



Generation of *Lactobacillus plantarum* strains with improved potential to target gastrointestinal disorders related to sugar malabsorption

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ABSTRACT

Malabsorption of dietary sugars is a common cause of gastrointestinal discomfort, affecting up to one in three people with debilitating symptoms, such as abdominal pain, osmotic diarrhoea, bloating and flatulence. Besides dietary interventions, it has been suggested that ingestion of lactobacilli may alleviate these symptoms. The objectives of this study were to generate strains with improved potential to ameliorate sugar malabsorption related gastrointestinal disorders. Initial selection was made from 183 natural isolates of lactic acid bacteria, on the basis of broad sugar fermentation ability, absence of gas production, gastrointestinal survival and susceptibility to important medical antimicrobials. Two strains of *L. plantarum* (KR6 and M5) exhibited favourable characteristics for all criteria, and were further optimised through random mutagenesis and selection approaches. Ultraviolet light (UV) exposure resulted in mutants characterized by better survival (for 1.9 log and 1.4 log) in gastrointestinal conditions. Subsequent exposure to ethyl methanesulfonate (EMS) provided mutants with greater tolerance to glucose induced catabolite repression. UV and UV-EMS mutants of *L. plantarum* M5 showed improved adhesion ability. As a result of this optimisation, *L. plantarum* MP2026 and *L. plantarum* MP2420 have been identified as promising candidates for probiotics, intended for alleviation of gastrointestinal discomfort originating from sugar malabsorption.

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1. Introduction

Lactic acid bacteria (LAB) are Gram-positive, non-sporulating bacteria which produce lactic acid as the major end product of carbohydrate fermentation (Axelsson, 2004). They are a heterogeneous group of bacteria, naturally present in nutrient rich environments, such as decomposing plants and milk products but also common among bacteria populating skin and mucosal tissues of humans and animals. The genus *Lactobacillus* is the largest among LAB and consists of over two hundred species, though this number still increases (NCBI Taxonomy Browser; Hammes & Hertel, 2006).

According to the type of anaerobic carbohydrate metabolism, lactobacilli can be divided into three large groups. First group are obligately homofermentative lactobacilli, which produce lactic acid as the primary by-product of carbohydrate fermentation. They cannot ferment

pentoses and gluconate and do not produce CO₂. The second group comprises facultatively heterofermentative lactobacilli which cannot produce CO₂ from glucose, because glucose is metabolized through the same pathway as in homofermentative lactobacilli. However, facultatively heterofermentative lactobacilli can produce CO₂ when cultivated on gluconate, and are also able to ferment pentoses into lactic and acetic acid. In the third group there are obligately heterofermentative lactobacilli, which in addition to lactic acid produce other end products such as acetic acid, ethanol and CO₂ when fermenting glucose (Margolles, Mayo, & Ruas-Madiedo, 2009). A wide variety of *Lactobacillus* strains have a long history of apparent safe use in traditional fermented food products. The whole taxonomical unit of *Lactobacillus* has GRAS (generally recognised as safe) and QPS (qualified presumption of safety) status (EFSA, 2015). Several *Lactobacillus* strains are widely used in modern food and feed fermentation and as probiotics (Fontana, Bermudez-Brito, Plaza-Diaz, Munoz-Quezada, & Gil, 2013). Probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001),

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where their safety and benefits to health has to be demonstrated *in vitro* and *in vivo* in properly controlled human studies (Hill et al., 2014). Evaluation of probiotics intended for food comprises several safety considerations, among which the absence of acquired antibiotic resistance has gained particular attention in recent times (FAO/WHO, 2002). Among functional traits, the survival in the gastrointestinal conditions and adhesion to intestinal epithelium are particularly desirable in terms of colonization and functionality in the gut. *Lactobacillus plantarum* is a Gram-positive species distributed in a variety of niches such as naturally fermented foods and feeds, gut, vagina, milk and others. *Lactobacillus plantarum* strains are also used in starter cultures and as probiotics (Fontana et al., 2013; Hammes & Hertel, 2006).

The composition of microbiota in different regions of the human gastrointestinal tract (GI) greatly depends on the availability of nutrients and particularly carbon sources. In the small intestine competition with the host for rapid uptake and subsequent fermentation of available simple carbohydrates is crucial for maintenance of bacterial microbiota, rich in *Streptococcus* sp., *Escherichia coli* and *Clostridium* sp. (Zoetendal et al., 2012). In the colon in contrast, complex plant derived polysaccharides, resistant to degradation by the host enzymatic machinery, and intestinal wall-derived mucins represent the majority of carbohydrates available to the microbes. Consequently, different resident bacterial species which are highly efficient degraders of complex polysaccharides are performing well in this environment, particularly *Bacteroides* representatives, with the genome of *B. thetaiotaomicron* alone encoding >260 glycosyl hydrolases (Mahowald et al., 2009). Fermentation of cellulose, xylenes, resistant starch and inulin by microbiota in the gut results in the production of short-chain fatty acids, mainly acetate, propionate and butyrate, and intestinal gases (Tremaroli & Backhed, 2012).

