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Anti-bacterial and anti-biofilm activity of probiotic bacteria against oral pathogens

Fadia Ben Taheur [a], Bochra Kouidhi [b], Kais Fdhila [a], Hamouda Elabed [a], Rihab Ben Slama [a], Kacem Mahdouani [a], Amina Bakhrouf [a], Kamel Chaieb [c, *]

[a] Laboratory of Analysis, Treatment and Valorization of Environmental Polluants and Products, Faculty of Pharmacy, Monastir University, Tunisia
[b] College of Applied Medical Sciences, Medical Laboratory Department, Yanbu el Bahr, Taibah University, Al Madinah Al Monawarah, Saudi Arabia
[c] College of Sciences, Yanbu el Bahr, Taibah University, Al Madinah Al Monawarah, Saudi Arabia

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ABSTRACT

In this study, three lactic acid bacteria (LAB), isolated from barley, traditional dried meat and fermented olive were characterized and tested for their anti-bacterial and anti-biofilm activities against oral bacteria.

Our results revealed that the tested LAB were γ-hemolytic and were susceptible to four antibiotics. All the strains were resistant to low pH, bile salt, pepsin and pancreatin. Furthermore, FB2 displayed a high aut-oaggregative phenotype (99.54%) while FF2 exhibited the best co-aggregation rate. Concerning the microbial adhesion to solvent, FB2 was the most hydrophobic strain (data obtained with chloroform and n-hexadecane). In addition *Pediococcus pentosaceus* FB2 and *Lactobacillus brevis* FF2 displayed a significant inhibitory effect against *Streptococcus salivarius* B468 (MIC = 10%). Moreover the selected strains were able to inhibit biofilm formation of *Bacillus cereus* ATCC14579 ($MBIC_{50}$ = 28.16%) and *S. salivarius* B468 ($MBIC_{50}$ = 42.28%).

The selected LAB could be considered as candidate probiotics for further application in functional food and mainly in the prevention of oral diseases.

1. Introduction

Numerous microorganisms colonizes the oral cavity of humans [1]. Poor hygiene, smoking, systemic diseases [2], conditions of low pH, or low saliva flow [3] alter this complex system and favor multiplication of pathogenic bacteria [4]. Dental caries is an infectious disease caused by the accumulation of oral flora forming biofilm on the tooth surface [5] and affecting the general health [6]. Furthermore, the interaction among oral microbiota, teeth, and dietary factors leads to some chronic diseases such as infective endocarditis and respiratory infections [7–9]. Early colonization of *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus sanguinis*, and *Streptococcus salivarius* on pellicle [10] may creates a favorable area for *Streptococcus mutans* growth and initial biofilm formation.

In order to maintain the stability of the oral ecosystem, probiotics microorganisms may be used for caries management. As defined by Joint [11], probiotic bacteria are live microorganisms that confer a health benefit on the host when administered in adequate amounts. Potential probiotics are expected to have some functional requirements like resistance to acid and bile, adherence to epithelial surfaces and inhibitory activity against some pathogens [12,13]. Likewise, probiotics must withstand to antibiotics, produce β-galactosidase, exhibit high auto-aggregation, co-aggregation with pathogens and surface hydrophobicity [14]. Recent research showed that *Streptococcus oligofermentans*, isolated from caries-free subjects, inhibited the *S. mutans* by the production of hydrogen peroxide [15]. Similarly, Samot and Badet [16], reported that *Lactobacillus reuteri* was able to inhibit the growth of *S. mutans*, *S. gordonii*, and *Actinomyces naeslundii* with different degree. Moreover some *Lactobacillus* species exhibited a significant inhibitory effects on *S. mutans* [3,17,18]. The ability to inhibit the growth or biofilm formation has been observed for other probiotic bacteria, including *Enterococcus faecium* WB2000, *Bifidobacterium adolescentis* SPM1005 and heat-inactivated *Bifidobacterium* BB12 [19–21].

The aim of the current study was to investigate the probiotic potencies of three lactic acid bacteria such as acid tolerance,

* Corresponding author.
E-mail address: chaieb_mo@yahoo.fr (K. Chaieb).

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antibiotic resistance, antimicrobial and antibiofilm activities, aggregation, co-aggregation, enzymatic activity and cell surface hydrophobicity.

2. Material and methods

2.1. Isolation of probiotic bacteria

Three lactic acid bacteria (LAB) designated as FB2, FF2 and FG1 used in this study were isolated from barley, fermented olive and traditional dried meat respectively. Briefly, ten grams of each sample were homogenized in 90 mL of Man Rogosa and Sharpe (MRS) broth and incubated at 37 °C for 48 h. Then, 0.1 mL of the culture was spread on MRS agar and incubated for 48 h at 37 °C. The obtained colony was selected using positive Gram stain, production of catalase and cytochrome oxidase. The biochemical characterization of the isolates was determined using the API 50 CHL (BioMerieux, France). The potential probiotic strains were conserved at −80 °C in MRS broth with 30% glycerol.

2.2. Molecular identification of the selected strains

Bacterial DNA was extracted using a Wizard Genomic Purification Kit (Promega, Lyon, France) according to the manufacturer's recommendations. The bacterial 16S rRNA gene sequence (1.5 Kb) was amplified by polymerase chain reaction using universal primers [22]. DNA sequencing reactions were performed in the DNA Engine Tetrad 2 Peltier thermal Cycler (Bio-Rad, Hercules, CA, USA) using the ABI BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Finally, DNA sequence homology search within the GenBank database was performed using BLAST.

