Effect of *Bifidobacterium thermophilum* RBL67 and fructo-oligosaccharides on the gut microbiota in Göttingen minipigs

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Modulating the gut microbiota via dietary interventions is a common strategy to enhance the natural defence mechanisms of the host. Several *in vitro* studies have highlighted the probiotic potential of *Bifidobacterium thermophilum* RBL67 (RBL67) selected for its anti-*Salmonella* effects. The present study aimed to investigate the impact of RBL67 alone and combined with fructo-oligosaccharides (FOS) on the gut microbiota of Göttingen minipigs. Minipigs were fed a basal diet supplemented with 8 g/d probiotic powder (1×10^9 CFU/g in skim milk matrix) (probiotic diet (PRO)), 8 g/d probiotic powder plus 8 g/d FOS (synbiotic diet (SYN)) or 8 g/d skim milk powder (control), following a cross-sectional study design. Faecal and caecal microbiota compositions were analysed with pyrosequencing of 16S rRNA genes and quantitative PCR. Metabolic activity in the caecum and colon was measured by HPLC. 16S rRNA gene amplicon sequencing revealed that minipig faeces show close similarity to pig microbiota. During the treatments and at the time of killing of animals, RBL67 was consistently detected in faeces, caecum and colon at numbers of 10^5–10^6 16S rRNA copies/g content after feeding PRO and SYN diets. At the time of killing of animals, significantly higher *Bifidobacterium* numbers in the caecum and colon of SYN-fed minipigs were measured compared with PRO. Our data indicate that the Göttingen minipig may be a suitable model for gut microbiota research in pigs. Data from this first *in vivo* study of RBL67 colonisation suggest that the combination with FOS may represent a valuable symbiotic strategy to increase probiotic bacteria levels and survival in gastrointestinal tracts for feed and food applications.

Key words: Probiotics: Synbiotics: Gut microbiota modulation: *In vivo* studies: Animal models

The gut microbiota provides a beneficial barrier function for the host, especially against common enteropathogens such as *Salmonella* or *Escherichia coli* that are encountered in pig farms. This barrier effect is possibly enhanced by probiotic bacteria that contribute to gut microbiota modulation. Specific characteristics attributed to probiotics include competition for nutrients and adhesion sites, stimulation of SCFA, production of antimicrobial substances and modulation of the immune response[1,2]. Probiotic traits are strain-specific, and must therefore be proven for any given strain. With safe history of use, strains of the genera *Bifidobacterium* and *Lactobacillus* are widely represented among probiotics[2]. *Bifidobacterium thermophilum* strains have been primarily isolated from the bovine rumen and animal faeces, mainly calf and pig[3,4], and have also been found in the faeces of babies[5,6].

The probiotic potential of the human faecal isolate *B. thermophilum* RBL67 (RBL67) was investigated in several *in vitro* studies. Strain RBL67 produces a bacteriocin-like substance[5,7], adheres to human intestinal cell lines[8] and exhibits protective effects on epithelial integrity in the presence of a competing human microbiota[9]. Furthermore, RBL67 showed adaptive and competitive traits in complex human intestinal ecosystems, and it led to an inhibition of *Salmonella* in an *in vitro* continuous fermentation model of the child proximal colon[10]. RBL67 has important technological features for application as a probiotic, such as being moderately oxygen-tolerant, growing at a low pH of 4 and up to high temperatures of 47°C by reaching high cell densities[11]. Recently, the genome of RBL67 was sequenced, providing information on the genetic background of the strain[12]. Probiotic effects can be potentiated by the use of prebiotics, a concept known as synbiotics[13]. Prebiotics are non-digestible food ingredients that largely escape digestion in the upper gastrointestinal tract that are readily fermentable in the colon and can stimulate the growth of beneficial bacteria, mainly lactobacilli and bifidobacteria, to confer a health benefit to the host[14]. Fructans such as fructo-oligosaccharides (FOS) and inulin

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Abbreviations: FOS, fructo-oligosaccharides; PRO, probiotic diet; qPCR, quantitative PCR; SYN, synbiotic diet.

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are among the most widely studied and applied prebiotics[15]. Inulin was shown to specifically enhance RBL67 growth in an *in vitro* continuous fermentation model of the child proximal colon[16]. FOS increased molar butyrate proportions in the caecum and proximal colon of piglets, although not affecting microbial populations[17]. A recent study showed a synergistic effect of RBL67 and FOS for reducing *Salmonella* colonisation in the porcine PolyFermS *in vitro* model while increasing the molar butyrate ratio[18,19].

Pigs are a close model of the human gastrointestinal tract[20,21] and are often affected by *Salmonella* infections[22]. Therefore, the anti-*Salmonella* effect of RBL67 is of particular interest for a targeted probiotic approach to reduce *Salmonella* in pigs. The Göttingen minipig holds promise as novel model for investigating the effect of nutritional compounds for food and feed applications. It has important advantages compared with pigs, including high control of breeding and origin, and is small in size, allowing easier handling and consequently reducing labour and costs[23,24]. The Göttingen minipig is already widely accepted as a non-rodent toxicology model, and extensive research of the comparative biology of minipigs to pigs and humans has been undertaken[25]. Nonetheless, information about the distal part of the intestine and gut microbiota composition of minipigs is very limited.

In this study, we aimed to study in detail the Göttingen minipig gut microbiota and test the *in vivo* impact of RBL67 alone or combination with FOS on the microbiota composition and metabolic activities.

Methods

Ethical statement

The minipig study was conducted at the INRA facilities of Clermont-Ferrand-Theix, France. All the experimental protocols were carried out according to the European directives on the protection of animals used for scientific purposes (2010/63/EU), and the laboratory procedures have been approved by the local ethics committee CEMEAA 02.

