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Screening and characterization of potential probiotic and starter bacteria for plant fermentations

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ABSTRACT

Probiotics are mostly consumed as fermented or fortified food products in Europe. There are two important factors in the selection of probiotic candidates regarding the potential health benefit; their viability and number when consumed, and their survival and persistence in the gastrointestinal tracts. This study focusses on the selection of potential probiotics to be used as starter culture in plant-based fermented foods. Lactic acid bacteria isolated from quinoa and amaranth were tested *in vitro* for their sensitivity to antibiotics, tolerance to gastrointestinal stress factors and adhesion to gut epithelial cells. Only five strains had suitable antibiotic profile to be used as probiotics and all of them were tolerant to lysozyme, bile salts, and had similar adhesion capacity. *Lactobacillus plantarum* Q823, administered as starter culture in a fermented quinoa drink, was selected for the human *in vivo* tests, because of its best *in vitro* tolerance to low pH. This strain was able to survive and persist at detectable levels (5–7 Log_{10} CFU/g feces) in the gastrointestinal tracts for at least seven days after the end of administration. Thus, *L. plantarum* Q823 has been identified as a suitable starter and a potential probiotic in fermented quinoa-based products.

1. Introduction

Probiotics are defined as living microorganisms which, when administered in sufficient numbers, confer a health benefits on the host (FAO/WHO, 2002). Probiotics are associated with the prevention or alleviation of several intestinal disorders such as antibiotic-associated diarrhea, irritable bowel disease, colon cancer, lactose intolerance, food allergies and more (Alander et al., 1999; Rijkers et al., 2010). These beneficial effects are related to one or more mechanisms, such as modulation of the intestinal microbiota, blockage of pathogen adhesion sites, modulation of the host immune responses and competition for nutrients (De Champs, Maroncle, Balestrino, Rich, & Forestier, 2003). Regardless of the mechanisms, probiotic bacteria should be able to survive the passage through the gastrointestinal (GI) tracts in order to provide health benefits, as well as persist in sufficient numbers in the gut to exclude pathogens and interact with the host epithelial and immune cells (Balgir, Kaur, Kaur, Daroch, & Kaur, 2013; Jacobsen et al., 1999; Sathyabama, Vijayabharathi, Devi, Kumar, & Priyadarisini, 2012).

Probiotic microorganisms can be introduced through consumption of fermented foods, as fortified food products or as pharmaceuticals. In Europe, because of the general attitude against medication, probiotics are mostly consumed components in food products, mainly as fermented milks (Yerlikaya, 2014). However, several factors have to be considered when using probiotics in fermented products, in particular, their viability and presence in high numbers at the time of consumption (Muller et al., 2013; Vinderola, Binetti, Burns, & Reinheimer, 2011). The strains mostly used as probiotics represent lactic acid bacteria (LAB) and bifidobacteria (Sathyabama et al., 2012; Vinderola et al., 2011). The recommended effective dose should be higher than 100 million CFU/

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dose. The numbers currently found in probiotics products and the numbers associated with significant outcomes in clinical trials are in the range of 1–10 billion CFU/dose (Naidu, Adam, & Govender, 2012; Reid, 2006; WGO, 2008).

Increasing rates in milk allergy and lactose intolerance, and the high content of saturated fatty acids are the major drawbacks associated with the consumption of dairy foods, including probiotic dairy products. Consequently, food industry is currently very interested in producing fermented or functional foods other than traditional milk-based products (Gupta & Abu-Ghannam, 2012; Rivera-Espinoza & Gallardo-Navarro, 2010). Also modern consumers are increasingly interested in the consumption of functional foods based on cereals, fruits and vegetables, because of their perceived beneficial nutritional values. Consequently, probiotic food products made from plant materials are nowadays in the focus of functional food product development (Mridula & Sharma, 2015).

In our search for novel plant materials suitable as functional foods quinoa (*Chenopodium quinoa* Willd) and amaranth (*Amaranthus* spp.), two Andean traditional crops with high nutritional values (Nascimento et al., 2014), were selected in this study, because these gluten-free crops could be used as a food matrix for new types of probiotic products.

Traditionally, most probiotic bacteria are of intestinal origin. However, probiotics used in plant-based fermented products should preferentially be isolated from plant materials, because plant-derived substrates present significant technological and physiological challenges to which strains of intestinal origin might not be well adapted (Gupta & Abu-Ghannam, 2012; Rivera-Espinoza & Gallardo-Navarro, 2010). However, for probiotic action, it is essential that plant-based potential probiotics are screened for their survival and persistence in the GI tracts.

There are several *in vitro* assays that are commonly used to check the intestinal survival and persistence of a potential probiotic strain based on exposing it to simulated gastrointestinal stress factors (low pH, digestive enzymes, bile acids) and checking its adhesion capacity to cultured gut epithelial cells or isolated mucus (Papadimitriou et al., 2015; Tuomola, Crittenden, Playne, Isolauri, & Salminen, 2001). Although these tests are important for a preliminary selection of potential probiotic strains, *in vivo* trials with the probiotic are needed in order to make a credible claim on the survival and persistence (De Champs et al., 2003; Oozeer et al., 2006). The aim of this study was to find LAB with potential probiotic properties from plant origin to be used as starter cultures in plant fermented food products. Therefore, indigenous isolated LAB were tested *in vitro* and *in vivo* to assess their ability to cope with the intestinal challenges and temporarily colonize the human gut.

