Research in Veterinary Science 108 (2016) 125–132

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Isolation, characterization and evaluation of probiotic lactic acid bacteria for potential use in animal production

Yaneisy García-Hernández [a,1], Tania Pérez-Sánchez [b,*,1], Ramón Boucourt [a], José L. Balcázar [c], Jacques R. Nicoli [d], João Moreira-Silva [d], Zoraya Rodríguez [a], Héctor Fuertes [b], Odalys Nuñez [a], Nereyda Albelo [a], Nabil Halaihel [b]

[a] Institute of Animal Science, Mayabeque, Cuba
[b] Faculty of Veterinary, Universidad Zaragoza, Zaragoza, Spain
[c] Catalan Institute for Water Research (ICRA), Scientific and Technological Park of the University of Girona, Girona, Spain
[d] Institute of Biological Science, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

ARTICLE INFO

Article history:
Received 17 June 2014
Received in revised form 3 June 2016
Accepted 22 August 2016

Keywords:
Lactic acid bacteria
Probiotics
Broilers
Lactobacillus pentosus

ABSTRACT

In livestock production, lactic acid bacteria (LAB) are the most common microorganisms used as probiotics. For such use, these bacteria must be correctly identified and characterized to ensure their safety and efficiency. In the present study, LAB were isolated from broiler excreta, where a fermentation process was used. Nine among sixteen isolates were identified by biochemical and molecular (sequencing of the 16S rRNA gene) methods as *Lactobacillus crispatus* ($n = 1$), *Lactobacillus pentosus* ($n = 1$), *Weissella cibaria* ($n = 1$), *Pediococcus pentosaceus* ($n = 2$) and *Enterococcus hirae* ($n = 4$). Subsequently, these bacteria were characterized for their growth capabilities, lactic acid production, acidic pH and bile salts tolerance, cell surface hydrophobicity, antimicrobial susceptibility and antagonistic activity. *Lactobacillus pentosus* strain LB-31, which showed the best characteristics, was selected for further analysis. This strain was administered to broilers and showed the ability of modulating the immune response and producing beneficial effects on morpho-physiological, productive and health indicators of the animals.

1. Introduction

Lactic acid bacteria (LAB) are non-sporulating, catalase-negative, Gram-positive, rod- or coccus-shaped and strictly fermentative organisms, with lactic acid as the major metabolic end product of carbohydrate fermentation (Holzapfel et al., 2001). Currently, this group comprises the following genera: *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Ahmed, 2003). They can be found in different habitats such as soil, water, animal and human gastrointestinal tract, as well as in food and fermented products (Holzapfel et al., 2001; Zacharof and Lovitt, 2012).

Several members of LAB, pure or mixed cultures, are commonly used as probiotics (microbial feed additives). However, the mode of action of probiotics is still not fully understood; thus, the evaluation of probiotics is essential to optimize their use (Awad et al., 2010). According to previous studies, these microbial additives may adhere and survive in the gastrointestinal tract of the animals, where act on the stability and protection of this ecosystem. They also contribute to the digestive and metabolic processes, as well as to the modulation of local and systemic immune response. These properties lead to an improvement in animal health, resulting in increased production yields (Isolauri et al., 2004) and reinforcing host immunity (Patel et al., 2015). However, the effectiveness of the probiotic preparations is species or strain dependent; therefore each candidate probiotic strain should meet a number of requirements, including safety (isolation from suitable habitats, correct identification and antimicrobial susceptibility), functional (intestinal mucosa adhesion and resistance to gastrointestinal environment) and beneficial (lactic acid production and antagonism against pathogens) characteristics (FAO/WHO, 2002). A suitable selection criterion should be considered to improve the process of developing better probiotics and when *in vitro* and *in vivo* properties are evaluated together, a substantial advantage can be achieved (Blajman et al., 2015).

