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ORAL MICROBIOLOGY
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# Molecular and biochemical characterizations of human oral lactobacilli as putative probiotic candidates

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**Introduction:** The objective of this study was to characterize the lactobacilli from the human oral cavity as a potential source of probiotic strains.
**Methods:** Samples were collected from four different locations within the oral cavity: surface of healthy tooth, oral mucous membrane, surface of tooth decay and deep tooth decay. On the basis of morphological and biochemical properties eight categories were formed and 26 isolates were selected for further characterization. The isolates were determined as *Lactobacillus* sp. using primers specific for 16S rDNA. Sequencing of 16S rDNA genes and repetitive sequence-based polymerase chain reactions were used for determination to species and subspecies levels.
**Results:** Predominant species were *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus salivarius* and *Lactobacillus paracasei* subsp. *paracasei*, while *Lactobacillus acidophilus*, *Lactobacillus cellobiosus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus gasseri* were also present. The isolates *Lactobacillus salivarius* BGHO1, *Lactobacillus fermentum* BGHO36 and BGHO64, *Lactobacillus gasseri* BGHO89 and *Lactobacillus delbrueckii* subsp. *lactis* BGHO99 exhibited antagonistic action on the growth of *Staphylococcus aureus*, *Enterococcus faecalis*, *Micrococcus flavus*, *Salmonella enteritidis*, *Streptococcus pneumoniae* and *Streptococcus mutans*, but not on growth of *Candida albicans.* Moreover, the isolates *L. salivarius* BGHO1 and *L. gasseri* BGHO89 were tolerant to low pH and high concentration of bile salts.
**Conclusion:** Taken together, these findings imply that *L. salivarius* BGHO1 and *L. gasseri* BGHO89 might be subjects for additional investigation as potential probiotic strains.

Key words: 16S rDNA; lactobacilli; oral microflora; probiotic; repetitive sequence-based polymerase chain reaction

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In recent years, selected probiotic strains have been thoroughly investigated for their specific health effects. The term probiotic was defined recently by an Expert Committee as 'Living microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition' (5). In particular, lactobacilli are considered safe based on their taxonomic position and are generally used as probiotics. Human origin is thought to be important for host-specific interactions by the probiotic. The main criteria for selecting probiotic strains are their acid and bile tolerance, survival through the gastrointestinal tract, ability to adhere to intestinal surfaces, temporary colonization, and antagonism against pathogens. Finally, potential probiotics need to have good technological properties (17).

Probiotics have traditionally been used to treat diseases related to the gastrointestinal tract. However, in principle any part of the body that harbours normal microflora can be a potential target for specific probiotics. The oral cavity has a microflora with a similar complexity to that of the

intestinal microflora. Here too, some of the members of the normal microflora have a detrimental effect on the host, for example causing dental caries or periodontal disease (13, 15).

Over the past 10 years the probiotic field has been exploited with a number of new strains isolated from different ecological niches. The bacterial species that are currently of commercial interest mainly belong to the genus *Lactobacillus*. Particular interest has been directed by the dairy industry to the survival and health effects of a relatively limited collection of strains of these bacteria. There have been some attempts to use intestinally derived bacteria such as lactobacilli for oral cavity probiotics. However, studies in humans reported that the bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus rhamnosus* GG, *Lactobacillus casei* or *Lactobacillus acidophilus*, all present in fermented milk products, cannot colonize the mouth (18, 26). It appears more likely that bacteria isolated directly from the oral microbiota, such as *Streptococcus salivarius*, will be useful for such purposes (3). Studies on probiotic strains of lactobacilli originating from the human oral cavity are limited (10). In this study we have collected putative probiotic strains of lactobacilli from the human oral cavity to select strains that could potentially be used and have investigated their interactions with oral and gut microflora *in vivo*, as well as their ability to colonize the human mouth and gastrointestinal tract.

## Materials and methods

### Subjects and sampling

Samples were collected from 14 healthy human subjects (six male and eight female, age 20–54 years, mean 27.6 years) who maintained their usual lifestyles. Subjects did not follow any special dietary regimen, and did not include yoghurt or other milk products in the diet for 24 h before sampling. Samples were collected from the oral mucous membrane, the surface of a healthy tooth, the surface of a carious lesion and a deep carious lesion and were transported immediately to the laboratory. A dilution series ($10^{-1}$ to $10^{-7}$) was made in sterile NaCl solution (9 g/l) and 100-µl portions of each dilution were inoculated onto MRS plates (Difco, Detroit, MI). The plates were incubated anaerobically in jars with Anaerocult A (Merck, Darmstadt, Germany) at 37°C for 48 h. After incubation 10 colonies were randomly selected from each sample and were subcultured onto MRS plates for further analysis. The number of randomly selected isolates examined per sample was chosen on the basis of the publication of Hartely et al. (6), who reported that 10 colonies gave an adequate representation of the major bacterial strains.

