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Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli

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Summary

To avoid detrimental interactions with intestinal microbes, the human epithelium is covered with a protective mucus layer that traps host defence molecules. Microbial properties such as adhesion to mucus further result in a unique mucosal microbiota with a great potential to interact with the host. As mucosal microbes are difficult to study *in vivo*, we incorporated mucin-covered microcosms in a dynamic *in vitro* gut model, the simulator of the human intestinal microbial ecosystem (SHIME). We assessed the importance of the mucosal environment in this M-SHIME (mucosal-SHIME) for the colonization of lactobacilli, a group for which the mucus binding domain was recently discovered. Whereas the two dominant resident Lactobacilli, *Lactobacillus mucosae* and *Pediococcus acidilactici,* were both present in the lumen, *L. mucosae* was strongly enriched in mucus. As a possible explanation, the gene encoding a mucus binding (*mub*) protein was detected by PCR in *L. mucosae*. Also the strongly adherent *Lactobacillus rhamnosus* GG (LGG) specifically colonized mucus upon inoculation. Short-term assays confirmed the strong mucin-binding of both *L. mucosae* and LGG compared with *P. acidilactici*. The mucosal environment also increased long-term colonization of *L. mucosae* and enhanced its stability upon antibiotic treatment (tetracycline, amoxicillin and ciprofloxacin). Incorporating a mucosal environment thus allowed colonization of specific microbes such as *L. mucosae* and LGG, in correspondence with the *in vivo* situation. This may lead to more *in vivo*-like microbial communities in such dynamic, long-term *in vitro* simulations and allow the study of the unique mucosal microbiota in health and disease.

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Introduction

The human intestinal tract is colonized by a complex microbial community, which is mostly (> 90%) dominated by microorganisms from two phyla, Bacteroidetes and Firmicutes (the latter including for instance Lactobacilli) (Eckburg *et al*., 2005). This organized microbiota renders multiple benefits to the host such as induction of immunological responses, breakdown of otherwise inaccessible food compounds and regulation of host metabolism (Backhed *et al*., 2005; Turnbaugh *et al*., 2006). On the other hand, as intestinal microbes may potentially also invade the epithelium, the host has developed an efficient mucosal defence barrier. A regularly replaced mucus layer traps antimicrobial peptides and other immune effectors, allowing the host to avoid direct microbial contact with the underlying epithelium and mucosal tissues (Lievin-Le Moal and Servin, 2006; Mukherjee *et al*., 2008).

As reviewed by Van den Abbeele and colleagues (2011), specific microbial characteristics including for instance adhesion to mucus, the ability to gain nutrients from host-derived glycans or resistance to host defence molecules, allow specific microbes to colonize this layer, resulting in a distinct mucosa-associated microbial community (MAMC) (Zoetendal *et al*., 2002; Macfarlane, 2008; Schreiber, 2010). Colonization of this mucus layer prolongs microbial colonization, as washout is counteracted and mucosal microbes are more protected from disturbances in the lumen. The MAMC is important for the

host as resident mucosal microbes might prevent pathogens from approaching and invading the epithelium, by excreting antimicrobial compounds in the mucus layer or by competing for adhesion (Kaur *et al*., 2002). Moreover, mucosal microbes can interact more closely with the epithelium than their luminal counterparts, which may be crucial for achieving immunomodulatory effects (Sun *et al*., 2010). Over the past few decades, strategies have been developed to optimize or restore the intestinal microbial balance. An important one is the use of probiotics, which include specific *Bifidobacterium* spp. and *Lactobacillus* spp. (FAO/WHO, 2002; O'Flaherty and Klaenhammer, 2010; Williams, 2010). Because of the mentioned potential health benefits of mucosal microbes, probiotics are often screened and selected for their adhesion capacity to the intestinal surface (Ouwehand *et al*., 1999; Tuomola *et al*., 2001).

Despite their physiological relevance, human intervention studies are restricted to end-point measurements regarding mucosal microbes (Alander *et al*., 1997; Zoetendal *et al*., 2002). Human studies are often restricted to faecal samples, which do not provide information on probiotic colonization of the mucus layer in different intestinal regions (Alander *et al*., 1999; Tuomola *et al*., 2001; Kankainen *et al*., 2009). In contrast, *in vitro* experiments are well suited to screen the adhering potency of candidate probiotic strains. Such experiments include adhesion assays to intestinal mucus (Ouwehand *et al*., 1999), mucins (Kinoshita *et al*., 2007; Van den Abbeele *et al*., 2009), colonic tissue (Ouwehand *et al*., 2002) and cell lines (Laparra and Sanz, 2009). However, these models only provide short-term information and often ignore the interaction between luminal and mucosal microbial communities. Long-term dynamic *in vitro* models do allow dynamic monitoring in different intestinal regions but typically do not incorporate a simulation of the mucosal environment, which limits their representative capacity. Indeed, the microbial community development dynamic *in vitro* models was recently shown to go along with distinct community shifts, such as increased Bacteroidetes/Firmicutes ratios, lower numbers of bacilli and an enrichment in propionate producers (*Clostridium* cluster IX) compared with butyrate producers (*Clostridium* clusters IV and XIVa) leading to altered SCFA ratios compared with the *in vivo* situation (Van den Abbeele *et al*., 2010). As incorporating a mucosal environment may avoid washout of adherent microbes (e.g. Lactobacilli), a more accurate *in vitro* simulation of the human intestinal microbial composition may be obtained. Additionally, a mucosal environment is characterized by a low shear stress that greatly activates the microbial gene expression (Nickerson *et al*., 2004), potentially resulting in a more *in vivo*-like behaviour of the individual microbes.

