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Characterization of *Lactobacillus* as probiotic from human intestine

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Absract

In order to perform a selective isolation of bacteria tightly bound to the human gut, ileal biopsies of healthy volunteers were treated to wash out the mucus layer and loosely bound bacterial cells. Rod-shaped, anaerobic bacteria that had remained attached to the epithelial cells were isolated and identified at the species level by biochemical tests. One isolate was identified as *Bifidobacterium breve*, while all the others were Lactobacilli of only two species, *Lactobacilus mucosae* and *L. gasseri*. Members of these species were found previously along with many others in intestinal samples but their predominance among bacteria strictly associated to the epithelium, as shown here, was not suspected before and suggested that these species may represent a specific sub-population of tissue-bound bacteria. A series of physiological tests was performed and indicated that all isolates were able to produce antimicrobial activity against selected pathogens and survive simulated gastric conditions. All isolates were able to grow and produce biofilm in intestinal fluid after exposure to gastric conditions. Those isolates can be proposed as potential probiotic strains for human use.

Keywords: *Lactobacillus*, gut, commensals, probiotics, mucus

توصيف عصيات اللبن المعزولة من أمعاء الانسان كمعززات حيوية

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الخلاصـة

جمعت عزلات بكتيرية مختارة عن طريق غسل الطبقة المخاطية وأخذ قطعة حية من معدة ولفائفي المتبرعين الاصحاء. تم عزل البكتريا الملتصقة بالخلايا الطلائية ذات الشكل العصوي واللاهوائية المعيشة، وتشخيصها بواسطة الاختبارات الكيموحيوية. شخصت عزلة واحدة تعود الى النوع *Bifidobacterium breve* بينما بقية العزلات البكتيرية تعود الى نوعين من Lactobacilli هما *L. mucosae* و *L. gasseri*، حيث تتواجد هذه الانواع من البكتريا في العينات المأخوذة من الامعاء، في حين يكون الشكل السائد لها هو الالتصاق بالطبقة الطلائية. ومما لاشك فيه ان هذه الانواع من البكتريا تمثل المجاميع المرتبطة بالانسجة. تشير الاختبارات الفسيولوجية الى ان هذه العزلات البكتيرية لها القابلية على انتاج مواد مضادة لنمو الممرضات وتتحمل البقاء في الظروف الموجودة في المعدة، اضافة الى قدرتها على تكوين طبقة من الغشاء الحيوي في السائل المعوي عند تعرضها للظروف الموجودة في المعدة وكذلك تعمل كمعززات حيوية فعالة في الانسان.

Introduction

The human intestinal microflora establishes a complex symbiotic interaction with epithelial and immune cells of the gastrointestinal tract (GIT). In this interaction the microbial role is essential in providing nourishment, forming a first line of defense against

invasion by pathogenic organisms, regulating epithelial development and inducing innate immunity (7). These contributions are reciprocated by stable conditions of temperature, pH, osmolarity and food supply for the microorganisms. Recent metagenomic experiments have indicated that the vast majority of the intestinal bacteria belong to two phyla, Firmicutes, including the large class of Clostridia and the lactic acid bacteria (LAB) and Bacteroidetes (7, 15). Most of these organisms are anaerobes and not cultivable in laboratory conditions and only 0.1% of the total gut bacteria are facultative anaerobes (7). However, the composition of the gut microbiota is known to vary transiently as a consequence of diet changes, enteral infections, antibiotic or anti-acid treatments and immune suppression (18). Recently, it has been shown that phylum-level changes in the microflora are associated with diseases, such as obesity (19). In particular, the coexistence of H2-producing bacteria and H2-utilizing methanogenic Archaea in the GIT of obese individuals induced to hypothesize that H2 transfer between Eubacteria and Archaeal species increases the energy uptake by the large intestine of obese individuals (23). At the species level a large diversity exists in the human gut. It is known that the type of neonatal feeding influences the species composition of the microflora (4) and that in the elderly a general reduction in species diversity occurs, with an increase of facultative anaerobes and a decrease of Lactobacilli and Bifidobacteria (11, 21). Lactobacilli and Bifidobacteria, were the most frequently used as probiotic products for human consumption. Lactobacilli, with all other LAB, are low G+C, gram-positive and belong to the phylum of the Firmicutes, while Bifidobacteria are high G+C, gram-positives and belong to the phylum of the Actinobacteria (20). Bifidobacteria, like all Actinobacteria, are not numerically important in the intestine, their number is higher in breast-fed than in formula-fed infants and decreases in adults (4, 18). In spite of their number, Bifidobacteria are thought to play a relevant role in keeping the gut health (13) and cells of Bifidobacterium longum have been shown in reduction of intestinal inflammation by efficiently inhibiting pancreatic and neutrophilic elastases (9). Not much attention has been given so far to whether bacteria isolated from the animal gut bind tightly or loosely to the epithelium and whether physical interactions between bacterial cells and animal tissue is relevant for probiotic activity of intestinal microflora. To gain insight into this problem we followed a previously reported procedure (17) to separate the mucus layer from the epithelial tissue of ileal biopsies of healthy human volunteers and found that only a small minority of ileal bacteria remains attached to the human cells after this treatment. Among these tightly bound bacteria we focused our attention on eleven anaerobic rod-shaped strains that would more likely display a probiotic function in the gut and report here on some of their relevant properties.