### Bacterial strains, media and culture conditions

The bacterial strains used in this study are listed in Table 1. *Lactobacillus* strains were generally grown on MRS (Difco) at 30°C or 37°C, depending on the strain. *Lactobacillus lactis* strains were grown in M17 (Merck) with 5 g/l glucose at 30°C. The indicator strains, other than lactic acid bacteria, were cultivated in the following media (all obtained from Torlak, Belgrade, Serbia): *Staphylococcus aureus* on Baird Parker agar, *Salmonella typhimurium* on Wilson–Blair medium, *Bacillus* sp. on Columbia agar with the addition of 5% horse blood, and *Streptococcus pneumonie* on blood agar with triptone–peptone (15 g/l), extract of bovine heart (3 g/l), NaCl (5 g/l) and 7% sheep blood. Other pathogenic strains were cultured on Mueller–Hinton medium (Torlak).

### Provisional identification of lactobacilli

Provisional identification was based on the ability of the isolates to grow in the MRS broth, and also on a gram-positive, rod-shaped, non-sporing cell morphology and a negative catalase reaction (7). Gram-positive and catalase-negative rods (110 in total) were initially characterized by morphology and simple physiological tests as follows:

1. colony morphology and pigmentation;
2. growth at 15 and 45°C in MRS broth (the tests were performed three times);
3. salt tolerance: 4%, 6.5% and 8% NaCl in MRS (the tests were performed three times);
4. production of carbon dioxide from glucose by sub-culturing the isolates in tubes with MRS broth containing Durham's bells;
5. citrate-utilizing bacteria and diacetyl production (4, 7, 14, 21, 23).

For identification of lactic acid bacteria API 50 CH tests (BioMérieux, Montalien-Vercien, France) were used, according to the manufacturer's instructions.

Subsequently, isolates were genetically identified using genus-specific primers specific for the V2–V3 region of the 16S rDNA of lactobacilli: P116S (5′-GGA ATC TTC CAC AAT GGA CG-3′) and P216S (5′-TGA CGG GCG GTG TGT ACA AG-3′). Total DNA from pure cultures was extracted as described previously (8). All polymerase chain reaction amplifications were performed with the *Taq* DNA polymerase kit (Fermentas, Vilnius, Latvia). Reaction mixtures consisted of 20 mM Tris–HCl (pH 8.4), 50 mM KCl,

*Table 1.* List of strains used in this study

| Strains | Relevant characteristics | Source or reference |
|---|---|---|
| *L. lactis* subsp. *lactis* BGSM1-19 | Natural isolate, $Bac^+$, $Bac^{Im}$ | Laboratory collection |
| *L. lactis* subsp. *cremoris* NS1 | $Bac^-$, $Bac^s$ | Laboratory collection |
| *L. paracasei* subsp. *paracasei* BGSJ2-8 | Natural isolate, $Bac^+$, $Bac^{Im}$ | Laboratory collection |
| *L. paracasei* subsp. *paracasei* BGUB9 | Natural isolate, $Bac^+$, $Bac^{Im}$ | Laboratory collection |
| *L. paracasei* subsp. *paracasei* BGHN14 | Natural isolate, $Bac^-$, $Bac^s$ | (9) |
| *L. paracasei* subsp. *paracasei* BGBUK2-16 | Natural isolate, $Bac^+$, $Bac^{Im}$ | (11) |
| *L. plantarum* A112 | $Bac^+$, $Bac^{Im}$ | (25) |
| *L. delbrueckii* subsp. *bulgaricus* BGPF1 | Natural isolate | Laboratory collection |
| *L. delbrueckii* subsp. *bulgaricus* BGTM2 | Natural isolate | Laboratory collection |
| *L. delbrueckii* subsp. *bulgaricus* BGTM3 | Natural isolate | Laboratory collection |
| *L. delbrueckii* subsp. *bulgaricus* BGJ1(398) | Natural isolate | Laboratory collection |
| *L. delbrueckii* subsp. *lactis* OLB 43b | | Laboratory collection |
| *L. delbrueckii* subsp. *bulgaricus* ATCC 9649 | | ATCC collection |
| *L. delbrueckii* subsp. *bulgaricus* CH3 | | CH collection |
| *Staphylococcus aureus* ATCC 25923 | | ATCC collection |
| *Staphylococcus aureus* MR 58 | | Laboratory collection |
| *Enterococcus faecalis* ATCC 29212 | | ATCC collection |
| *Streptococcus pneumoniae* ATCC 49619 | | ATCC collection |
| *Streptococcus mutans* | Clinical isolate[1] | This work |
| *Bacillus cereus* ATCC 11778 | | ATCC collection |
| *Bacillus subtilis* ATCC 8 | | ATCC collection |
| *Bacillus fragilis* ATCC 25285 | | ATCC collection |
| *Micrococcus flavus* ATCC 10240 | | ATCC collection |
| *Candida albicans* ATCC 24433 | | ATCC collection |
| *Salmonella enteritidis* | Clinical isolate[1] | This work |

$Bac^+$, bacteriocin producer; $Bac^{Im}$, bacteriocin immune; $Bac^-$, bacteriocin non-producer; $Bac^s$, bacteriocin-sensitive.
[1]Determined by sequencing of the 16S rDNA gene.

