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Lactobacillus plantarum, *Lactobacillus casei*, local proteolytic strains evaluated for their probiotic potential

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ABSTRACT

Probiotics are microorganisms that, in sufficient quantity, exert a positive effect on health. They have an important role in improving digestion and bowel function, maintaining the balance of intestinal flora and the acid-base balance in the colon. Probiotic strains must also have good technological properties. Indeed, this study aims to evaluate the probiotic ability of a collection of lactic acid bacteria strains with proteolytic character, isolated from cow's milk and goat local Algerian populations, in different growth conditions (pH survival 2.5 and 2, the resistance to 0.3% bile salts, and evaluating the hydrophobicity of the cell surface cultures against Xylene). All the strains studied and their associations have shown significant resistance against the acid pH and high concentration of bile salts (0.3%). In addition, the percentages of xylene strains adhesion indicates the hydrophobicity of their surfaces. In the end, three strains were selected because they show a most interesting probiotic profile characterized by a strong antibacterial effect against pathogenic strains. They were identified at the molecular level by 16S rDNA sequencing as Lactobacillus plantarum C7, C8 and Lactobacillus casei C5.

Key words: Lactic acid bacteria, proteolytic activity, probiotic, selection criteria.

INTRODUCRION

Lactic acid bacteria have techno food interests as they help to preserve food by producing lactic acid and bacteriocins and contribute to the development of their organoleptic quality by producing a number of flavors involved the characteristics of the product. Many studies are also attached to select strains of nutritional or technological interest [1].

Lactic acid bacteria, in particular lactobacilli and bifidobacteria, are widely known for their probiotic and nutritional potential [2]. In addition, for select probiotic strains must overcome some barriers and to perform some functions including, for example, resistance to gastrointestinal condition such as gastric acidity and bile toxicity, ability of adherence to to the host's epithelial cells [3, 4]. Probiotic strains must also have good technological properties [5]. Their beneficial effects can be exercised at changing the food matrix constituents, for example, contribute to improve the digestibility of certain macronutrients or bioavailability of micronutrients that can therefore have beneficial health effects by helping to protection against certain diseases or prevent certain nutritional deficiencies [6] such as prevention of common allergic conditions in children under 3 years, due to poor digestion of milk protein [1]. Although the efficacy of probiotics must be demonstrated and controlled by clinical trials, their primary preselection is based on *in vitro* tests [3, 7].

The objective of this study was to evaluate the probiotic profile *in vitro*, of a collection of local lactic bacteria with proteolytic character by tolerance to bile salts, resistance to gastric acidity and hydrophobicity of the cell surface of strains against xylene.

MATERIALS AND METHODS

Microorganisms Strains

The collection of strains used in this study is: *Lb.delbrueki lactis* (V4); *Lb.plantarum* (C7, C8 and C10); *Lb.casei* (C5, V2); *Lc.lactis subsp lactis* (C1, V1); *Pc.parvulus* (C3); *Lc.pseudomesenteroides* (V3); and *Sc.thermophilus* (C12).

These strains were isolated from cow's milk (V) and goat (C) of local Algerian population in the region of khemis miliana, Aindefla, and identified the phenotypic level by conventional microbiological tests in the laboratory of microbiology of University Djilali Bounaama, Khemis Miliana, Algeria.

The indicator strains for antibacterial activity are: *Pseudomonas aerugenosa*; *Niesseria gonrrheae*; *Klebsiella pneumoniae*; *Escherichia coli*, pathogenic strains supplied by the private laboratory analyzes of doctor Zibouche, Aindefla.

Proteolytic activity

The proteolytic activity of different cultures [V1, V2, V3, V4, C1, C3, C5, C7, C8, C10, C12 and mixed cultures CM1 (V1, V2 and V4), CM2 (C5, C7 and C12), CM3 (C10 and C8) and CM4 (C1 and C3)], was determined and compared to the medium Agar at 1% of milk [8], as well as another richer MRS medium supplemented with skimmed milk 10% [9].

Tolerance to Gastric Acidity and Resistance to Bile salts

We chose the study of the survival of bacteria to the passage of the digestive tract, or more specifically to acid pH and the presence of bile salts, mainly because of the definition of probiotics which includes only living microorganisms[6].

