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Viability of probiotic micro-organism *Lactobacillus acidophilus* in dairy chocolate dessert and its action against foodborne pathogens

Viabilidade de microrganismo probiótico *Lactobacillus acidophilus* em sobremesa láctea de chocolate e ação sobre patógenos alimentares

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ABSTRACT

*The ability to produce antimicrobial factors is considered an important feature of probiotic microorganisms. Bacteriocins, hydrogen peroxide, acetic acid and lactic acid are examples of these substances. The present research aimed to develop probiotic dairy desserts (DD) with **Lactobacillus acidophilus** and evaluate the viability of this strain, as well as its action on food pathogens. Treatments with and without interactions between **L. acidophilus** and pathogenic Gram-negative bacteria (**Salmonella** sp. and **Escherichia coli** O157:H7) and Gram positive (**Bacillus cereus** and **Staphylococcus aureus**) were produced. The products were stored at a temperature of 8°C and analyzed at the times 24, 48, 72 hours, 7 days and 28 days (at 28 days, only T1 was analyzed because the other products were deteriorated). In an analysis of the potential for development of new products, the dairy dessert with **L. acidophilus** was considered a probiotic product. Assessment of the counts of pathogens in dairy desserts with or without **L. acidophilus** showed different behaviors of these products in response to pathogens, which could be justified by a possible action of bacteriocins or microbial competition, but there has been no overall reduction or reduction up to a safe level. It is concluded that the probiotic products developed reduced significant food pathogens, but not up to safe levels. Thus, we emphasize the importance of the use of quality tools in the development and monitoring of dairy desserts.*

Key words: *probiotics, dairy dessert, **Lactobacillus acidophilus**.*

RESUMO

*A capacidade de produzir fatores antimicrobianos é considerada uma importante característica dos microrganismos probióticos. Bacteriocinas, peróxido de hidrogênio, ácido acético e ácido lático, são exemplos destas substâncias. Com o presente trabalho, objetivou-se desenvolver sobremesas lácteas (SL) probióticas, acrescidas de **Lactobacillus acidophilus** e avaliar a viabilidade desta cepa, além da ação frente a patógenos alimentares. Foram produzidos tratamentos com e sem interações entre o **L. acidophilus** e bactérias patogênicas Gram negativas (**Salmonella** sp e **Escherichia coli** O157:H7) e Gram positivas (**Bacillus cereus** e **Staphylococcus aureus**). Os produtos foram armazenados em temperatura de 8ºC e analisados nos tempos 24, 48, 72 horas, 7 dias e 28 dias (apenas T1 por deterioração dos demais produtos neste tempo). A sobremesa láctea com **L. acidophilus** foi considerada produto probiótico, verificando o potencial de desenvolvimento de um novo produto. Analisando as contagens dos patógenos nas sobremesas lácteas com e sem adição de **L. acidophilus**, observaram-se diferentes comportamentos diante dos patógenos, o que poderia ser justificado por uma possível ação de bacteriocinas ou competição microbiana, porém não houve uma redução total ou até um nível considerado seguro. Conclui-se que os produtos probióticos desenvolvidos reduziram os patógenos alimentares de importância, porém não a níveis considerados seguros. Dessa forma, ressalta-se a importância de ferramentas de qualidade no desenvolvimento e monitoramento de sobremesas lácteas.*

Palavras-chave: *probióticos, sobremesa láctea, **Lactobacillus acidophilus**.*

INTRODUCTION

According to FAO's/WHO's (2002) reports, probiotics are defined as living microorganisms that, when administered in adequate amounts, confer benefits to host health, through a positive action on the intestinal microbiota. Additionally, the probiotics are associated to reduction of lactose intolerance, cancer, allergies, hepatic disease, ***Helicobacter pylori*** infections, urinary tract infections, hyperlipidemia and increased immunity (EJTAHED et al., 2011; LOLLO et al., 2013; NABAVI et al., 2014).

