Probiotic characterization of a commercial starter culture used in the fermentation of sausages

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Abstract

Probiotic starter culture does not only improve the safety and shelf-life of products but also extends health benefits to the consumer. This study investigated the probiotic potential of a commercial starter culture used in the fermentation of meat sausages. The starter culture tested, composed of *Lactobacillus sakei*, *Staphylococcus xylosus*, and *Staphylococcus carnosus*, was evaluated for resistance to antimicrobials, low pH values and bile salts; production of gas and capsules; acidification capacity; and growth after exposure to different pH values, temperatures, and curing salts. The antagonistic capacity was also assessed against *Escherichia coli* ATCC25922, *Salmonella Enteritidis* ATCC13076, *Vibrio parahaemolyticus*, *Staphylococcus aureus* ATCC43300, *Enterococcus faecalis* ATCC29212, and *Listeria monocytogenes* CERELA. The starter culture was susceptible to all tested antimicrobials and strongly inhibited pathogenic strains, with inhibition halos diameters > 30 mm. The culture was resistant to all concentrations of bile salts tested, did not produce gas or capsules, and could grow within a temperature range of 15 °C to 35 °C in saline medium containing healing salts (nitrite/nitrate). Although, the inability of the culture to withstand low pH, indicating intolerance to stomach acidity, limits its use as a live probiotic, beneficial health effects may be derived from the inactivated culture.

**Keywords:** antagonism; bile salts; antimicrobial susceptibility.  

**Practical Application:** Probiotic starter cultures contribute to the production of functional fermented foods.

1 Introduction

Today, consumers are increasingly looking for foods that, in addition to their basic nutritional function, can provide health benefits such as reducing the risk of chronic and degenerative diseases (Behera & Panda, 2020). The high demand for healthier foods has encouraged researchers and the meat processing industry to develop products with new characteristics to attract consumers, for example, with the use of probiotic cultures in fermented products such as sausages (Slima et al., 2018).

According to a recent concept, probiotics can be defined as viable or inactivated microbial cells (vegetative or spore; intact or broken) healthy for the host (Zendeboodi et al., 2020). Probiotics produce a wide range of bioactive compounds, such as bacteriocins, enzymes, amino acids, peptides, short-chain fatty acids, vitamins, antioxidants, anti-inflammatory agents, immunomodulators, and exopolysaccharides (Chugh & Kamal-Eldin, 2020). Collectively, these metabolites act on the human body by strengthening the immune system, increasing nutrient absorption, decreasing blood cholesterol levels, blood pressure and heart rate, and improving digestion, food allergies, brain function, and inflammation (Guimarães et al., 2020; Roobab et al., 2020). Driven by health benefits, it is estimated that by 2023, the global probiotic market will earn about US $ 69.3 billion, with the food sector responsible for generating greater economic value (Barros et al., 2020).

Currently, the most consumed probiotic products are yogurts and fermented milk (Behera & Panda, 2020). Although dairy products are the main vehicle for probiotics, studies have also demonstrated the feasibility of applying probiotic cultures in the preparation of fermented meat products, such as salami, representing an alternative for consumers intolerant to lactose and milk protein (Pavli et al., 2020).

In the preparation of sausages, initial cultures are generally added which are defined as microbial preparations, with a large number of cells from at least one species of microorganism, added to a raw material to produce a fermented food. These preparations accelerate and direct the fermentation process, improving product safety and extending shelf life by controlling pathogens and other microorganisms through competition, and producing new sensory properties (Farnworth & Champagne, 2016). In addition to these food benefits, starter cultures can confer health benefits to the consumer. However, to fully exploit these potential health benefits, the probiotic characteristics of starter cultures must be fully known.

Considering the potential benefits of adding probiotic starter cultures to food products, this study aimed to verify the probiotic potential of a commercial starter culture commonly used in the production of fermented sausages.
2 Materials and methods

2.1 Starter culture and growth conditions

The lyophilized starter culture, composed of Staphylococcus xylosus, S. carnosus, and Lactobacillus sakei, was acquired from Açogueiro Online, located in Jundiaí - São Paulo, and their probiotic characteristics were evaluated. The starter culture was activated in MRS broth (Man, Rogosa, and Sharpe) and inoculated in MRS agar and Baird-Parker agar for optimum growth of the three species. Confirmation of the growth of each species was carried out through morphological characterization and biochemical tests of fermentation of arabinose, sucrose, maltose, and xylose. The starter culture was activated in MRS broth at 37 °C for 24 hours, centrifuged (10,000 ×g for 10 min at 4 °C), washed twice in peptone water (0.1%), and stored in a medium supplemented with 20% glycerol at -20 °C (Kongkiattikajorn, 2015).