To determine this survival capacity in strains V1, V2, V3, V4, C1, C3, C5, C7, C8, C10, C12 and mixed cultures CM1 (V1, V2 and V4), CM2 (C5, C7 and C12), CM3 (C10 and C8) and CM4 (C1 and C3), the effect of exposure to an acid medium in the absence or in the presence of 0.3% bile salts on these bacteria was determined using the technique described by [10]. The bacterial pellet youth cultures was recovered after centrifugation at 13,000 rpm / 4min, then the cells were suspended in MRS broth with or without 0.3% of bile salts and at different pH (pH 2, pH2.5) for 120 minutes. The results are expressed in percentage in comparison with a control (pH6.5).

Hydrophobicity test

The hydrophobicity of the lactic bacteria was conducted according to the method described by [11]. Briefly, the bacterial pellet of 18 h culture after centrifugation (12,000 rpm for 5 min) was recovered re-suspended in 1.2 ml of Magnesium Urea Phosphate Buffer (pH 6.5) and adjusted, if necessary, to OD_{450} 1.0 ($OD_{initial}$). Then, xylene (0.6 ml) was gently added to 3 ml of the bacterial suspension and incubated at 37°C for 10 min. This mixture was correctly mixed and let to settle (~15 min). The aqueous phase was collected to measure once again the OD_{450} (OD_{final}). The hydrophobicity cell surface (H%) was calculated using the following equation:

$$H\% = [(OD_{initial}-OD_{final})/OD_{initial}] \times 100$$

Sequencing of the 16S rDNA gene of the selected strains

The bacterial genomic DNA was extracted from the cultures in MRS broth according to [12] and used as template for amplification of 16S rRNA genes by the polymerase chain reaction (PCR). The universal primers SSU for (3'-TGCCAGCAGCCGCGGTA-5 ') and SSU rev (5'GACGGGCGGTGTACAA-3') were used. PCR products were resolved by electrophoresis in 1% (w / v) agarose gels and visualized by ethidium bromide. The gels were analyzed with the use of a GelDoc apparatus (BioRad) and of QuantityOne image analysis software.). The DNA sequencing was performed by the company Beckman Coulter Genomics .A homology search was performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments were performed using ClustalW program and Bioedit DNAbaser.

Antibacterial activity

The method of the discs described by [13] was applied: it is to flood the surface Mueller-Hinton medium with the indicator strain (OD_{660} is between 0.08 and 0.1). After incubation for 30 min at 37°C, sterile disks (5 mm diameter)

were deposited on the surface of the agar. Each disk receives 10µl of lactic youth culture. Once the dishes are dried at room temperature, they were incubated at 4°C for 4h, thereafter incubated at 37°C for 24h. The inhibition of the indicator strain results in the formation of clear zones around the discs. The supernatant effect was tested by [14]. The antimicrobial activity is revealed by the appearance of zones of inhibition around the discs [15]. The diameters of the inhibition zones appearing around the discs were measured, the result is positive if the diameter of the inhibition zone (Z) is greater than 2mm [16]:

Zi (mm) = diameter of the zone of inhibition obtained (mm) - well diameter (5mm)

Statistical analysis

The data were calculated with mean values and standard deviations (mean ± SD) from triplicates. Analysis of variance (ANOVA) was used and statistical significance was attributed to ($P < 0.05$).

RESULTS AND DISCUSSION

Proteolytic activity

Proteolysis is one of the most important biochemical processes involved in the manufacture of many fermented milk products. The ability to produce extracellular proteases is a very important feature of lactic bacteria. These enzymes hydrolyze milk protein by providing the essential amino acids for growth. It is known that the proteolytic system of lactic acid bacteria degrade proteins and therefore changes the texture, taste and aroma of fermented products [17].

Enzyme extracts showed proteolytic activity reflected by zones of proteolysis around the wells (Figure 1(a) and (b)), so these extracts have the ability to hydrolyse the casein of the milk. Proteolytic behaviors of these different cultures vary in medium to another. The proteolytic activity depends in part on the chemical composition of the culture medium [18, 19, 20, 21].