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Moreover, probiotics have possible mechanisms of action that produce a response to pathogens. This is justified by the competition for binding sites, forming a physical barrier to pathogens, competition with pathogens for nutrients, inactivation of toxins and receptors through the stimulation of phagocytosis and of specific and nonspecific immune responses against pathogens (WENDLING & WESCHENFELDER, 2013). The capacity to produce antimicrobial factors is another possible characteristic of probiotic microorganisms. Bacteriocins, hydrogen peroxide, acetic acid and lactic acid are examples of these substances (RASTALL et al., 2005). PUUPPONEN-PIMIÄ et al. (2002) suggest that the use of probiotics in the diet reduces the proliferation of potentially harmful bacteria, enhancing natural defense mechanisms of the host.

The main probiotic foods are dairy products, especially yogurts and fermented milks (EJTAHED et al., 2011; LOLLO et al., 2013; KANMANI et al., 2013; NABAVI et al., 2014; MAGANHA et al., 2014). CRUZ et al., (2012) developed yogurts with glucose oxidase enzyme, as a potential alternative to increase the survival of probiotic bacteria in fermented milks, minimizing oxidative stress in probiotic yogurts. Cheeses are less frequently used as sources of probiotic products, e.g. Minas Frescal, Feta and curd cheeses, despite their advantages such as higher pH and fat, as well as firmness, providing a favorable environment for the survival of probiotic bacteria in the gastrointestinal tract (GOMES et al., 2011; KARIMI et al., 2012). In studies on alternative products such as dairy desserts, many researchers have stressed that they are a vehicle for the incorporation of probiotic strains and other functional ingredients (ARAGON-ALEGRO et al., 2007; CARDARELLI et al., 2008; MAGARIÑOS et al., 2008, FERNANDES et al., 2013). Consumer preferences regarding texture and flavor are essential in the elaboration of a dairy product and, consequently, impact the acceptability of a given product (TARREGA & COSTELL, 2006). The technologies used in the dairy industry and the innovative ingredients have stimulated the production of desserts with new sensory characteristics and higher nutritional value (NIKAEDO, 2004). The influence of probiotic microorganisms on the behavior of foodborne pathogens has been little studied, given that each pathogen may act differently depending on the type of food. (MADUREIRA et al., 2011). Besides, more in-depth studies are needed on the viability of probiotic microorganisms (CARDARELLI et al., 2008). Therefore, the present study aimed to develop probiotic dairy desserts with addition of ***Lactobacillus acidophilus***, and assess the viability of this strain at the times 24, 48, 72 hours, 7 days and 28 days after preparation, as well as the competition in response to important foodborne pathogens.

MATERIAL AND METHODS

The lyophilized probiotic culture used in the preparation of the dairy dessert was ***Lactobacillus acidophilus*** (LA-05, direct vat set –DVS, CHR HANSEN, Juiz de Fora, Minas Gerais, Brasil). The pre-activation was performed according to a methodology described by BURITI et al. (2007) with adaptations. It was done as follows: 0.02g of LA-05 DVS probiotic culture were added to 40mL of reconstituted skimmed milk (m/v). Then the culture was incubated at 37ºC for 150 minutes.

The following ingredients were used in the preparation of the probiotic dairy dessert: 81.5% UHT whole milk, 8.5% sucrose, 4% cocoa powder, 4% Global Food® formulation and 2% skimmed milk powder. After weighing, the dry ingredients were mixed and UHT milk was added. The product was homogenized during 30 minutes in mix (Pérola Plus Britânia) and heated to 80ºC for 3 minutes. Subsequently, the temperature was reduced to 40ºC to incorporate the milk enriched with the probiotic strain ***L. acidophilus*** (SILVA et al., 2012 with modifications).

The Gram positive and Gram negative pathogens used were ***Escherichia coli*** O 157:H7 (CDC EDL-933), ***Salmonella*** sp. (ATCC 00150), ***Staphylococcus aureus*** (ATCC 00358) and ***Bacillus cereus*** (ATCC 14579). The identification of these microorganisms was confirmed by biochemical, morphological tests and growing on selective and differential media. The microorganisms were maintained at -80°C, in Eppendorf tube containing *Brain Heart Infusion (*BHI, Difco) broth with addition of glycerol (80:20). In each experiment, the cultures were activated and subcultured twice in succession in 10mL of TSB broth and incubated at 35±1°C for 18 hours. Decimal dilutions were performed so that the final concentration of pathogens in each dessert was 6log C/g. The concentrations were confirmed in all the experiments by plating in specific media. The study was composed of nine treatments: Treatment (T)1: Dairy dessert (DD) with addition of ***L. acidophilus***, T2: DD with ***E. coli*** O157:H7, T3: DD with ***Salmonella*** sp., T4 DD with ***S. aureus***, T5: DD with ***B. cereus***, T6: DD with ***L. acidophilus*** and ***E. coli*** O157:H7, T7: DD with ***L. acidophilus*** and ***Salmonella*** sp., T8: DD with ***L. acidophilus*** and ***S.***