3 mM $MgCl_2$, 50 mM each of the four deoxynucleoside triphosphates (dNTP), 1 U *Taq* polymerase, 5 pmol each primer and 1 μl of template DNA in a final volume of 50 μl. The samples were amplified in a GeneAmp polymerase chain reaction System 2700 (Applied Biosystems, Foster City, CA) programmed as follows: initial denaturation of DNA for 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C; and extension of incomplete products for 7 min at 72°C. polymerase chain reaction products were quantified by electrophoresis on a 1% (weight/volume) agarose gel containing ethidium bromide.

Polymerase chain reaction products obtained from the selected isolates by using primers P116S and P216S specific for the V2–V3 region of the 16S rDNA of lactobacilli were purified on a QIAquick polymerase chain reaction Purification KIT (Qiagen, GmbH, Hilden, Germany), and sequenced by CRIBI-BMR servizio sequenziamento DNA (Universita di Padova, Padua, Italy). The sequence was checked in the National Center for Biotechnology Information (NCBI) database using the standard nucleotide–nucleotide homology search BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

For repetitive sequence-based (rep-) polymerase chain reaction analysis products were generated with polymerase chain reaction primer $(GTG)_5$ (5′-GTG GTG GTG GTG GTG-3′). The samples were amplified in a GeneAmp polymerase chain reaction System 2700 (Applied Biosystems) programmed as follows: initial denaturation of DNA for 7 min at 95°C, 32 cycles of 1 min at 94°C, 1 min at 53°C, and 8 min at 65°C; and extension of incomplete products for 16 min at 65°C. Polymerase chain reaction products were quantified by electrophoresis on a 1.5% (weight/volume) agarose gel containing ethidium bromide in a horizontal gel electrophoresis system (Bethesda Research Laboratories, Gaithersburg, MD) and visualized with a CCD camera Biometra BDR2/5/6 (Bio Doc Analyze GmbH, Göttingen, Germany). Amplified polymerase chain reaction fragments were analyzed by horizontal electrophoresis on 1.5% agarose gels, in 1X TAE [2 mol/l Tris base, 1 mol/l glacial acetic acid, 0.05 mol/l EDTA (pH 8.0)], at a constant voltage 60 V and a temperature of 4°C for 20 h (24).

#### Antimicrobial activity assay

For detection of antimicrobial activity, an agar well diffusion assay was used (22). Soft MRS agar (0.75%, weight/volume) containing approximately $10^5$ cells/ml of indicator strains sensitive to antimicrobial activity, *L. lactis* subsp. *cremoris* NS1, *Lactobacillus paracasei* subsp. *paracasei* BGHN14, were overlaid onto GM17 or MRS plates (depending on the indicator strain). Wells were made in the lawn of GM17 or MRS soft agar. For strains other then lactococci and lactobacilli, corresponding soft agars and plates were used. Aliquots (100 μl) of fresh overnight cultures (16 h) were poured into the wells. Plates were incubated for 48 h at appropriate temperatures for the growth of indicator strains. After the 48-h incubation, inhibition zones were read. A crystal of pronase E (non-specific protease mixture, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was placed close to the edge of the well containing the bacteriocin sample to confirm the proteinaceous nature of bacteriocin. A clear zone of inhibition around the well but not in the vicinity of the pronase E crystal was taken as a positive signal for bacteriocin production. The pH values of the supernatants were also measured. MRS broth pH 3.7 was used as negative control. The test for the effect of catalase on the antimicrobial activity of the isolates was performed as described previously (11) using catalase (Sigma-Aldrich; final concentration 1 mg/ml) in 50 mM sodium phosphate (pH 6.5). Reaction mixtures were incubated at 37°C for 1 h. The remaining antimicrobial activity was tested using the agar well diffusion assay. Enzyme-free buffer and supernatants with buffer, incubated at 37°C for 1 h, were used as controls. Each test was performed twice. The natural isolates from home-made cheeses, *L. lactis* subsp. *lactis* BGSM1-19 and *L. paracasei* subsp. *paracasei* BGSJ2-8 were used as positive controls for antimicrobial activity as the producers of the class II bacteriocins (unpublished data).