The objective of this study was therefore to incorporate a mucosal environment, containing mucin-covered microcosms, in a dynamic *in vitro* gut model, i.e. the simulator of the human intestinal microbial ecosystem (SHIME), and assess its importance for the colonization of Lactobacilli within the background of a complex microbial community. We focused on the Lactobacilli as a mucus binding domain has recently been described for several species belonging to this group (Boekhorst *et al*., 2006). The aims of this study were (i) to quantify, identify and isolate the dominant Lactobacilli, (ii) to evaluate the *in vitro* adhesion capacity of the isolated bacteria to mucin agar, (iii) to characterize the colonization capacity of the model probiotic *Lactobacillus rhamnosus* GG (LGG), and (iv) to investigate the resilience of the Lactobacilli community after administration of an antibiotic pulse.

Results

Colonization of the luminal and mucosal environment in the M- and L-SHIME

One day after inoculation, DGGE analysis for the total bacterial community revealed distinct differences between the microbiota of the luminal and the mucosal environment (< 60% similarity between mucus and lumen) (Fig. 2A). Interestingly, both the M- and L-SHIME contained fairly similar microbial communities in their luminal environment (≈ 90% similarity).

Analysis of the *Lactobacillus*-subgroup demonstrated the distinct nature of the luminal and mucosal microbiota. Plating of samples of the M- and L-SHIME on *Lactobacillus*-specific growth medium revealed two predominant colony morphologies (Fig. 1). When these isolates were analysed with *Lactobacillus*-specific DGGE, both strains were found to cover the entire *Lactobacillus*-specific DGGE profile (Fig. 2B). The first species was associated with one band in the DGGE profile and was identified as *Pediococcus acidilactici* (99.7%), a species that strictly speaking should be regarded as a coccoid Lactobacilli. The second species covered three bands within the DGGE profile and was identified as *Lactobacillus mucosae* (99.9%). DGGE analysis showed that the colonization of the mucosal environment occurred species specifically: both strains were detected in the luminal content of the M- and L-SHIME (density band *P. acidilactici* = 15.8%; density bands *L. mucosae* = 84.2%), whereas only *L. mucosae* was able to establish in the mucin-adhered microbial community (density band *P. acidilactici* = 1.4%; density bands *L. mucosae* = 98.6%) (Fig. 2B).

Colonization of L. rhamnosus *GG (LGG)*

As the colony morphology of LGG, *P. acidilactici* and *L. mucosae* on LAMVAB growth medium was distinguish-

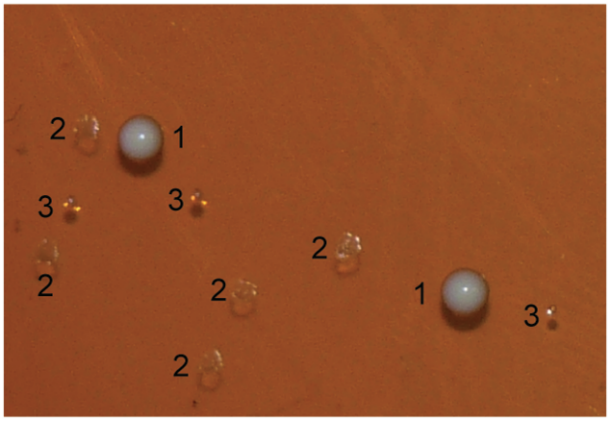

Fig. 1. Suspensions from both the M- and L-SHIME, plated on *Lactobacillus*-specific growth medium (LAMVAB) revealed two predominant colony morphologies, identified as *Pediococcus acidilactici* (1; big, smooth and circular) and *Lactobacillus mucosae* (2; rough and irregular) by means of the 16S rRNA gene sequencing. Upon inoculation, also *Lactobacillus rhamnosus* GG had a distinct colony morphology (3; small, smooth and circular).

able (Fig. 1), it was possible to quantitatively estimate the amounts of these three strains using plate counts. Moreover, after addition of LGG on day 3, the three *Lactobacilli* species covered the entire range of the *Lactobacillus*-specific DGGE profile. The results of the plate counts were thus representative for the shifts within the Lactobacilli community (Fig. 2B/C). One day after the administration (day 4), LGG was washed-out from both SHIME systems at a comparable rate (≈ factor 20 decrease) (Table 1). However, 3 days after inoculation (day 6), planktonic LGG was detected in slightly higher abundances in the M-SHIME compared with the L-SHIME (0.4% compared with 0.1% of total Lactobacilli, respectively). The presence of LGG within the mucus was a factor 26 higher compared with the lumen (10.7% compared with 0.4% of total Lactobacilli, respectively). DGGE analysis confirmed these results as 3 days after inoculation (day 6), LGG could only be detected in the mucus layer of the M-SHIME (Fig. 2C). Finally, 5 days after administration of LGG (day 8), the abundance of LGG in the mucus layer was also below the detection limit of DGGE. Interestingly, the single dose of LGG on day 3 resulted in *P. acidilactici* to be outcompeted within 3 days from the luminal content of both SHIME units.