aureus, T9: DD with ***L. acidophilus*** and ***B. cereus***. The products were transferred to plastic containers with lids, then stored at a temperature of 8ºC, analyzed at the times 24, 48, 72 hours, 7 days and 28 days (at 28 days, fonly T1 was analyzed because the other products were deteriorated).

pH determination was performed with digital pH meter (Micronal, B-375) by direct insertion of the electrode in the sample (MARSHALL, 1993). Titratable acidity was determined by the method described by BRASIL (2006) and expressed in lactic acid in g $100g^{-1}$ of the product. Viability of the probiotic ***L. acidophilus*** was determined according to a methodology recommended with the use of Agar medium De Man Rogosa and Sharpe (MRS) with addition of 0.15% of bile (VINDEROLA; REINHEIMER, 1999) and pathogen count was performed according to the recommendations: ***E. coli*** O157:H7 (ZADIK et al., 1997), ***S. aureus*** (LANCETTE; BENNETT, 2001), ***Salmonella*** spp. (ANDREWS et al., 2001), ***B. cereus*** (BENNETT; BELAY, 2001). The results were expressed in log CFU g^{-1}.

Statistical analyzes

Base 10 logarithms were used for normal distribution of the data obtained with the counts. Three independent experiments were performed in duplicate, and statistical treatment was obtained by analysis of variance (ANOVA) in entirely randomized design, with significance level of 5% ($P<0.05$). Tukey's multiple comparison tests were used for mean comparison, through the software Prism 5.01 GraphPad (GraphPad Software, San Diego, CA, EUA).

RESULTS AND DISCUSSION

Regarding the dessert with addition of ***L. acidophilus*** (T1), on the first day of storage, the count was 8.25 log CFU $25g^{-1}$ and at the subsequent times they ranged from 8log CFU $25g^{-1}$ (24 hours) to 8.70 CFU $25g^{-1}$ (28 days). Thus, the final count of the probiotic strain, considering the daily dose of the product of 120 grams, (BRASIL, 2003), is in accordance with the Brazilian legislation. Probiotic products or products claimed to be probiotic can be considered those that have microorganisms, e.g. ***L. acidophilus***, at minimum concentrations of 10^8 to 10^9CFU (8 to 9log CFU) of probiotic microorganisms per portion of the product (BRASIL, 2008). In their experiment of production of a dairy dessert with addition of ***L. acidophilus***, FERNANDES et al. (2013) and VASCONCELOS et al. (2013), reported similar results for microbial counts.

For desserts T2, T3, T4, T5, T6, T7, T8 and T9, the analyzes were performed until the time 7 days, because at the time 28 days the products showed markedly altered sensory characteristics such as color, smell and general aspect, being considered deteriorated, and, so, it was not possible to carry out the analyzes (Figure 1).

In their study with probiotic dairy dessert (LA-05) with coconut flavor and assessment of the behavior of this strain in response to ***Listeria monocytogenes***, FERNANDEZ et al. (2013) did not observe deterioration after 28 days of storage. However, it should be noted that the ingredients and pathogen used in the referred study are different from those in the present study. The pH values of the desserts ranged from 6.3 to 6.81 conferring low acidity to the culture (Figure 2). The pH values of the desserts are not characterized as a factor associated to the inhibition of pathogens, as demonstrated by several authors (PEREIRA & GÓMES, 2007; WENDLING & WESCHENFELDER, 2013) in studies with fermented milks.