Figure 1(a) : Proteolytic activity of different cultures on MRS milk

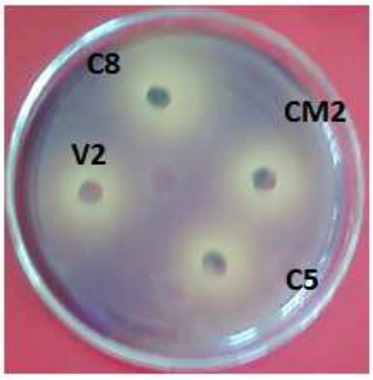
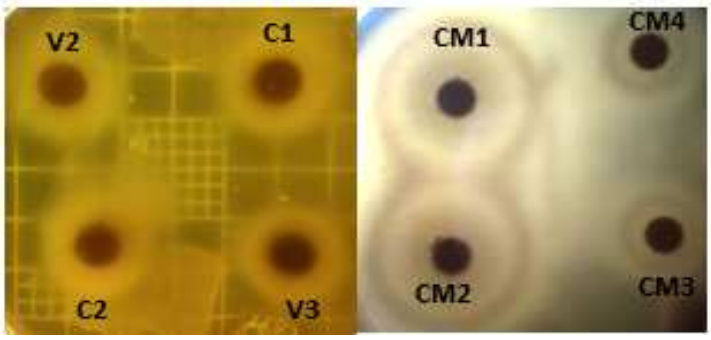


Figure 1(b): Proteolytic activity of different cultures on agar milk



Resistance to acidity and bile salts

Bacterial survival conditions of the digestive tract is a key criterion for selecting probiotic strains [22], however, is a complex function involving many mechanisms allowing the bacteria to survive successive environments very various (low pH, the presence of bile salts) [6].

All cultures have suffered significant losses of viability when subjected to gastric conditions compared to their survival at pH 2 and 2.5 over the (survival rate superior to 100%). Furthermore, the number of viable cells is still significant and greater than 55% survival rate recorded by the strain *Sc. thermophilus* C12 to pH2 (Figure2). *Lactobacillus plantarum* C7 showed better resistance to low pH, followed by C8, C5 and cultures that compose them.