To determine the type of bacteriocin produced by *Lactobacillus gasseri* BGHO89, primers A01 (5′-GTC TAG ACG CTA AAT TAG TCA CTT TTC C-3′) and A02 (5′-GTC TAG ACG CTG CCT ACA GTC AAT TAG A-3′) specific for the chromosomal DNA of *L. gasseri* LF221 and containing the nucleotide sequence of the structural gene of acidocin LF221 A were used (12).

#### Kinetics of antimicrobial substance production

To remove bacteriocin, cells were collected from 1 ml of overnight culture (16 h incubation), and washed twice. Pelleted cells were resuspended in 1 ml MRS and this was used as inoculum for a new culture in MRS broth with approximately $10^5$ cells/ml. The culture was incubated at 37°C and 100-μl subsamples were taken every hour. Cells were pelleted and supernatants were filtered and loaded onto indicator strains.

#### pH and bile tolerance

To test the growing ability and survival of strains BGHO1 and BGHO89, 3 ml MRS (pH 2.5), MRS containing 0.3% bile salts (Torlak, Belgrade, Serbia) or MRS (pH 6.8) were inoculated (1% inoculum) with the bacteria to be tested. Survival (measured as colony-forming units/ml) was determined after 4 and 24 h of incubation at 37°C in MRS (pH 2.5) and MRS containing 0.3% bile salts, by plating onto MRS agar plates and further incubation for 48 h at 37°C.

## Results

#### Isolation, identification and distribution of the isolates in the oral cavity

Microscopic examination of the 158 isolates with unknown identity [selected from 560 colonies on the basis of colony morphology: 2–5 mm, convex, entire, opaque and without pigment (7)] revealed that 110 (69.6 %) of them were rods. On the bases of the morphological and biochemical tests for the provisional identification of the isolates, eight categories were formed containing morphologically and biochemically similar isolates, from which 26 isolates (three or four randomly chosen from each group) were selected for further characterization. Subsequently, these 26 isolates were genetically identified using genus-specific DNA primers for *Lactobacillus* sp. and all strains were identified as lactobacilli. Twenty-six isolates were identified further both phenotypically and genotypically to the species level using API CH 50 and rep-polymerase chain reaction: six isolates were identified as *Lactobacillus fermentum* (25%), four as *Lactobacillus plantarum* (16.7%), four as *Lactobacillus salivarius* (16.7%), three as *L. paracasei* subsp. *paracasei* (12.5%), two as *Lactobacillus acidophilus* (8.3%), two as *L. cellobiosus* (8.3%), two as *L. delbrueckii* subsp. *lactis* (8.3%) and one isolate as *L. gasseri* (4.2%). Five isolates, selected as producers of antimicrobial substances, were further identified by sequencing of the V2–V3 region of the 16S rDNA: *L. salivarius* BGHO1, *L. fermentum*

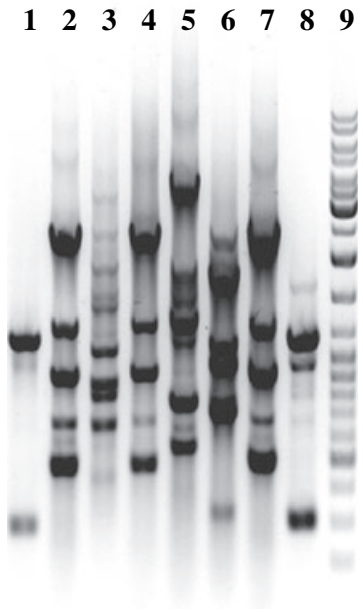


*Fig. 1.* Clustering of the rep-polymerase chain reaction patterns of lactobacillus isolates obtained by using $(GTG)_5$ primer. Lane 1, human oral isolate BGHO99; lane 2, *Lactobacillus delbrueckii* subsp. *bulgaricus* BGPF1; lane 3, *L. delbrueckii* subsp. *bulgaricus* BGTM2; lane 4, *L. delbrueckii* subsp. *bulgaricus* BGTM3; lane 5, *L. delbrueckii* subsp. *bulgaricus* ATCC 9649; lane 6, *L. delbrueckii* subsp. *bulgaricus* CH3; lane 7, *L. delbrueckii* subsp. *bulgaricus* J1(398); lane 8, *L. delbrueckii* subsp. *lactis* OLB 43b; lane 9, GeneRuler DNA Ladder mix.

BGHO36 and BGHO64, *L. gasseri* BGHO89 and *L. delbrueckii* BGHO99. The isolate BGHO99 was determined to the subspecies level by $(GTG)_5$ rep-polymerase chain reaction analyses as *L. delbrueckii* subsp. *lactis* (Fig. 1).