Preparation of probiotic powder

For production of the probiotic powder, two parallel fermentations with RBL67 (our culture collection) were carried out. Bioreactors (Multifors; INFORS HT) were filled with 1 litre sterile MRS broth (online Supplementary Table S1) supplemented with 0·05 % L-cysteine hydrochloride (VWR International AG) (MRS-C). RBL67 was inoculated at 2 % (v/v) after being propagated twice overnight in the same medium. Batch fermentations were performed at 37°C with constant flushing of the headspace with CO_2 to ensure anaerobic conditions. The pH was maintained at 6·0 by adding 5 M NH_4OH with constant stirring at 150 rpm. Fermentations were stopped after 14 h, and culture broths from the two fermentations were combined. Cells were collected by centrifugation (7000 ***g***, 10 min, room temperature) and washed in PBS, supplemented with 0·05 % L-cysteine hydrochloride. After another centrifugation step, cells were resuspended in 20 % (w/v) reconstituted skim milk (RSM; Lonza Ltd) for 1 h at 4°C under constant stirring. Cells in RSM were stored at –80°C for 2 d before freeze-drying (Christ Alpha 1–4 LD plus; Martin Christ Gefriertrocknungsanlagen GmbH). The dried biomass was subsequently milled to a particle size of approximately 600 μm and blended with skim milk powder to yield an average cell count in the final probiotic powder of log 9·36 (SD 0·07) CFU/g. Aliquots of 8 g, corresponding to 2 % probiotic powder supply in the daily minipig diet, were prepared under vacuum and stored at 4°C, protected from light, until usage.

Animals and procedures

The present study included eight female Göttingen minipigs (20–21 months old, average body weight 28 kg), randomly allocated into two treatment groups (A and B) in a cross-sectional study design (Fig. 1). All animals were housed in separate units (1 × 1·50 m) in a ventilated and temperature-controlled room (22–23°C). Animals received 400 g/d of basal diet (18 % protein (N × 6·25), 2 % fat, 5 % cellulose and 6 % ash (Porcyprima, Sanders Nutrition Animale)) before the start of the study and during the wash-out periods. During treatment periods, the basal diet was supplemented with 8 g/d skim milk powder (control (CON)), 8 g/d probiotic powder (probiotic diet (PRO)) or 8 g/d probiotic powder plus 8 g/d FOS (Fibrulose F97; Cosucra Groupe) (synbiotic diet (SYN)), according to the experimental design of the feeding trial (Fig. 1). Animals had access to water *ad libitum* throughout the study period. Faecal samples were collected daily and immediately stored at –80°C for subsequent DNA extraction and metabolite analyses[26].

At the end of the trial, pigs were euthanised with an injection of pentobarbital (Doléthal, 125 mg/kg body weight; Vétoquinol).
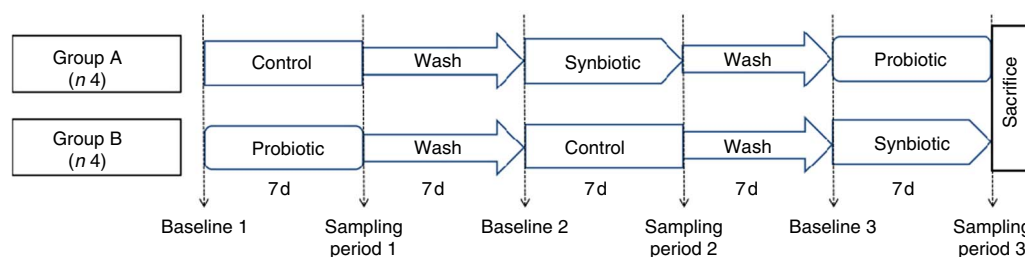

Fig. 1. Experimental set up of the feeding trial using two groups of four Göttingen minipigs. The diets included were as follows: 400 g of basal diet supplemented with 8 g/d milk powder (control), 8 g/d probiotic powder with average *Bifidobacterium thermophilum* RBL67 cell count of log 9·36 (SD 0·07) colony-forming units/g (probiotic) and 8 g/d probiotic powder + 8 g/d Fibrulose F97 (synbiotic). During wash periods, minipigs received 400 g/d basal diet without supplement.

Four minipigs (two minipigs of each group) were euthanised on day 36, whereas the remaining four minipigs were euthanised on day 37. After euthanisation, caecum and colon (proximal, middle and distal) contents were collected and immediately stored at − 80°C until further analysis.

Faecal samples as well as caecum and colonic (proximal, middle and distal) contents were analysed for bacterial populations by quantitative PCR (qPCR) and for metabolite concentrations by HPLC analysis. The microbial profiles of faecal samples before the start of the study (day 1) as well as caecal content samples were also analysed for their microbial profiles with 16S rRNA gene-based amplicon sequencing.

Microbial composition by quantitative polymerase chain reaction

Genomic DNA for qPCR was extracted using the FastDNA SPIN Kit for soil (MP Biomedicals) according to the manufacturer's instructions. qPCR was performed using an ABI PRISM 7500-PCR sequence detection system (Applied Biosystems) for the enumeration of predominant bacterial groups of the swine gut microbiota[27], including *Bifidobacterium* spp., *Lactobacillus/Pediococcus/Leuconostoc* spp., *Bacteroides–Prevotella*, Enterobacteriaceae, *Roseburia* spp./*Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Streptococcus* spp. and *Eubacterium ballii*, using specific primers (online Supplementary Table S2). Standard curves for each target group were prepared as described previously[28], and assays were carried out using the KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Inc.) in a reaction volume of 25 μl. A TaqMan assay was carried out for the enumeration of *B. thermophilum* with specific primers (online Supplementary Table S2) using the RT-QP2X-03WOULR Mastermix (Eurogentec S.A.) and reaction conditions set by Mathys *et al.*[6].