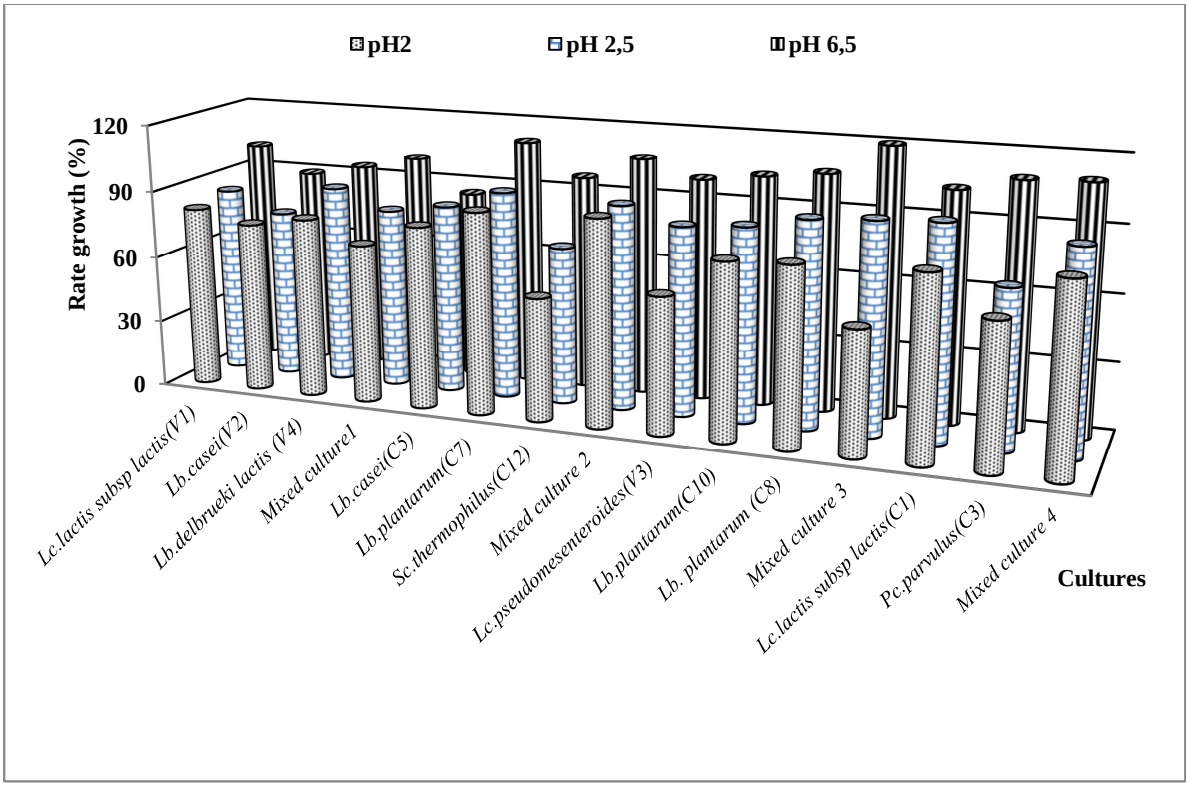


Figure 2: resistance of different cultures to acid medium

Furthermore, all cultures showed variable sensitivity against bile salts and acids in different media. At pH 2.5, the survival rate decreased to a rate of 65.66 ± 2.08% for *P. parvulus* C3. The difference between the strains was significant (P> 0.05). Whereas at pH 2, has not been a significant reduction in survival rate compared to pH 2.5 and even between all the pure strains and mixed cultures, with the exception of *Lc.lactis subsp. lactis* V1, C1, and *P. parvulus* C3 where their viability has decreased significantly (Figure3). Note that the combination of strains had no significant effect on increasing the chance of survival of lactic acid bacteria tested in acidic media and in the presence of bile salts, but resistance remains high (superior than 62 %), which makes possible living passage of these cultures in the digestive tract.

Same result was found by [23] which showed that the highest rate of resistance to pH 1 was observed with *L. plantarum* F12 strain with good tolerance to bile salts (0.3%). Several studies have shown that human stem the species. *L. acidophilus, L. brevis, L. fermentum, L. plantarum* and *L. casei* group have tolerance stomach Conditions [3, 24, 25, 26, 27, 28]. The work of [29] suggested that lactococci can survive to reach the human gastrointestinal tract or animal. [30] showed good resistance of the strain *Lc. lactis ssp. cremoris* NCDO 712 at acidic pH whose survival was 100% at pH 4 after 2h incubation. They reported that the majority of the lactic acid bacteria possess an acidic medium tolerance mechanism; they are capable of surviving at lethal concentrations of acid.

The work of [31] showed a high tolerance of *Lactobacillus* sp. against the pH 2.5 and pH 2 after 2 hours of incubation. Similarly, [32] showed a viability of up to 93% At pH 3 of isolates of Jebneh Darfiyeh *Leuconostoc mesenteroides spp. dextranicum* (JD1 and JD3). [33, 34] showed that bacterial competitiveness acid medium lactobacilli is an advantage over other bacteria because of their tolerance to acidity.