Distribution of the species at the different locations of the oral cavity was also calculated. According to the results three species were present on the oral mucous membrane: *L. fermentum* (50%), *L. acidophilus* (25%) and *L. plantarum* (25%); six species were present at the surface of healthy teeth: *L. plantarum* (30%), *L. paracasei* subsp. *paracasei* (20%), *L. salivarius* (20%), *L. fermentum* (10%), *L. cellobiosus* (10%) and *L. delbrueckii* subsp. *lactis* (10%); three species at the surface of the carious lesions: *L. fermentum* (50%), *L. cellobiosus* (25%) and *L. salivarius* (25%); and five species in the deep carious lesion: *L. plantarum* (32%), *L. fermentum* (17%), *L. acidophilus* (17%), *L. paracasei* subsp. *paracasei* (17%) and *L. gasseri* (17%).

### Test for probiotic characteristics

The 26 isolates identified as *Lactobacillus* sp. were tested for the various probiotic characteristics. Antimicrobial activities of the 26 strains were investigated using an agar well diffusion assay against *L. lactis* subsp. *cremoris* NS1 and *L. paracasei* subsp. *paracasei* BGHN14 indicator strains. Twelve isolates showed antimicrobial activity; among them, five isolates, determined as *L. salivarius* BGHO1, *L. fermentum* BGHO36 and BGHO64, *L. gasseri* BGHO89 and *L. delbrueckii* subsp. *lactis* BGHO99, showed strong antimicrobial activity against the indicator strains, and were further tested for antimicrobial activity against known bacteriocin-producing strains (Table 2) and against putative pathogens (Table 3). Reciprocal inhibitory activity of oral isolates was observed (Table 2). The same relationship existed between the strains *L. salivarius* BGHO1, *L. fermentum* BGHO36 and BGHO64 known bacteriocin producers, whereas the strain *L. delbrueckii* subsp. *lactis* BGHO99 showed an inhibitory effect only on the growth of the *L. plantarum* A112 control strain, and *L. gasseri* BGHO89 had no effect on the growth of the tested bacteriocin producers. Results revealed that all the tested isolates exhibited antagonistic action on the growth of *S. aureus* ATCC 25923, *S. aureus* MR 58, *Enterococcus faecalis* ATCC 29212, *S. pneumoniae* ATCC 49619, *S. mutans* (clinical isolate), *Micrococcus flavus* ATCC 10240, and *Salmonella enteritidis* but not on *Candida albicans* ATCC 24433. Tests with pronase E showed that the *L. salivarius* BGHO1, *L. fermentum* BGHO36 and *L. gasseri* BGHO89 strains produced antimicrobial substances that were proteinaceous in nature, while *L. fermentum* BGHO64 and *L. delbrueckii* subsp. *lactis* BGHO99 produced antimicrobial substances that were non-proteinaceous. To elucidate the compound(s) responsible for the antimicrobial activity of *L. fermentum* BGHO64 and *L. delbrueckii* subsp. *lactis* BGHO99, the strains were screened for production of hydrogen peroxide and the pH values of the cultures were measured. The results revealed that the antimicrobial activity of these isolates was not related to the production of hydrogen peroxide. The pH values of the cultures were pH 4.04 for BGHO64 and pH 3.98 for BGHO99. In this study we used MRS broth pH 3.7 as the negative control and no zones were obtained.

Moreover, polymerase chain reaction analysis with primers designed on the basis of homology with chromosomal DNA of *L. gasseri* LF221 and containing the nucleotide sequence of the structural gene of acidocin LF221 A (12) suggested that the isolate *L. gasseri* BGHO89 produced the variant of acidocin A with 49% nucleotide sequence homology. Interestingly, depending on the indicator strain, all the tested isolates showed two zones of inhibition one of which was cleared and

