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In vitro resistance of *Enterococcus faecium* and *Lactobacillus helveticus* against stress conditions, sodium chloride and curing salts: preliminary study aimed application as starter culture in fermented sausage

Catharina Calochi Pires de Carvalho
ccalochi@hotmail.com
Food Technology Department, São Paulo State University (UNESP), São José do Rio Preto, São Paulo, Brazil.

Vidiany Aparecida Queiroz Santos
vidianyqueiroz@yahoo.com
Chemistry Department, Federal University of Technology (UTFPR), Campus Pato Branco, Paraná, Brazil.

Catierine Hirsch Werle
catierinewerle@gmail.com
Genetics, Evolution and Bioagents Department, Campinas State University (UNICAMP), Campinas, São Paulo, Brazil.

Raquel Gutierres Gomes
rgutti02@gmail.com
Chemical Engineering Department, State University of Maringá (UEM), Maringá, Paraná, Brazil.

Fernando Leitte Hoffmann
hoffmann@ibilce.unesp.br
Food Technology Department, São Paulo State University (UNESP), São José do Rio Preto, São Paulo, Brazil.

ABSTRACT

The objective of this study was to evaluate the *Enterococcus faecium* (ATCC 8459) and *Lactobacillus helveticus* (ATCC 15009) *in vitro* resistance against stress conditions (acidity and bile salts); curing salts (sodium nitrate and nitrite) and sodium chloride, aiming application as starter culture in fermented sausage. Acid resistance test was carried out at pH values (3, 4 and 5) and bile salts (0, 0.15 and 0.30%), both monitored at times 0 and 4 hours. For resistance to curing salts was used concentration of 0.1% and sodium chloride concentrations tested were 1.0, 2.0 and 3.0%. *Enterococcus faecium* and *Lactobacillus helveticus* were resistant to bile salts; however, *E. faecium* was more sensitive to acidity. In presence of sodium chloride and curing salts, *Lactobacillus helveticus* showed the highest sensitivity. Both microorganisms had satisfactory results for this test, indicating that they may be used as starter culture in the preparation of meat products, such as fermented sausages. Moreover, these products compositions may affect positively the starter culture viability in final product.

KEYWORDS: lactic acid bacteria; embedded meat product; microbial viability; probiotic potential.

INTRODUCTION

Fermented sausages are produced by biochemical, physical and microbiological transformation of meat mixture, composed of lean and fat meat, curing salts, sodium chloride, sugars and spices, that are stuffed in casings and then was submitted to maturation under controlled conditions of temperature and humidity (COLORETTI *et al.*, 2014; ESSID; HASSOUNA, 2013). Microbial fermentation can be realized spontaneously by autochthonous microorganisms in raw material or by addition of starter culture to meat mixture *(*CHEN *et al.*, 2016; SIMION *et al*., 2014).

Starter culture are composed by microbial mixture (bacteria, fungi or both) that by fermentation produces compounds responsible by sensorial and technological characteristics in fermented foods. This microorganism's use enhances the maturation speed and reduces risk of pathogenic and spoilage bacteria ensuring shelf-life and security by fermented products (SIMION *et al*., 2014; ESSID; HASSOUNA, 2013; LORENZO; GÓMEZ; FONSECA, 2014).

Commercial starter cultures used in fermented sausage, are composed by lactic acid bacteria or gram positive cocci (LEROY; DE VUYST, 2004; BA *et al.*, 2016). Lactic acid bacteria are a gram positive microbial group, non-sporulating; catalase-negative; anaerobic and strictly fermentative that produces lactic acid as major metabolite (TEUBER, 1993).

According Ahmed (2003) and Hernández *et al.* (2016) lactic acid bacteria include a microbial group genetically diverse, that comprises different species of the genus *Carnobacterium, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus, Weissella, Enterococcus* and *Lactobacillus*.

Enterococcus and *Lactobacillus* are the most extensively genus used as starter culture and probiotic strains in fermented meat products (GUCUKOGLU; KUPLULU, 2010; GARDINI *et al.*, 2008; RUBIO *et al.*, 2013; SAWITZKI *et al.*, 2009).