Microbial composition by 16S rRNA gene-based amplicon sequencing

For 16S rRNA gene-based amplicon sequencing analysis, DNA from individual faecal samples (*n* 8) before the start of the study (day 1) and caecal samples (*n* 8) at the end of the study was extracted using the FastDNA SPIN Kit for soil. Extracted DNA was sent to DNAVision for high-throughput sequencing of the hypervariable V5–V6 region of the entire 16S rRNA gene pool using a 454 Life Sciences Genome Sequencer FLX instrument (Roche AG), according to procedures described previously[29]. Resulting sequencing reads were quality-filtered according to the following three criteria: maximum of one mismatch in barcode and primers, at least 240 nucleotides length and a maximum of two undetermined bases per sequence. Sequencing reads passing the quality check were assigned at the phylum, family and genus level using the Ribosomal Database Project (RDP) Bayesian classifier (v2.1)[30] with a confidence threshold of 80 %. Sequences were further assigned into operational taxonomic units (OTU) based on nearest neighbour clustering using mothur software package[31]. Chao1 richness and Shannon diversity were calculated based on the numbers of OTU. The complete 16S rRNA gene-based amplicon sequencing data set has been deposited to the National Center for Biotechnology Information Sequence Read Archive under accession number SRP044704.

Metabolite analysis

Samples for HPLC analyses were prepared according to the protocol described by Dostal *et al.*[32]. In brief, caecum and colonic content (proximal, middle and distal) samples (100–200 mg) were homogenised with 1 ml of 0·15 M H_2SO_4, centrifuged (4°C, 9000 ***g***, 20 min) and the resulting supernatants were filtered through a 0·45 μm nylon filter (Infochroma AG) before injection. Total metabolites (acetate, propionate, butyrate, valerate, isovalerate, isobutyrate, formate and lactate) as well as glucose and lactose were quantified in duplicate by HPLC (LaChrome; Hitachi High-Tech) using an Aminex HPX-87H column (Bio-Rad Laboratories AG) at a flow rate of 0·4 ml/min and with 10 mM H_2SO_4 as the eluent. Data are expressed as means from duplicate analyses in mM/g caecal and colonic contents.

Statistical analysis

All the statistical analyses were performed using PASW Statistics for Windows (v.18.0; SPSS, Inc.). qPCR data were $\log_{10}$ transformed before analysis. To test the effects of experimental diets in faecal samples, mean qPCR data of group A (*n* 4) and B (*n* 4) at the end of each treatment period (Fig. 1) were compared using ANCOVA. The bacterial target group was treated as a dependent factor, the treatment group as an independent factor and the baseline of the corresponding treatment period (Fig. 1) was taken as the covariate. qPCR data from faecal samples are expressed as mean values with their standard errors from four minipigs of group A (*n* 4) and B (*n* 4).

A factorial repeated measures ANOVA was performed to test the effects of experimental diets (between-subjects factor; PRO and SYN), intestinal segment (within-subject factor: caecum, proximal, middle and distal colon) and their interactions on bacterial groups targeted by qPCR and metabolite concentrations in the caecum and colon. If assumption of sphericity was not met, the Greenhouse–Geisser correction was applied and significant effects of intestinal segments were further tested using pairwise comparison, with *post hoc* Bonferroni's correction for multiple comparisons.

In order to compare differences in microbial composition and diversity using 16S rRNA gene-based amplicon sequencing, the relative abundance data at genus and family levels from PRO and SYN in the caecum at the time of killing of animals were compared using the non-parametric Mann–Whitney *U* test with exact significance.

qPCR, HPLC and 16S rRNA gene-based amplicon sequencing data from caecum and colon are expressed as mean with standard deviation for PRO (*n* 4) and SYN (*n* 4).

For all statistical tests, results were considered significant at $P \leq 0.05$.

Results

Faecal microbiota composition and diversity of Göttingen minipigs

Faecal microbiota composition and diversity were assessed before the start of the study (baseline 1) with qPCR targeting predominant bacterial groups of the swine gut microbiota and 16S rRNA gene-based amplicon sequencing. At baseline 1, the mean (n 8) total 16S rRNA gene copy number by qPCR was log 11·8 (sd 0·2) copies/g faeces (Table 1). The most abundant bacterial groups targeted were *Bacteroides–Prevotella* (log 10·9 (sd 0·2) copies/g) and Enterobacteriaceae (log 10·9 (sd 0·7) copies/g), followed by *Lactobacillus/Leuconostoc/Pediococcus* (log 10·3 (sd 0·4) copies/g), *Streptococcus* (log 9·6 (sd 0·6) copies/g), *F. prausnitzii* (log 9·3 (sd 0·2) copies/g), *E. hallii* (log 9·3 (sd 0·2) copies/g) and *Roseburia/E. rectale* (log 8·5 (sd 0·3) copies/g). *Bifidobacterium* was least abundant with log 7·4 (sd 0·2) copies/g, whereas *B. thermophilum* was not detected in both groups at baseline.

Microbiome analysis on the V5–V6 region of the entire 16S rRNA gene pool from faecal samples of minipigs (n 8) before the start of the study generated a total of 72 048 quality-filtered reads with a mean of 9991 (sd 3864) reads/sample and a mean read length of 258 (sd 1) bp. Richness (Chao1 index: 2369 (sd 769)) and diversity (non-parametric Shannon index: 4·50 (sd 0·88)) were estimated from the number of OTU with a similar cut-off of 0·03 % (1068 (sd 329)).

