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Probiotic Potential of Autochthone Microbiota from *São Jorge* and *Parmigiano-Reggiano* Cheeses

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Abstract

For a long time, ripened cheeses have been officially recognized as having human health beneficial properties. However, to date only a few studies have investigated the complex microbiota of *São Jorge* and *Parmigiano-Reggiano* cheeses in order to identify microorganisms with probiotic potential. Hence, with the purpose of analyzing the probiotic potential of autochthone bacteria from *São Jorge* and *Parmigiano-Reggiano* cheeses, lactic acid bacteria isolated from both products were isolated and their direct biotic interaction against three bacteria found in the human intestine evaluated. From a total of 225 bacterial autochthone isolates, 67 had synergetic behavior with *Bifidobacterium animalis*, 34 had no synergetic behavior with *Shigella dysenteriae* and 33 had antagonistic activity with this human pathogen. Biochemical tests were used to identify these cheese isolates with probiotic potential. *Lactobacillus paracasei* subsp *paracasei Lactobacillus rhamnosus* were found in *São Jorge* cheese and *Lactobacillus rhamnosus*, *Lactobacillus buchneri* and *Lactobacillus curvatus* in the *Parmigiano* cheese. Overall, initial exploratory studies with *São Jorge* cheese seem to show a higher number of potential probiotic agents than those found in the *Parmigiano-Reggiano* cheese. However, in order to better understand the functional potential of this traditional Portuguese cheese, further confirmatory studies should be pursued.

Keywords

Probiotic Potential, Autochthone Microbiota, *S. Jorge* Cheese, *Parmigiano-Reggiano* Cheese

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1. Introduction

Foodborne diseases result from the ingestion of contaminated water or food, the main manifestation being gastrointestinal disorders. Diarrhea alone is estimated to kill 2.2 million people globally every year, many of which children from developing countries [1]. Foodborne diseases constitute a heavy burden for economies all over the world due to associated mortality, morbidity and disability. Despite improvements in sanitary and living conditions, their incidence and outbreaks continue increasing globally.

Nowadays, consumers are aware of the fact that diet can play a positive role in disease risk management and also increase life quality. Pre-, pro- and symbiotic products aimed at improving health by modifying microbiota composition have already become widely available and acceptance of these products appears to be on the rise [2].

The main potential source of probiotic bacteria started to be the gastrointestinal tract, but the scientific community has recently focused attention on fermented foods. A large number of previous studies highlight the potential benefic effects of dairy products and their mechanisms of action on consumer health [3]-[10]. Currently, the ingestion of food containing probiotics is being increasingly used in the prevention and treatment of gastrointestinal disorders [11].

The probiotic potencial of hard and semi-hard cheeses has already been addressed. In this context, *São Jorge* and *Parmigiano-Reggiano* cheeses constitute valid candidates for the selection of autochthone bacteria with probiotic potential.

São Jorge is a hard/semi-hardcheese from the Portuguese and of *São Jorge*, located in the Azores. Produced from unpasteurized cow's milk this cheese holds a Protected Designation of Origin (PDO) certification. For the manufacture the cow's milk is mixed with whey from the previous batch, acidification occurs at 30˚C, followed by curd cooking, whey draining, curd pressing and the addition of salt. Ripening takes between 6 and 16 months.

Parmigiano-Reggiano, or parmesan cheese, is a hard, granular cheese produced from unpasteurized cow's milk; its PDO status limits its producing areas to the provinces of Parma, Reggio Emilia, Bologna, Modena and Mantova, Italy. The manufacture is similar to *São Jorge* cheese, but the maturation period is longer, taking more than 12 months.

For the present study both aforementioned cheeses were selected due to their common features; both are produced from raw cow milk using similar manufacture procedures, in which the fermentation relies on autochthone microorganisms, either present in the whey, raw milk or introduced during the manufacturing processes. Previous studies performed on these products showed that the dominant microorganisms belong to the lactic acid bacteria [12]-[16]. Therefore, the objective of the current study here to isolate and identify new probiotic candidates in a bacterial collection obtained from *São Jorge* and *Parmigiano-Reggiano* cheeses. Furthermore, their interaction with three bacterial agents usually found in the human intestine, including a pathogen (*Shigella dysenteriae*) and a beneficial (*Bifidobacterium animalis* subsp. *lactis*), where also assessed.

2. Material and Methods

2.1. Sampling and Bacteria Isolation

Ten samples of hard cheeses were collected in retail outlets: five *Parmigiano-Reggiano* samples (five different brands, 12 months of cure) and 5 *São Jorge* samples (five different brands, 4 - 7 months of cure).