According Barbosa et al. (2014), *Enterococcus* sp., have main habitat the gastrointestinal tract of humans and warm-blooded animals. This genus are involved in the development of the typical organoleptic characteristics due to glycolytic, proteolytic and lipolytic activities in a variety of fermented foods (FRANZ *et al.*, 2011; HUGAS; GARRIGA; AYMERICH, 2003). *E. faecium* and *E. faecalis* strains are more used as starter culture in fermented meat products (GARDINI *et al.,* 2008; RUBIO *et al.,* 2013; RUIZ-MOYANO *et al.*, 2009).

Lactobacillus sp. are the main bacterial genus isolated from dry sausages and species as *L. sakei, L. plantarum, L. pentosus* and *L. casei* are most used as commercial starter cultures in fermented meat products (SENTER *et al.,* 2015; AMMOR; MAYO, 2007; HAMMES; HERTEL, 1998).

A microorganism with probiotic potential could to show same specific characteristics as viability during food processing and shelf life; provide therapeutic efficacy; reach the intestinal tract in adequate concentration and produce beneficial effects on the human body (FAO, 2002; STANTON *et al.*, 2005). However, these microorganism need remain viable during passage through the gastrointestinal tract, specially to stomach acid and bile salts, which may interfere on viability and on beneficial effects exercised by microbial strain (ERKKILÄ;

PETÄJÄ, 2000; JOSE; BUNT; HUSSAIN, 2015; KECHAGIA *et al.,* 2013; MATTILA *et al*., 2002).

In this context, the objective of this study was to evaluate *in vitro* resistance of *Enterococcus faecium* (ATCC 8459) and *Lactobacillus helveticus* (ATCC 15009) against stress conditions (acidity and bile salts), sodium chloride and curing salts as a preliminary study to application as starter culture in fermented sausage.

MATERIALS AND METHODS

MICROORGANISMS

Enterococcus faecium (ATCC 8459) and *Lactobacillus helveticus* (ATCC 15009) were purchased from Tropical Culture Collection of "André Tosello" Foundation.

SIMULATION TESTS CONDITIONS OF STRESS

Acidity resistance

Firstly *Enterococcus faecium* was reactivated in BHI broth at 37 °C/24 hours and *Lactobacillus helveticus* in MRS broth at 37 °C/48 hours. Then, for each microorganism, cell suspension (1 mL) were inoculated into the test tubes containing sterile saline solution 0.9% (9 mL) and pH solution was adjusted to 3, 4 and 5, using 8 Mol.L^{-1} hydrochloric acid (ERKKILÄ; PETÄJÄ, 2000). Tubes were incubated at 37 °C and counting of viable cells number was performed after both bacteria exposure to saline solution at 0, 1, 2, 3 and 4 hours, using Petri dishes containing BHI agar (*E. faecium)* and MRS agar (*L. helveticus)*. Counts were made after incubation at 37 °C for 48 hours and results were expressed in log CFU.mL^{-1}.

Bile salts resistance

Resistance test to bile salts was performed by reactivation of bacterial strains, as described previously. Then, 1 mL of each bacteria suspension was inoculated into the test tubes containing 9 mL of BHI broth (*E. faecium*) and MRS (*L. helveticus*) and 0; 0.15 and 0.30% of bile salts (ERKKILÄ; PETÄJÄ, 2000). Tubes were incubated at 37 ^{o}C and viable cells counting were performed after 0, 1, 2, 3 and 4 hours of exposure to bile salts, using Petri dishes containing BHI agar or MRS, as described in the topic acidity resistance. Counts were made after incubation at 37 °C for 48 hours and the results were expressed in log CFU. mL^{-1}.