Materials and methods

Collection of ileal samples

Mucosal samples were collected by forceps biopsy in the distal ileum from 7 adult human volunteers (M/F 4/3, mean age ± SD 45.0 ± 13) undergoing routine diagnostic endosocopy for colorectal cancer (CRC) screening. All patients recruited gave their informed consent to the study. The patients did not follow any special dietary regimen, and had not recently received any antibiotic or probiotic treatment. Samples were stored in phosphate-buffered saline (PBS) containing 15% glycerol before subsequent analysis. Endoscopic appearance as well as histology of the ileum was normal in all patients.

Bacterial isolation and culture conditions

Ileal samples (10-20 mg/each) were treated with DTT as previously reported (18), extensively washed with PBS to eliminate loosely attached bacteria. Tissue samples after the washes, as well as buffer recovered from each wash, were plated on deMan, Rogosa and Sharpe medium (MRS) (Difco) in anaerobic condition to isolate lactic acid bacteria. Anaerobic conditions were obtained by incubating liquid and solid cultures in an anaerobic chamber (Oxoid).

Physiological and biochemical analysis

Exponentially growing cells of the various isolates were used for biochemical analysis by the use of API 50 CHL Kit (Biomerieux) following the manufacturer's instructions. For biofilm formation bacteria were grown in modified TSB (mTSB) medium as described by Lebeer et al., (2007): 15 g/liter TSB (BD Biosciences) enriched with 20 g/liter Bacto peptone No. 3 (BD Biosciences). Antimicrobial activity was determined as previously described (2) with the following modifications: 10 µl of each culture in stationary growth phase were spotted on the surface of an MRS agar plate and the spots air dried. A 100 µl of an exponential culture of the indicator bacterial strain obtained from laboratories of hospitals, were mixed with 5 ml of soft agar (0.7%) and poured over the plate. The plates were incubated aerobically overnight at

37°C and the inhibition halos were measured and expressed in mm.

Simulated gastric and intestinal fluids

Gastric and intestinal fluids (SGF and SIF) were simulated as previously reported (8). Exponentially growing cells were washed, resuspended in SGF (PBS 0.5% w/v, pepsin 3 g/l, pH 2.0) or SIF (PBS 0.5% w/v, pancreatin 1 g/l, pH 8.0) and incubated 1 hour at 37°C. Cells were then diluted, plated on MRS plates and incubated at 37°C in anaerobic conditions. To monitor growth in simulated intestinal condition after gastric treatment, exponentially growing cells were washed, resuspended in SGF (PBS 0.5% w/v, pepsin 3 g/l, pH 2.0) and incubated 1 hour at 37°C. Then cells were diluted at 0.05 OD600nm in MRS supplemented with pancreatin (1 g/l, pH 8.0), incubated at 37°C in anaerobic conditions and growth monitored after 18 hours.

In vitro biofilm assay

Biofilm formation was assayed as in reference (15). Briefly, a platform carrying 96 polystyrene wells was filled with 200 µl medium. Approx. 3x107 CFU were added and incubated without shaking for 24 h at 37°C. To quantify biofilm formation, the wells were briefly washed in phosphate-buffered saline (PBS). The remaining attached bacteria were stained for 30 min with 200 µl 0.1% (w/v) crystal violet in an isopropanol-methanol-PBS solution (1:1:18 v/v). Excess stain was washed with water. Wells were air dried (30 min), the dye bound to the wells was extracted with 200 µl ethanol-acetone (80:20 v/v) and the optical density (OD) was measured at 570 nm. Each strain was tested in at least three independent experiments, each with three biological replicates. Data were normalized to the indicated positive control (LGG), which was taken as 100% to compare different experiments. Additionally, sterile medium was always included (negative control)