At the phylum level, mean relative abundance data revealed the predominance of four major phyla (Fig. 2). The phylum Firmicutes was the most abundant (approximately 75 %) followed by Bacteroidetes (approximately 14 %), Proteobacteria (approximately 4 %) and Spirochaetes (approximately 3 %), whereas 4 % of the reads could not be assigned to any phylum. Inter-individual variation was observed in all four phyla, mostly in Bacteroidetes and Proteobacteria, with relative abundances ranging from 4 to 22 % and from 0·16 to 17 %, respectively. At the family level, the most abundant phylum, Firmicutes, encompassed the families Streptococcaceae (25 %), Ruminococcaceae (11 %), Lactobacillaceae (8 %), Clostridiaceae (5 %), Lachnospiraceae (4 %) and Erysipelotrichaceae (4 %). The phylum Bacteroidetes was almost exclusively represented by the family Prevotellaceae (7 %), whereas Porphyromonadaceae and Rikenellaceae, the two other families detected from this phylum, displayed low relative abundances (both at 1 %). Two other families, Enterobacteriaceae (phylum Proteobacteria) and Spirochaetaceae (phylum Spirochaetes), were detected (both approximately 3 %), and unclassified reads accounted for approximately 24 % of all reads at the family level. In all, eleven predominant genera represented at least 1 % relative abundance in at least one minipig, whereas unclassified reads accounted for 44 % (Fig. 3). The highest relative abundance was observed for the genera *Streptococcus* (25 %), *Lactobacillus* (8 %) and *Clostridium* (5 %). The genus *Acinetobacter* was detected in only one pig (pig B7), whereas the genus *Escherichia/Shigella* was missing in one pig (pig A2) out of eight. The remaining nine genera were detected in all minipigs. At the genus level, inter-individual variations were observed, most prominently within the genera *Streptococcus*, *Lactobacillus* and *Clostridium*.

Table 1. Bacterial concentrations (log copies/g) in faeces at different time points during the trial for each treatment group detected by quantitative PCR†
(Mean values and standard deviations)

Target	Baseline 1 A Mean	sd	B Mean	sd	Period 1 CON Mean	sd	PRO Mean	sd	Baseline 2 A Mean	sd	B Mean	sd	Period 2 SYN Mean	sd	CON‡ Mean	sd	Baseline 3 A‡ Mean	sd	B Mean	sd	Period 3 PRO Mean	sd	SYN Mean	sd
Total 16S rRNA gene copies	11·8	0·2	11·8	0·2	12·0	0·2	12·0	0·2	11·1	0·7	11·4	0·2	11·0	0·4	11·1	0·4	11·4	0·1	11·4	0·2	11·5	0·1	11·7*	0·1
Bacteroides–Prevotella	10·9	0·3	10·9‡	0·1	11·0	0·2	11·1	0·1	10·6	0·7	10·8	0·1	10·5	0·5	10·5	0·4	11·0	0·2	10·8	0·2	10·9	0·1	10·8	0·2
Enterobacteriaceae	10·8	0·7	11·0	0·8	11·4	0·2	11·1	0·4	11·0	0·4	11·1	0·2	10·6	0·5	10·6	0·4	11·3	0·3	11·2	0·3	11·2	0·2	11·1	0·3
Lactobacillus/Pediococcus/Leuconostoc spp.	10·3	0·5	10·4	0·3	9·4	0·8	10·0	0·4	9·3	0·6	8·6	1·0	8·9	0·9	8·9	0·9	9·5	0·4	9·3	0·2	10·5*	0·3	9·1	0·5
Streptococcus spp.	9·7	0·5	9·6	0·8	9·8	0·1	10·0	0·8	10·1	1·0	10·4	0·9	9·5	0·2	9·1	1·2	9·4	0·5	9·9	0·4	9·7	0·3	10·2	0·2
Bifidobacterium thermophilum	ND		ND		ND		6·1‡	0·5	ND		ND		5·6	0·4	ND		ND		ND		5·9‡	0·3	6·3	0·2
Bifidobacterium spp.	7·4	0·1	7·5	0·3	6·9	0·2	7·0	0·4	7·2	0·2	6·9	0·4	6·7	0·1	6·7	0·1	6·5	0·1	7·0	0·5	6·4	0·2	7·1	0·4
Roseburia spp./*Eubacterium rectale*	8·5	0·4	8·6	0·1	8·7	0·4	8·4	0·2	8·8	0·4	8·5	0·5	8·6	0·4	8·5	0·3	9·0	0·2	8·5	0·2	8·6	0·1	8·4	0·2
Faecalibacterium prausnitzii	9·3	0·3	9·3	0·1	9·5	0·2	9·3	0·3	9·5	0·3	9·1	0·3	9·2	0·5	9·0	0·4	9·3	0·2	9·1	0·3	9·0	0·1	9·6	0·3*
Eubacterium hallii	9·2	0·2	9·4	0·1	9·1	0·1	9·3	0·4	9·5	0·3	9·3	0·3	9·3	0·5	9·0	0·8	9·6	0·4	9·5	0·5	9·3	0·1	9·6	0·3*