Sensibility test to curing salts and sodium chloride

Enterococcus faecium and *Lactobacillus helveticus* were tested as the resistance to sodium chloride (1.0; 2.0 and 3%) and curing salts mixture (Commercial Mixture of nitrite/sodium nitrate), in concentration of 0.1%. This concentration of the curing salts mixture was determined according to producer instructions and to standards established by current legislation (BRASIL, 1998). For

both tests, microorganisms were sub cultured in test tubes containing BHI (*E. faecium*) and MRS (*L. helveticus*) and incubated at 37 °C/24 and 48 hours. Then, both microorganisms were standardized by dilution in peptone water (0.1%) until 10^{-8} CFU. mL^{-1} and then, this last dilution (1 mL) was inoculated in Petri dishes, by spread plate method, containing BHI or MRS agar previously added of the different salt concentrations. As control, it was seeded 1 mL of standardized microorganisms in agar (BHI or MRS) without salts. Petri dishes were incubated in bacteriological incubator at 37ºC for 48 hours and results were expressed in log CFU. mL^{-1} and compared to the control assay (CARR; CHILL; MAIDA, 2002).

STATISTICAL ANALYSIS

Results were statistically analyzed by variance analysis (ANOVA) and Tukey (at 5% significance).

RESULTS AND DISCUSSION

RESISTANCE TO ACIDITY

Enterococcus faecium and *Lactobacillus helveticus* tolerance to acidity were evaluated by exposure test as described by Erkkilä e Petaja (2000).

Figures 1 and 2 show the microorganisms behavior in different pH values during 4 h of exposure. *Enterococcus faecium* initial count was 10.3 ± 0.01 log CFU. mL^{-1} and after 4 hours were found values of 3.4 ± 0.006; 9.8±0.006 and 10.6± 0.002 log CFU. mL^{-1} in pH 3, 4 and 5, respectively. On the other hand, *Lactobacillus helveticus* strain had an initial concentration of 10.8 CFU. mL^{-1} log and after 4 hours of exposure there were scores of 7.2 ± 0.01; 10 ± 0.006 and 10.2± 0.005 log CFU. mL^{-1} in pH 3.4 and 5.0, respectively.

E. faecium after 4 hours of exposure showed greater sensitivity to high acidity (pH 3), showing 3.4 ± 0.006 log CFU. mL^{-1}. This result had statistical significant difference ($p < 0.05$) in relation to *L. helveticus*, which did not show the same behavior, keeping value of 7.2 ± 0.01 log CFU. mL^{-1}. Both microorganisms, in pH 4 and 5, exhibited stability during exposition by 4 hours, as shown in the figures 1 and 2.

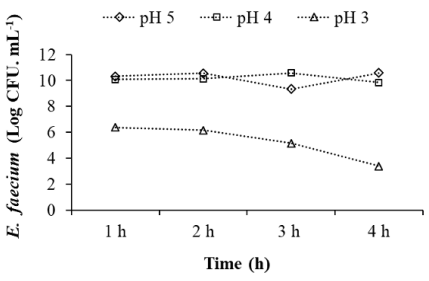

Figure 1 - *Enterococcus faecium* behavior in different pH values.

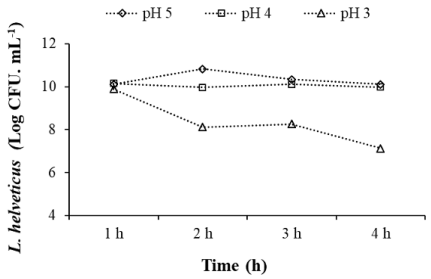

Figure 2 - *Lactobacillus helveticus* behavior in different pH values.

Acidity is one of the most important factors to bacterial viability and proliferation. This behavior was verified by bacterial development reduction in pH values below 4.5. This fact is related to undissociated lactic acid form which penetrates through the cell membrane and promotes collapse in concentration gradient, causing a bactericidal effect (AMMOR; MAYO, 2007).

This may be possible cause for cellular concentration reduction of *Enterococcus faecium* when exposed to high acidity of the solution at pH 3 (Fig. 1). Collado and Sanz (2006) reported that due to sensitivity of the lactic bacteria to stomach acidity, they should be ingested in food which act as buffering agent. Työppönen et al. (2003) highlight that meat and dairy products have capacity to act as buffers in an acid environment, protecting the probiotic microorganisms of the inhibitors agents.