2.2 Probiotic characterization of the starter culture

Antimicrobial resistance

The resistance of the starter culture to the antimicrobials ampicillin (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), nitrofurantoin (300 µg), tetracycline (30 µg), and vancomycin (30 µg) was determined by the disk-diffusion method, according to the recommendations of the Brazilian Committee on Antimicrobial Susceptibility Testing (2017). The disks were added to the surface of the MRS agar and the plates incubated at 37 °C for 24 hours in anaerobiosis. The results were expressed as sensitive (S), intermediate (I) and resistant (R) according to the standards recommended by Acar & Goldstein (1991).

Antagonistic activity

The starter culture was activated in MRS broth and incubated at 37 °C for 24 hours. Aliquots of 5 µL were inoculated onto plates containing MRS agar and incubated for 24 hours at 37 °C. A Brain Heart Infusion (BHI) agar overlay, containing indicator cultures (Escherichia coli ATCC25922, Salmonella Enteritidis ATCC13076, Vibrio para-hae-molyticus (isolated from oysters), Staphylococcus aureus ATCC43300, Enterococcus faecalis ATCC29212, and Listeria monocytogenes CERELA), was added to the surface of the plates and incubated again for 24 hours at 37 °C. Antimicrobial activity was observed by the formation of an inhibition zone against indicator cultures (Vieira et al., 2020).

Determination of resistance to low pH values and bile salts

The resistance of the starter culture to low pH values and pH salts was investigated according to the methodology described by Muñoz-Quezada et al. (2013), with the following adaptations. 900 µL of MRS broth buffered to different pH values (2, 2.5, 3 and 7) and containing varying concentrations of bile salts (0%, 0.3%, 0.5%, and 0.7%) were inoculated with 100 µL of the standardized starter culture at 10⁸ CFU mL⁻¹ (colony forming units per mL). Subsequently, 100 µL of each treatment was diluted in peptone water (0.1%) at times 0, 0.5, 1, 1.5, and 2 hours. 10 µL of each dilution was plated onto MRS agar. Colony counting was performed after 48 hours of anaerobic incubation at 37 °C.

Gas production, acidification capacity and capsule production

Gas production and acidification capacity were determined according to Laslo et al. (2019). Capsule production was evaluated according to Hitchener et al. (1982), through negative staining by the Gims method.

Growth at different pH values and temperatures

The growth capacity of the starter culture was checked at pH adjusted to 3, 4, 5, and 6 with 5 M hydrochloric acid (HCl), and at temperatures of 4 °C, 15 °C, 25 °C, 35 °C, and 45 °C (Laslo et al., 2019).

Sensitivity to different concentrations of sodium chloride, and to the healing salts nitrite and sodium nitrate

For the resistance tests, sodium chloride was added to the MRS agar at concentrations of 1.5%, 2.5% and 3.0%. Tests for resistance to nitrite and sodium nitrate were performed using the same procedure with concentrations of 100, 120, 150 and 100, 200, 300 ppm, respectively (Bis-Souza et al., 2020).

Statistical analysis

Significant differences were assessed through analysis of variance (ANOVA) and Tukey's test using the RStudio program (RStudio Team, 2015), with a significance threshold of p < 0.05. Graphic analysis was performed with the aid of the Orange version 3.26.0 program (Orange, 2020).

3 Results and discussion

3.1 Probiotic characterization of the starter culture

Susceptibility to antimicrobials

The starter culture was susceptible to all tested antimicrobials, consistent with the findings of Müller et al. (2016), in which all S. carnosus isolates from starter cultures were susceptible to several antimicrobials, including ampicillin, ciprofloxacin, gentamicin, imipenem, tetracycline, and vancomycin, demonstrating that S. carnosus strains are widely susceptible to antimicrobials. Resistance to clinically relevant antimicrobials (Rychen et al., 2018) by Lactobacillus spp. involved in the fermentation of sausages were reported by Fraqueza (2015) and Rozman et al. (2020). Although the resistance of starter cultures to antimicrobials does not pose a direct risk to consumers because they are not pathogenic, susceptibility to antimicrobials is a desirable trait in probiotic cultures, as it ensures that the organisms do not contribute to the transmission of resistance genes to pathogens or commensal bacteria in the intestines (Zarzecka et al., 2020).