CON, control; PRO, probiotic diet; SYN, synbiotic diet; ND, not detected.
* Significantly different between groups within a treatment period and for the bacterial target group ($P \leq 0.05$).
† Reported are values (log copies/g faeces) from wash-out periods (baseline) and treatment periods per group (n 4).
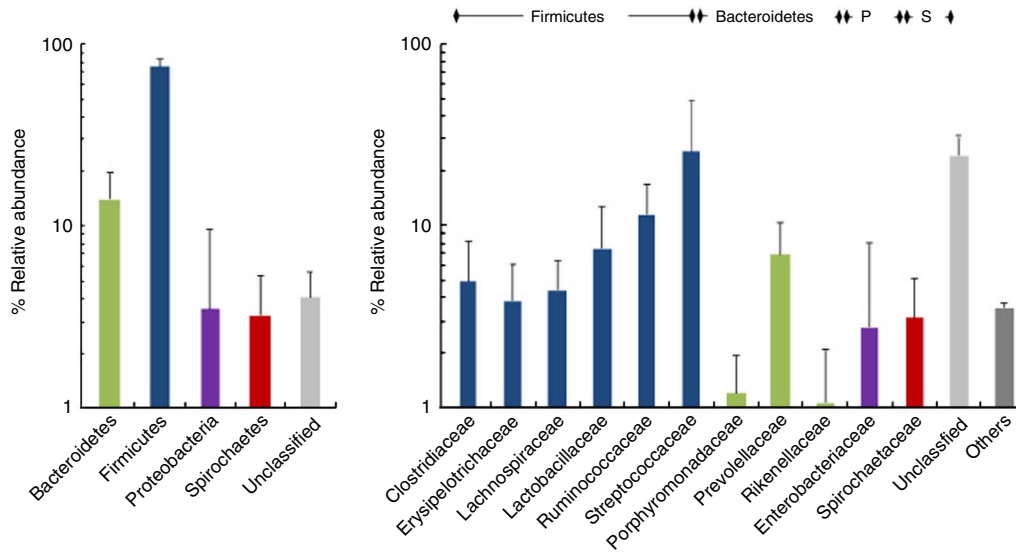‡ n 3.

Fig. 2. Mean relative 16S rRNA gene abundances detected in faeces from Göttingen minipigs at the phylum and family level using 454 pyrosequencing. Values are means with standard deviation for all minipigs (n 8) before the start of the study (baseline 1) and with a relative abundance >1 % in at least one minipig. Relative abundances <1 % are summarised in the group 'others'. P, Proteobacteria; S, Spirochaetes.
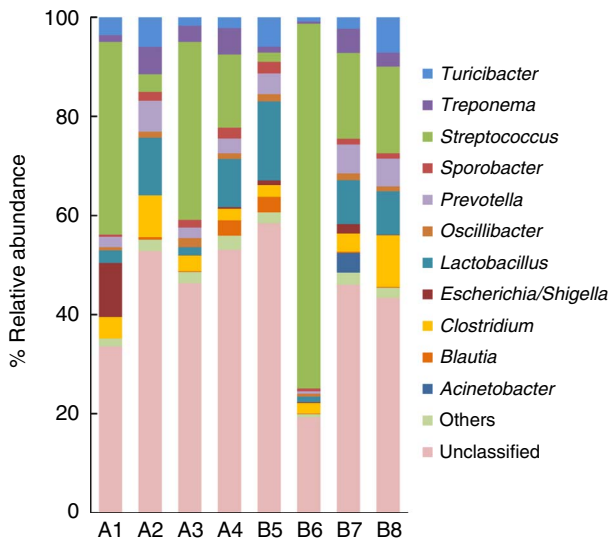

Fig. 3. Relative 16S rRNA gene abundance detected in faeces from Göttingen minipigs at the genus level using 454 pyrosequencing. Values are given for each minipig individually before the start of the study (baseline 1). Relative abundances <1 % are summarised in the group 'others'.

Effect of probiotic and synbiotic diets on faecal bacterial concentrations

Differences in faecal bacterial concentrations upon feeding experimental diets PRO or SYN were assessed by qPCR targeting predominant bacterial groups of the swine microbiota. Throughout the study (during wash-out and treatment periods), high and stable concentrations of total 16S rRNA gene copies (log 11·5 (sd 0·4) copies/g faeces), Enterobacteriaceae (log 11·0 (sd 0·3) copies/g faeces) and Bacteroides–Prevotella (log 10·8 (sd 0·2) copies/g faeces) were measured in both groups. Stable concentrations during wash-out and treatment periods were

also observed for Streptococcus (log 9·8 (sd 0·4) copies/g faeces), Roseburia/E. rectale (log 8·6 (sd 0·2) copies/g faeces) and Bifidobacterium (log 6·9 (sd 0·3) copies/g faeces). B. thermophilum was not detected during wash-out periods and in the CON groups, but was detected in the treatment groups that included RBL67 (PRO or SYN). Experimental diets in periods 1 and 2 did not significantly change faecal bacterial numbers of the groups targeted by qPCR compared with the CON group. In period 3, faecal Lactobacillus/Pediococcus/Leuconostoc numbers were significantly higher with PRO (log 10·5 (sd 0·3) copies/g) compared with SYN (log 9·1 (sd 0·5) copies/g) (P=0·007). In contrast, total 16S rRNA (P=0·011), F. prausnitzii (P=0·001) and E. hallii (P=0·043) numbers were significantly higher with SYN compared with PRO in the third treatment period.

Effects of probiotic and synbiotic diets on bacterial concentration and composition in the caecum and colon

The effect of experimental diets (PRO and SYN) on bacterial composition in the caecum and colon was assessed with qPCR and 16S rRNA gene-based amplicon sequencing (Table 2, Fig. 4). A significant effect of experimental diets in the caecum and colon was observed for Bifidobacterium (P=0·021) with higher numbers in the SYN group compared with the PRO group. For all the other bacterial groups (total 16S rRNA gene copies, Bacteroides–Prevotella, Enterobacteriaceae, Streptococcus, B. thermophilum, Lactobacillus/Leuconostoc/ Pediococcus, Roseburia/E. rectale, F. prausnitzii and E. hallii), no significant effect of experimental diets (PRO and SYN) was observed for bacterial gene copy numbers in the caecum and the different colon segments.