Adhesion of Lactobacilli isolates to mucin agar

In order to compare the adhering potency of the isolated Lactobacilli to mucins, a short-term *in vitro* adhesion assay was performed. The first isolate was designated *P. acidilactici* LB1 and the second *L. mucosae* LB2. Whereas adhesion of *L. mucosae* LB2 to the mucin agar (8.0 ± 1.4%) was comparable with that of LGG (10.9 ± 2.0%), *P. acidilactici* LB1 adhered to a much lower extent (1.8 ± 0.4%) (Fig. 3).

Detection of a gene encoding for a mucus binding protein in L. mucosae *LB2*

It has earlier been reported that *L. mucosae* strains can carry mucus binding proteins similar to Mub in *Lactobacillus reuteri* 1063 (Roos *et al*., 2000). The presence of genes encoding Mub1 and Mub2 repeats in *L. mucosae* LB2 was analysed by PCR. The mub1 primers generated fragments of the same sizes from both *L. mucosae* LB2 and *L. reuteri* 1063 (approximately 600 bp), while the mub2 primers gave a larger fragment from *L. mucosae* LB2 (approximately 550 bp) than from *L. reuteri* 1063 (approximately 280 bp). The mub1 fragment from *L. mucosae* LB2 was sequenced and the sequence was shown to have 97.1% identity with the corresponding gene from *L. reuteri* 1063. Thus, a gene with considerable similarities to *mub* is present in *L. mucosae* LB2.

Resilience of the Lactobacilli communities after administration of an antibiotic pulse

When determining the minimal inhibitory concentration (MIC) values of amoxicillin, tetracycline and ciprofloxacin for *P. acidilactici* LB1 and *L. mucosae* LB2 (Table 2), it was observed that *P. acidilactici* LB1 was more resistant

Table 1. Abundance of *Pediococcus acidilactici* (*P. ac*), *Lactobacillus mucosae* (*L. muc*) and *Lactobacillus rhamnosus* GG (LGG) (% of total amount of lactobacilli) as determined with plate counts on a *Lactobacillus*-specific growth medium (LAMVAB) in the M-SHIME (lumen and mucus) and L-SHIME (lumen), on day 3 (before and after administration of LGG), 4 and 6.

	M-SHIME						L-SHIME		
	Lumen			Mucus			Lumen		
Time (days)	*P. ac*	*L. muc*	LGG	*P. ac*	*L. muc*	LGG	*P. ac*	*L. muc*	LGG
3 (before LGG)	7.7	92.3	0.0	6.6	93.4	0.0	12.4	87.6	0.0
3 (after LGG)	5.7	59.8	34.4	nd	nd	nd	3.7	29.0	67.3
4	1.0	97.0	2.0	0.5	97.8	1.7	3.5	93.3	3.2
6	0.1	99.4	0.4	0.2	89.2	10.7	0.0	99.9	0.1

