Microbiological Research 200 (2017) 1–13

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Selection of new lactic acid bacteria strains bearing probiotic features from mucosal microbiota of healthy calves: Looking for immunobiotics through *in vitro* and *in vivo* approaches for immunoprophylaxis applications

Sávio Sandes[a,*], Luige Alvim[a], Bruno Silva[a], Leonardo Acurcio[b], Cinara Santos[a,b], Márcia Campos[a], Camila Santos[a], Jacques Nicoli[b], Elisabeth Neumann[b], Álvaro Nunes[a]

[a] *Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Campus Pampulha, Av. Antônio Carlos 6627, 31270-901, Belo Horizonte, MG, Brazil*
[b] *Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Campus Pampulha, Av. Antônio Carlos 6627, 31270-901, Belo Horizonte, MG, Brazil*

ARTICLE INFO

Article history:
Received 17 January 2017
Received in revised form 15 March 2017
Accepted 23 March 2017
Available online 24 March 2017

Keywords:
Lactobacillus
Weissella
Probiotic features
Cytokines
Immunomodulation

ABSTRACT

From the birth, since their mucosal microbiota and immune system are not fully developed, newborn calves are susceptible to several mucosal pathogenic microorganisms. Operating through humoral and non-humoral mechanisms in the host, several lactic acid bacteria strains bearing probiotic features are often employed in livestock as food supplement, improving animal production performance, promoting health and reducing the severity of mucosal infections. Accordingly, we isolated, species-level identified and screened for their probiotic potentials seventy lactic acid bacteria strains from upper airway, vaginal and intestinal mucosa of healthy calves. Based on *in vitro* approaches, we selected three strains: *Lactobacillus fermentum* V3B-08 isolated from upper airway mucosa, *Weissella hellenica* V1V-30 isolated from vaginal mucosa and *Lactobacillus farciminis* B4F-06 isolated from intestinal mucosa were used to mono-colonize germ-free mice in the same site in which these strains were isolated, aiming to characterize their immunomodulatory features. These strains were able to colonize germ-free mice mucosa and trigger sIgA synthesis at a local level, in addition to stimulating, in different ways, adaptive immune responses at a systemic level.

1. Introduction

Lactic acid bacteria (LAB) belong to mammalian resident microbiota, colonizing skin, respiratory, vaginal and intestinal mucosa. They are commonly employed in food manufacturing and preservation (Castro et al., 2016; Guo et al., 2016; Zanirati et al., 2015), being generically recognized as safe to their host's health. Additionally, several strains have been categorized as probiotic, which are defined as live microorganisms that when administered in adequate amounts, confer a health benefit to the host (FAO/WHO, 2002; Hill et al., 2014). Indeed, several LAB strains have some functional and beneficial features which give them the potential to be employed as probiotics, protecting their human and animal hosts against mucosal infections by humoral and non-humoral mechanisms (Alvim et al., 2016; Gaggìa et al., 2010; Tsai et al., 2012; Varankovich et al., 2015). For this reason, probiotics have been receiving special attention from farmers, who seek alternatives to the use of traditional antibiotics as growth promoters.

From the birth, since their mucosal microbiota and immune system are not fully developed, newborn calves are susceptible to several mucosal pathogenic microorganisms. Etiological agents include *Escherichia coli*, *Salmonella enterica* and *Clostridium perfringens* type C, which are associated to bovine post-weaning diarrhea and calf scour, as well as *Fusobacterium necrophorum* and *Mannheimia haemolytica,* which are associated with calf diphtheria and pneumonia, respectively. Additionally, newborn calves that fail in ingesting colostrum or have been submitted to a diet change, long distance transportation, exposure to adverse weather, nutritional privation, and any other kind of stress are more susceptible to pathogenic microorganisms, due to an impaired immunity

* Corresponding author at: Universidade Federal de Minas Gerais, Campus Pampulha, Av. Antônio Carlos, 6627, 31270–901, Belo Horizonte, MG, Brazil.
E-mail address: savio.cicco@gmail.com (S. Sandes).

http://dx.doi.org/10.1016/j.micres.2017.03.008
0944-5013/

development. In this context, probiotics have been used in animal production as food supplement, especially in newborn animals (Castillo et al., 2012), where reduction of neonatal mortality and improvement of animal performance have been observed (Frizzo et al., 2011; Naqid et al., 2015; Yeoman and White, 2014).

Several mechanisms are associated to beneficial effects of probiotics, such as stabilization of mucosal barrier, mucus production by goblet cells stimulation, promotion of adhesion between epithelial cells, competition with pathogenic microorganisms for adhesion sites and nutrients in mucosa (competitive exclusion), production and secretion of antagonistic substances, as well as local and systemic immunomodulation (Castillo et al., 2012; Collins et al., 2009; Frizzo et al., 2011; Kemgang et al., 2014; Lebeer et al., 2008). Regarding immunomodulation, it was already shown that ingestion of probiotic strains kept the host immune system primed to respond faster and more effectively to microbial infections (Fagundes et al., 2011), being a promising tool for calve creation, once these strains could be used as food supplement, promoting immunoprophylaxis in cattle (Frizzo et al., 2011).

Accordingly, the goal of this study was to isolate LAB strains from healthy calves' upper airway, vaginal and intestinal mucosa for further selection, based on their functional and beneficial characteristics, the best strains for potential use as probiotic and immune adjuvant for veterinary purposes.