The treatment with probiotics has shown improved production values in broilers and may have potential as an alternative to antibiotics. Taking into consideration that antibiotic resistance has become a public health concern, the scientific community and public authorities should collaborate for the implementation of strategies, policies and programs that will limit the use of antibiotics (Marti et al., 2014). In poultry and livestock production, regulatory pressures have limited antibiotic usage and there is a need to evaluate alternatives to both increase production and improve disease resistance (Huff et al., 2015). The aim of this study was, therefore, to characterize and select autochthonous LAB for potential use as probiotics in broiler production.

* Corresponding author at: Faculty of Veterinary, Universidad Zaragoza, C/Miguel Servet 177, 50013 Zaragoza, Spain.
E-mail address: taniaper@unizar.es (T. Pérez-Sánchez).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.rvsc.2016.08.009
0034-5288/

2. Materials and methods

2.1. Isolation and culture conditions

LAB were isolated from broiler chicken feces as previously described (García-Hernández et al., 2012). Briefly, the fecal samples were homogenized in peptone water, tenfold serially diluted and plated on De Man, Rogosa and Sharpe agar (MRS; Pronadisa, Madrid, Spain). After an incubation period for 48–72 h at 37 °C, colonies showing different morphologies were randomly selected and plated on MRS agar. All isolates were subcultured and stored at −80 °C in MRS broth supplemented with 15% (v/v) glycerol.

2.2. Biochemical and molecular identification of LAB

Each bacterial strain was subjected to Gram staining and catalase and oxidase tests to select catalase- and oxidase-negative, Gram-positive cocci and bacilli. Selected strains were then identified using API 50 CHL and API 20 Strep kits, according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France), and the results were interpreted using the APILAB Plus software version 3.3.3 (bioMérieux).

The biochemical identification was confirmed by sequencing of the 16S rRNA gene as previously described (Pérez-Sánchez et al., 2011). Briefly, bacterial cultures, grown on MRS agar for 24 h at 37 °C, were homogenized with 50 μL of phosphate buffered saline (PBS, 10 mM phosphate, pH 7.4), and total DNA was then extracted and purified using the illustra tissue & cells genomic Prep Mini Spin kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's recommendations. The 16S rRNA gene was amplified and sequenced on a MegaBACE 500 sequencer following the manufacturer's protocols (Amersham Biosciences, Buckinghamshire, UK).

Nucleotide sequences were compared against the sequences available in the GenBank/EMBL/DDBJ databases. Phylogenetic analysis was performed using the MEGA software version 4.0 (Tamura et al., 2007). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method was determined by using bootstrap values based on 1000 replications.

2.3. Characterization and selection of LAB as potential probiotics

2.3.1. Production of lactic acid

Strains were grown in MRS broth for 18 to 24 h at 37 °C and shaking at 120 rpm. From these cultures, suspensions in MRS broth with $OD_{600\ nm}$ of 0.125, equivalent to 10^7 colony forming units (CFU) mL^{-1}, were prepared. Five milliliters of each suspension were inoculated in Erlenmeyer flasks containing 45 mL of MRS broth and incubated under the same conditions as described above. The pH of the culture medium and the production of lactic acid were then determined at the initial time and after a 24-h incubation period to determine changes in these variables. Briefly, the samples were centrifuged to 5000 *g* for 10 min and supernatants were kept at −20 °C until their use to measure the amount of lactic acid by the colorimetric method proposed by Taylor (1995). The pH was measured using a pH meter (Crison Instruments, Alella, Spain), and value lower than 5.5 after 24 h of incubation was used as a selection criterion for further analyses (Rondón et al., 2008).

2.3.2. Growth characteristics of LAB

Conditions to determine growth parameters were the same as previously mentioned. Briefly, samples (1 mL) were collected and subjected to tenfold serial dilutions in saline solution, plated on MRS agar, and CFU mL^{-1} were determined after incubation for 24–48 h at 37 °C. Growth kinetic assays were performed in microplates to determine specific growth rate and doubling time. For this, bacterial cultures were used in stationary phase and their concentrations were adjusted spectrophotometrically to an $OD_{600\ nm} = 0.6$ with PBS. The cultures were inoculated in MRS broth at a 1/10 ratio (v/v) and $OD_{600\ nm}$ was then measured every 30 min for 12 h at 37 °C using a microplate spectrophotometer system (Molecular Devices, Sunnyvale, USA). Specific growth rate (μ) was determined as the slope of the best-fit equation corresponding to the exponential phase of growth. Doubling time (T_d) was calculated from this value using the equation: $T_d = \ln 2/\mu$ (El-Mansi et al., 2012).