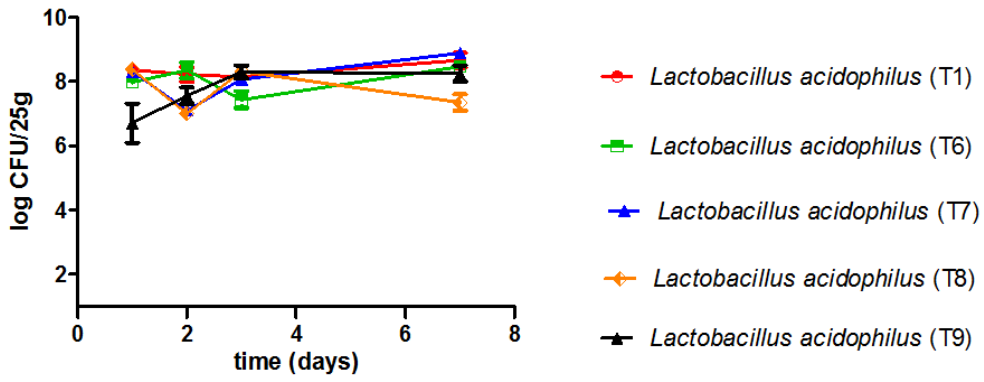

Figure 1 - Microbial counts of ***L. acidophilus*** in dairy desserts.

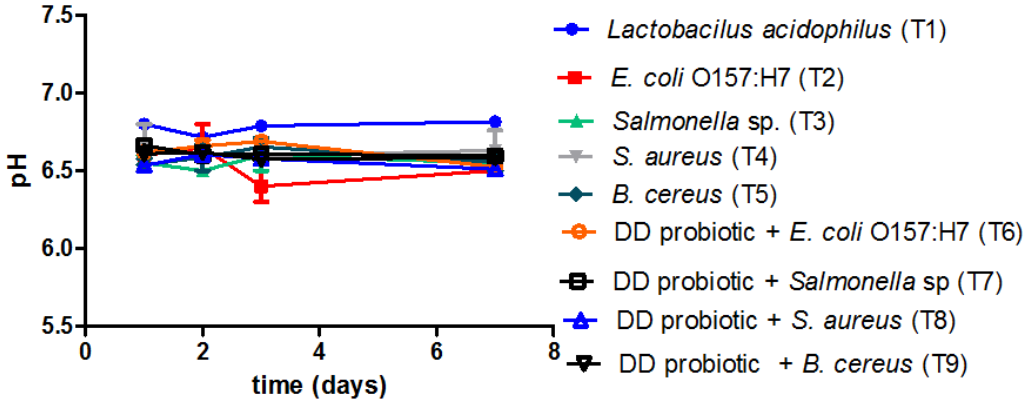

Figure 2 - Changes in pH values of dairy desserts up to day 7.

According to the counts of pathogens in dairy desserts with and without the addition of ***L. acidophilus***, it can be seen that for all Gram negative pathogens, there was no significant difference at 24 hours of storage (Figures 3 and 4). Except for ***E. coli*** that showed no significant difference at the time 48 hours, there were significant differences ($P<0.05$) at all the other times between the pathogens in the presence or absence of the probiotic. Also, the difference in the behavior of ***E. coli*** at the last time assessed compared to ***Salmonella*** sp. deserves mention: both showed reduction, but it was more significant for ***E. coli*** (Figure 3). This can be due to the differences between genera and other factors that deserve more in-depth studies.

In Gram positive pathogens, ***Bacillus cereus*** showed a significant difference at all the analyzed times. As for ***S. aureus*** only the time 48 hours showed a significant difference ($P<0.05$) between the pathogens in the presence of absence of the probiotic. Also, bacteriocins, active proteins produced by some microorganisms, can have antimicrobial action, as well as some lactic bacteria with bactericidal action (ZACHAROF & LOVITTB, 2012; YANG et al., 2012; PEREZ et al., 2014), and that also compete for nutrients. Some bacteriocins have already shown to be active against several microorganisms such as ***Bacillus cereus***, ***Clostridium perfringens***, ***Listeria monocytogenes*** and ***S. aureus*** (CLEVELAND et al., 2001).

