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Research Article

Technological Properties of Probiotic Bacteria Obtained from Raw Milks

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Abstract

Five strains isolated from different raw milks, obtained from different farms, were characterized in respect to their technological properties such as the acidifying, proteolytic and lipolytic activities, gas and exopolysaccharides productions. Also, they have been tested for their growth at different non-optimal temperatures.

In fact, acceptable acidification and low proteolytic and lipolytic capacities were detected for all strains. In addition, they were able to produce exopolysaccharides even at 40% and grow at 45°C, 4°C and -20°C.

Moreover, the Principal Component Analysis (PCA) of all the technological properties measured on the five probiotic bacteria contributed to the selection of two best-performing strains such as *Lactobacillus plantarum* (BA12) and *Lactobacillus fermentum* (CABA16). After that, these bacteria may be used further for production of functional foods and studying their added effect as probiotic starters on the quality of these products.

Keywords: Probiotic Bacteria; Raw Milks; Lactobacillus plantarum; Lactobacillus fermentum

Introduction

The importance of lactic acid bacteria in food industries is increasing due to its beneficial contribution and its ability to prevent foods. Actually, increasing attention to the health benefits of consuming probiotic micro-organisms has been increased worldwide [1]. In fact, these bacteria play an important role in fermented foods and beverage due to their long and safety application [2].

There are many micro-organisms that can be classified as probiotics belonging numerous bacterial strains particularly the species *Lactobacillus plantarum* and *Lactobacillus fermentum* [3]. They have many physiological, biochemical and genetic characteristics. In addition, these bacteria can produce antimicrobial compounds such as peptides, organic acids such as, acetoin reuterin, reutericyclin, hydrogen peroxide and diacetyl [4]. The physiological properties of probiotic micro-organisms associated with pH reduction, production of some digestive enzymes, vitamins and antibacterial substances [5].

Given the diversity of possible applications, these bacteria must be able to adapt to different conditions, like acid challenges, thermal stresses and freezing, encountered during manufacturing and storage of products [6].

Despite that the main role of lactic acid bacteria in fermented products is related with lactic acid production, it possess additional important characteristics that need to be taken into account in order to select them as starter cultures and improve the bioavailability of food [7].

Therefore, the main objective of this work was to attain better knowledge on the overall ability of probiotic bacteria to withstand environmental challenges and to gain new indications on how to improve their exploitation in new technological applications in probiotic food. In this work, five strains of *Lactobacillus* species, characterized for successful technological exploitation, were studied.

Materials and Methods

Probiotic strains and culture conditions

Five probiotic strains, two isolated from camel milk *Lactobacillus fermentum* (CAT19 and CABA16) [8], one isolated from cow's milk (BA12), one isolated from goat milk (CT28) and one isolated from sheep milk (OSO47) (Research Unit "Bioconservation et Valorisation des Produits Agro-alimentaires UR 13AGR 02", ESIAT, Tunis, Tunisia), were selected taking into account their probiotic potentials such as their resistance to gastro-intestinal conditions, adhesion properties, antioxidant and hypocholesterolemia and symbiotic effect with prebiotics. The cultures were stored at 20% of glycerol at - 80°C. For this, the probiotic cultures were activated for three times in de Mann Rogosa Sharpe (MRS) broth (Biokar Diagnostics, France) using 1% of inoculum and incubated at 37°C for 18h.

Technological properties

Acidification activity

The acidification activity of the five probiotic strains was measured using titratable acidity and pH measure according to Ben Moussa., *et al* [9]. The two parameters were determined at 6, 8, 24 and 48h of incubation at 37°C.

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Gas production

All probiotic strains were tested for gas production according to the method described by Greco., *et al* [10]. The strains were seeded in 10 mL of MRS broth in the presence of a Durham bell. After incubation at 37°C for 48h, the rise of the bell accompanied by turbidity of the broth is synonymous with a production of CO_2.

Extracellular proteolytic activity

The proteolytic activity was tested using the agar method on PCA agar (Biokar Diagnostics, France), supplemented with 10% of skim milk or 4% of bovine gelatin. Also, this activity was performed by spectrophotometric method at 345 nm and 340 nm, using azocasein and o-phthaldialdehyde respectively (Sigma, France) as substrates [11].