Moreover, 16S rRNA gene-based amplicon sequencing indicated significantly (P≤0·05) lower relative abundance of

Table 2. Bacterial concentrations (log copies/g) detected by qualitative PCR in the caecum and colon (proximal, middle and distal) after killing of animals of the probiotic diet (PRO) and synbiotic diet (SYN) groups
(Mean values and standard deviations)

Target	Caecum				Proximal				Middle				Distal				P: effect of the diet	P: effect of intestinal segment
	PRO		SYN		PRO		SYN		PRO		SYN		PRO		SYN			
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
Total 16S rRNA copies	11.1	0.2	11.3	0.1	11.1	0.2	11.3	0.1	11.4	0.2	11.6	0.1	11.5	0.3	11.7	0.1	0.076	0.004**
Bacteroides–Prevotella	10.4	0.3	10.5	0.2	10.5	0.3	10.5	0.2	10.5	0.1	10.5	0.2	10.6	0.2	10.7	0.2	0.772	0.090
Enterobacteriaceae	11.1	0.3	11.0	0.3	11.2	0.2	11.0	0.2	11.2	0.2	11.0	0.1	11.1	0.1	11.1	0.1	0.242	0.974
Lactobacillus/Pediococcus/Leuconostoc spp.	10.8	0.4	10.4	0.4	10.8	0.4	10.5	0.3	10.8	0.4	10.4	0.3	10.9	0.4	10.4	0.3	0.159	0.429
Streptococcus spp.	8.0	0.8	9.2	0.1	8.3	1.0	9.2	0.2	8.3	1.0	9.1	0.3	8.3	1.2	9.3	0.3	0.094	0.472
Bifidobacterium thermophilum†	5.8	0.6	6.4	0.7	5.9	0.6	6.0	0.3	5.4	0.5	5.7	0.3	5.9	0.6	6.0	0.4	0.505	0.005**
Bifidobacterium spp.	6.5	0.3	7.1	0.4	6.6	0.2	7.0	0.3	6.6	0.2	7.0	0.3	6.6	0.3	7.0	0.2	0.021*	0.648
Roseburia spp./Eubacterium rectal	9.3	0.3	9.7	0.3	9.2	0.2	9.4	0.3	9.3	0.2	9.3	0.3	9.3	0.1	9.2	0.4	0.449	0.028*
Faecalibacterium prausnitzii	9.3	0.3	9.4	0.1	9.3	0.2	9.5	0.2	9.3	0.1	9.4	0.2	9.4	0.2	9.5	0.2	0.205	0.507
Eubacterium hallii	9.5	0.5	9.7	0.3	9.5	0.3	9.8	0.5	9.6	0.2	9.7	0.4	9.6	0.2	9.7	0.5	0.481	0.926

* $P \leq 0.05$, ** $P \leq 0.01$ (factorial repeated-measures ANOVA using Greenhouse–Geisser correction if assumption of sphericity was not met).
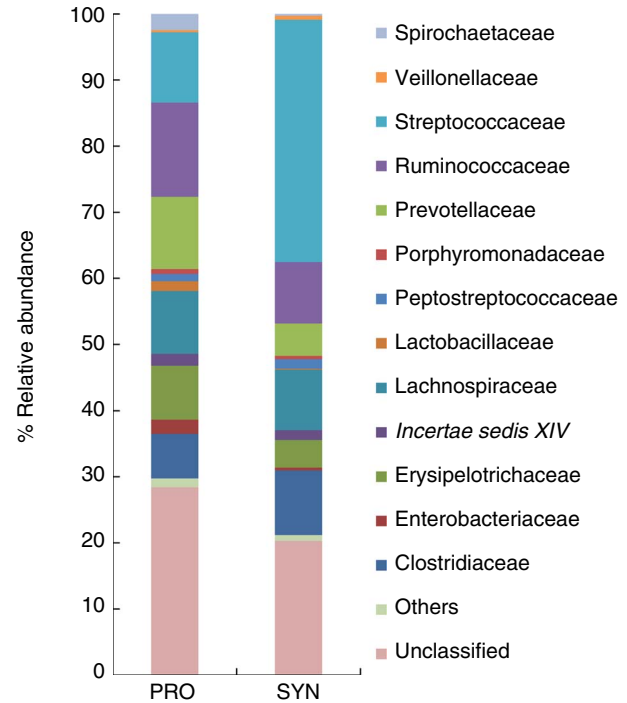† No B. thermophilum detected in one minipig of the SYN group.

Fig. 4. Caecal microbiota composition of minipigs in the probiotic (PRO, n 4) and synbiotic (SYN, n 4) groups analysed by 454 pyrosequencing. Relative abundance is depicted at the family level. Families <1 % are summarised in the group 'others'.

Lactobacillaceae (0·15 %) and Spirochaetaceae (0·27 %) for minipigs from the SYN group compared with the PRO group (Lactobacillaceae 1·48 %; Spirochaetaceae 2·47 %) (Fig. 4).

Intestinal segments displayed a statistically significant difference in the total 16S rRNA gene copies ($P = 0.004$), B. thermophilum ($P = 0.005$) and Roseburia/E. rectale ($P = 0.028$). Post hoc pairwise comparisons revealed lower total 16S rRNA gene copies in the first two segments (caecum and proximal colon) compared with the third (middle colon), whereas B. thermophilum concentrations were higher in the caecum compared with the middle colon. For Roseburia/E. rectale pairwise comparisons failed to detect the location effects. For all the bacterial groups, no significant interaction between experimental diets and intestinal segments was observed.