2. Material and methods

2.1. Microorganisms and growth conditions

The LAB strains used in this study were isolated from different quinoa varieties and amaranth seeds obtained from INTA (Instituto Nacional de Tecnología Agropecuaria), Hornillos (Jujuy, Argentina) (Ruiz Rodriguez et al., in press). Seeds (25 g) were suspended in 225 ml MRS broth (LabM, UK) supplemented with cycloheximide (0.1 g/L) (Sigma–Aldrich, Germany) and incubated at 30 °C during 24 h. Then, decimal dilutions were prepared and plated on MRS-5 agar (Meroth, Walter, Hertel, Brandt, & Hammes, 2003) incubated at 30 °C for 48 h, aerobically and under anaerobiosis (AnaeroGen and AnaeroJar, Oxoid, UK) for LAB isolation. Gram-positive and catalase negative (determined by transferring fresh colonies from a Petri dish to a glass slide and adding H_2O_2 5%, v/v) were considered as presumptive LAB and further purified by successive subculturing (2–3 times) in MRS-5 broth, harvested and stored in MRS-5 containing 20% of glycerol at −80 °C for further experiments. The isolated strains were deposited at CERELA Culture Collection (Tucumán, Argentina).

For the fingerprinting of the strains, LAB isolates (212 from quinoa varieties and 32 from amaranth seeds) were subjected to RAPD-PCR analysis using primers RAPD2 (5′-AGCAGCGTCG-3′) and M13 (5′-GAGGGTGGCGGTTCT-3′) in separate reactions. The PCR conditions used for amplification experiments are those reported by Fontana, Cocconcelli, and Vignolo (2005). DNA extraction from isolates and reference strains (*Lactobacillus sakei* CRL1463, *Lactobacillus curvatus* CRL1465 and *Lactobacillus plantarum* CRL1481 from CERELA, as well as *Lactobacillus paracasei* UC8808, *Leuconostoc lactis* UC8020, *Leuconostoc mesenteroides* UC8232 from UCSC culture collections) was performed using Microlysis (Labogen, UK).

A total of 18 isolated strains were selected for further studies based on the genomic profiling. Taxonomical identification was carried out by species-specific PCR and by sequencing the 16S rDNA genes. Primer sequences, conditions and references are shown in Table 1. The sequences of the 16S rDNA gene amplicons have been deposited in the Gen Bank database under accession numbers listed in Table 2. Two commercial probiotic strains were used as controls in various *in vitro* assays; *Lactobacillus rhamnosus* GG (LGG) and *L. rhamnosus* LC705 (LC705). The strains and their source of isolation are listed in Table 2. All strains were routinely grown in MRS agar or broth (Oxoid, Italy) at 30 °C for 24–48 h.

2.2. Antibiotic resistance

The minimum inhibitory concentrations (MICs) of nine antibiotics (gentamicin, kanamycin, streptomycin, erythromycin, chloramphenicol, tetracycline, ampicillin, clindamycin and vancomycin) were determined for all strains according to ISO 10932:2010 standard. Epidemiological break point values were based on the recommendation of the committee on Antimicrobial Susceptibility Testing (EUCAST) and EFSA Panel on Additives and Products or Substance used in Animal Feeding (FEEDAP).

2.3. In vitro screening of probiotic properties

2.3.1. The potential to survive the passage through the gastrointestinal tracts

The resistance of the strains to some host defense mechanisms, namely lysozyme, bile and low pH, was determined for the isolates and controls. The resistance of the strains to lysozyme was tested as described by Kimoto-Nira, Suzuki, Kobayashi, and Mizumachi (2008). Briefly, overnight cultures of the strains grown in MRS broth were harvested and washed. One mL of cells was suspended in phosphate-buffering saline (PBS) supplemented with 100 μg/mL of lysozyme (Sigma–Aldrich) for 1 h. The degree of cell lysis was measured spectrophotometrically by calculating the change in absorbance at OD 620 nm.

The tolerance to bile and low pH was determined by standard plating of broth cultures onto MRS plates after different exposure times. For bile tolerance, 1% of overnight cultures of the bacterial strains were inoculated into MRS broth supplemented with 0.3% oxgall (Sigma–Aldrich) and incubated at 37 °C for 4 and 24 h pH tolerance was determined by adjusting the MRS broth to pH 2.5 (with 10 M HCl), inoculating 1% of the overnight culture cells and incubated for 1, 2 and 4 h. After the bile or pH challenge, the cells were plated on MRS plates. The plates were incubated for 48 h at 30 °C. All tests were done in duplicates.

2.3.2. Adhesion capacity to a human epithelial cells

The capacity of the isolates to adhere to a human epithelial colorectal adenocarcinoma cell-line, Caco-2 (ATTC HTB-37), was