Sampling consisted of aseptically collecting 2 g of each cheese, transferring to sterile plastic bags, adding 8 ml of buffered peptone water solution (Scharlau, Barcelona, Spain) and homogenizing for two minutes using a Stomacher blender (Stomacher Lab-Blender 400). Subsequently, 0.1 ml of each cellular suspension was strike on the surface of Man, Rogosa and Sharpe -MRS- agar (Oxoid, CM0361) and Muller Hinton -MH- agar (Oxoid CM0337) for detection of non-lactic acid bacteria, seeded in duplicate. One series of boxes with MRS and MH were incubated aerobically and other series under anaerobiosis. Cultures develop at 37˚C for 48 h. After incubation, differential isolated colonies were recovered and maintained at −80˚C in Brain Heart Infusion broth with 20% glycerol for subsequent screenings.

2.2. Evaluation of the Interaction of Cheese Isolates with Selected Bacteria

Suspensions of challenge bacteria of human intestinal origin, including *Shigella dysenteriae* NCTC 4837 (serotype 1) and *Bifidobacterium animalis* subsp. *lactis* BB12 (private collection), were added to molten agar and then poured into Muller Hinton plates. Subsequently, the cheese isolates were stab-inoculated in duplicate and the bacterial cultures incubated at 37˚C for 48 h. After the incubation period the plates were screened for positive inhibition (transparent halo around the stabs) or synergism.

2.3. Microbial Identification

Isolates showing synergism with *Bifidobacterium* sp. and inhibitory behavior against *S. dysenteriae* were further identified using standard microbiological procedures, including cell morphology, Gram staining, catalase/oxidase tests, assessment of glucose fermentation activity, growth in the presence of NaCl (4% and 6.5%) and in distinct incubation temperatures (15˚C and 45˚C). Representative isolates were further identified using the API 50 CHL system.

3. Results

From the ten cheese samples tested in this work, it was possible to obtain a total of 225 bacterial isolates (**Table 1**).

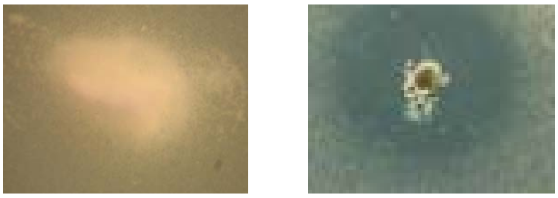
From the 225 isolates, 67 presented potential probiotic effect, by presenting a synergistic effect regarding *B. animalis lactis* and an inhibitory effect or an absence of synergism for *S. dysenteriae*, as illustrated in **Figure 1**. From the selected set, 41 (61%) isolates were obtained from *São Jorge* cheese samples and 26 (39%) from *Parmigiano-Reggiano*.

Interestingly, although the number of bacteria originally obtained from *Parmesan* was low, their synergistic interaction with *Bifidobacterium* sp. was higher. Of the 62 isolates obtained, 42% (26) showed a synergistic effect for *Bifidobacterium* sp., while only 25% (41) from the 163 isolates from the *São Jorge* cheese presented the same effect.

Regarding bacterial identification, all 67 selected isolates were Gram-positive bacilli, non-sporulated, catalase-negative and produced acid in the presence of glucose. Growth ability at different temperatures (15˚C and 45˚C) and NaCl concentrations (4% and 6.5%) was also evaluated. Twenty-three isolates (13 from *São Jorge* cheese and 10 from *Parmigiano-Reggiano*) were able to gowth in all the conditions tested. These features, along with the results from the inhibitory and synergistic effects, allowed the distribution of isolates in 10 groups. Isolates were then identified using the API 50 CHL system (**Table 2**).

4. Discussion

To assess the potential effect of probiotic microorganisms of the ripened cheeses or fermented milks, many studies have been developed in order to characterize the specific beneficial effect on health and the chemical nature or physical proprieties of specific structures or metabolites by those agents [10] [17].



(a) (b)

Figure 1. Representation of a cheese isolate presenting synergism with *B. animalis lactis* (a) and an inhibitory effect against *S. dysenteriae* (b).

Table 1. Number of bacterial isolates obtained from *Parmigiano-Reggiano* and *São Jorge* cheeses samples tested in this study.

	Incubation conditions			
Cheese	MH + aerobiosis	MRS + aerobiosis	MRS + anaerobiosis	Total
Parmigiano-Reggiano	17	21	24	62
São Jorge	43	69	51	163

Table 2. Identification results from the different bacterial groups.

Group	API identification	Origin
1	*Lactobacillus paracasei* subsp. *paracasei* 3	*São Jorge*
2	*Lactobacillus paracasei* subsp. *paracasei* 1	*São Jorge*
3	*Lactobacillus paracasei* subsp. *paracasei* 1	*São Jorge*
4	*Lactobacillus paracasei* subsp. *paracasei* 1	*São Jorge*
5	*Lactobacillus paracasei* subsp. *paracasei* 1	*São Jorge*
6	*Lactobacillus paracasei* subsp. *paracasei* 1	*São Jorge*
7	*Lactobacillus paracasei* subsp. *paracasei* 1	*São Jorge*
8	*Lactobacillus buchneri*	*Parmigiano-Reggiano*
9	*Lactobacillus rhamnosus*	*São Jorge*; *Parmigiano-Reggiano*
10	*Lactobacillus paracasei* subsp. *paracasei*	*Parmigiano-Reggiano*

In this study we evaluated for the first time the probiotic potential of a hard to semi-hard Portuguese cheese, *São Jorge*, and also of *Parmigiano-Reggiano*. It was possible to obtain a total of 225 bacterial isolates, which biotic effects were assessed against *Shigella dysenteriae*, a strictly human pathogen, and *Bifidobacterium animalis lactis*, a probiotic agent often incorporated in commercial foods.