Extracellular lipolytic activity

This activity was tested on Nutritive agar (Biokar Diagnostics, France) supplemented with 1% Tween 20, 1% Tween 80 [12]. Also, this assay was determined by titration method as indicated by Ben Moussa., *et al* [9].

Texturing power: Exopolysaccharides production

The five probiotic strains were grown in tubes containing 20 mL of MRS broth added with glucose (2% (w/v)) at 37°C for 3 days. After centrifugation (6000 rpm, 4°C, 20 minutes), two volumes of cold ethanol (Merck) (95% (v/v)) were added to one volume of culture supernatant. After that, precipitates were recupered by filtration under vacuum, dried at 60°C and measured to determine the weight of exopolysaccharides (EPS) produced [13].

Growth at different non-optimal and cold temperatures

The five probiotic strains were tested for their ability to grow at 35, 37, 40, 42, 45 and 50°C in MRS agar (pH 6.8) containing bromocresol purple (0.16 g/L) (Sigma, France). For cold shock tolerance, the cultures were exposed at + 4°C and - 20°C for 24h. After that, the cultures were incubated at 37°C for 24h. The OD at 595 nm was measured after the cold treatment and again after incubation [14].

Statistical analysis

The software used was SPSS version 20.0. The Student's test was also used and the threshold differences ($P < 0.05$) were considered statistically significant. Also, the principal component analysis (PCA) was performed for all the data measured on the main techno-functional properties of the tested probiotic lactic acid bacteria strains.

Results and Discussion

Technological properties

Titrable acidity and pH

Initially, the five probiotic strains were able to acidify the milk with a significant difference ($P < 0.05$) giving values not exceeding 102 ± 0.01°D, obtained by the strain *L. fermentum* (CAT19). Similarly, the pH values of milk inoculated with probiotic bacteria decreased to 4.2 ± 0.01 pH units below 4.6 (iso-electric casein pH). In fact, the production of lactic acid following the decrease in pH gives a specific aroma and increases the sensitivity of certain micro-organisms to the acidity of the medium [15].

Gas production

The five probiotic strains produced CO2 from D-glucose after incubation for 48h at 37°C in MRS broth in the presence of a Durham bell. These strains may not interfere with the texture of fermented dairy products. Moreover, Mathieu [16], García-Ceja., *et al.* [17] and Senaka Ranadheera., *et al.* [18] inoculated respectively *L. plantarum, L. reuteri* (58% *L. fermentum*) and *Propionibacterium jensenii,* producing gas, in yogurt without affecting its texture or having undesirable effects. Therefore, it can be pointed out that our probiotic strains may be suitable candidates for the production of probiotic yoghurt fermented milk.

Proteolytic activity

The proteolytic activity of each probiotic strains was demonstrated on agar medium and spectrophotometric method revealed by azocasein as a substrate. Clear zones were obtained after incubation of probiotic strains on skim milk agar. Whereas, no clear halo was observed on medium supplemented with bovine gelatin. All isolates were able to hydrolyze milk casein ($P < 0.05$). On the other hand, the two strains *L. plantarum* (BA12) and *L. fermentum* (CABA16) were considered the most proteolytic compared to the other strains (Figure 1), producing proteolysis zones of 3.44 ± 0.56 and 5 ± 0.12 mm respectively. As concern the spectrophotometric methods, we found important proteolytic powers ranging from 0.37 ± 0.21 to 0.66 ± 0.42 obtained by the o-phthaldialdehyde method from 0.142 ± 0.07 to 0.294 ± 0, 33 mg azocasein.

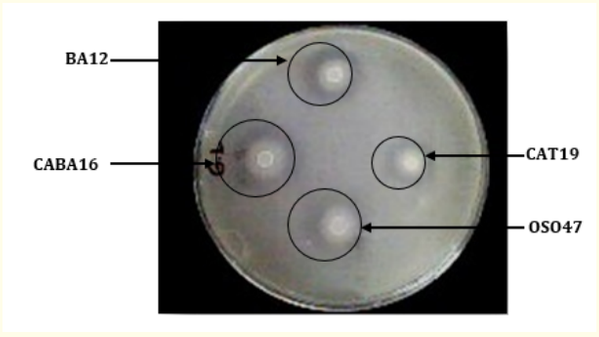

Figure 1: Proteolytic activities of four probiotic strains on nutrient agar supplemented with 10% of skim milk

These activities varied significantly ($P < 0.05$) depending on the strain.

