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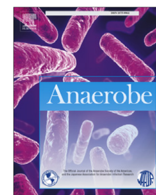

Clinical microbiology

Isolation and characterization of faecal bifidobacteria and lactobacilli isolated from dogs and primates

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ABSTRACT

Although bifidobacteria and lactobacilli have been suggested beneficial for the host and are components of many probiotics and competitive exclusion mixtures, the knowledge on abundance, metabolic and probiotic characteristics in isolates from dogs and monkeys is still limited. The present study was aimed to isolate *Bifidobacterium* and *Lactobacillus* strains (faeces of 22 dogs and of 5 primates: *Cebus apella*, *Eulemur fulvus*, *Erythrocebus patas*, *Macaca fascicularis*, *Papio hamadryas*) with the MALDI-TOF identification system *Lactobacillus murinus*, *Bifidobacterium animalis* and *Pediococcus acidilactici* were more frequently isolated species in dogs while *Lactobacillus plantarum* was isolated in several species of primates. Ten strains of 6 species were assayed for enzymatic activities (only *Lactobacillus reuteri* strains showed no undesirable enzymatic activity), antimicrobial susceptibility (detected higher minimum inhibitory concentration levels for tetracycline and gentamicin), and inhibitory activity against 15 indicator bacteria. All strains inhibited Gram-negative indicators while lactobacilli showed larger inhibition zones than bifidobacteria. *L. reuteri* II/3b/a (isolate from *M. fascicularis*) showed the best antimicrobial properties. Resistance to bile (0.3% w/v) was observed in all tested strains (no decrease of CFU/ml) whereas the decrease of 68.4–94.4% (after 90 min exposition) and 78.4–99.9% CFU/ml (after 180 min) depending on the strain was detected in the artificial gastric juice.

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1. Introduction

The microbiota of the gastrointestinal tract (GIT) is a complex of bacteria, viruses, yeasts and protozoa; it plays a crucial role in the host health through its implementation in the nutritional, physiological and immunological functions [1]. The metabolic activity of the intestinal microbiota is believed to be comparable to that of the liver and comprises especially fermentation of exogenous and endogenous carbon and energy sources [2]. Well known factors such as diet composition (e.g. fiber, protein content), age, diseases, and stressful factors affect the composition and activity of the human as well as animal gut microbiota [3]. It is clearly evident that bacterial populations differed between individuals [3]. Large differences among detected quantitative and qualitative microbial composition also in dogs are caused most probably by the use of different samples (luminal vs. faecal) and different methods for the microbiota detection (culture-based vs. molecular). Handl et al. [4] detected in canine faeces 10 different species of lactic acid bacteria in 83% of dogs and 6 species of bifidobacteria in 67% of dogs using 16S rRNA gene pyrosequencing. Ley et al. [5] using the same molecular method demonstrated that the canine microbiota is closely related but distinct from the microbiota of other mammals such as humans. The genera *Lactobacillus* and *Bifidobacterium* were present in only less than 1% of all identified sequences in faeces of dogs and cats evaluated by pyrosequencing [6]. Concerning the species occurrence, *Bifidobacterium animalis* [7], *Bifidobacterium subtile* and *Bifidobacterium bifidum* [4] were more frequently isolated species while *Bifidobacterium angulatum* was detected in faeces of wild living chimpanzees [8]. Among lactobacilli, *Lactobacillus aviarius*, *Weissella cibaria* in dog faeces [4], *Lactobacillus murinus* and *Lactobacillus reuteri* in jejunal chyme [9], *Lactobacillus acidophilus* and *Lactobacillus fermentum* in the cecum and colon mucosa [10] were detected as the most prevalent species in dogs. The strains of both genera *Lactobacillus* and *Bifidobacterium* were found to have many beneficial effects and are frequently incorporated in probiotic preparations with the main aim to restore the balance of intestinal microbiota easily disturbed by many factors. The beneficial effects

Abbreviations: CFU, colony forming unit; GIT, gastrointestinal tract; MALDI-TOF, matrix assisted laser desorption/ionization-time of flight mass spectrometry; MIC, minimum inhibitory concentration; MRSC, de Man–Rogosa–Sharpe + L-cysteine HCl.

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attributed to probiotic strains include the inhibition of pathogens (*Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, clostridia, etc.), reduction of colonic transit time (prevention of constipation), alleviation of lactose intolerance, immunomodulation, cholesterol assimilation, cancer prevention [11–14]. Interestingly, a lot of probiotic functional effects are observed despite no or small changes in the abundance of intestinal microbial groups [6].

The effects of only few probiotic bifidobacteria [15] and lactobacilli [16–18] on intestinal and overall health in dogs have been studied, therefore limited data are available concerning their effects. Similarly, probiotic and metabolic features of these bacterial genera – isolates from pets or primates – detected under *in vitro* conditions are studied rarely. The objective of this study was to examine harmful enzymatic activities, antibiotic susceptibility, the ability to inhibit intestinal pathogens and the ability to survive at low pH and in the presence of bile in strains isolated from faecal samples of dogs and primates.

