Fermented sausage production using *E. faecium* as starter culture: Physicochemical and microbiological profile, sensorial acceptance and cellular viability

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ABSTRACT. Fermented sausages are defined as a mixture of lean meat and fat, curing salts, sucrose and spices, stuffed in a natural or artificial casing and submitted to fermentation and air-drying process. Starter culture and ripening process may affect the quality and acceptability of the final product. Current research evaluates the use of *Enterococcus faecium* as starter culture in fermented sausage production and its physicochemical and microbiological profile during maturation process, coupled to sausage sensory acceptance after ripening. *Enterococcus faecium* showed 10.9 log CFU g^{-1} and remained viable after the ripening period with 8.32 log CFU g^{-1}. Fermented sausage was monitored during the ripening period by physicochemical (pH control, water activity and weight loss) and microbiological (analysis of coagulase-positive *Staphylococcus*, coliforms and *Salmonella* spp.) analyses. All tests complied with standards established by Brazilian legislation and did not interfere in final product quality. Results showed that *E. faecium* was resistant to curing salt and sodium chloride, maintaining its viability during ripening and conferring beneficial effects on fermented sausage technological characteristics. *E. faecium* also proved to be *in vitro* resistant to simulate passage through the human digestive tract. Fermented sausage containing *E. faecium* had better sensory acceptance than commercial sausage evaluated.

Keywords: bacterial fermentation, ripening, acceptance, salami.

Produção de embutido cárneo fermentado por *E. faecium* como cultura *starter*: caracterização físico-química e microbiológica, aceitação sensorial e viabilidade celular

RESUMO. Os embutidos cárneos fermentados são uma mistura de carne magra e gordura, sais de cura, sacarose e especiarias, embutidos em envoltório natural ou artificial e submetidos à fermentação e secagem. A cultura *starter* e o processo de maturação podem afetar as características físico-químicas e sensoriais do produto. Neste sentido, o presente estudo teve como objetivos a utilização de *Enterococcus faecium* como cultura *starter* para a produção de embutido cárneo fermentado; avaliar as características físico-químicas e microbiológicas durante a maturação e, ainda, avaliar a aceitação sensorial do produto após a maturação. *Enterococcus faecium* demonstrou concentração inicial de 10,9 log UFC g^{-1} e permaneceu viável até o final da maturação, com 8,32 log UFC g^{-1}. Durante a maturação foram monitoradas as características físico-químicas (pH, atividade de água e perda de peso) e os contaminantes microbiológicos (*Staphylococcus* coagulase-positiva, coliformes e *Salmonella* sp.) e todos estiveram em acordo com os padrões estabelecidos e não interferiram na qualidade do produto final. *Enterococcus faecium* apresentou resistência aos sais de cura e cloreto de sódio e manteve-se viável durante a maturação do embutido. A cepa apresentou resistência *in vitro* à simulação de passagem pelo trato digestório humano e, ainda, apresentou maior aceitação sensorial quando comparado a uma amostra comercial.

Palavras-chave: fermentação bacteriana, maturação, aceitação, salame.

Introduction

Although meat is an important source of proteins in human diet and is consumed worldwide, it is also a rich culture medium for microbe development and fast deterioration due to its high nutrient concentration (Font-i-Furnols & Guerrero, 2014). Thus, the development of meat products as embedded meat is a strategy to increase shelf life and, consequently, new and safe products to consumers.

Brazilian legislation describes embedded meat as products elaborated with fresh meat, edible offals and seasonings, embedded in natural or artificial casings and submitted or not to cooking, drying or

ripening (Brasil, 1997). Sausage is an example of embedded meat and is produced by microbial fermentation of lean and fat meat, curing salts (nitrite and nitrate), sodium chloride, sugars and spices (garlic, pepper, nutmeg, etc.), stuffed in natural or synthetic casings followed by ripening under controlled conditions of moisture and temperature (Coloretti et al., 2014, Essid & Hassouna, 2013).

Sausages are traditionally prepared with spontaneous fermentation of raw material microorganisms, following by ripening, although technological and sensorial quality and safety are not ensured (Simion, Vizireanu, Alexe, Franco, & Carballo, 2014, Lorenzo, Gómez, & Fonseca, 2014). So that the problem may be solved, selected microbiological cultures, called starters, are employed. In fact, they are extensively cited in specialized literature (Tabanelli et al., 2012, Essid & Hassouna, 2013; Simion et al., 2014; Coloretti et al., 2014; Lorenzo et al., 2014; Chen et al., 2016).