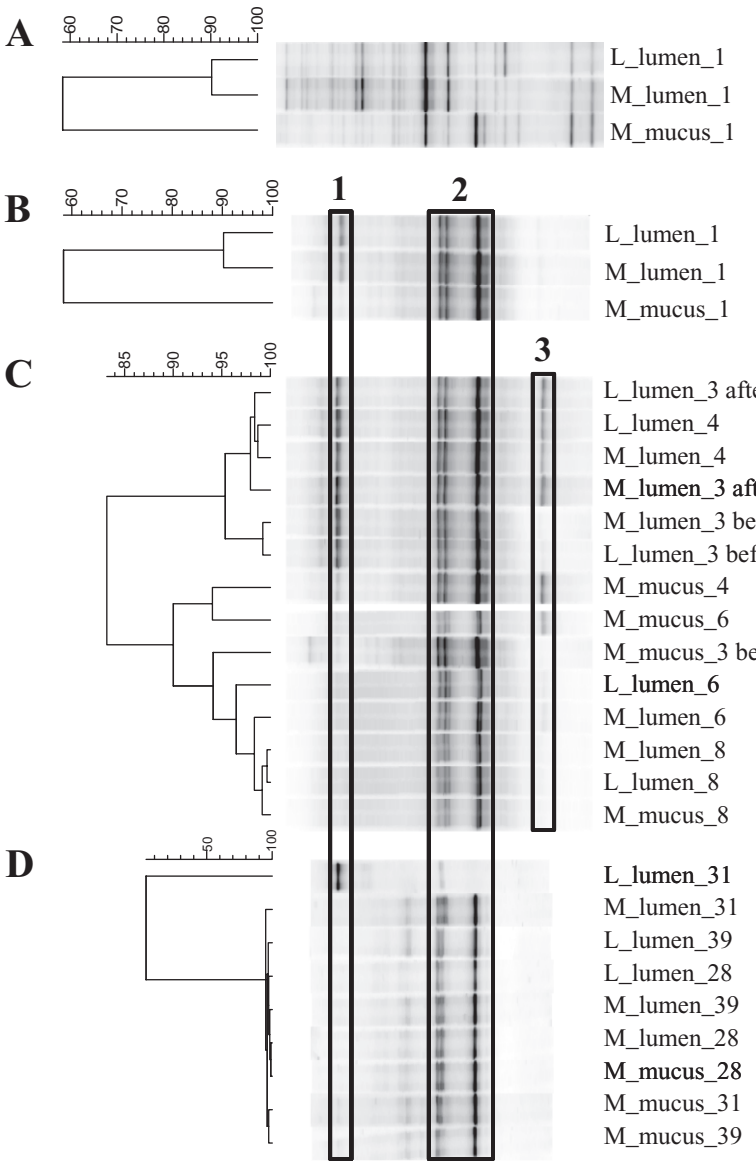

Fig. 2. Clustering tree based on Pearson and UPGMA correlation of total bacterial (A) *Lactobacillus*-specific (B/C/D) DGGE profiles of samples from the M-SHIME (lumen and mucus) and L-SHIME (lumen) on day 1 (A/B). On day 3, both the M- and L-SHIME were inoculated with LGG and samples were taken on day 4, 6 and 8 (C). Finally, a long-term stabilized SHIME was treated with 10 μg l^{-1} tetracycline, amoxicillin and ciprofloxacin on two consecutive days (day 28 and 29) and samples were analysed on day 28 (before treatment), 31 and 39 (D). The bands corresponding to pure cultures of *Pediococcus acidilactici* (1), *Lactobacillus mucosae* (2) and *Lactobacillus rhamnosus* GG (LGG; 3) are indicated with rectangle 1, 2 and 3 respectively.

to these three antibiotics compared with *L. mucosae* LB2. Especially amoxicillin inhibited the growth of *L. mucosae* LB2 (MIC = 0.25 μg ml^{-1}) while *P. acidilactici* LB1 was quite resistant to this antibiotic (MIC = 4 μg ml^{-1}).

Upon antibiotic supplementation (10 μg ml^{-1} of each antibiotic) to the continuous model (day 28 and 29) (Fig. 4), the amount of *L. mucosae* decreased below detection limit (= 2 log cfu ml^{-1}) in both the M- and L-SHIME. In contrast, *P. acidilactici* was much less affected by the antibiotic treatment and even increased after the antibiotic treatment ($P = 0.037$ for both M- and L-SHIME). One week after the antibiotic pulse, the Lactobacilli communities in both units returned to their initial composition. The presence of a mucosal compartment allowed a faster and more complete recovery after the antibiotic pulse. Interestingly, a 3 week stabilization period allowed *L. mucosae* to dominate over *P. acidilactici* within the M-SHIME, while the inverse was true for the L-SHIME. The level of *L. mucosae* was significantly higher in the M-SHIME compared with the L-SHIME ($P = 0.009$).

Table 2. MIC (μg ml^{-1}) of *Pediococcus acidilactici* LB1 and *Lactobacillus mucosae* LB2 for tetracycline, amoxicillin and ciprofloxacin, determined on Mueller-Hinton agar.

Antibiotic	*Pediococcus acidilactici*	*Lactobacillus mucosae*
Tetracycline	16	8
Amoxicillin	4	0.25
Ciprofloxacin	16	8

The MIC value is the lowest concentration with no visible growth.

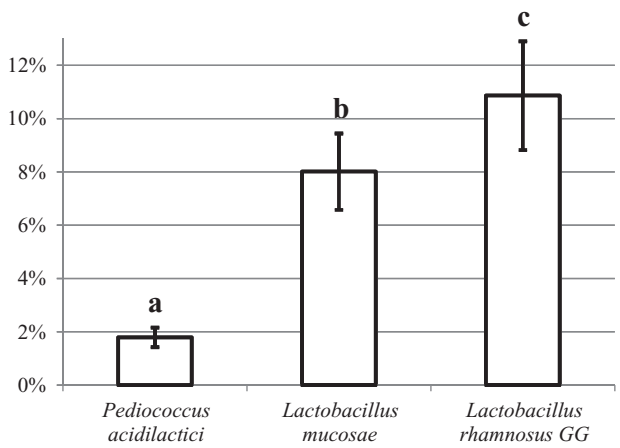

Fig. 3. Proportion of bacteria (%) that adhered to mucin agar for *Pediococcus acidilactici, Lactobacillus mucosae* and *Lactobacillus rhamnosus* GG. Significant differences are indicated with a different superscript.

Discussion

In this study, we designed a dynamic gut model that is more representative for the *in vivo* situation than earlier models that only mimic the luminal microbiota and its associated functionalities. Incorporation of a mucosal environment allowed colonization and development of specific microorganisms that benefit from mucosal adhesion, in correspondence with the *in vivo* environment. This method allows evaluating the long-term colonization of the luminal and mucosal region in a multistage gastrointestinal *in vitro* simulator. In contrast to previous *in vitro* adhesion studies, this strategy allowed to unravel the adhesion capability of (potential) probiotic Lactobacilli, while accounting for their long-term interaction with the resident, luminal and mucosal intestinal microbial communities.

To evaluate the *in vitro* mucosal environment consisting of mucin-covered microcosms, colonization of the model probiotic and generally strongly adherent LGG was examined. Human *in vivo* studies showed that LGG effectively colonizes the colonic mucus layer after oral administration (Alander *et al*., 1997). Interestingly, LGG remained present in the mucus layer of most subjects 1 week after the end of LGG administration, whereas it was hardly detected in faeces (Alander *et al*., 1999). LGG thus needs a surface to which it can adhere in order to increase its ecological fitness. This *in vivo* finding was consistent with the observations in our M-SHIME model. Three days after inoculation, LGG was strongly enriched in the mucosal compartment of the M-SHIME compared with the lumen of both M- and L-SHIME (Table 1 and Fig. 2C). The strong mucus-adhering potency of LGG has been attributed to a SpaC pilin, which is located on the top of the pili and exerts a strong mucus binding activity, thus allowing LGG to colonize within an established mucosa-associated microbiota (Kankainen *et al*., 2009). The preferential colonization of the mucus by LGG was thus confirmed in the newly developed *in vitro* model.