Effect of probiotic and synbiotic diets on microbial activity in the caecum and colon

Metabolite concentrations were analysed in the caecum and colon content samples from PRO (n 4) and SYN (n 4) at the time of killing of animals by HPLC. Total SCFA and measured metabolite concentrations did not significantly differ between the two experimental diets (Fig. 5, online Supplementary Table S3). A significant effect of the intestinal segment was recorded for total SCFA ($P = 0.029$), acetate ($P = 0.032$), propionate ($P = 0.043$) and butyrate ($P = 0.029$), reflecting a higher metabolic activity in the caecum (total mean metabolite
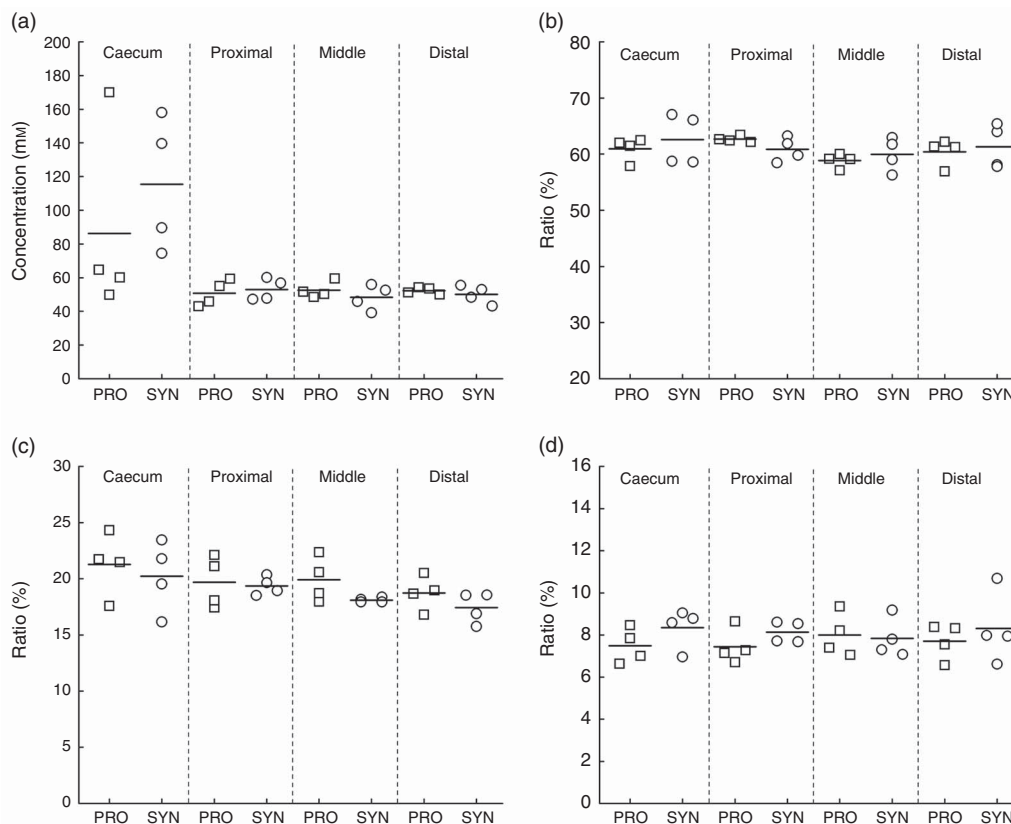
Fig. 5. Total metabolite concentrations (mM) and metabolite ratios (% of total metabolites) detected in the caecum and subsequent colon sections (proximal, middle and distal) in probiotic (PRO) and synbiotic (SYN) groups after killing of animals using HPLC. (a) Total metabolite concentration, (b) acetate ratio, (c) propionate ratio and (d) butyrate ratio. Depicted are mean values (line) and individual values (□ PRO and ○ SYN).

production of 86 (sd 56) mM for PRO and 115 (sd 40) mM for SYN), but also large inter-individual variations within groups. In the proximal colon, total metabolite concentrations decreased to 51 (sd 8) mM (PRO) and 53 (sd 6) mM (SYN) and remained stable in the middle and distal colon regions. The ratios of SCFA were similar for PRO and SYN (acetate 61 %:propionate 19 %: butyrate 8 %) and remained stable along the caecum and colon sections.

Minor metabolites, isobutyrate, isovalerate and valerate also exhibited similar ratios for PRO and SYN in the caecum and colon sections (online Supplementary Table S3). Interestingly, a considerable amount of residual glucose in the caecum of one minipig from PRO (17 mM) and two from SYN (17 and 8 mM) was detected, which also displayed the highest total metabolite concentration.

Discussion

The present study investigated the impact of the probiotic RBL67 alone or in combination with FOS on faecal, caecal and colonic microbiota composition and activity in Göttingen minipigs, a potential new model for characterising the effect of nutritional compounds for feed and food applications on the gut microbiota and gut health.

The Göttingen minipig is particularly advantageous over conventional pig models due to its small size, facilitating handling

and reducing costs[24], and its high control in breeding and origin[23]. However, information on the gut microbiota composition of Göttingen minipigs is scarce. Therefore, in this study, microbiota composition and activity in faeces and different colonic sections were measured using qPCR and next generation sequencing methods and HPLC, respectively. The results at baseline obtained by qPCR and 16S rRNA gene-based amplicon sequencing showed that members of the Firmicutes (*Streptococcus*, *F. prausnitzii*, *E. hallii*, *Roseburia/E. rectale*, *Lactobacillus/Leuconostoc/Pediococcus*), Bacteroidetes (*Bacteroides–Prevotella*) and Proteobacteria phylum (Enterobacteriaceae) were dominant. However, quantitative comparison between qPCR and 16S rRNA gene-based amplicon sequencing should be considered with caution due to inherent biases associated with each method. For instance, the 16S rRNA gene copy number is affected by primer specificity bias in qPCR and a relatively low sensitivity is associated with 16S rRNA gene-based amplicon sequencing. As such, *Bifidobacterium* with the lowest abundance in qPCR likely fell below the detection limit of 16S rRNA gene-based amplicon sequencing. Thus, qPCR targeting specific populations and 16S rRNA gene-based amplicon sequencing should be used in a complementary approach to accurately analyse microbiota composition. In general, the faecal microbiota composition in Göttingen minipigs revealed by 16S rRNA gene-based amplicon sequencing was in accordance with recent data of the same minipig model[33]. Minor differences between the studies can be explained by the

site-specificity of the samples (ileum, caecum, colon *v.* faeces), variations in diet and environment and methodological differences of the two studies (different variable 16S rRNA region targeted, different pipeline for reads assignment)[34].