Erkkila and Petaja (2000) reported that during hydrochloric acid release the pH of stomach is about 0.9 and undergoes increase during digestion until pH 3 due to the presence of food. Stomach pH remains at that level for a period of 2 to 4 hours, so the probiotic bacteria survival directly depends of the ability of tolerate low pH. In addition, Klaenhammer (1993) explains that differences in pH tolerance can also vary among different probiotic bacteria. This behavior was observed in this study, with different resistance percentage among the bacterial strains studied (Fig. 1 and 2).

In contrast to that was observed in this study, Macedo, Planzer and Land (2005) evaluated *Lactobacillus paracasei* resistance to acidic solutions (pH 3.0; 4.0 and 5.0). It was observed initial values $1x10^9$, $7.5x10^8$ and $1x10^9$ CFU. mL^{-1}, respectively. After exposure during 4 hours, values were 1.2, 1.5 and $2.7x10^9$ CFU. mL^{-1} at pH 3.0; 4.0 and 5.0 respectively, showing that microbial growth was not affected by low pH. In this study, it was not observed by *E. faecium* and *L. helveticus* when subjected to pH 3 as demonstrated in Figures 1 and 2. Higher resistance to acidic conditions demonstrated by *Lactobacillus helveticus* (Fig. 1) can be explained by the fact that some microorganisms have buffering cytoplasm (pH 3.72 to 7.74) favoring the strength and stability under acidic conditions (RIUS *et al.,* 1994).

BILE SALTS RESISTANCE

Effect of *Enterococcus faecium* and *Lactobacillus helveticus* exposure in different bile salts concentrations during 4 hours are showed in Figures 3 and 4.

Enterococcus faecium initial concentration was 10.1 ± 0.03 log CFU. mL^{-1} and after exposure to bile salts (0; 0.15 and 0.30%) during 4 hours, were found values of 11.2 ± 0.04 log CFU. mL^{-1}; 10.9 ± 0.04 log CFU. mL^{-1} and 10.5 ± 0.01 log CFU. mL^{-1}, respectively (Fig. 3).

As observed to *E. faecium, Lactobacillus helveticus* demonstrated 10.8 ± 0.01 log CFU. mL^{-1} in initial concentration of biliar salts and after exposure by 4 hours, results were overtime 11.2 ± 0:03 log CFU. mL^{-1}; 10.0 ± 0:02 log CFU. mL^{-1} and 9.9 ± 0.02 log CFU. mL^{-1} by same bile salt concentrations evaluated by *E. faecium* (Fig. 4).

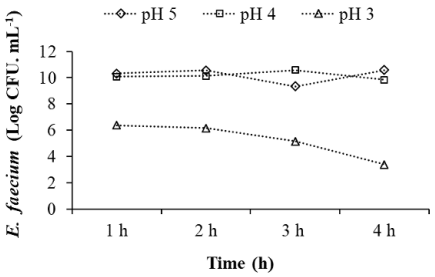

Figure 3 - Behavior of *Enterococcus faecium* in different bile salts concentrations.

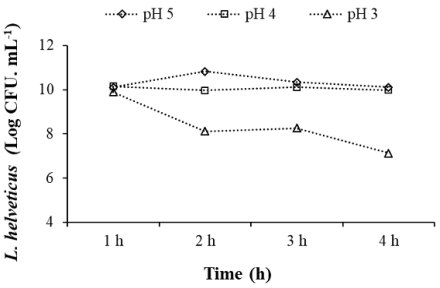

Figure 4 - Behavior of *Lactobacillus helveticus* in different bile salts concentrations.

These results demonstrate that *Enterococcus faecium* and *Lactobacillus helveticus* were not affected by the bile salts presence, once after exposure for 4 hours, microbial counts remained close to that observed in initial test, as showed in Figures 3 and 4. Microbial counts to both bacteria were statistically significant ($p < 0.05$).