Besides the preference of LGG to colonize the mucosal environment, a species-specific colonization of the mucosal environment was also demonstrated for other Lactobacilli. Both *L. mucosae* and *P. acidilactici* were detected in the luminal environment, but only *L. mucosae* was detected in the mucus (Fig. 2B). These results were confirmed during a short-term adhesion assay to mucin agar (Fig. 3), showing that *L. mucosae* adheres at a comparable rate as LGG, while *P. acidilactici* does not display significant adhesive potential. Interestingly, almost every study on *L. mucosae* spp. of human origin has pointed out the preference of this species to reside in the mucosal or tissue-bound intestinal microbiota. *L. mucosae* was previously isolated from human biopsies together with 29 other *Lactobacillus* strains and was found to be the second most adhesive strain (Kinoshita *et al*., 2007). Furthermore, when isolating Lactobacilli attached to the *in vivo* intestinal epithelium, 11 strains were isolated of which 8 were identified as *L. mucosae* (Fakhry *et al*., 2009). The strong mucus adhesion of *L. mucosae* was reported to be

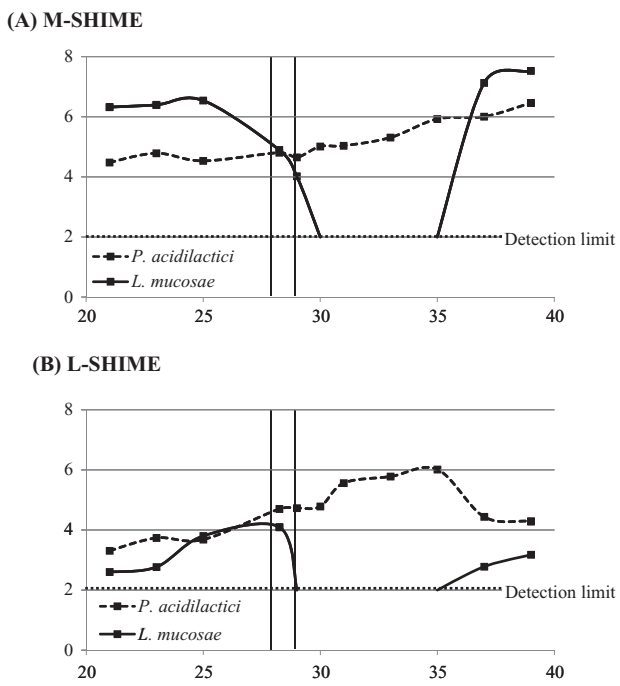

Fig. 4. Abundance of *Pediococcus acidilactici* and *Lactobacillus mucosae* (log cfu ml^{-1}) as determined with plate counts on a *Lactobacillus*-specific growth medium (LAMVAB) in the luminal content of the M-SHIME (A) and L-SHIME (B). Results are represented in function of the time after inoculation (days). An antibiotic pulse with 10 $\mu g\ l^{-1}$ tetracycline, amoxicillin and ciprofloxacin was applied on two consecutive days (day 28 and 29 after inoculation). The detection limit of the plate count-method was 2 log cfu ml^{-1}.

related to the mucus binding (*mub*) gene, which encodes for a cell-surface protein with mucus binding activity (Roos and Jonsson, 2002), probably common among all *L. mucosae* strains. Roos and colleagues (2000) assumed that *L. mucosae* is the source of the *mub* gene and (few) other Lactobacilli such as some *L. reuteri* strains were recipient during the course of evolution. In the present study we could also show that *L. mucosae* LB2 harbours a gene with considerable similarities to *mub* from *L. reuteri* and it is hypothesized that this gene is involved in the adhesion of LB2 to mucus and its colonization of the mucosal environment. *L. mucosae* is thus well equipped to reside in the MAMC. These traits of *L. mucosae* indicate that specific adhesion mechanisms are involved in the colonization and development of the *in vitro* mucosal microbiota.

The mucosal environment also influenced the long-term colonization of *L. mucosae* in the luminal compartment and enhanced its stability upon antibiotic treatment. Twenty-eight days post inoculation, the M-SHIME was enriched with the strongly adhesive *L. mucosae*, while the less adhesive *P. acidilactici* was more abundant in the L-SHIME. *L. mucosae* decreased below detection limit during the antibiotic treatment, while it was again detected when the antibiotic stress was removed. The recovery in the M-SHIME was more sudden and complete compared with the L-SHIME where it was more delayed (Fig. 4). This shows that the support of a mucosal environment might enhance community stability upon antibiotic treatment.

During the antibiotic treatment, the antibiotic-resistant *P. acidilactici* increased in abundance, while its abundance decreased afterwards. This finding is consistent with the recently proposed existence of an evolutionary stable strategy within the intestinal microbiota (Van den Abbeele *et al.*, 2011), meaning that the intestinal microbiota are a resilient microbial association. In the case of an antibiotic treatment, the antibiotic will directly or indirectly affect a vast amount of microbes so that their corresponding functions are endangered (e.g. *L. mucosae*). In contrast, functionally redundant microbes that were initially present in low amounts become more abundant and thus compensate this loss of function in order to maintain community functionality (e.g. *P. acidilactici*). After the perturbation, the existing evolutionary stable strategy may lead to a restoration of the initial community composition. This suggests that after further characterization *P. acidilactici* might be used during antibiotic treatments in order to maintain the Lactobacilli community and its associated functionalities.

