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Production and structural characterization of exopolysaccharides from newly isolated probiotic lactic acid bacteria

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ABSTRACT

In this work, four exopolysaccharide-producing lactic acid bacteria (LAB) strains, newly isolated from Tunisian spontaneously fermented foods and beverages, namely bovine and turkey meat sausages (BMS and TMS), date palm sap (DPS) and cow milk (CM), were identified as *Leuconostoc citreum*-BMS, *Leuconostoc mesenteroides*-TMS, *Pediococcus pentosaceus*-DPS and *Leuconostoc pseudomesenteroides*-CM, respectively. The isolated strains showed the ability to withstand simulated human gastrointestinal (GI) tract conditions (low pH, lysozyme, bile salts, pepsin and pancreatin) and showed high surface hydrophobicity (79–90%), besides their ability to act against *Escherichia coli* and *Listeria monocytogenes* and to produce exopolysaccharides (EPS). Therefore, these isolates can be served as potential probiotics. The produced EPS were growth-associated suggesting that they are primary metabolites. The molecular weights were higher than 10^6 Da using HPLC-SEC. 2D-NMR results indicated that all the samples were mixtures of dextran and levan, except for EPS-CM which was a levan-type EPS. Furthermore, the EPS samples showed an abitlity to inhibit and to disrupt pathogenic biofilms and showed high thermostability studied *via* differential scanning calorimetry (DSC) with melting points higher than 224 °C making them promising to be used in thermal processed foods.

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1. Introduction

Nowadays, taste and nutritional needs of foods and beverages are no more the only purpose of consumers, but also their specific health benefits. Currently, improving the activity and the balance of the intestinal microbiota is among the main objectives of the functional foods market, which can be attained by the consumption of food products containing live bacteria, known as 'Probiotics'. According to FAO/WHO [1], probiotics are live microorganisms which confer health benefit on the host when administered in sufficient amounts. These beneficial effects include hypocholesterolemic, anti-mutagenic, anti-osteoporosis, anti-hypertensive and immunomodulatory activities [2]. Moreover, it has been reported that probiotics are able to alleviate some intolerances (such as lactose intolerance), to prevent or reduce the prevalence of allergies in susceptible individuals and to improve the intestinal microbial balance of the host and thus to lower the risk of gastro-intestinal diseases [2,3]. Since lactic acid bacteria (LAB) are known to be food-grade bacteria due to their generally recognized as safe status (GRAS), they can be easily used in foods as probiotics in a juridical point of view. Obviously, this will be greatly beneficial combined with the current knowledge of their ability to produce exopolysaccharides (EPS) with health-promoting properties. In fact, it has been shown that EPS may contribute to the human health as prebiotics or as immunomodulating, anti-ulcer, anti-biofilm agents to prevent pathogenic bacteria adhesion or anti-tumour agents [4]. Dilna et al. [4] found that EPS from probiotic *Lactobacillus plantarum* RJF4 was able to inhibit cancer cell lines but was non-toxic to normal cell lines. Similarly, Ismail et al. [5] reported that the EPS from probiotic *Lactobacillus plantarum* MTCC 9510 can be used as an anti-cancer agent taking into account its non-toxicity towards normal cells. Moreover, microbial EPS have shown multitude of functional effects with enormous range of applications as viscosifers, biothickeners as well as emulsifying and stabilizing agents [6].

As regards their physiological role, EPS exhibit diverse functions including cell to-cell interactions, adhesion and cell protection against environmental conditions [7]. Based on their monomeric

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composition, EPS are generally classified as homopolysaccharides and heteropolysaccharides [8]. Homopolysaccharides are formed by only one type of monosaccharide repeating unit where monomer residues are linked to form either linear structures (levan, curdlan, pullulan or bacterial cellulose) or ramified structures (dextran). Nevertheless, heteropolysaccharides are made of two or more types of monosaccharides and are usually present as multiple copies of oligosaccharides, containing three to eight residues [8]. Jolly et al. [9] reported that sugar composition, chain length, sugar linkages, presence of repeated units and substitutions, affect the biological and technological properties of EPS. Thus, the chemical characterization of novel EPS is crucial for predicting their potential application.

In this work, EPS were produced by four LAB strains newly isolated from Tunisian spontaneously fermented foods and beverages. The probiotic potential of these LAB strains was evaluated together with structural characterization of their produced EPS.

2. Materials and methods

2.1. Isolation and identification of EPS-producing LAB strains

Different Tunisian spontaneously fermented foods and beverages, namely bovine and turkey meat sausages (BMS and TMS, respectively), date palm sap (DPS) and cow milk (CM) were used to isolate EPS-producing LAB. Serial dilutions of homogenized samples with stomacher in sterile saline water were spread on MRS agar plates and then incubated for 48 h at 30 °C. Mucoid colonies were selected and purified on MRS agar. Stocked cultures were stored at −80 °C in MRS broth with 20% (v/v) glycerol (Merck, Darmstadt, Germany) for further studies.

The isolates were characterized on the basis of morphological, biochemical and microscopic observations. The pure cultures were initially tested for their Gram staining, cell morphology, motility and catalase reaction. The isolates were further characterized taking into account the following biochemical tests: mannitol fermentation and mobility test, growth at 4, 15, 37 and 45 °C in MRS broth, tolerance to NaCl by growth in MRS broth containing 4, 6, 8 and 10% NaCl, growth at pH 3.9 and pH 9.6 in MRS broth, gas production from glucose and production of hydrogen peroxide [10].