According to WHO [18], *Shigella* is a major cause of dysentery outbreaks throughout the world, being responsible for symptoms such as fever, diarrhea, mucosal ulceration, abdominal pain and tenesmus. It affects specially children under 5 years [19], being a characteristic disease from developing countries, due to poor hygiene and lack of potable water.

Bifidobacteria is a major group of commensal microbiota, being well established that their presence in the intestine is associated with the human host good health status. *Bifidobacterium animalis lactis* was isolated from feces of animal and human origin, and has the ability to colonize and multiply the intestine, to promote immune modulation, to prevent diarrhea and to present antimicrobial activity in presence of pathogens [6] [10] [20].

From the 225 isolates obtained, 67 presented a potential probiotic effect, by presenting a synergistic effect regarding *B. animalis lactis* and an inhibitory effect or an absence of synergism for *S. dysenteriae*. From the selected set, most isolates were obtained from *São Jorge* cheese samples, showing that the probiotic potential of this cheese should be further evaluated. Those properties should include: higher resistance to gastric acidity and bile salts, antimicrobial activity in the presence of enteropathogens, antimicrobial resistance and adhesion or competition by specific receptors in human intestinal cells [21] [22].

Relevant isolates from *São Jorge* island cheese belong to species *Lactobacillus paracasei* subsp. *paracasei* and *L. rhamnosus*. Several authors have already evaluated the addition of probiotic *L. paracasei* strain in cheese production, with very promising results regarding stability and viability of bacteria during storage and also regarding cheese storage [23] [24]. This strain has been also isolated from cheeses, especially at the end of the ripening period, and often considered the dominant bacteria species [25].

L. paracasei subsp. *paracasei* isolated from fermented dairy products has a broad antimicrobial spectrum, which includes *Salmonella Salmonella enterica* subsp. *enterica*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Enterobacter sakazakii*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Shigella flexneri*, *Shigella dysenteriae*, *Listeria innocua*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* Typhi, *Staphylococcus aureus* and *Porphyromonas gingivalis* [26]-[28]. The antagonistic ability was associated with the production of different bacteriocins [29]. Its immunomodulating properties have also been described [30].

The antagonistic spectrum activity of *L. rhamnosus* is also very wide, including *Escherichia coli*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Salmonella* Typhimurium and *Listeria monocytogenes* [6] [31]. Its antimicrobial properties are probably due to the presence of bacteriocin-like proteins, stable to temperature variations and with a broad action spectrum [32] [33]. *L. rhamnosus* has been shown to possess immunomodulatory properties with an beneficial impact for the consumer [10]. It also has the ability to inhibit pathogenic bacteria adhesion to intestinal cells by competing for their receptors [34].

Relevant bacteria obtained from *Parmigiano* cheese were *L. rhamnosus*, *L. buchneri* and *L. curvatus*. *L. buchneri* has been previously isolated in matured cheeses, but it was neves defined as the dominant population [25] [35]. It is often used as inoculum in silage, participating in the fermentation process by the production of lactic and acetic acid, allowing the control of unwanted microorganisms [2] [36]. This bacterium has shown a strong antagonist activity against *Campylobacter jejuni* [7] and produces a bacteriocin capable of inhibit *Listeria*, *Bacillus*, *Enterococcus*, *Lactobacillus*, *Leuconostocs*, *Micrococcus*, *Pediococcus* and *Streptococcus* [37]. *L. buchneri* have been shown to be able to resist to gastrointestinal conditions and have shown potential in reducing serum cholesterol [38].

L. curvatus has already been isolated in milk for *Parmigiano-Reggiano* production, but its presence at the end-product has not been documented [39]. This bacterium has been isolated from several cheeses, such as Gouda [40], Lebanese Darfiyeh [41] and Cheddar [25]. This bacterial species probiotic potential has been related to the production of a bacteriocin active against *Listeria monocytogenes* and *E. faecalis* [42]; the production of hydrogen peroxide active against *E. coli* [43]; the survival at conditions of the gastro-intestinal tract [44]; and to its potential immunomodulating effect [45] [46].

5. Conclusions

The differences observed between these two cheeses can be attributed to the specificity of the raw materials and manufacture settings, which are product-specific and carefully defined by the PDO status.

Overall, results obtained suggest a putative probiotic effect of the predominant autochthone microbiota of *S. Jorge* and *Parmigiano-Reggiano*. Evaluation of the biological functionalities involved requires a more detailed research analysis.

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