Results

Isolation of Lactobacilli from ileal epithelial cells

In order to restrict our isolation to bacteria tightly associated to the human tissue, samples of ileal biopsies of healthy human volunteers, collected as described in Materials and Methods, were treated with DTT and extensively washed with PBS to eliminate the mucus layer, as previously reported (18). Biopsy samples, as well as buffers recovered from each wash, were used to isolate lactic acid bacteria (LAB) by plating on MRS medium in anaerobic condition. The majority of the bacteria present in the ileal samples were found in the buffer recovered after the washes with an average of 3.54x103/mg and 2.21x103/mg of aerobic bacteria and LAB, respectively. A lower number of bacteria with an average of 16/mg and 10/mg of aerobic bacteria and LAB, respectively, were found tightly attached to ileal epithelial cells. Bacteria able to grow anaerobically on MRS medium were analyzed for their colony morphology on MRS plate, Gram-staining and catalase phenotype. Only one colony of those apparently identical from each ileal sample was selected, analysed for their cell shape under the light microscope and divided into two groups: round and rod-shaped bacteria. By this procedure we ended up with 24 isolates: 13 round-shaped and 11 rod-shaped. With the aim of characterising potential probiotic Lactobacilli, we focused our attention on the rod-shaped bacteria that were characterised at the species level by biochemical tests (API gallery). As a result of the eleven rod-shaped isolates, one isolate was identified as Bifidobacterium breve, while the remaining ten were belonged to Lactobacilli, containing L. mucosae (eight isolates) and L. gasseri (two isolates) (Table 1), only strains SF1087, SH1091 and SH1108, belonging to the same species and coming from the same ileal biopsy, could be potential siblings.

Table 1. Rod-shaped bacteria tightly associated to ileal epithelial cells

Biopsy No.	Strain name	Species
1	SH1031	*Lactobacillus mucosae*
1	SH1036	*Bifidobacterium breve*
2	SH1087	*Lactobacillus mucosae*
2	SH1091	*Lactobacillus mucosae*
2	SH1108	*Lactobacillus mucosae*
2	SH1109	*Lactobacillus gasseri*
3	SH1111	*Lactobacillus mucosae*
4	SH1146	*Lactobacillus mucosae*
5	SH1183	*Lactobacillus gasseri*
6	SH1232	*Lactobacillus mucosae*
7	SH1233	*Lactobacillus mucosae*

[a] Assessed on the base of biochemical tests.

Production of antimicrobial activity

All Lactobacilli were analysed for the production of antimicrobial molecules active against selected pathogens (Table 2). An exponential culture of each of the 11 isolates was used to 'spot' sterile LB plates. As previously reported (2), the spots were air-dried and used to overlay soft agar (0.7%) containing exponential cells of one of the indicator strains obtained. Solidified plates were then incubated at the appropriate temperature for 18-24 hours and the appearance of a growth-inhibition halo taken as an indication of the presence of an antimicrobial activity. As summarized in Table (2), all strains produced antimicrobial molecules, active against the Gram-positive and the Gram-negative pathogens used in our study. As a control we used strains LGG, It is interesting to note that, while all our ileal isolates produced similar antimicrobial activities, the three *Lactobacillus* strains from other sources showed a more heterogeneous profile of production of antimicrobial molecules.

Table 2. Antimicrobial activity against selected pathogens

Strain	*Bacillus cereus*	*Staphylococcus aures*	*Listeria monocytogenes*	*Salmonella typhimurium*	*Shigella sonni*	*Escherichia coli*
SH1031	++	++	++	++	++	+
SH1036	++	++	++	++	++	++
SH1087	++	++	++	++	++	+
SH1091	++	++	++	++	++	++
SH1108	+	++	++	++	+	++
SH1109	++	++	++	++	++	++
SH1111	++	++	++	++	++	++
SH1146	+	++	++	++	++	++
SH1183	++	++	++	++	++	++
SH1232	++	++	++	++	++	++
SH1233	+++	+++	++	++	++	++
LGG	++	+	++	+/-	+/-	++

