *P*<0.001.

change in the *Firmicutes:Bacteroidetes* ratio and weight gain or loss. Also, in many recent studies the relationship between *Firmicutes:Bacteroidetes* and weight differences were not investigated. It is significant that most studies have been conducted with faecal samples because of difficulties in collecting intestinal samples during *in vivo* studies on humans and animals. As the faeces represent more closely the condition of the distal intestinal microbiota (colon including the rectum) rather than that of the small intestine, it can hardly serve as a reliable parameter for assessing the effect of probiotic feeding on the small intestinal ecosystem as major target of viable probiotic strains. Moreover, it should be kept in mind that the small intestine is the most important energy absorption site in vertebrates. Therefore, comparing microbiota of the small intestine after feeding of a probiotic can provide valuable and more relevant information towards understanding the relationship between microbiota and obesity. In our study, probiotic feeding more effectively modulated the murine small intestinal microbiota than that of the faeces (Figure 1). The probiotic strains LGG and *L. sakei* NR28 could not induce any significant weight loss, although the weight gain of the NR28 group was significantly lower than that of the control or LGG groups. The white adipose tissue, especially epididymal fat mass, was reduced (Figure 3), concomitantly with a small intestinal shift in the major microbial groups studied, i.e. the *Firmicutes, Bacteroidetes* and *Clostridium* cluster XIVab. In particular, the numbers of *Clostridium* cluster XIVab were significantly lowered in response to probiotic feeding. Antagonistic characteristics of *Lactobacillus* spp. against *Clostridium* spp. have been reported by by some authors (Matijašic *et al.*, 2006; Naaber *et al.,* 2004) and may explain why the population of *Firmicutes* decreased when feeding *Firmicutes* representatives such as *Lactobacillus* spp. in this study. Likewise, the *Firmicutes:Bacteroidetes* ratio was decreased in the small intestine (Figure 2). Bifidobacteria appeared to be present in insignificant numbers of ≤0.01% of the total population, both in the small intestine and in the faeces.

Probiotic feeding of the experimental mice resulted in a significant reduction in epididymal fat and obesity related biomarkers such as FAS, ACC and SCD-1 from the liver. These results suggest that probiotic consumption may significantly modulate the gut microbiota, especially in the small intestine, with a concomitant impact on host energy metabolism.

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