In their experiment with a dessert with ***L. acidophilus*** La-05 and ***Listeria innocua***, FERNANDES et al. (2008) demonstrated that the count of both strains increased at the end of the storage of the product. This

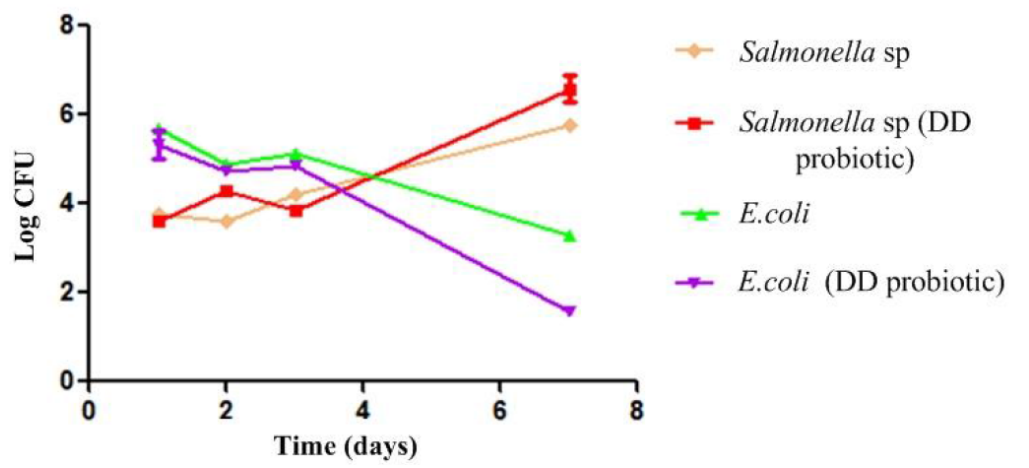

Figure 3 - Microbial counts of Gram negative pathogens in dairy dessert (DD) and probiotic dairy dessert (probiotic DD).

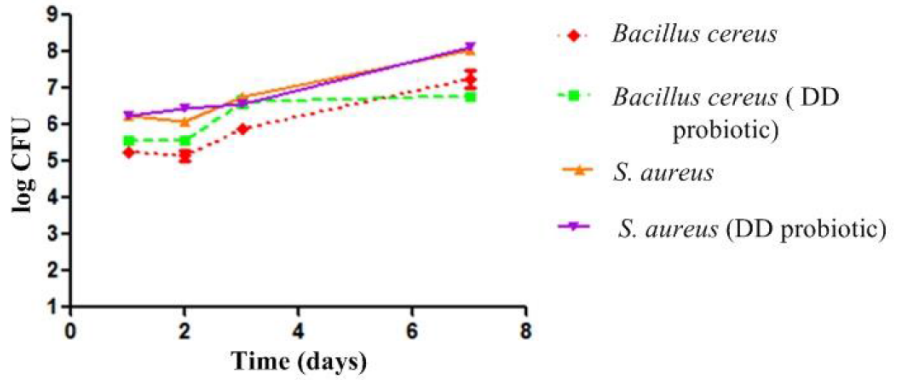

Figure 4 - Microbial counts of Gram positive pathogens in dairy dessert (DD) and probiotic dairy dessert (probiotic DD).

can be caused by the mutual benefit between these microorganisms. It should be stressed that the probiotic strain may have benefited from metabolites released by ***L. innocua***, such as peptides and aminoacids.

Brazilian legislation RDC 12/2001 (BRASIL 2001) that establishes the Microbiological Standards for Food Health, has the following parameters: ***B. cereus***, Coliforms at 45ºC, coagulase-positive staphylococci and ***Salmonella*** sp. for dairy desserts, which justifies the selection and importance of these microorganisms used in the present study. Of all the pathogens analyzed, only ***E. coli*** O157:H7, both in the non-probiotic and probiotic products, was reduced at th 7th day, though without significant differences between the treatments.

CONCLUSION

It is concluded that dairy chocolate dessert allows the development of probiotics and has great economic potential. However, it also allows the development of pathogens, with or without the presence of probiotics. Probiotic products developed reduced important foodborne pathogens but not up to safe levels. Therefore, we emphasize the importance of the use of quality tools in the development and monitoring of dairy desserts.

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