*Table 2.* Cross-inhibition of representative human oral isolates

| | Indicator strain | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Producer | BGHO1 | BGHO36 | BGHO64 | BGHO89 | BGHO99 | BGSJ2-8 | BGBUK2-16 | BGUB9 | BGA112 |
| BGHO1 | ND | $0.8 \pm 0.5^1$ | $1.5 \pm 0^1$ | $1.2 \pm 0.2^1$ | $1.5 \pm 0.5^1$ | $2 \pm 0^1$ | $2.5 \pm 0^1$ | $2.2 \pm 0.2^1$ | $2.6 \pm 0.2^1$ |
| BGHO36 | $1 \pm 0^1$ | ND | $1.5 \pm 1^1$ | $0.8 \pm 0.5^1$ | $2.5 \pm 0.5^1$ | $1.5 \pm 0.5^1$ | $2.5 \pm 0.5^1$ | $1.8 \pm 0.2^1$ | $2.5 \pm 0^1$ |
| BGHO64 | $1.5 \pm 0.5$ | $2 \pm 0.5$ | ND | $1.8 \pm 0.5$ | $1.8 \pm 0.5$ | $3 \pm 0$ | $2.5 \pm 0.5$ | $2 \pm 0$ | $3 \pm 0$ |
| BGHO89 | $0.5 \pm 0.2^1$ | $2 \pm 0^1$ | $1.5 \pm 0.5^1$ | ND | $2 \pm 0^1$ | – | – | – | – |
| BGHO99 | $1.5 \pm 0.5$ | $1.5 \pm 0.5$ | $1 \pm 0$ | $1.5 \pm 0.5$ | ND | – | – | – | $1.5 \pm 0.5$ |
| BGSJ2-8 | $2.5 \pm 0.5^1$ | $1 \pm 0.5^1$ | $2 \pm 0^1$ | – | $1.2 \pm 1^1$ | ND | – | $2.5 \pm 0.5^1$ | $2.0 \pm 0.5^1$ |
| BGBUK2-16 | – | – | $1.2 \pm 1^1$ | $1.2 \pm 1^1$ | $3 \pm 1^1$ | – | ND | – | $1.5 \pm 0.5^1$ |
| BGUB9 | $1 \pm 0.5^1$ | $0.8 \pm 0.5^1$ | $1.2 \pm 1^1$ | – | $2.5 \pm 0.5^1$ | $1.5 \pm 0.5^1$ | $1.8 \pm 0.5^1$ | ND | $1 \pm 0^1$ |
| BGA112 | – | – | – | – | – | $1.8 \pm 0.5^1$ | – | $1 \pm 0^1$ | ND |

BGHO1, *L. salivarius* BGHO1; BGHO36, *L. fermentum* BGHO36; BGHO64, *L. fermentum* BGHO64; BGHO89, *L. gasseri* BGHO89; BGHO99, *L. delbrueckii* subsp. *lactis* BGHO99; BGSJ2-8, *L. paracasei* subsp. *paracasei* BGSJ2-8; BGBUK2-16, *L. paracasei* subsp. *paracasei*; BGUB9, *L. paracasei* subsp. *paracasei* BGUB9; BGA112, *L. plantarum* BGA112.

The results are the averages of two independent experiments represented (given in mm of inhibition zone ± SD)

[1]A zone sensitive to pronase E (given in mm); ND, not determined; –, without a zone.

*Table 3.* Antimicrobial activity of human oral isolates against pathogens

| | Indicator strain | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Producer | *Staphylococcus aureus* ATCC 25923 | *Staphylococcus aureus* MR-58 | *Enterococcus faecalis* ATCC 29212 | *Streptococcus pneumoniae* ATCC 49619 | *Streptococcus mutans* | *Bacillus cereus* ATCC 11778 | *Bacillus subtilis* ATCC 8 | *Bacillus fragilis* ATCC 25285 | *Micrococcus flavus* ATCC 10240 | *Salmonella enteritidis* |
| BGHO1 | 4.2 ± 1.2 | 4.0 ± 2.0 | 4.0 ± 2.0 | 9.0 ± 1.0 | 7.8 ± 2.8 | 1.5 ± 0.5 | 5.0 ± 1.0 | 10.0 ± 2.0 | 3.5 ± 1.5[1] | 1.9 ± 0.4[1] |
| BGHO36 | 3.4 ± 0.6 | 2.0 ± 2.0[1] | 2.0 ± 1.0 | 4.0 ± 2.0 | 4.0 ± 3.0 | – | 4.0 ± 0.0 | 2.0 ± 2.0 | 2.0 ± 0.0[1] | 2.5 ± 0.0 |
| BGHO64 | 3.2 ± 1.8 | 2.0 ± 2.0[1] | 2.0 ± 1.0 | 4.0 ± 2.0 | 5.0 ± 1.0 | – | – | – | 4.5 ± 1.5[1] | 1.2 ± 0.3 |
| BGHO89 | 3.4 ± 1.6 | 3.0 ± 1.0[1] | 4.5 ± 2.5 | 6.0 ± 2.0 | 3.5 ± 1.5 | 2.5 ± 0.5[1] | 2.5 ± 0.5 | 8.0 ± 4.0 | 4.0 ± 2.0[1] | 1.8 ± 1.2[1] |
| BGHO99 | 3.3 ± 1.3 | 5.0 ± 1.0 | 4.5 ± 3.5 | 9.0 ± 1.0 | 4.0 ± 3.0 | 4.0 ± 1.0[1] | 4.5 ± 1.5 | 12.0 ± 4.0 | 4.0 ± 2.0[1] | 2.2 ± 0.8[1] |

Inhibition zones are given in mm; –, no inhibition.
The results are the averages of two independent experiments represented (given in mm of inhibition zone ± SD)
[1]Presence of a diffuse zone in addition to a clear one was noticed.

the other diffuse by appearance, suggesting the putative presence of at least two antimicrobial substances in the isolates.