Different *L. mucosae* strains have been shown to possess several characteristics, which support also their potential use as probiotic. First, *L. mucosae* could reach the colon upon oral administration as it survives the acidic conditions in the stomach and bile secretions in the small intestine (Beasley *et al.*, 2006; Fakhry *et al.*, 2009). In addition, we confirmed the strong mucus binding capability of *L. mucosae*, allowing the bacterium to reside in the mucus layer and prolong its colonization. Moreover, as *L. mucosae* has been show to produce antimicrobial compounds (other than pH lowering) towards pathogenic bacteria (Tzortzis *et al.*, 2004; Beasley *et al.*, 2006; Fakhry *et al.*, 2009) it could secrete these antimicrobial agents in the mucus layer, thus impacting the composition of the mucosal microbiota and avoiding interaction between pathogenic bacteria and the host. Additionally, *L. mucosae* can also bind to epithelial cells *in vitro* (without cytotoxic effects) and *in vivo* (Fakhry *et al.*, 2009), therefore having a great potential to closely interact with the host and modify the host's immune system. Although probiotic effects should not be generalized among strains of the same species, these findings suggest that *L. mucosae* could be a next-generation probiotic.

The new methodology using mucin-covered microcosms allowed a more relevant study of the long-term *in vitro* microbial colonization of the mucus layer, in the presence of a complex intestinal microbiota. The selective colonization of the mucosal region by the probiotic LGG was a first validation of our *in vitro* model. With this approach, we also found a species-specific colonization of the mucosal compartment by *L. mucosae*, a strain with promising probiotic properties. It will be interesting to unravel how other microbial groups colonize this *in vitro* model in order to obtain a more *in vivo*-like overall microbial community composition and activity. The model may be particularly useful when studying the composition and function of the mucosal microbiota during treatments with antibiotics or functional foods. Further, by inoculating with samples of healthy and diseased human subjects, mucosal microbes associated with particular diseases may be investigated. The model may also be applied to study mutants of microbes isolated from humans as it is ethically not allowed to test such mutants in their natural human host. In conclusion, incorporation of a mucosal environment in dynamic gut models may be a powerful tool to obtain a more realistic view on processes that drive the gastrointestinal microbiota.

Experimental procedures

Preparation of growth media and bacterial solutions

Unless stated otherwise, chemicals were obtained from Sigma (Bornem, Belgium). The SHIME feed contained (in g l^{-1}) arabinogalactan (1.0), pectin (2.0), xylan (1.0), starch (3.0), glucose (0.4), yeast extract (3.0), peptone (1.0), mucin (4.0) and cystein (0.5). Pancreatic juice contained (in g l^{-1}) $NaHCO_3$ (12.5), bile salts (6.0) (Difco, Bierbeek, Belgium) and pancreatin (0.9). Mucin agar was prepared by boiling autoclaved distilled H_2O containing 5% porcine mucin type II and 1% agar. The pH was adjusted to 6.8 with 10 M NaOH.

Pure cultures of Lactobacilli were grown overnight in MRS medium at 37°C under aerobic conditions.

Dynamic gut model (SHIME) and simulation of a mucosal environment

The SHIME is a dynamic *in vitro* model of the human intestinal tract, composed of five double-jacketed vessels, respectively simulating the stomach, small intestine and the three colon regions. In this experiment, only the first colon compartment was used (Fig. 5). Two SHIME units were used in parallel ('Twin-SHIME') in order to obtain identical environmental conditions and identical microbial composition and activities for both units (Van den Abbeele *et al.*, 2010). Whereas the first unit consisted of the conventional set-up that only harbours luminal microbes (= luminal SHIME or L-SHIME), the second unit was modified by incorporating a mucosal environment (= mucosal SHIME or M-SHIME). In order to achieve a representative mucosal surface in the M-SHIME, 100 mucin-covered microcosms were added per 500 ml luminal suspension. The microcosms (length = 7 mm, diameter = 9 mm, total surface area = 800 m^2/m^3, AnoxKaldnes K1 carrier, AnoxKaldnes AB, Lund, Sweden) were coated by submerging them in mucin agar. To simulate the renewal of the mucus layer, half of the mucin-covered microcosms were replaced daily by sterile ones.

The ascending compartment (500 ml) from both SHIME units was inoculated with 40 ml of a 1:5 dilution of fresh stools provided by a healthy human volunteer (25 years) who had no history of antibiotic treatment 6 months before the study. Inoculum preparation was done as previously described by Possemiers and colleagues (2004). Three times per day, 140 ml SHIME feed and 60 ml pancreatic juice were added to the stomach and small intestine respectively.

Experimental design

In a first experiment, the luminal and mucosal microbiota were sampled 1 day after inoculation for total and *Lactobacillus*-specific community analysis. The dominant Lactobacilli were isolated and the following characteristics were determined: (i) preferential colonization of mucosal environment compared with luminal content, (ii) adhesion capacity to mucin agar, and (iii) MIC for tetracycline, amoxicillin and ciprofloxacin. In a second experiment, the luminal and mucosal microbiota were allowed to stabilize during 3 days after inoculation after which a single dose (5×10^7