2. Materials and methods

2.1. Animals, sampling and LAB strains isolation

LAB strains were isolated from upper airway, vaginal fluid and feces from healthy female calves, younger than thirty days old, belonging to Holstein Friesian breed and free of any antibiotic therapy. Saliva and vaginal fluid were sampled with Salivette (Sarstedt AG & Co., Nümbrecht, Germany), which was introduced and maintained until saturation into oral and vaginal cavities. The fecal samples were collected with rectal stimulus and stored in sterile flasks. Samples were maintained at 4 °C until analysis, which were performed on the same day of the sampling.

At the laboratory, samples were serially diluted in phosphate buffered saline (PBS) and cultivated in De Man, Rogosa and Sharpe agar (MRS – Merck, Darmstadt, Germany) for 48 h at 37 °C in an anaerobic chamber (Forma Scientific, Marietta, United States), containing an atmosphere of 85% N_2, 10% H_2, and 5% CO_2. Colonies with distinct morphology were isolated and submitted to Gram staining. Only Gram-positive strains with bacilli or coccobacilli morphology were selected and preserved in MRS broth with 20% of glycerol for −80 °C storage.

2.2. Species-level identification of LAB strains

LAB strains were identified to species-level by amplified ribosomal DNA restriction analysis (ARDRA) of the 16S-ITS-23S *locus* according to Sandes et al. (2014). Chromosomal DNA was isolated from LAB cultivated cultures in 1 ml of MRS broth. It was then centrifuged at 8000*g* for 10 min, and the bacterial pellet was incubated with 5 mol l^{-1} LiCl followed by another centrifugation and incubation with protoplasting buffer containing Lysozyme (25 mmol l^{-1} sucrose, 50 mmol l^{-1}, Tris-HCl pH 8.0, 10 mmol l^{-1}M EDTA, and 10 mg ml^{-1}) at 37 °C. Sample was centrifuged and the genomic DNA was isolated using a NucleoSpin Tissue XS kit (Macherey-Nagel, Düren, Germany), according to manufacturer's instructions.

The 16S-ITSI-23S region was amplified using 10 ρmol of 16-1A (5′ GAA TCG CTA GTA ATC G 3′) and 23–1 B (5′ GGG TTC CCC CAT TCG GA 3′) primers, described by Tilsala-Timisjarvi and Alatossava (1997), PCR Master Mix (Promega Corporation, Madison, United States) containing 0.2 mmol l^{-1} of each deoxyribonucleotide triphosphate, 1.5 mmol l^{-1} of $MgCl_2$, 1.5 units of *Taq* DNA polymerase, and 10 ng of template DNA. The amplification was performed with 35 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min after the initial denaturation (95 °C for 2 min) and finished by the final extension (72 °C for 5 min). The amplicons were digested using 12 endonucleases (*Sph*I, *Nco*I, *Nhe*I, *Ssp*I, *Csp*45I, *Eco*RV, *Dra*I, *Vsp*I, *Hinc*II, *Eco*RI, *Hind*III – Promega, Madison, United States and *Avr*II – New England Biolabs Inc., Ipswich, United States) and were electrophoresed in a 1.4% agarose gel.

When necessary, species-level identification was performed by rRNA 16S gene sequencing analysis. For this, the full-length of this *locus* was amplified using 10 ρmol of 27F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1492R (5′ GGT TAC CTT GTT ACG ACT T 3′) primers, described by Lane (1991). The amplification conditions were the same for the 16S-ITSI-23S *locus*. The amplicons were purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, United States) before sequencing with the DYEnamic ET Dye Terminator Cycle Sequencing kit (GE Healthcare, Piscataway, United States) for the automatic sequencing system MegaBACE 1000 (GE Healthcare, Piscataway, United States). Species-level identification was performed using Seqmatch algorithm (RDP – Ribosomal Database Project) taking into account only sequences of good quality that belonged to type strains.

2.3. Functional and probiotic features screening – establishment of selection criteria

A selection criteria was established based on the study of Silva et al. (2013) and Silva et al. (2016), taking into account the cell surface hydrophobicity by microbial adhesion to solvents (MATS), antagonistic activity against bacterial pathogens, hydrogen peroxide production, antibiotics susceptibility, gastric juice susceptibility (GJS) and bile salts susceptibility (BSS).

2.4. Cell surface hydrophobicity

Cell surface hydrophobicity was assessed by microbial adhesion to solvents (MATS) approach described by Pelletier et al. (1997). LAB cultures in stationary phase were centrifuged, washed twice in PBS and adjusted to an OD_{600nm} of 0.6 with 0.1 mol l^{-1} of KNO_3 pH 6.2 (A0). Next, Xylene was added to bacterial suspensions, forming a two-phase system. The aqueous phase was removed and OD_{600nm} was measured (A1). MATS was calculated by the percentage of LAB associated to Xylene according to the formula: $MATS = [(A_0\text{-}A_1/A_0)]x100$. The strains were classified as hydrophobic for MATS ≥ 55.00%, amphiphilic for 45.00% ≤ MATS ≤ 54.99% or hydrophilic for MATS ≤ 44.99%.

2.5. Antagonistic activity against bacterial pathogens

Five microliters of LAB cultures in stationary phase were spotted onto MRS agar and incubated in anaerobic conditions for 18 h at 37 °C. Next, cells were killed by exposure to chloroform vapor for 20 min. In Brain Heart Infusion (BHI – Acumedia Neogen Corp., Lansing, United States), seven pathogenic bacteria (*Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 15313, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25922, *Salmonella enterica* ATCC 14028, and *Pseudomonas aeruginosa* ATCC 27853) were cultured until stationary phase. These microorganisms were inoculated in BHI soft agar, which was used to overlay the surface of MRS plates containing dead LAB spots. After incubating at 37 °C for 24 h, LAB antagonistic activity was determined by measuring the growth inhibition zone using a digital pachymeter (Mitutoyo Sul Americana Ltd., São Paulo,