The kinetics of antimicrobial activities was analysed for strains that exhibited strong antimicrobial activity against putative pathogens *L. salivarius* BGHO1, *L. gasseri* BGHO89 and *L. delbrueckii* subsp. *lactis* BGHO99. Maximal antimicrobial activities were observed after 16 h for strains *L. salivarius* BGHO1 and *L. delbrueckii* subsp. *lactis* BGHO99, and after 14 h for strain *L. gasseri* BGHO89. When *L. lactis* subsp. *cremoris* NS1 was used as the indicator strain, an additional spike of antimicrobial activity was observed for all three strains examined, indicating that each of these strains most probably produces two putative antimicrobial substances (Fig. 2).

### pH and bile tolerance

Survival of the isolates *L. salivarius* BGHO1, *L. gasseri* BGHO89 and *L. delbrueckii* subsp. *lactis* BGHO99 at pH 2.5 and in MRS broth containing 0.3% bile salts was tested. Results showed that the isolates *L. salivarius* BGHO1 and *L. gasseri* BGHO89 survived after 4 h incubation in MRS broth at pH 2.5 and in MRS broth containing 0.3% bile salts, while the isolate *L. delbrueckii* subsp. *lactis* BGHO99 did not (Fig. 3).

## Discussion

This work aimed to study the population of lactobacilli in the oral cavity to find a strain(s) that possessed potential probiotic characters. In this study we presented bacterial enumeration of lactobacilli in different locations of the oral cavity, determination of selected strains, their antimicrobial activity against putative pathogens and their tolerance to low pH and bile salts.

Generally, lactobacilli represent a small part of the oral microflora. Hence, selective culture media and phenotypic tests enable lactobacilli to be differentiated from morphologically similar bacteria. The accurate identification of *Lactobacillus* species can be accomplished by reference to the 16S rDNA sequence. Using genus-specific primers for polymerase chain reaction-based identification, 26 of the 110 isolates from the human oral cavity were tested and confirmed to be *Lactobacillus* sp. In the past decade, various methods have been developed for the identification and typing of microorganisms to the species level (19). The rep-polymerase chain reaction analysis belongs to the category of selective techniques. Eight different species were genotypically identified (*L. fermentum*, *L. plantarum*, *L. salivarius*, *L. paracasei* subsp. *paracasei*, *L. acidophilus*, *L. cellobiosus*, *L. delbrueckii* subsp. *lactis* and *L. gasseri*). The predominant *Lactobacillus* species in the oral cavity in these experiments was found to be *L. fermentum* (25%). A similar diversity in oral lactoflora was described previously and the most frequently observed species from the healthy human mouth were *L. fermentum*, *L. plantarum*, *L. salivarius*, *L. gasseri* and *L. rhamnosus* (1, 4, 10, 20).

Screening lactobacilli from different sources for their antibacterial activity is usually the first step in searching for putative probiotic strains. We found that some of the oral isolates showed strong antimicrobial activity against known bacteriocin-producing strains and putative pathogens. The isolate *L. salivarius* BGHO1 exhibited the strongest antimicrobial activity and had acted antagonistically on the growth of all putative pathogens and all tested bacteriocin producers. Moreover, the isolates *L. gasseri* BGHO89 and *L. delbrueckii* subsp. *lactis* BGHO99 exhibited strong antimicrobial activity against putative pathogens and a narrow antimicrobial spectrum against closely related species. Finally, the *L. fermentum*

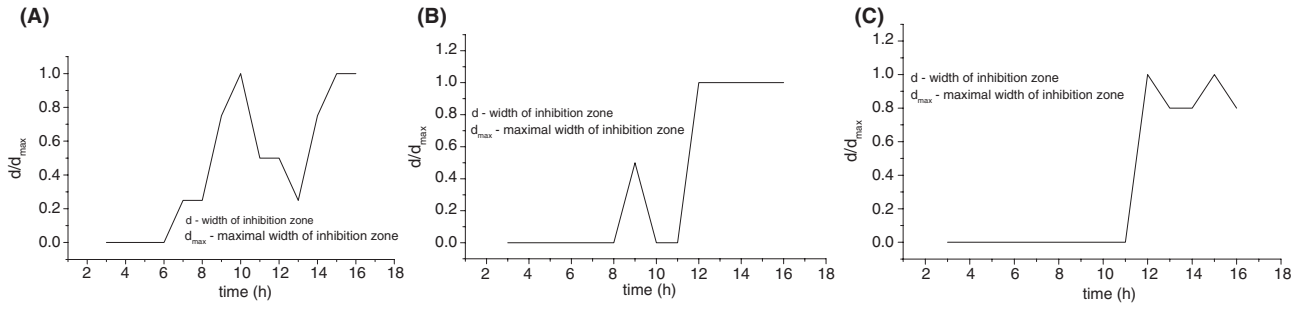


*Fig. 2.* Kinetics of antimicrobial activities of BGHO1 (A), BGHO89 (B) and BGHO99 (C). *Lactobacillus lactis* subsp. *cremoris* NS1 was used as the indicator strain.