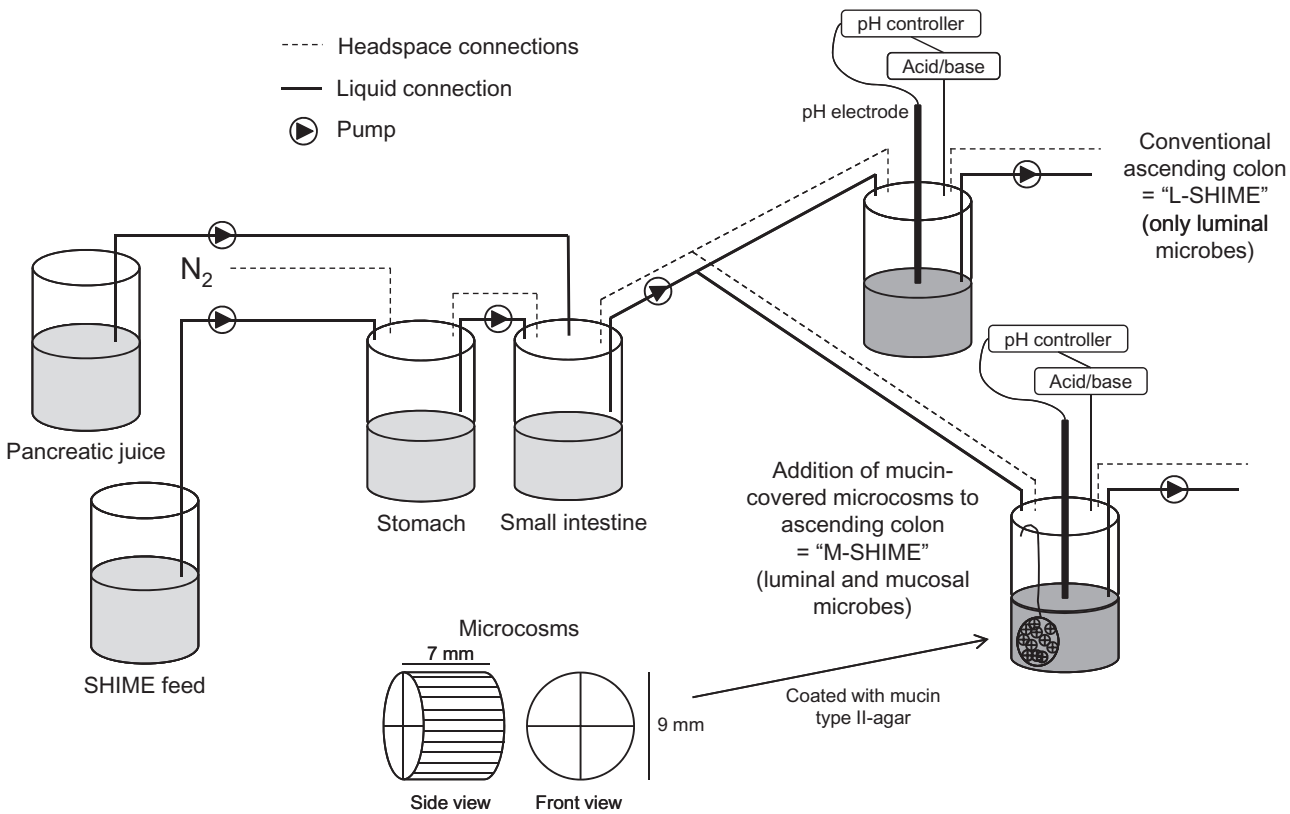

Fig. 5. The new experimental design was based on the SHIME, a dynamic *in vitro* model of the human gastrointestinal tract, composed of several double-jacketed vessels, simulating the stomach, small intestine and three main colon regions. In this experiment, only the first colon compartment (ascending colon) was used and inoculated with a human faecal microbiota. The first ascending colon unit consisted of the conventional set-up that only harbours luminal microbes (= luminal SHIME or L-SHIME), whereas the second unit was modified by incorporating a mucosal compartment (= mucosal SHIME or M-SHIME), which contained 100 mucin-covered microcosms per 500 ml suspension. Both units were run in parallel in order to attain identical environmental conditions and identical microbial composition and activities for both units.

cfu ml^{-1}) of *L. rhamnosus* GG (LGG – LMG 18243) was administered. Samples for Lactobacilli community analysis were collected on day 3 (both before and after inoculation of LGG), 4, 6 and 8. For a third experiment, the microbiota were allowed to stabilize during 28 days after which an antibiotic pulse was applied on two consecutive days (28 and 29). For the antibiotic pulse, 1 ml of stock solution (1 mg ml^{-1} tetracycline, 1 mg ml^{-1} amoxicillin and 1 mg ml^{-1} ciprofloxacin) was added per 100 ml SHIME suspension, thus reaching a final concentration of 10 μg ml^{-1} for each antibiotic. During all experiments, the luminal and mucosal Lactobacilli communities were monitored by means of culture-based quantification (plate counts) and DNA-based fingerprinting analysis (DGGE).

Microbial community analysis: plate counts, DGGE and flow cytometry after live–dead staining

Lactobacilli were isolated or quantified by selective plating on LAMVAB agar (Hartemink *et al.*, 1997). Luminal samples were serially diluted in physiological solution (8.5 g l^{-1} NaCl), transferred to the plates and incubated aerobically at 37°C during three days ($n = 3$). Mucosal samples were suspended in physiological solution (2 g in 20 ml) and homogenized with a stomacher before preparing the serial dilutions.