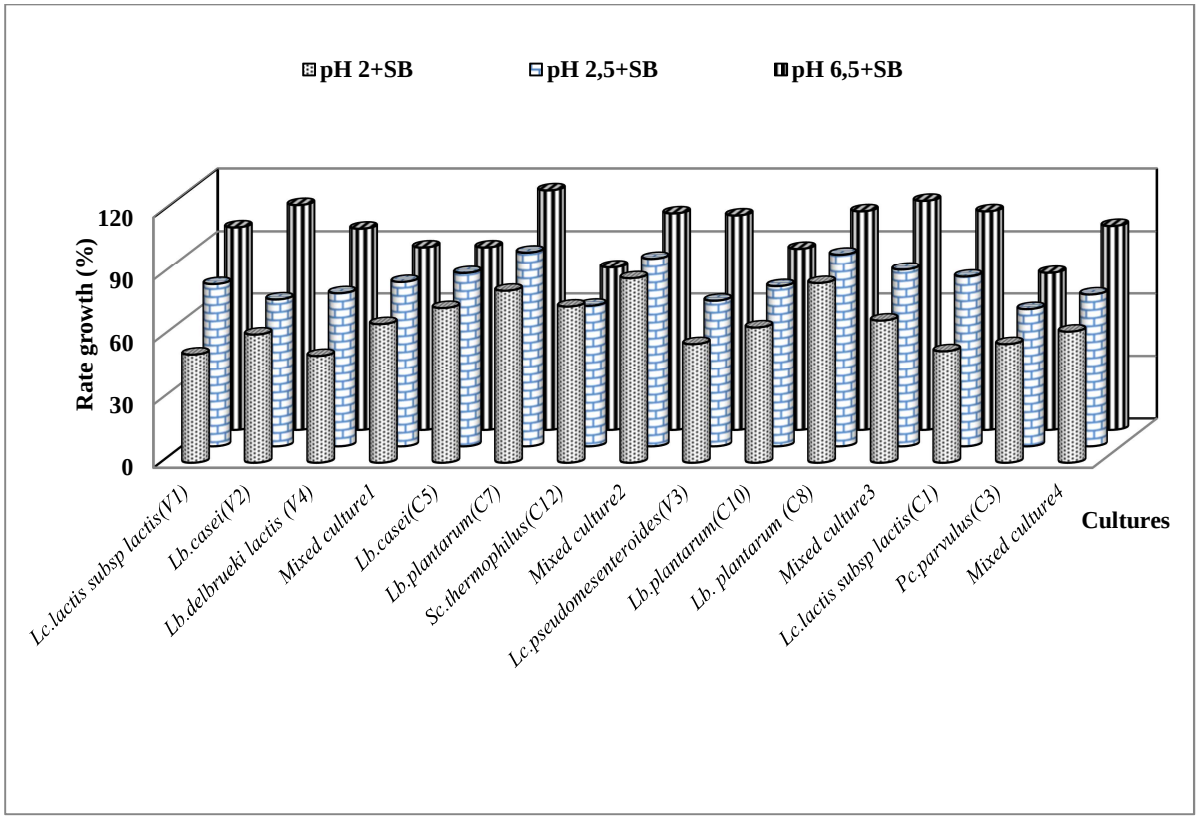


Figure 3 : resistance of different cultures to bile salts at 0.3%

Lactobacilli are capable of metabolizing bile acids which protect against bile. One of the mechanisms of resistance; is deconjugation of bile acids by enzymes biles salts hydrolase (BSH). The hydrolysis releases the wisteria and / or taurine core steroid which has the effect of reducing the solubility of bile at low pH and reduce detergent activities [35, 36]. This ability to detoxify allowed bile salts which would increase the survival and intestinal persistence producing strains thus make them robust to extreme conditions prevailing in the gastrointestinal tract [37]. The selection of strains with a high tolerance to acidity is very interesting for the food industry [38].