2.3.3. Tolerance to different pH conditions

The method described by Prasad et al. (1998), and adapted to microplate, was used to determine the tolerance of LAB to different pH conditions. Briefly, bacterial cultures in stationary phase, based on our previous results (Section 2.3.2), were pelleted by centrifugation at 5000*g* for 10 min, washed three times with PBS (pH 7.4) and resuspended in 3 mL of the same buffer solution. The $OD_{600\ nm}$ was adjusted spectrophotometrically to 0.6 with PBS (pH 7.4). This suspension was inoculated at a 1/10 ratio (v/v) in PBS previously adjusted to pH 7.4 and pH 2.5 with NaOH (1 M) and HCl, respectively. These treated suspensions were incubated at 37 °C for 3 h, and then centrifuged, washed and resuspended in MRS broth. Recovery of the cells was determined using the $OD_{600\ nm}$ in a 96-well microplate. Aliquots of 100 μL of these bacterial suspensions were inoculated in each well. $OD_{600\ nm}$ reading was measured every 30 min for 20 h of incubation at 37 °C in a microplate spectrophotometer system (Molecular Devices). The strains that tolerated pH 2.5 after 3 h of exposure were selected for the next steps.

2.3.4. Tolerance to bile salts

The method described by Walker and Gilliland (1993) was used with minor modifications. Briefly, $OD_{600\ nm}$ of bacterial cultures in stationary phase was adjusted to 0.6. The cultures were inoculated into MRS broth supplemented with 0 and 0.3% (w/v) of bile salts (Oxoid, Basingstoke, UK). $OD_{600\ nm}$ was measured every 30 min for 12 h of incubation at 37 °C using a microplate spectrophotometer system (Molecular Devices). Survival rate (S) was calculated using the following equation: $\%\ S = [OD_{MRS\ +\ Salts} / OD_{MRS}] \times 100$, where: $OD_{MRS\ +\ Salts}$ corresponds to the OD of the culture in MRS broth with the addition of bile salts, whereas OD_{MRS} corresponds to the OD of MRS broth culture (Rondón et al., 2008). Strains that survived to bile salts exposure in >50% after 3 h were selected for the next steps.

2.3.5. Cell surface hydrophobicity

The hydrophobicity was determined as the ability of bacteria to adhere to hydrocarbons (MATS: Microbial Adhesion to Solvents), according to the methodology described by Vinderola and Reinheimer (2003), but using toluene as solvent. Bacterial cultures in stationary phase were pelleted by centrifugation at 5000*g* for 10 min, washed twice with PBS (pH 7.4) and resuspended in 3 mL of the same buffer solution. The bacterial concentration was adjusted with PBS (pH 7.4) to $OD_{560\ nm} = 1.0$ (A_{b0}) according to the methodology previously described, and 1 mL of this suspension was added to 0.2 mL of toluene (Lab-Scan, Dublin, Ireland) and mixed for 2 min. After an incubation period at 37 °C for 1 h, the aqueous phase was removed and the $OD_{560\ nm}$ was determined again (A_{b1}). Percentage of MATS was calculated using the following equation: $\%\ MATS = (A_{b0} - A_{b1}) / A_{b0} \times 100$. Isolates with MATS above 50% were considered as hydrophobic according to Moreira (2005).

2.3.6. Antimicrobial susceptibility

Antibiotic susceptibility was determined by disk diffusion according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012). The following antibiotics were tested: ampicillin (10 μg), chloramphenicol (30 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), streptomycin (10 μg), tetracycline (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg) and vancomycin (30 μg), which were chosen according to the recommendations proposed by the European Food Safety Authority (EFSA Panel on Additives and Products or Substances used in Animal