Identification of selected strains was done by using 16S rRNA gene sequencing. Total DNA was isolated using QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions and amplified using the primers NW9- GCTAACTACGTGCCAGCAG and NW17-TAAGGGCCATGA(T/G)GA(C/T)TTGAC. The PCR amplification products were about 710 pb. PCR was performed in 50 μL reaction mixture containing 3 μL of extracted DNA, 0.5 μM of each primer, 1 U of flexi Taq DNA polymerase (Promega, Madison WI, USA), 0.2 mM dNTP. PCRs were performed using the Gene Amp PCR System 9600 thermocycler under the following conditions: 2 min of denaturation at 95 °C, followed by 35 cycles of amplification at 95 °C for 30 s, 53 °C for 40 s and 72 °C for 30 s. The 16S rRNA PCR products were visualized after electrophoresis in 1% agarose gels by ethidium bromide staining. The resulting sequences were compared to the 16S rRNA gene sequences of Genbank using BLAST.

2.2. Assessment of probiotic potential of EPS-producing LAB strains

2.2.1. Antimicrobial activity

The antimicrobial activity of the isolated LAB strains was assessed against *Escherichia coli*, *Listeria monocytogenes* and *Enterococcus faecalis* using the well diffusion assay according to Argyri et al. [11] with slight modifications. 50 μL of 5 h grown test pathogen cells were added to 5 mL of soft LB medium and poured on LB agar plates. 100 μL of the cell free supernatant, obtained by harvesting 24 h old cultures of each LAB strain from MRS broth by centrifugation (at 8000 rpm, 15 min at 4 °C), was added into the wells (cut with sterile 6 mm borer) of the seeded LB plates with each of the pathogen cells. The supernatant was allowed to diffuse into agar at 4 °C for 2 h and for 24 h at 37 °C. The antimicrobial activity was then recorded as growth-free inhibition zones (diameter) around the well.

2.2.2. Antibiotic susceptibility assay

Overnight bacterial cultures were diluted to approximatively 10^7 CFU/mL and streaked on MRS agar plates. Antibiotic discs (the API-ZYM system (BioMérieux, Montalieu-Vercieu, France)) were placed onto solid media. Growth inhibition was read by measuring the diameter of the inhibition zones after 24 h of incubation at 37 °C [12].

2.2.3. Bile salts resistance

The ability of the isolated strains to grow in the presence of bile salts was studied. Each strain was inoculated (2% v/v) into 10 mL MRS broth containing 0.3% (w/v) of Oxbile (Sigma,USA) along with a control i.e., without bile salts and all the tubes were incubated at 37 °C. After 24 h of incubation, the bacterial concentration was checked by viable count determination on MRS agar by plating suitable dilutions [13].

2.2.4. Resistance to gastrointestinal conditions

The influence of the successive transit through artificial *saliva*, gastric and intestinal juices on the viability of the tested strains was evaluated as reported by Pinto et al. [14]. Reconstituted skim milk (15% w/v, Merck) was inoculated with approximately 2×10^8 CFU/mL of an overnight culture. A 1 mL aliquot was removed, serially diluted and spread-plated onto MRS agar to determine the CFU/mL at time zero. To simulate the dilution and possible hydrolysis of bacteria in the human oral cavity, the suspension was diluted 1:1 in a sterile electrolyte solution containing 6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$ and 1.2 g/L $NaHCO_3$ to which lysozyme was added to a final concentration of 100 ppm, and incubated for 5 min at 37 °C. The sample was subsequently diluted 3:5 with an artificial gastric fluid, consisting of the electrolyte solution mentioned above adjusted at pH 2.5 and with 0.3% pepsin added. If required, pH was readjusted to pH 2.5 with 5 M HCl. After 1 h-incubation at 37 °C, another 1 mL aliquot was removed, serially diluted and spread-plated onto MRS agar. To simulate the dilution in the small intestine, the remaining volume was diluted 1:4 using an artificial duodenal secretion (pH 7.2) consisting of 6.4 g/L $NaHCO_3$, 0.239 g/L KCl, 1.28 g/L NaCl, 0.5% bile salts and 0.1% pancreatin. One milliliter aliquots were again removed after 2 and 3 h-incubation at 37 °C, serially diluted and plated onto MRS agar to determine the CFU/mL.

2.2.5. Cell surface hydrophobicity

Cell surface hydrophobicity is generally defined as the ability of bacteria to adhere to hydrocarbons (MATS: Microbial Adhesion to Solvents) and was determined as described by Garcia-Hernandez et al. [15] with slight modifications. Bacterial cultures (18 h) were centrifuged at 9000 rpm for 10 min and the collected cells were washed twice with PBS (pH 7.4) and re-suspended in 3 mL of the same buffer solution.

The suspension concentration was adjusted to OD 560 nm = 1.0 (Ab_0) with PBS (pH 7.4) and 1 mL of this suspension was added to 0.2 mL of toluene. The mixture was vortexed for 2 min and then incubated at 37 °C for 1 h. The absorbance at 560 nm of the organic

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