Our samples displayed higher relative abundance of 75 % Firmicutes compared with 49 % revealed by Pedersen *et al.*[33]. Large variations in relative abundance of Firmicutes have also been reported in studies characterising the caecal microbiota of weanling pigs ranging from approximately 37 %[35] to up to approximately 75 %[36]. Further, in our study, lower relative abundances of Spirochaetes (3·2 *v.* 6 %) and Tenericutes (<1 *v.* 3·8 %) but higher relative abundance of Proteobacteria (3·5 *v.* <1 %) were observed compared with the study by Pedersen *et al.*[33]. At the family and genus level, our study revealed a similar microbiota composition in minipigs[33] and pigs[35–39]. A remarkable high relative abundance of the genus *Streptococcus* was recorded in three out of eight faecal samples, which is in accordance with several other pig studies[37–39]. In contrast, Pedersen *et al.*[33] and other studies on pig caecal micro-biota[35–39] did not find a predominance of the genus *Strepto-coccus* in the caecum, suggesting a dominance of streptococci in faecal material but not in the proximal colon. The high fraction of unclassified reads (up to 58 % of total) presumes that a large fraction of bacteria of the faecal microbiota in minipigs is not yet deciphered, which is in contrast with the well-studied pig microbiota. Therefore, to support the use of the minipigs as model for gut microbiota research, the microbiota composition of minipigs should be further deeply studied using culture-dependent approaches for isolation of potential new microbes and culture-independent approaches using long-length ampli-cons. In our study, *B. thermophilum* was not detected in minipig faeces in the CON groups and during wash-out periods. Consequently, we assume that the general *B. thermophilum* population is below detection limit or absent, and data of the qPCR test used to detect *B. thermophilum* enumerated the supplemented strain RBL67. After feeding experimental diets (PRO and SYN), RBL67 was consistently detected in faecal, caecal and colonic content. *Enterococcus faecium* NCIMB 10415, an authorised probiotic strain for use in pig feed in the European Union[40], was recovered at $1\cdot8 \times 10^5$ CFU/g wet weight faeces in pregnant sows fed the probiotic for 80 d at approximately 1×10^9 CFU/kg feed[40,41], which is consistent with our data on *B. thermophilum*, despite the largely different physiological features of these two strains.

The SYN group displayed significantly higher total *Bifidobacterium* numbers in the caecum and colon com-pared with the PRO group. In addition, the analysis of *B. thermophilum* after killing of animals revealed highest numbers of *B. thermophilum* in the caecum of the SYN group. These data suggest that RBL67 is viable and growth in the caecum is stimulated by the concomitant administration of FOS, implying the synbiotic concept. The simultaneous stimulation of *Bifidobacterium* and RBL67 by SYN explains why the relative percentage of *B. thermophilum* in the caecum as a proportion of the total *Bifidobacterium* population is higher in the PRO group (36 %) compared with the SYN group (22 %). FOS has previously been reported to selectively stimulate the growth of bifidobacteria[42], although it has been established nowadays

that other bacteria (e.g. members of the genera *Roseburia*, *Bacteroides*, *Salmonella*) are able to utilise prebiotics as well[15,43,44].

No differences between PRO and SYN were observed in metabolite concentrations measured by HPLC of the caecum and colonic samples. This is in agreement with a previous study where piglets had *ad libitum* access to experimental diets including 10 g/kg FOS or transgalactooligosaccharide[45]. However, other studies have reported a significant increase in butyrate and decrease in acetate concentrations when including 4 % FOS[17] or 3 % inulin to piglet diets[46]. Different factors including prebiotic substrate (degree of polymerisation, inclusion rate), feeding frequency (*ad libitum*, restricted), pig breed and basal diet[42] may contribute to the variable outcome of the different studies and complicate direct comparisons between studies. SCFA concentrations do not provide a direct marker of metabolic activity due to their rapid absorption by the host or utilisation by other members of the gut microbiota[46,47]. The rapid absorption of SCFA is demonstrated by a decrease of SCFA concentrations from the caecum to the proximal colon, as reported previously[48] and also shown in our study. It is possible that differences in absorption and high inter-individual variations of SCFA profiles have masked the effect of FOS on metabolite concentrations, leading to non-significant differences between treatment groups. Another hypothesis may be that FOS is partly digested in the upper gastrointestinal tract, as previously shown by Loh *et al.*[46], where 20–50 % of the supplemented inulin was digested in the jejunum of pigs.

In conclusion, Göttingen minipigs may be a suitable model for pig microbiota study, although the high fraction of assigned reads at the family and genus levels emphasise the need for additional characterisation of gut microbe diversity. We demonstrated for the first time the *in vivo* recovery of the probiotic candidate RBL67 by qPCR upon feeding minipigs, with a stimulation effect of FOS on the bifidobacteria popula-tion and specifically RBL67 in the caecum. Our data suggest that Göttingen minipigs can be a suitable model for investigat-ing the impact of feed and food additives for gut health. Additional *in vivo* investigations using *Salmonella*-infected pigs must be carried out to confirm the antimicrobial efficacy of the symbiotic formulation of RBL67 and FOS against enteropathogens.

Supplementary Material

For supplementary material/s referred to in this article[(49–53)], please visit http://dx.doi.org/10.1017/S0007114515002263

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