Starter culture comprises non-pathogenic microorganisms capable of fermenting raw material which contains nitrogen compounds and sugar (e.g. meat batter), improves sausage technological (color), sensorial characteristics (flavor, odor) and also maintains the traditional product profile. Further, starter culture promotes reduction in the ripening time and reduces the microbiological contaminant levels, increasing the product´s shelf life and safety (Tabanelli et al., 2012, Lorenzo et al., 2014).

Lactic acid bacteria (*Lactobacillus acidophilus*, *L. plantarum*, *L. sakei*, *Lactococcus lactis* ssp. *Lactis),* *Pediococcus acidilactici* and *Staphylococcus* coagulase negative species *(S. xylosus, S. equorum*, and *S. carnosus*) are often used as starter culture in sausage production (Cirolini et al., 2010; Coloretti et al., 2014; Ruiz, Villanueva, Favaro-Trindade, & Contreras-Castillo, 2014; Sidira, Karapetsas, Galanis, Kanellaki, & Kourkoutas, 2014). According to Essid and Hassouna (2013), starter culture directly affects sausage technological and sensorial quality and thus the choice of adequate culture is an important step to produce high quality and microbiologically safe sausages.

Current research evaluates the use of *Enterococcus faecium* as starter culture in fermented sausage production, assesses the physicochemical and microbiological profile during ripening process and evaluates the sensorial acceptance of sausage after ripening.

Material and methods

Microorganism and material

Enterococcus faecium (ATCC 8459) was obtained from Tropical Culture Collection André Tosello Research and Technology Foundation in Campinas, São Paulo, Brazil. Spices and curing salts used were purchased from Fego® Company, while pork meat (ham and bacon) was purchased in a local market in São José do Rio Preto, São Paulo, Brazil.

Fermented sausage production

A standard meat batter was used in fermented sausage manufacturing process, as shown in Table 1. *Enterococcus faecium* was reactivated in a BHI broth (50 mL) and incubated at 37ºC for 24 hours. The reactivated bacterium was then inoculated in a fresh BHI broth (500 mL) and incubated in same conditions above. *E. faecium* biomass was recuperated by centrifugation at 8000 rpm 5 min^{-1} at 4°C and the cell pellet was suspended in sterilized water (30 mL) and added to meat batter.

Table 1. Meat batter composition.

Raw material	Quantity (%)
Ham	80.0
Bacon	20.0
Spices (Fego®)	4.0
Curing salts (Fego®)	0.1

Fermented sausage contained meat batter composed of ham, bacon, spices, curing salts and *Enterococcus faecium* as starter culture. Sausages were manually embedded in artificial collagen casings and incubated in heated ripening chamber for 28 days under controlled temperature and relative humidity, as shown in Table 2.

Table 2. Ripening conditions for sausage incubation.

Time (days)	Temperature (°C)	Relative humidity (%)
1-2	20	90
3-4	20	85
5-9	20	80
10-28	20	75

Physicochemical analysis

Physicochemical tests were performed after 1, 7, 14, 21 and 28 days of fermented sausage ripening. pH was measured using a digital pH-meter; Water activity (wa) was determined with Novasina-Axair/Switzerland-WA Sprint TH-500; Weight loss by fermented sausage was measured during every ripening period (1, 7, 14, 21 and 28 days). Proximal composition, comprising protein, lipids, moisture and ash, was determined in fermented sausage after 28 days of ripening, according to Cecchi (2001).

Microbiological analysis

All microbiological tests were performed after 1, 7, 14, 21 and 28 days of fermented sausage ripening. *Staphylococcus* coagulase-positive; Coliform thermotolerant and *Salmonella* sp. were assessed, according to Silva et al. (2007) and Brasil (2001). *Enterococcus faecium* viability was determined with Brain Heart Infusion agar (BHI) and incubated at 37ºC for 24 hours.

Enterococcus faecium survival in gastrointestinal conditions

Survival in gastrointestinal conditions after 28 days of ripening was estimated according to Liserre, Re, and Franco (2007), with modifications. Samples of fermented sausage were ground in a multiprocessor and 10 g were added in Erlenmeyer flasks containing 0.5% saline solution (90 mL^{-1}) and four (10 mL^{-1}) aliquots was pipetted into previously sterilized test tubes. Further, pH was adjusted to 2.0-2.5 with 0.5 N HCl and solutions of pepsin (3.0 g L^{-1}) and lipase (0.9 mg L^{-1}) were added in the test tubes and incubated at 37ºC under agitation. *E. faecium* resistance was determined by CFU containing BHI agar using the pour plate method.