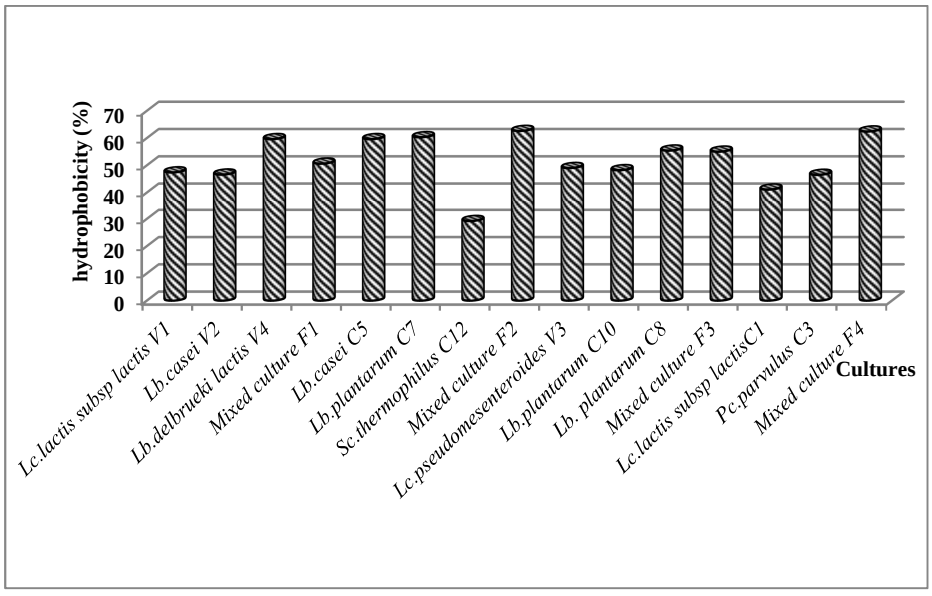


Figure 4: hydrophobicity of different cultures against xylene

Hydrophobicity test

This test evaluates the hydrophobicity of the cell surface of mixed cultures and pure strains against xylene may reflect the colonization potential of enzymes to intestinal mucus. The distribution of cells between the aqueous phase and xylene results from the hydrophobic interaction between microorganisms and hydrocarbons. The percentages of adhesion pure cultures and mixed cultures xylene indicate the hydrophobicity of their surface (Figure 4).

To exert their beneficial effects, probiotics must adhere to epithelial cells or intestinal mucus and persist in the gut [39, 27]. The results show that cultures put to test exhibit good hydrophobicity, this shows good selectivity of membrane surfaces. The highest values were recorded with pure strains of *L.plantarum* C7, C8 and *L.casei* C5 followed by their mixed cultures CM2, and CM3. The difference recorded between the pure strains and mixed cultures is significant (P <0.05).

[23] found that the *L. plantarum* F12 strain showed the highest adherence to Caco-2 cells. [40] found a hydrophobicity of 45% for *Lactococcus lactis cremoris* SBSP CNRZ 107. The hydrophobicity of several strains of *Lc. lactis* dairy sources was assessed by [41] who found values varied between 5% and 88%. In our study *Sc.thermophilus* was the only strain that has low hydroohobicity against xylene with a percentage of 29%. Similar results were obtained by [11] on two Streptococci with about 20%.

Antibacterial activity

Based on the results of our study, three strains were identified as having high potential probiotic. BLAST analysis of 16S rDNA sequences corresponding to strains C5, C7 and C8 confirmed their prior phenotypic identification as *L. plantarum* C7, C8 and *L. casei* C5.

Via all tested criteria, the three strains were selected for the study of antibacterial activity. The antibacterial activity of a probiotic is paramount to the successful colonization of the intestinal mucosa. It ensures a barrier effect and defense against pathogens [42]. The study of autochthonous lactic acid bacteria allows to select the best candidates to improve the microbiological safety of traditional food products and may increase their shelf life [43]. The extracellular fraction of the supernatant has a strong antibacterial power, which confirms the production of antimicrobial agent by the strains tested in the medium(Table 1).

Table 1: Antimicrobial activity of probiotic strains selected against bacterial pathogens

	L.plantarum C7		*L.plantarum* C8		*L. casei* C5	
	Culture	NS	Culture	NS	Culture	NS
E.coli	08	08	7.5	09	10	10
P.aerugenosa	12	17	12	12	09	09
N.gonorrheae	10	11	10	10	11	11
K.pneumoniae	11	11	11	11	10	10

NS: Native supernatant

[44] Found that *Lactobacillus plantarum* showed the most antibacterial potency to *Escherichia fergusoni* other studies have shown that the extracellular fraction contains substances responsible for this interaction [45]. Lactic acid bacteria are known to produce a variety of antimicrobial compounds: organic acids, bacteriocins, diacetyl and hydrogen peroxide [46].

CONCLUSION

However, it is important to note that each strain is unique and the mechanisms associated with specific strains can't be extrapolated to all probiotic microorganisms [47].

The initial selection of strains using *in vitro* methods remains a useful preliminary step in the detection of probiotic candidates, despite the difficulties to characterize reliable probiotic strains in this way [48]. In this study, we selected three strains with proteolytic character: *Lactobacillus plantarum* (C7 and C8) and *Lactobacillus casei* (C5) able to have certain technological properties (proteolysis), functional health interest of both probiotic and nutritional.

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