Antagonistic activity

The starter culture inhibited all pathogens tested, presenting inhibition zones with diameters greater than 30 mm. Greater
zones of inhibition were observed against Gram-positive bacteria *L. monocytogenes* and *S. aureus* (Table 1). Lactic acid bacteria isolated from sausages by Laslo et al. (2019) also showed an inhibitory effect against *S. aureus* (16.63 mm) and *E. coli* (14.47 mm), although with significantly smaller zones of inhibition than that found in this work.

The antagonistic activity of viable bacteria can occur through various mechanisms, such as competition for nutrients and adhesion sites, and the production of acidic compounds (lactic, acetic, and propionic acid), carbon dioxide, diacetyl, hydrogen peroxide and bacteriocins (Costa et al., 2018). However, inactivated bacteria can release bacterial components with antagonistic properties against pathogens, such as lipoteichoic acids, peptidoglycans, or exopolysaccharides (Sarkar & Mandal, 2016; Castro-Bravo et al., 2018). The inhibition of pathogens by the starter culture is important in the production of sausage-type sausages as it guarantees the microbiological safety of the product, in addition to its fundamental characteristic of modulating the intestinal microbiota, conferring benefits to the health of the host (Oliveira et al., 2018).

**Resistance to low pH values**

The effects of different pH values (2, 2.5, 3 and 7) on the growth of the starter culture are shown in Figure 1. The starter culture was not able to survive at pH 2. At pH 2.5, the culture could not survive above 30 minutes (count at last time-point (*t* = 0): 5.73 log CFU mL⁻¹), and at pH 3 it resisted up to 1 hour of incubation (count at last time-point (*t* = 0.5): 3.84 log CFU mL⁻¹).

![Table 1. Average diameter of the inhibition halos (in mm) created by the starter culture when challenged with common food pathogens.](image)

| Pathogens                  | Inhibition zones (mm) |
|----------------------------|-----------------------|
| *Staphylococcus aureus*    | 36.02                 |
| *Escherichia coli*         | 31.18                 |
| *Enterococcus faecalis*    | 30.75                 |
| *Salmonella Enteritidis*   | 30.25                 |
| *Listeria monocytogenes*   | 37.33                 |

![Figure 1. Growth of commercial starter culture at low pH values for 2 hours. Means followed by the same letters indicate no significant difference (p < 0.05), as analyzed using Tukey's test.](image)

At pH 7, the viability of the culture was not affected, even after 2 hours (count at last time-point (*t* = 2): 7.10 log CFU mL⁻¹). Therefore, the starter culture showed intolerance to acidic pH, which represents stomach conditions, limiting its use as a live probiotic culture (Kandylis et al., 2016). In fact, strains of the genus *Lactobacillus* often have high sensitivity to acidic conditions, impairing their survival in adverse environments such as the stomach and fermented foods (Soares et al., 2019).

The observed bacterial suppression can be explained by the strong oxidizing action of the acid against biomolecules such as fatty acids, proteins, cholesterol, and DNA (Almada et al., 2016). Attractive options to reduce the deleterious effects of extreme gastric acid conditions (pH 1.5-3.5) and improve the performance and functionality of cultures may include protecting microorganisms through encapsulation or strategies based on adaptation mechanisms, where cells are previously exposed to low pH for a short period to induce tolerance and avoid acid stress (Chen et al., 2017; Kavitake et al., 2018; Zhao et al., 2020).

Studies have shown that most of the health benefits of probiotics can be produced by both viable and inactivated cells. There is evidence that preparations containing dead cells can exert relevant biological responses such as restoring intestinal homeostasis, inhibiting pathogens, and improving anxiety and stress (Nishida et al., 2017b; Vandenplas et al., 2017; Aguilar-Toalá et al., 2018; Piquè et al., 2019). In addition, probiotics retain their immunomodulatory activity even after the loss of cell viability (Rossoni et al., 2020), presenting a better immunological effect than live probiotics (Barros et al., 2020; Shripada et al., 2020). The modulation of the host's immune response seems to be associated with the structural components of dead cells, mainly the constituents of the cell wall (Rossoni et al., 2020).

Research also showed that one of the methods of inactivating probiotic microorganisms is a change in pH to induce cell membrane damage, chemical changes in fundamental components (ATP and DNA), and enzyme inactivation (Almada et al., 2016). In addition, studies pointed to the possibility of using food as a vehicle for delivering inactivated probiotics (Sawada et al., 2016, 2019; Sugawara et al., 2016; Nishida et al., 2017a). Thus, it is possible that the inactivation of the starter culture due to stomach acidity does not completely eliminate the beneficial health effects of probiotics.