The availability of carbohydrates in GI can change dramatically if dietary sugars are not absorbed by the human host. Malabsorption of dietary sugars can occur due to lack of appropriate enzymes in the human digestive system to digest certain oligosaccharides, natural decline of enzyme expression in case of lactose malabsorption or pathological deficiency of carrier molecules in patients suffering fructose malabsorption (Melchior, Gourcerol, Dechelotte, Leroi, & Ducrotte, 2014; Misselwitz et al., 2013; Raithel et al., 2013; Seo, Kim, & Oh, 2013). Unabsorbed sugars are transferred to the large intestine where they are rapidly fermented by resident microbes, leading to increased osmotic pressure and production of large amounts of gases, such as hydrogen, CO₂ and methane (Misselwitz et al., 2013; Raithel et al., 2013). This often leads to symptoms such as abdominal pain, osmotic diarrhoea, bloating and flatulence, which can be even more pronounced in patients suffering from functional gastrointestinal disorders such as irritable bowel syndrome (IBS) (Goebel-Stengel et al., 2014).

Random mutagenesis with ultraviolet light (UV) or chemicals including ethyl methanesulfonate (EMS) is an already established approach in the strain improvement of industrial LAB (Derkx et al., 2014; Kuipers, 2015). In contrast to the bacterial strains improved by recombinant DNA technology, those modified by the random mutagenesis are not considered GMO (genetically modified organisms) which presents an advantage in terms of the requirements related to the tight regulation on the introduction of novel strains in the food chain and in terms of the acceptance by the consumers.

In this study we aimed to select probiotic candidates particularly suited to colonize GI tract of patients suffering from sugar malabsorption symptoms. Initially we investigated the capability of 183 strains of LAB to ferment lactose and fructose, dietary sugars involved in most malabsorption symptoms, as well as raffinose as an example of plant oligosaccharide that passes undigested to the lower intestine. The selection was based on the ability of the strains to rapidly consume these three sugars, the absence of gas production, the ability to grow in the environment with low amino acid concentrations, good survival in GI tract conditions, and sensitivity to antibiotics important in human and veterinary medicine. Using random mutagenesis, we further developed two novel strains (*L. plantarum* MP2026 and *L. plantarum* MP2420) with

improved survival in GI conditions and reduced catabolite repression by glucose. These novel strains represent excellent candidates for clinical trials in patients suffering from malabsorption of dietary sugars (lactose, fructose) and abdominal bloating.

2. Materials and methods

2.1. Bacterial strains

A total of 183 bacterial strains were tested in this study (Table A.1). Strains were isolated from sour milk, fermented turnip or fermented cabbage (this study), gained from microbial collection of autochthonous isolates of Laboratory for Antibiotic, Enzyme, Probiotic and Starter Cultures Technology at Faculty of Food Technology and Biotechnology, University of Zagreb, from microbial collection of Institute of Dairy Science and Probiotics at Biotechnical Faculty, University of Ljubljana and from commercial microbial collections.

Isolation of new strains from sour milk, turnip or fermented cabbage was carried out by plating the diluted samples on de Man, Rogosa and Sharpe (MRS) agar (Biolife, Milano, Italy) plates and incubated at 37 °C for 48 h in anaerobic conditions (GENbox anaer; BioMérieux, Marcy l'Etoile, France). All the white round colonies were purified with streaking to single colonies and stored at –80 °C in MRS broth with 30% glycerol.

2.2. Growth and gas production in different media

All the strains were first tested for production of gas during growth in MRS broth (Biolife, Milano, Italy) with Durham tubes. Strains were inoculated (1% v/v) and checked for gas production after 12 to 18 h.

The growth in modified MRS broth differing in the content of carbon sources, nitrogen sources or bile, was examined during 8 h incubation of inoculated broths in microtiter plates at 37 °C. Inoculum was prepared from the pellet of overnight cultures of LAB isolates in MRS broth (inoculum 1% v/v). The pellet was resuspended in the initial volume of sterile saline solution (0.9% NaCl). The medium used in the growth assay was either original MRS broth or MRS broth with one of the following modifications: without sugar (i), glucose replaced with fructose, 2% (w/v) (ii), glucose replaced with raffinose, 2% (w/v) (iii), glucose replaced with lactose, 2% (w/v) (iv), content of nitrogen sources reduced to ¼ of original concentration (v), 0.3% of bile (Bile bovine B3883, Sigma-Aldrich Chemie, Germany) added or 0.6% of bile added. Each well contained 10 µL of bacterial suspension and 190 µL of culture broth. Optical density (O.D._{600 nm}) was measured (Tecan Infinite M200, Switzerland) each hour. All strains were tested twice, in two replicates. Percentage of growth in different media in comparison with the growth in medium with glucose was calculated.

2.3. Antibiotic susceptibility

Minimal inhibitory concentrations (MIC) of antibiotics recognised by EFSA (2012) as of human and veterinary importance (ampicillin (AM), vancomycin (VA), gentamicin (GM), kanamycin (KM), streptomycin (SM), erythromycin (EM), clindamycin (CM), chloramphenicol (CL) and tetracycline (TC)) were determined by E-test® system (bioMérieux, France). Agar plates (radius 9 cm) consisting of 90% Mueller Hinton agar and 10% MRS agar with pH adjusted to 6.7 contained 25 mL of agar medium. LAB cells from overnight cultures were collected with centrifugation and resuspended in sterile saline solution to reach O.D._{600 nm} around 0.3. One hundred microliters of suspension was spread with sterile cotton bud on each plate. Plates were incubated for 48 h on 37 °C in anaerobic conditions (GENbox anaer, bioMérieux, France) and results were read in accordance with manufacturer's instructions.

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