Pennacchia *et al.* (2004) reported that bile salts are released in small intestine after ingestion of fatty foods, emulsifying fats and facilitating digestion. These salts can inhibit microorganisms in the intestinal tract, since the cell membrane of microorganisms is also composed of lipids and fatty acids.

Charteris *et al.*(2000) reported that bile has an important role in gut defense and its inhibitory action is determined by bile salts concentration. These salts destroy microbial lipid layer and cell membrane fatty acids. However, some

Lactobacillus promote bile salts hydrolysis by enzyme production (bile salt hydrolase) (PAPAMANOLI *et al.*, 2003).

This capacity may explain the resistance of *Lactobacillus helveticus* against bile salts in different concentrations observed in this study (Fig. 4).

Erkkila and Petaja (2000) reported that during the meat products processing starter culture are protected by meat and fat matrix and this helps in bacterial survival during gastrointestinal tract passage and present greater efficiency than the isolated microbial exposure in acidity and bile salts conditions.

CURING SALTS AND SODIUM CHLORIDE RESISTANCE

Results obtained by *Enterococcus faecium* and *Lactobacillus helveticus* resistance to sodium chloride (0.1; 2 and 3%) and curing salts (0.1%) are showed in Figures 5 and 6.

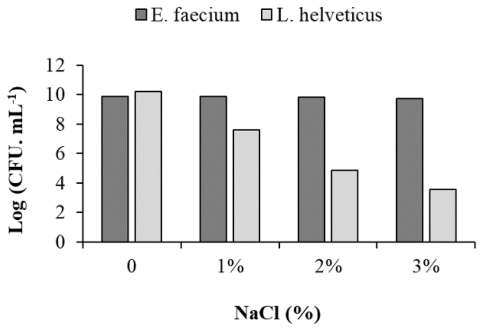

Figure 5 - *E. faecium* and *L. helveticus* behavior against sodium chloride concentrations.

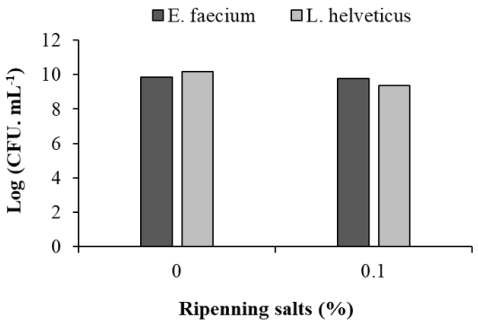

Figure 6 - *E. faecium* and *L. helveticus* behavior against curing salts.

Sensitivity test to different concentrations of sodium chloride demonstrated that *L. helveticus* bacteria was sensitive to all tested concentrations, with greater inhibition at 3% (highest tested concentration). On the other hand, *E. faecium* were resistant to all sodium chloride tested concentrations, as shown in Figure 5. In relation to curing salt resistance test (0.1%) was observed similar behavior to verify

to sodium chlorite, that is, *L. helveticus* showed sensitivity and *E. faecium* was resistant as demonstrated in Figure 6.

E. faecium showed positive results to both tests, showing ability to remain viable in meat products containing curing salts and sodium chloride. However, *L. helveticus* showed sensitivity and cannot survive during meat products production.

Macedo *et al.* (2005) tested resistance of mixed probiotic culture (*Lactobacillus rhamosus, Lactobacillus paracasei* and *Lactobacillus casei)* against sodium chloride concentrations (0 to 3%) and sodium nitrate (0 to 200 ppm). These authors observed good viability, with scores higher than recommended for a probiotic effect. This behavior was not observed by *L. helveticus* evaluated in this study (Fig. 5 and 6), even though similar genus. To be considered probiotic potential, a microorganism must have many characteristics, among which to be able to resist the adverse conditions provided by the food (in the case of a probiotic food) and also the stress conditions encountered during passage through the digestive tract until reaching the intestinal tract (place of colonization).

According to Anvisa (BRASIL, 1998), a probiotic food must have a minimum concentration of probiotic microorganisms between 8 and 9 log CFU. mL^{-1}, proven until the end of shelf life product's and still resist gastric acidity and the presence of bile salts. In this research, bacterial strains were submitted *in vitro* to stress conditions which included: exposure to different pH values, bile salts in different concentrations and the maximum concentration established by the legislation for curing salts.