**Resistance to bile salts concentrations**

At time 0, there was no significant difference (p > 0.05) between the effects of treatments containing 0.3% bile salts (7.17 log CFU mL⁻¹) and the control treatment (0% bile salts) (7.41 log CFU mL⁻¹). However, significant differences (p < 0.05) did emerge from the treatments of 0.5% (6.99 log CFU mL⁻¹) and 0.7% (7.00 log CFU mL⁻¹) bile salts, compared to control, demonstrating loss of viability of culture at these concentrations (Table 2). After 30 minutes, none of the bile salt treatments differed significantly from the control (p > 0.05), even after 2 hours of incubation (Table 2). This was corroborated by Han et al. (2017), who also found that *Lactobacillus* isolates from sausages showed high tolerance to bile salts. Bile salts...
are important in the defense mechanism of the intestine, with normal physiological concentrations ranging from 0.3 to 0.5% (Muñoz-Quezada et al., 2013).

In the present study, the starter culture showed strong resistance to bile salts in concentrations of up to 0.7%. Bacteria can use several defense mechanisms against bile, including special transport mechanisms, the production of exopolysaccharides, or the synthesis of various types of surface proteins and fatty acids. In addition, several bacterial genera have the ability to enzymatically hydrolyze bile salts (Horáčková et al., 2018).

Tolerance to biliary stress is crucial for the survival of probiotics in the gastrointestinal tract. Evidence indicated that viable probiotic cultures are able to alter the synthesis of bile acids causing cholesterol reduction, which is beneficial in the treatment of hypercholesterolemia and hypertension (Sivamaruthi et al., 2020).

**Technological characteristics**

A probiotic culture must be able to survive food production conditions. In the case of fermented sausages, such as salami, the cultures must be able to acidify the medium, in addition to surviving varied temperatures and the presence of curing salts (Cruxen et al., 2019).

The results of the evaluation of the technological characteristics of the starter culture are shown in Table 3. The starter culture was able to tolerate temperatures of 15 °C, 25 °C and 35 °C, pH 5 and 6, different concentrations of sodium chloride (1.5%, 2.5% and 3%), nitrite (100, 120, and 150 ppm) and nitrate (100, 200, and 300 ppm), conditions commonly used in the production of sausages.

The ability of microorganisms to grow under adverse conditions has been shown to be dependent on species and lineage. The addition of 150 ppm of nitrite or sodium nitrate and the temperature of 15 °C proved to be a limiting factor to the growth of a strain of *Lactobacillus casei* (Bis-Souza et al., 2020), while *Staphylococcus* spp. generally survive NaCl concentrations of up to 15%, 150 ppm sodium nitrate, and temperatures between 15 and 40 °C (Cruxen et al., 2019). In addition, the starter culture reduced the pH of the medium from pH 7 to pH 4.88, after 48 hours of incubation. Rapid acidification is an important characteristic of cultures used in the manufacture of salami, as the drop in the pH of the meat gives stability to the product (Kunrath et al., 2017).

The starter culture did not produce gas (Table 3). The lack of gas production in sausage cultures is particularly important, as gas is associated with the formation of cavities within the product (Laslo et al., 2019). In addition, the starter culture did not produce a capsule (Table 3). Capsule production may be desirable for some fermented foods. However, during the processing of sausages, encapsulated microorganisms can adhere to the equipment and become a source of contamination for other products (Hitchener et al., 1982).

One of the great challenges of the food industry is to maintain the viability of starter cultures during the production process of fresh sausages and the storage period, an essential characteristic to preserve the food since these sausages do not undergo heat treatment. Mafra et al. (2019) demonstrated that a studied starter culture resisted the production conditions of a salami that used tilapia meat as a raw material, promoting an efficient reduction in pH (6.8-5.95). The acidic environment during the sausage fermentation process constitutes an obstacle to the survival of decay-causing and pathogenic microorganisms, providing microbiological stability, reducing water retention and, consequently, firm texture and feasibility to the product (Savoldi et al., 2019).

**4 Conclusion**

The commercial starter culture investigated in this study presents technological characteristics expected for application in sausage maturation processes. Although the culture did not present the characteristics for use as live probiotics due to its sensitivity to stomach acidity, it may still be considered a potential probiotic culture, since bacterial viability is not essential in the human health benefits afforded by probiotics. Future studies should focus on the use of inactive functional cells in food as...
an important alternative for cases in which probiotics cannot survive processing, extensive shelf-life, or passage through the gastrointestinal tract. In addition, future research should determine the level of protection that adaptation mechanisms and encapsulation of probiotics can provide, allowing the use of starter cultures sensitive to acidic pH in the development of new products with functional properties.

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