2. Materials and methods

2.1. Isolation and identification of bacterial strains

The faecal samples from healthy dogs ($n = 22$, age 0.5–10 years, various breeds and sex) and primates ($n = 8$, *Cebus apella*, *Eulemur fulvus*, *Erythrocebus patas*, *Macaca fascicularis*, *Papio hamadryas*, provided by Zoo, Košice–Kavečany) were homogenized in Ringer buffer (Oxoid, UK) using stomacher, properly diluted and plated on de Man–Rogosa–Sharpe agar (MRS, Merck, Germany) and TOS-propionate agar plates (Merck). Plates were incubated anaerobically (Bactron Shel Lab II-1, Sheldon Manufacturing Inc., USA, atmosphere composition 90% N_2 + 5% H_2 + 5% CO_2) at 37 °C for 48–72 h. After incubation, morphologically different colonies were picked up and re-streaked for several generations in order to isolate purified individual bacterial strains. Isolates were identified using protein "fingerprints" determined by MALDI-TOF mass spectrometry (Bruker Daltonics MALDI Biotyper, USA) as described by Bessède et al. [19].

2.2. Antimicrobial susceptibility (MIC)

Strains were grown overnight in LSM + cysteine broth [90% Iso-Sensitest broth, 10% MRS broth and 15 g/L agar, supplemented with 0.3 g/L L-cysteine-HCl (Sigma–Aldrich, USA)] under anaerobic conditions at 37 °C. Bacterial cultures were then inoculated on LSM + cysteine agar plates with appropriate M.I.C. evaluator strip (Oxoid, UK) and incubated for 48 h under the same conditions. The minimum inhibitory concentrations (MICs) of the following antimicrobial agents were determined: tetracycline (TE 256 μg/mL), vancomycin (VA 256), ampicillin (AMP 256), metronidazole (MTZ 256), erythromycin (E 256), penicillin (P 32), ampicillin/clavulanic acid (AMC 256), oxacillin (OX 256), gentamicin (CN 1024). The MIC was determined as the lowest concentration of antimicrobial agent at which no visible growth was recorded.

2.3. Enzymatic activities

Strains were assayed for enzyme production using the API-ZYM system (bioMérieux, France) following the manufacturer's recommendations. The inocula (65 μL) of the McFarland standard 1 suspensions were pipetted into each well of the kit. Enzyme activities were evaluated after 4 h of anaerobic incubation at 37 °C and after the addition of Zym A and Zym B reagent. Color intensity values from 0 to 5 and their relevant value in nanomoles were assigned for each reaction according to a color chart with the kit.

2.4. Resistance to low pH and bile

The tolerance of the strains to low pH and bile was determined following the procedure of Arboleya et al. [20]. Cells harvested after centrifugation of overnight cultures (grown in 5 mL of MRSC broth, 37 °C, anaerobically) were washed twice with Ringer buffer (Oxoid) and resuspended in 500 μL of the same buffer. Prepared bacterial suspension (100 μL) was added to 900 μL of simulated gastric juice [125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, and 3 g/L pepsin (Sigma), adjusted to pH 2.5 with HCl] or bile juice [45 mM NaCl, 1 g/L pancreatin (Sigma) and 3 g/L Oxgall (Sigma), adjusted to pH 8.0 with NaOH]. Suspensions were then incubated for 90 and 180 min and samples of 0 h, 90 and 180 h were inoculated at MRSC (Merck) to detect viable counts (CFU/mL). The assay was performed in triplicate.

2.5. Antimicrobial activity

The agar spot test described by Jacobsen et al. [21] was used to evaluate the ability of the strains to inhibit the growth of indicator bacteria (Table 4). A 3 μL of an overnight culture (in MRSC broth, Merck) was spotted onto MRSC agar plates (1.2% w/v agar) and incubated for 24 h at 37 °C under anaerobic conditions. The spots were then covered with TSA or MRSC soft agar (0.8% w/v) inoculated with the indicator bacteria (1% v/v), the plates were incubated at 37 °C aerobically except *Bacillus* sp. Inhibition of bacterial growth was determined measuring the inhibition zone surrounding the bacterial spots. The present test was performed in duplicate.

The well-diffusion assay was used to test the antibacterial activity of strain supernatants [22]. Nutrient agar (20 mL, 1.2% w/v agar) at 45 °C was mixed with 200 μL of an overnight culture of the indicator strain and poured into Petri dishes. Wells were made in the agar layer (5 mm diameter) and filled with 50 μL of supernatant obtained after centrifugation of 24 h cultures (14,000 rpm for 5 min). Plates were incubated at 37 °C for 24 h aerobically. Supernatants were concentrated (10-fold) and treated with pepsin (1 mg/mL, Sigma–Aldrich, USA), proteinase K (1 mg/mL), boiling for 20 min and neutralized with 1 M NaOH. Analytical Merckoquant peroxide test strips (Merck) were used to measure hydrogen peroxide production (detection scale 0 and 25 mg/L).

3. Results and discussion

The MALDI-TOF identification revealed *L. murinus*, *B. animalis* and *Pediococcus acidilactici* as more frequently isolated species in dogs while *Lactobacillus plantarum* was isolated in several species of primates (*E. patas*, *C. apella*, *M. fascicularis*, Table 1). The species

Table 1
Species of lactic acid bacteria isolated from dogs and primates.

	Number of isolates
Dog ($n = 25$)	
Lactobacillus murinus	13
Bifidobacterium animalis	5
Pediococcus acidilactici	5
Lactobacillus reuteri	3
Bifidobacterium choerinum	1
Lactobacillus plantarum	1
Lactobacillus oris	1
Lactobacillus saerimneri	1
Lactobacillus agilis	1
Primates ($n = 8$)	
Lactobacillus plantarum	3
Lactobacillus reuteri	2
Lactobacillus murinus	1
Bifidobacterium animalis	1
Bifidobacterium longum	1

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