Resistance to simulated GIT conditions

We measured the survival of all Lactobacilli isolates in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) as previously reported (8). Almost identical numbers of cells were recovered on MRS plates from treated and untreated cells of all 11 isolates, indicating an almost total resistance to both treatments. We then analysed whether cells exposed to simulated gastric fluid were still able to grow in simulated intestinal conditions. To this aim exponentially growing cells of all 11 isolates were exposed to SGF for 1 hour, then diluted to 0.05 OD_{600nm}, and incubated anaerobically in MRS (pH8.0) supplemented with pancreatin (1 g/l, pH 8.0) and the optical density measured every hour for 18 hours. All strains were able to grow and very similar data were collected for all strains. Figure (1) reports the growth curves of treated and untreated cells for four representative strains. In all cases a long lag phase was observed, but once that growth was started the growth rates appeared similar to that of untreated cells. We cannot exclude the possibility that pancreatin inhibited growth and that after 8-14 hours the enzyme was no longer active, thus allowing the cells to enter the exponential growth phase. However, in such case we would expect cell growth to start at similar time points in all samples. As can be observed in Figure (1), for the various isolates cell growth started at different time points (i.e. about after 8 hours for B. breve, about 11 hours for L. mucosae SH1233 and about 13 hours for L. mucosae SH1091 and L. gasseri). Since we did not observe any mortality on plates due to the treatment in SGF conditions, we believe it is unlikely that the long delay is due to reduced number of live cells after the SGF treatment. For these reasons we favor the idea that the long lag phase experienced by cells subjected to SGF and then grown in SIF was due to the need of treated cells to adapt their metabolism to the new growth conditions.

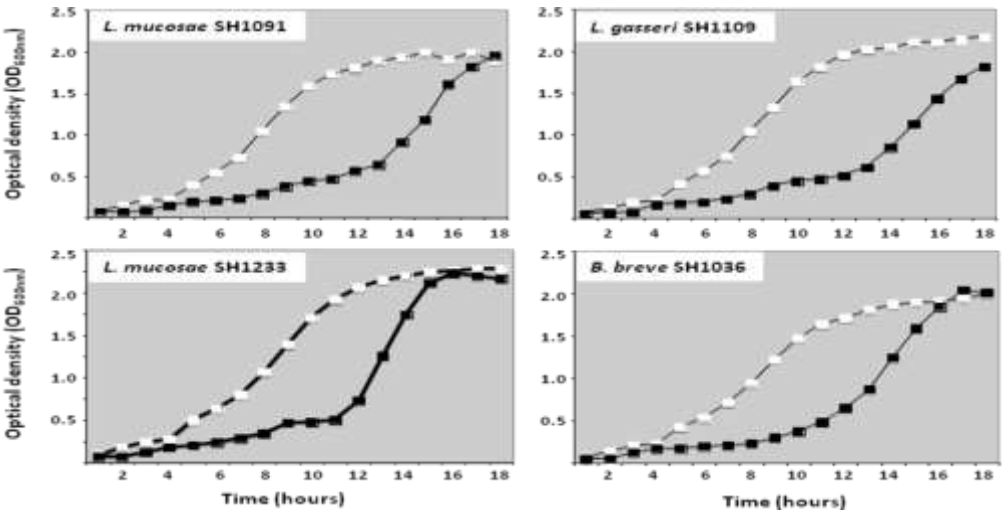

Figure 1: Growth curves of treated (closed symbols) and untreated (open symbols) cells. Overnight cultures of the various strains were either treated with pepsin at pH 2 for 1 hour at 37°C or left in MRS broth at 37°C, then diluted to 0.05 OD_{600nm}, and used to inoculate MRS (open symbols) or MRS pH 8-supplemented with pancreatin (closed symbols) and OD measured every hour for 18 hours.

Biofilm formation

All 11 isolates of Lactobacilli were tested for their ability to form biofilm in microtiter plate assays (14). *L. rhamnosus* GG (LGG), one of the clinically best-studied probiotic organisms (3) and a known biofilm producer (14), was used as a reference strain. Since biofilm formation is known to depend on environmental conditions (14), we measured the production of biofilm after exposure of our isolates to simulated gastric fluid and in simulated intestinal conditions, as described above. As reported in Figure (2), all isolates produced a biofilm and the SGF-SIF treatments did not affect significantly the amount of biofilm produced. Production of biofilm, when bacteria was transited the stomach tract and colonize the intestine was an important feature for a potential probiotic strain, since biofilms have protective and adhesive properties and have been associated to a longer persistence of bacteria in the GIT of animals (8, 14).