This intra- and inter-specific variability observed in proteolysis is frequently reported for strains isolated from natural sources [19]. Our results are in perfect agreement with Almeida-Junior., *et al*. [20] improving that all *Lactobacillus* strains isolated from goat's milk are able to hydrolyze casein. Although, lactic acid bacteria are considered to be weakly proteolytic. Their proteolytic system is essential, on the one hand, for optimal growth in milk and contributes significantly to the development of flavor in fermented dairy products [21]. On the other hand, proteolysis could also contribute to the prevention of frequent allergies in children under three due to the poor digestion of milk proteins [22].

Lipolytic activity

For this, we observed that all probiotic strains tested did not reveal extracellular lipolytic activities on agar media supplemented with Tween 20 (1%), Tween 80 (1%) and tributyrin (1%).

In addition, the probiotic strains lack extracellular lecithinases. In addition, the lipolytic activity, expressed as a percentage of oleic acid, is very low for all strains with a maximum of 2.3 ± 0.03% (Figure 2). These results are consistent with the literature data that lactic acid bacteria have weak lipolytic powers [23].

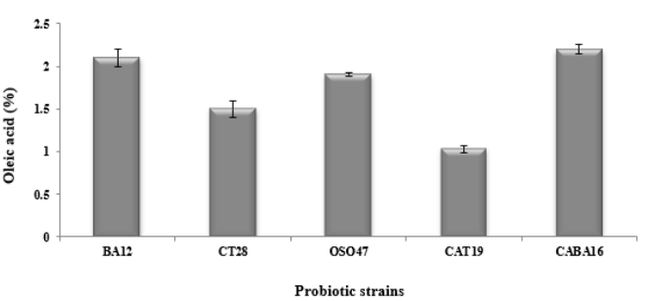

Figure 2: Percentage of oleic acid produced by the five probiotic strains.

Exopolysaccharides production

The results for the EPS levels produced by the five probiotic strains are shown in figure 3. Significant percentages ($P < 0.05$) varied from 33.33 ± 0.05 mg/L obtained with the strain (OSO47) to 40.01 ± 0.03 mg/L for the strain (CABA16). These probiotic strains have acceptable texturing powers and are similar to those found by Almeida-Junior., *et al.* [20] showing an average EPS production of 27.60 mg/L enregistrated by probiotic bacteria. It can

be justified that EPS levels produced by bacteria could be strongly influenced by growing conditions, such as acidity, temperature, and environmental composition [24]. These EPS contents have an immunomodulatory effect on intestinal epithelial cells and can be used as texturing agents in fermented dairy products [25].

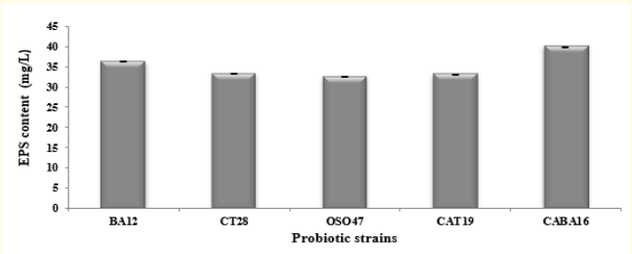

Figure 3: Exopolysaccharide levels produced by the five probiotic strains.

Growth at non-optimal temperatures

None of the five probiotic strains is capable of growing at 50°C. On the other hand, they were able to grow at 45°C, and this resulted in the bend of the color of the culture medium from purple to yellow. Our results are similar to the T_{max} noted in the work of Reale., *et al*. [14] who reported that four strains of *L. casei* which developed even at 48 °C, whereas the strain *L. paracasei* showed T_{max} values below 45°C. On the other hand, Minervini., *et al*. [26] demonstrated that *L. rhamnosus* strain exhibited tolerance even at 55°C for 10 minutes which is higher than other mesophilic strains tested. It should be noted that our strains behave like thermophilic bacteria and could be used in the formulation of fermented probiotic dairy products such as yoghurt.