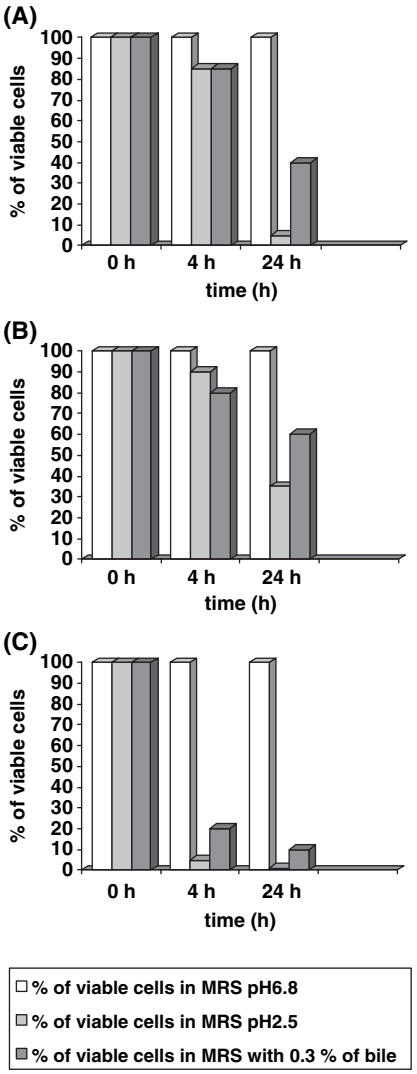


*Fig. 3.* pH and bile tolerance. Survival following 4 and 24 h of incubation in MRS broth at pH 2.5 and in MRS broth with 0.3% bile salts was observed for strains BGHO1 (A), BGHO89 (B) and BGHO99 (C).

strains BGHO36 and BGHO64 showed weak antimicrobial activity against putative pathogens and a broad antimicrobial spectrum against closely related species. None of the isolates tested showed antimicrobial activity against *C. albicans*. In general, lactobacilli in the oral cavity are considered to be cariogenic bacteria. In the present study, good antimicrobial activity of lactobacilli against *S. mutans*, previously described as the predominant species in dental caries (15), was observed. Näse et al. (16) have shown that long-term consumption of milk containing *L. rhamnosus* GG reduced the risk of dental caries in children, suggesting that in spite of the cariogenic potential of lactobacilli some of them may be associated with oral health; this possibility therefore needs further investigation. Tests with pronase E suggested that the isolates *L. salivarius* BGHO1, *L. fermentum* BGHO36 and *L. gasseri* BGHO89 produced bacteriocins, while *L. fermentum* BGHO64 and *L. delbrueckii* subsp. *lactis* BGHO99 produced antimicrobial substances of a non-proteinaceous nature. *L. gasseri* BGHO89 produced the variant of acidocin LF221 A (12). The antimicrobial activity of the BGHO64 and BGHO99 isolates could be the result of the production of an organic acid, such as lactic or acetic acid, upon fermentation of glucose, with a consequent decrease in pH (2), or could be related to the production of other antimicrobial factors such as hydrogen peroxide. Our results revealed that the antimicrobial activity of these isolates was not related to the low pH or to the production of hydrogen peroxide. However, the antimicrobial activity could be result of the presence of low-molecular-weight components, or of the synergistic activity of a mixture of organic acids (2). Future work will aim to determine the type and nature of the antimicrobial substances in these isolates.

In recent years, the commercial manufacture and marketing of functional foods (foods that affect the function of the body in a targeted manner so as to bring about positive effects on physiology and nutrition), particularly probiotic (*acidophilus–bifidus*) yoghurts, has spread from the well-established Japanese niche marketplace into the expanding European Union marketplace. As stated earlier, several research groups have recommended that the assessment of potential probiotics should involve the assessment of resistance to gastric acidity and bile toxicity, adhesion to gut epithelial tissue, ability to colonize the gastrointestinal tract, production of antimicrobial substances, and the ability to modulate immune responses (5, 17). Therefore, preliminary experiments were completed to determine the degree of acid and bile acid resistance exhibited by human oral lactobacilli isolates. The results revealed that the isolates *L. salivarius* BGHO1 and *L. gasseri* BGHO89 survived after 4 h of incubation at pH 2.5 and were found to be tolerant to the presence of bile salts. The results suggested that these human oral isolates could successfully transit the human stomach and may be capable of reaching the intestinal environment and functioning effectively there.

On the basis of the results presented in this study we suggest that the human oral isolates *L. salivarius* BGHO1 and *L. gasseri* BGHO89 should be subjected to clinical trials to investigate their effect on human oral and gut microflora. Moreover, the same strains could be used as starter cultures for dairy products to make a probiotic product and the health effect of these isolates on human volunteers could be followed.

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