In relation to resistance to acid pH values, as shown in figures 1 and 2, both strains during exposure to pH values of 4 and 5 showed counts above of minimum limit to be considered as potential probiotic (greater than 8 log CFU. mL^{-1}). However, at pH 3 only *L. helveticus* maintained the minimum concentration of viable cells up to 3 hours of incubation. *E. faecium*, on the other hand, was strongly inhibited when exposed to pH 3, and was not able to maintain the cell concentration above 8 log CFU. mL^{-1} at no time evaluated.

When submitted to the presence of different concentrations of bile salts, both bacteria remained in cell concentration above 8 log CFU. mL^{-1} up to four hours of exposure (Figures 3 and 4), demonstrating high resistance to the presence of bile salts, which is an important factor for studies of probiotic potentialities.

E. faecium and *L. helveticus* showed high resistance to curing salts presence with a cellular concentration above 8 log CFU. mL^{-1} (Figure 5). However, when exposed to different concentrations of sodium chloride, only the *E. faecium* showed resistance, also presenting values greater than 8 log CFU. mL^{-1} in all tested concentrations. *L. helveticus* showed a high sensitivity to the presence of sodium chloride from the lowest to the highest concentration tested, presenting cell counts well below 8 log CFU. mL^{-1} (Figure 6). Such sensitivity may be a major problem for possible applications of *L. helveticus* in meat sausages, since they are made with a high concentration of sodium chloride.

CONCLUSION

Enterococcus faecium and *Lactobacillus helveticus* were resistant to bile salts. *Lactobacillus helveticus* showed greater acidity resistance. E. *faecium* was more

resistant than *Lactobacillus helveticus* in the presence of curing salt and sodium chloride. Both bacteria showed viability under the assessed conditions and demonstrated potential by application as starter culture in fermented sausage production. Bacterial strains evaluated must undergo more validation tests to be considered probiotic.

Resistência *in vitro* de *Enterococcus faecium* e *Lactobacillus helveticus* frente a condições de stress e sais de cura: Estudo preliminar objetivando aplicação em embutido cárneo fermentado

RESUMO

O objetivo deste estudo foi avaliar a resistência *in vitro* de *Enterococcus faecium* (ATCC 8459) e *Lactobacillus helveticus* (ATCC 15009) frente a condições de estresse (acidez e sais biliares); sais de cura (nitrato de sódio e nitrito) e cloreto de sódio, visando aplicação destes microrganismos como cultura *starter* para a produção de embutido cárneo fermentado. Os ensaios de avaliação da resistência a condições ácidas foram realizados em valores de pH de 3, 4 e 5 e concentração de sais biliares de 0; 0,15 e 0,30%, ambos sendo monitorados em tempos de 0 e 4 horas. Para a avaliação da resistência a presença de sais de cura foi utilizada concentração de 0,1% e cloreto de sódio em concentrações de 1, 2 e 3%. *Enterococcus faecium* e *Lactobacillus helveticus* foram resistentes a presença de sais biliares. *E. faecium* foi mais sensível a condições ácidas enquanto *Lactobacillus helveticus* demonstrou alta sensibilidade à presença de cloreto de sódio e saios de cura. Ambos os microrganismos apresentaram resultados satisfatórios para os testes avaliados, indicando que os mesmos podem ser utilizados como culturas *starters* na preparação de produtos cárneos, como os embutidos cárneos fermentados. Além disso, tais aditivos podem afetar positivamente a viabilidade da cultura starter no produto final.

PALAVRAS-CHAVE: Bactérias ácido-láticas; embutido cárneo; viabilidade microbiana; potencial probiótico.

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Correspondência:
Catharina Calochi Pires de Carvalho
Food Technology Department, São Paulo State University (UNESP), Rua Cristovão Colombo, 2265, Bairro Jardim Nazareth, São José do Rio Preto, São Paulo, CEP: 15054-000, Brazil.