Furthermore, pH was adjusted at 4.3-5.2 and solutions of pancreatin (1 g L^{-1}) and bile salts (10 g L^{-1}) were added. The tubes were incubated at 37ºC during 240 min, under agitation, whilst cell viability was determined as described previously. Finally, pancreatin (1 g L^{-1}) and bile salts solutions (10 g L^{-1}) were added and pH was adjusted in 6.7-7.5. Test tubes were then incubated during 360 min and *E. faecium* viability was evaluated as described above. For this analysis, was performed an independent fermentation.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy was determined following Madi-Ravazzi (2009). Samples, fixed in 1% (v v^{-1}) osmium tetroxide (0.1 M cacodylate buffer, pH 7.2) during 1 hour, were washed in distilled water and treated with increasing acetone concentrations (30, 50, 70, 90 and 100% (v v^{-1}) for 10 min. They were afterwards passed through the critical point, dried (K550, Emitech) and mounted on SEM stub with copper tape and sputter coated with gold/palladium. Images were analyzed by scanning electron microscope (LEO 435 VPi SEM, Zeiss).

Sensorial evaluation

Following (Brasil, 2005), current research was submitted to health risk evaluation by the Committee for Ethics in Research of the São Paulo State University (Unesp - Ibilce), São José do Rio Preto, São Paulo, Brazil, with protocol 13014913.7.0000.5466. Sensorial acceptation of sausage samples was assessed by a taste panel made up of one hundred 18-40 year-old untrained tasters.

Acceptance test evaluated appearance, color and aroma of two sausage samples (commercial sausage and sausage produced with *E. faecium* as starter culture). Sausage samples were presented in a monadic form, aleatory, coded by three-digit numbers in individual cabins. Acceptability index (AI) was evaluated according to the above-mentioned attributes. Tasters evaluated whether they liked or disliked the product through a nine-point hedonic scale (9 = 'I liked it extremely'; up to 1 'I disliked it extremely').

Statistical analysis

Physicochemical and microbiological parameters were submitted to statistical analysis, applying variance test (ANOVA) and Tukey's test at 5%.

Results and discussion

Physicochemical analysis

Proximal composition of fermented sausage after 28 days of ripening complied with limits determined by Brazilian legislation (Brasil, 2000) for all evaluated parameters (Table 3).

Table 3. Proximal composition of fermented sausage after ripening for 28 days.

	Moisture (%)	Protein (%)	Lipids (%)	Ash (%)
Sausage sample	37.00±0.79	32.97±1.90	27.20±1.35	3.07±0.51
Brasil (2000)	Max. 40	Min. 25	Max. 32	-

Meat in the manufacture of fermented sausages showed pH rates between 5.8 and 5.9 and thus the raw material was qualified for the elaboration of a safe product. According to Ordoñez et al. (2005) and Andrade (2006), pH rates for normal meat products must not exceed 6.0; rates outside this limit may indicate chemical and/or microbial deteriorations in the product and thus unfit for consumption.

Fermented sausage with *E. faecium* displayed pH rates between 5.96±0.005 and 5.03 ±0.001 during 28 days of ripening. Significant differences ($p < 0.05$) were reported after seven days of ripening, or rather, pH rates decreased (Figure 1), due to hexoses fermentation and lactic acid formation from lactic acid bacteria. Lücke (2000) highlighted that decreases in pH rates promoted inhibition of undesirable microorganisms, maintaining the product´s microbiological safety. In addition, pH evaluation of meat products is extremely important for the formation of sensorial

properties and for the maintenance of the final product´s microbiological safety (Terra, 1997).
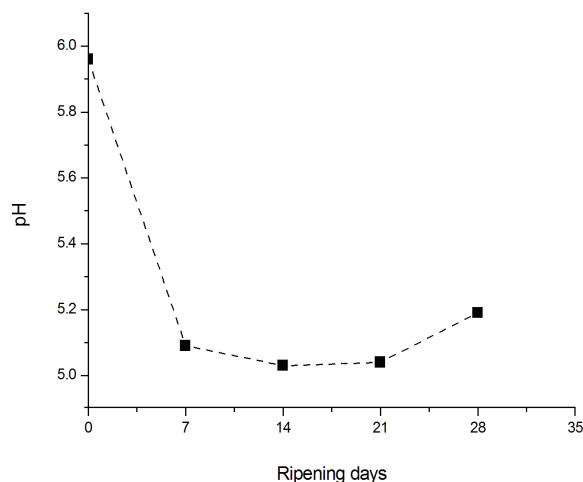

Figure 1. Evolution of pH rates in fermented sausage during ripening process.