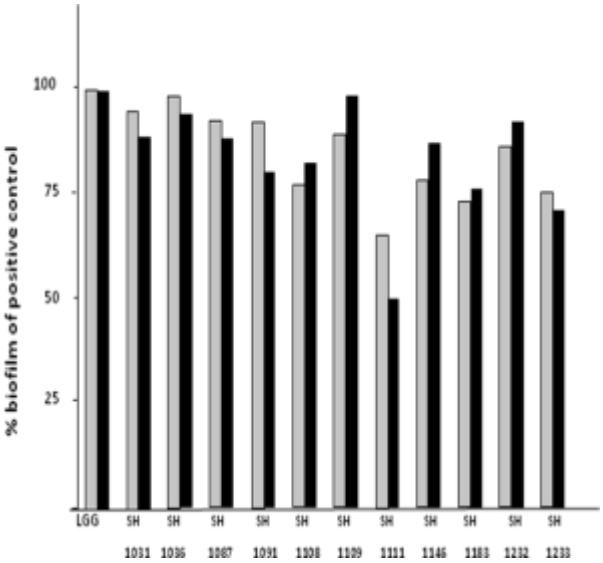

Figure 2: Biofilm formation. The abilities of biofilm formation by the various isolates are expressed in comparison with that of strain LGG. Values obtained for strain LGG were taken as 100%. Biofilm formation was monitored in MRS broth (grey bars) and in MRS pH 8-supplemented with pancreatin (black bars). The data shown are representative of at least three independent experiments, each with three biological replicates.

Discussion

Ileal biopsies from healthy human volunteers were used to isolate anaerobic bacteria specifically and tightly bound to the ileum. Following a previously reported procedure (17) we separated the epithelial cells from the mucus layer. Most bacteria were associated to the mucus and only a minority remained attached to the epithelial cells. We characterized the anaerobic bacteria attached to the epithelial cells and, in particular, focused our attention on the rod-shaped ones. Only one colony of those apparently identical from each ileal sample was selected for further characterisation. As a result, eleven rod-shaped isolates were identified to the species level as *Bifidobacterium breve* (1 isolate), *Lactobacillus gasseri* (2 isolates) and *Lactobacillus mucosae* (8 isolates). Since most isolates either come from different ileal samples or belong to different bacterial species (Table 1) only strains SH1087, SH1091 and SH1108 could be potential siblings. However, further characterization of those three strains indicated that SH1108 behaves differently with respect to the other two for mucin-degradation activity (Table 3) and therefore, only SH1087 and SH 1091 could be potential siblings. *L. mucosae* and L. gasseri are normal inhabitants of the gastrointestinal tract of humans (10) and various other mammals (6, 12, 16). *L. gasseri* is a well characterised species, known to represent the major homo fermentative Lactobacillus species of the human intestine (14, 19). *L. mucosae* is, instead of a poorly characterized species, firstly isolated in a study aimed at the isolation of L. reuteri (18). The similarities between *L. reuteri* and *L. mucosae* also depend on the presence in both species of the mub gene, encoding a cell-surface protein with mucin-binding activity. It is interesting that the G+C content of mub of *L. gasseri* is similar to the overall G+C content of *L. mucosae* and different from that of other *L. reuteri* genes. Based on this and on the observation that the mub gene has been found in all *L. mucosae* but only in some *L. reuteri* strains, Ross et al., 2000 suggest that *L. mucosae* may be the source of mub and *L. reuteri* is a recipient of the gene at some point during the course of evolution. In all studies in which *L. mucosae* and *L. gasseri* have been found associated to intestinal samples, they had never been indicated as predominant in number with respect to other Lactobacillus species. In a study by Kinoshita et al., (12), for example, *L.* gasseri and *L. mucosae* were identified as 10 and 3% of the total LAB population, respectively, in human colonic mucin. Although the low number of isolates of our study does not allow us to draw statistically relevant conclusions, we propose that the extensive washing and DTT treatment we performed on the ileal samples removed most of the loosely attached bacteria, allowing us to isolate a sub-population of bacteria tightly associated to the epithelial cells. *L. mucosae* and *L. gasseri* would then be predominant species among those bacteria tightly attached to the epithelial cells of the ileal tract of the human intestine. Our hypothesis is supported by the extremely similar physiological properties that characterize this sub-population of intestinal bacteria: i) all produce antimicrobial molecules similarly active against all tested pathogens. This is a peculiar feature since LAB from different sources have a more heterogeneous profile of antimicrobial activity (see strain LGG); ii) all are resistant to simulated gastric fluid and able to grow efficiently in simulated intestinal fluid after exposure to gastric conditions; iii) all produce biofilm also in conditions that mimick gastric and intestinal environments. The tight association with the epithelial cells, presumably indicative of a direct interaction with the host cell, together with the physiological properties discussed above makes the sub-population of intestinal bacteria that we isolated very promising as probiotic strains for human use. A future challange will be to verify their presence and biologic behaviour in human inflammatory conditions such as Inflammatory Bowel Diseases.

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