Denaturing Gradient Gel Electrophoresis (DGGE) was applied to monitor qualitative shifts within the mixed microbial community. DNA extractions on luminal (1 ml) and mucosal samples (0.5 g) were performed on according to Boon and colleagues (2003). DGGE was applied to separate PCR products of 16S rRNA genes of the total community obtained with general bacterial primers (338F-GC and 518R) or PCR products of the Lactobacilli community obtained with nested PCR (PCR with *Lactobacillus*-specific primers SGLAB0159F SGLAB0667R, 1:100 dilution, PCR with general bacterial primers) (Possemiers *et al.*, 2004). Gels had a denaturating gradient from 45% to 60% and were run on an Ingeny PhorU apparatus (Ingeny International, Goes, The Netherlands). Further analysis was carried out using BioNumerics software version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). Pearson correlation and UPGMA clustering algorithm were used to calculate dendrograms, taking into account both band position and band density.

Pure cultures were quantified with flow cytometry after live–dead staining. 10 μl bacterial suspension was added to 960 μl filter-sterilized (0.22 μm) Evian mineral water, 10 μl Na_2EDTA (500 mM, pH 8.0), 10 μl Cytocount beads (Dako, Glostrup, Denmark) and 10 μl staining stock solution (1 ml = 970 μl filtered DMSO, 10 μl SYBR Green and 20 μl Propidium Iodide) (Invitrogen, Merelbeke, Belgium). After 15 min in the dark, live/dead cells and beads were counted with a CyAnADP flow cytometer (Dako, Glostrup, Denmark).

Adhesion experiment on mucin agar

Overnight-grown microbial cultures were diluted to an optical density (610 nm) of 0.5 with filter-sterilized Evian using a Sunrise multi-well spectrophotometer (Tecan, Mechelen, Belgium). The mucin adhesion assay was performed as recently described (Van den Abbeele *et al.*, 2009). Briefly, bacterial cells were washed three times with physiological solution and immediately thereafter, 1 ml of bacterial suspension and 2 ml of physiological were added to six-well microtitre plates covered with mucin agar. The bacteria were allowed to adhere to this mucin layer under anaerobic conditions, at 37°C and under slight agitation. After 80 min incubation, non-adhered bacteria were removed, each well was rinsed three times and the remaining adhered bacteria were detached using Triton X-100. The amount of initially added and adhered bacteria was quantified using flow cytometry after live–dead staining.

Determination of the MIC

Minimal inhibitory concentration values for tetracycline, amoxicillin and ciprofloxacin were determined on Mueller-Hinton agar plates containing twofold serial antibiotic dilutions ranging from 128 to 0.06 μg ml^{-1}. The antibiotics were dissolved in appropriate solvents and further diluted in distilled water as outlined in the National Committee for Clinical Laboratory Standard guidelines (NCCLS). The reference strains used for determination of MIC values were *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213). Bacteria were suspended in physiological solution to an optical density of 0.5 and diluted 1:10. Approximately 10^5 cfu were inoculated on the plates, which were incubated for 24 h under anaerobic conditions and at 37°C. The MIC was defined as the lowest concentration producing no visible growth.

16S rRNA gene sequencing

To obtain phylogenetic information of the isolates, almost full 16S rRNA gene sequences were determined as described by Eeckhaut and colleagues (2008). Briefly, DNA of colonies was extracted with an alkaline lysis procedure and amplicons were obtained using primers fD1 and rD1. Purified amplicons were sequenced using the BigDye Terminator sequencing kit (primers pD, Gamma*, 3 and O*) on an ABI PRISM 310 Genetic Analyzer. Sequences with the highest similarity were found using the National Centre for Biotechnology BLAST search. Sequences generated in the study were submitted to the European Nucleotide Archive under accession numbers FR693800 (*L. mucosae* LB2) and FR693801 (*P. acidilactici* LB1).

Detection of mucus binding protein genes

Presence of the mucus binding protein gene, mub, earlier characterized in *L. reuteri* 1063 (Roos and Jonsson, 2002) were examined by PCR using primers slightly modified from MacKenzie and colleagues (2010). Bacteria were grown anaerobically on MRS agar for 16 h at 37°C. Bacterial colonies (1 μl) were collected with a sterile plastic loop and suspended in 100 μl sterile water. The PCR was run using PuReTaq Ready To Go PCR beads (GE HealthCare) and primers detecting mub1 (MucB1-RVIfm 5′-CAAGAAGCTC AAGCCATC-3′ and MucB2-RVIrm 5′-ATCAAGCTTCTTGT AGGT-3′) and mub2 repeats (MucB2-R4fm 5′-GGTACGAA GACGCTGAC-3′ and MucB2-R4rm 5′-GGCATCAGCCGTG TAGA-3′) (0.4 μM of each). Bacterial suspension (0.5 μl) was

added to the PCR mix and the reaction included following program 95°C, 5 min; 35 × (95°C, 30 s; 48°C, 30 s; 72°C, 60 s); 72°C, 10 min. *L. reuteri* 1063 was used as a positive control. The PCR products were separated and visualized using standard agarose gel electrophoresis. The fragment obtained with the mub1 repeat primers was sequenced using the same primers as in the PCR and the sequence of *L. mucosae* LB2 has been deposited to GenBank and has the accession number HQ326232.

Statistics

All data were analysed using SPSS 16 software (SPSS, Chicago, USA). Before investigating probability of intergroup differences, normality and homogeneity of variances were studied with a Kolmogorov–Smirnov and Levene test respectively. If so, an Anova with (*post hoc*) Bonferroni test was performed, while otherwise a Kruskal–Wallis with Mann–Whitney test was applied. Differences were considered significant if $P < 0.05$.

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