2.3. Test for safety considerations of LAB

2.3.1. DNase test

The selected LAB were grown in MRS broth than transferred onto DNase Agar containing toluidine blue. The DNase activity was described by observing a clear zone around the colonies.

2.3.2. Hemolytic activity

Fresh cultures of LAB were streaked on Columbia agar plates, containing 5% (w/v) sheep blood, and incubated for 48 h at 37 °C [23]. Plates were examined for signs of β-hemolysis (clear zones around colonies), α-hemolysis (green-hued zones around colonies) or γ-hemolysis (no clear zones around colonies). *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 35218 were used as positive control for β - and for α -hemolysis respectively. The assay was performed twice.

2.3.3. Antibiotics susceptibility of the selected strains

The antibiotic susceptibility of LAB was tested against Penicillin (6 μg), Amoxycillin (20 μg), Piperacillin (10 μg), Streptomycin (10 μg), Streptomycin (500 μg), Kanamycin (30 μg), Novobiocin (5 μg), Rifampicin (30 μg), Ofloxacin (5 μg), Aztreonam (30 μg) and Cefalotin (30 μg) using the agar disc diffusion assay [24].

Bacterial suspensions (10^8 CFU/mL) were inoculated in Muller Hinton agar using sterile cotton swabs. The plates were air dried for 15 min, then discs containing antibiotics were deposited on the plates.

After 18 h of incubation at 37 °C, inhibition zone diameters around each disc were measured and the strains were categorized as resistant, intermediate resistant, or susceptible to the antimicrobial agents based on the inhibition zone size [24,25]. Mean values of three independent assays were reported.

2.4. Resistance of lactic acid bacteria to the simulated gastrointestinal conditions

2.4.1. Acid and bile salt tolerance

LAB were grown in MRS broth at 37 °C for 18 h, harvested by centrifugation (10.000 g, 5 min, 4 °C) and transferred to phosphate buffer saline (PBS (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2)) with a pH values adjusted to 1, 2 and 3 followed by incubation at 37 °C during 0, 1 and 3 h, reflecting the time spent by food in the stomach. Acid tolerance was evaluated by comparing the viable cells counts of bacteria in MRS agar before and after the incubation in medium at various pH [23,26]. The bile salt tolerances of LAB were determined in MRS broth containing 0.3, 0.5 and 1% (w/v) of ox gall bile (Sigma). After 4 h of incubation at 37 °C, the bile salt resistance was assessed by enumeration of survival cell using MRS agar medium [27].

2.4.2. Resistance to pepsin and pancreatin

An overnight cultures of LAB were collected by centrifugation (10.000 g, 5 min, 4 °C), washed twice with PBS buffer (pH 7.2), re-suspended in PBS solution (pH 2 and pH 3) containing pepsin (3 mg/mL), pH 8 or pancreatin (1 mg/mL). After incubation at 37 °C, viable cells were counted using MRS agar medium by the plate count method for 0, 1 and 3 h with pepsin and for 0 and 4 h with pancreatin. Those time correspondent to period spent by food in the stomach and small intestine, respectively. Survival rate was determined as follow:

Survival rate (%) = [cell number (log CFU/mL) survived in PBS modified pH/cell number (logCFU/mL) of initial inoculated cell] × 100 [27].

2.5. Aggregation activity

2.5.1. Auto-aggregation

Auto-aggregation capabilities were performed by the method of Del Re et al. [28]. LAB was harvested by centrifugation at 5000*g* for 15 min. Then, pellets were washed twice with phosphate-buffered saline PBS, re-suspended in the same buffer and adjusted to 10^8 CFU/mL. Cell suspension (4 mL) was mixed, and auto-aggregation was monitored during 5 h of incubation at room temperature.

Every hour, 0.1 mL of the upper suspension was transferred to another tube with 3.9 mL of PBS, and the absorbance (A) was measured at 600 nm. The auto-aggregation percentage was expressed as followed: $1 - (A_t/A_0) \times 100$, where A_t represents the absorbance at time t = 1, 2, 3, 4 or 5 h and A_0 the absorbance at t = 0.

2.5.2. Co-aggregation

To investigate the co-aggregation of LAB, five potential pathogenic strains (*Staphylococcus aureus* ATCC 25923, *Salmonella enterica* serovar Typhimurium ATCC14028, *Enterococcus feacalis* ATCC 29212, *Bacillus cereus* ATCC 14579 and *Escherichia coli* ATCC 35218) were used as coaggregation partners. Cell suspension was prepared as described for autoaggregation assay. Then, equal volumes (2 mL) of each cell suspension were combined in pairs by vortexing for 10 s. Tubes containing 4 mL of each bacterial suspension on its own were used as control. During incubation at room temperature for 5 h, absorbance at 600 nm was measured. The percentage of coaggregation was determined according to [29]. % Coaggregation = $[(A_{\text{test bacteria}} + A_{\text{lactic acid bacteria}}) - 2 \times (A_{\text{mixed strains}})/(A_{\text{test bacteria}} + A_{\text{lactic acid bacteria}})] \times 100$. Each experiments were conducted twice.