The study of the behavior of lactic bacteria with regard to the cold is very important because during the storage of the fermented products, the micro-organisms are subjected to the stress "cold" which can affect their survival. The ability to grow at 37°C after pre-incubation at + 4°C and - 20°C for 24h was studied. Analysis of the results shows that all probiotic strains were able to survive significantly ($P < 0.05$) at both + 4°C and -20°C. Moreover, they are less affected by refrigeration than by freezing. In fact, refrigeration was less stressful after 24h of incubation. The effects of refrigeration and freezing were also studied to evaluate their influence on the probiotic activities of *L. casei* and *L. rhamnosus* strains [27]. It should be noted that refrigeration is the most appropriate means for preserving food products containing probiotic bacteria.

Selection of the most technologically efficient probiotic strains by ACP

A statistical analysis in PCA was performed on all the analytical data obtained during the study of the technological properties of the five probiotic strains isolated from raw milks (Figure 4).

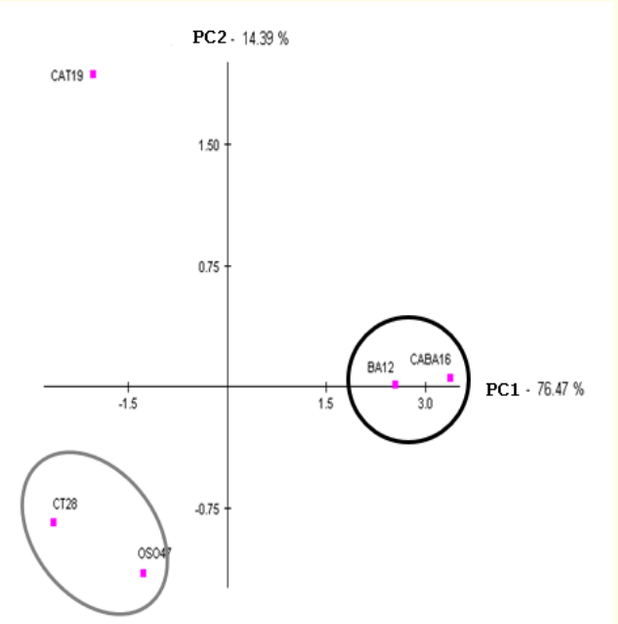

Figure 4: Principal Component Analysis performed on the technological parameters of the five probiotic strains.

The factorial map 1 - 2 is defined by the first two principal components (factor 1 and factor 2), explaining 90.86% of the total variance, which is very satisfactory for an interpretation of the results. The dispersion of these data on the factorial card 1 explains about 76.47% of the total inertia.

The objective is therefore to analyze the relationships between the technological parameters (pH, acidity, proteolysis degrees, fat free acidity, texturizing activity (EPS), growth capacities at refrigeration (+ 4°C) and freezing (-20°C)).

By examining the position of the five strains of selected probiotic lactic acid bacteria with respect to CP1, we can easily discriminate strains (BA12 and CABA16) from other strains, underlining that the technological properties of these two probiotic strains were considered more important, as previously discussed and this both strains have significantly similar characteristics, regardless of the trait considered.

Moreover, the analysis of the data of the factorial map 1 showed the existence of strong positive correlations within a first group including variables such as pH, proteolytic and texturing activities (EPS), free fat acidity (% oleic acid), the growth at + 4°C and at -20°C. On the other hand, a single parameter (acidity) is correlated with all the parameters examined of the first group in a negative way. These same observations were found by examining the data of the matrix of simple linear correlation coefficients calculated between the different parameters measured on the five strains of probiotic lactic acid bacteria. Thus, we noted that acidity is the main technological parameter significantly influencing ($P < 0.05$) the degree of degradation of azocasein, the rate of lysis (oleic acid), and the growth at refrigeration and freezing.

Conclusion

Finally, the two strains *Lactobacillus plantarum* (BA12) and *Lactobacillus fermentum* (CABA16), retained for their high probiotic potentialities, revealed acceptable acidifying and proteolytic properties and also prove to be non-lipolytic. Also, they have been able to produce high levels of exopolysaccharides and develop at non-optimal temperatures of 45°C, + 4°C and - 20°C. However, these probiotic strains proved to be the most efficient, which led us to formulate a functional foods that could bring beneficial effects while preserving its overall quality.

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Conflict of Interest

The analyses were supported by unity "Bioconservation et Valorisation des Produits Agro-alimentaires" (UR 13AGR 02) (ESIAT). The authors declare that they have no conflict of interest. The authors are responsible for the content of the manuscript.

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