Initial mean water activity rate in raw meat was 0.99. Since raw meat naturally has higher water activity, it is susceptible to microbial spoilage and consequently yo a shorter shelf life. Since relative moisture is an important factor in the ripening process of fermented sausages, control started from 90% and reduced every two days until 75%, as Table 2 shows. Relative humidity was maintained at 75% to facilitate the drying and water activity reduction process.

Figure 2 shows water activity (wa) rates during 28 days of fermented sausage ripening. Water activity on initial ripening day was 0.99±0.0005, followed by 0.95±0.002; 0.95±0.0005; 0.91±0.003 and 0.89 ±0.005 respectively on 7, 14, 21 and 28 ripening days.
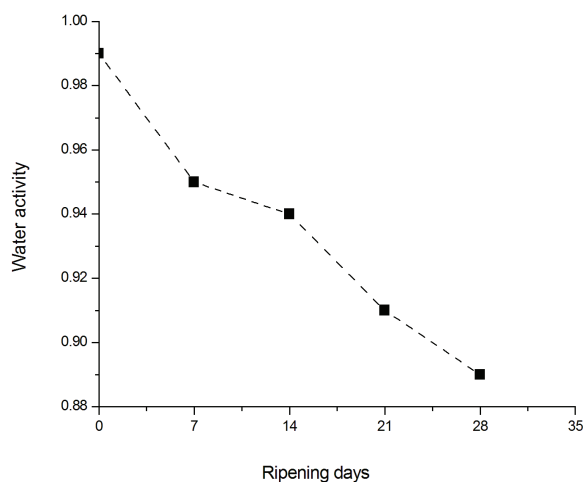

Figure 2. Water activity profile in fermented sausages during the ripening process.

Mauriello, Casaburi, and Villani (2004) reported that water activity decrease is related to pH decrease, or rather, when pH rates are close to the isoelectric protein point, the water retention capacity (WHC) decreases and dehydration occurs.

As shown in Figure 3, weight loss in the first seven ripening days reached 16.67%, followed by 23.9, 28.87 and 32.5% in 14, 21, 28 ripening days, respectively, with a statistically significant difference ($p < 0.05$) between the days evaluated.
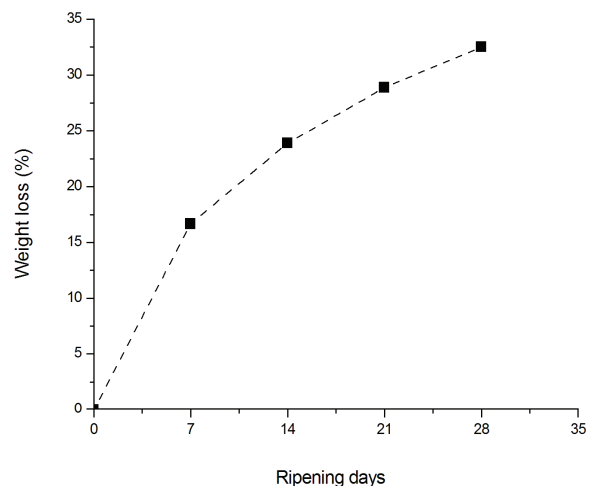

Figure 3. Sausage weight loss profile in fermented sausage during ripening process.

Sausages´ weight loss is a natural consequence during the ripening process and depends on such factors as pH, sausage diameter and incubation conditions in the ripening chamber. Terra (1998) mentioned that dehydration during fermented sausage ripening is essential for the product´s safety, quality and sensorial characteristics.

At the end of the ripening process, fermented sausage had 32.5% weight loss (Figure 3). Coelho, Santana, Terra, and Morandini (2000) reported that sausages may lose up to 40% of their weight during processing. Higher losses may interfere in texture, with the product´s deformation.

Microbiological analysis

Microbiological contaminants were monitored during the ripening process and results are shown in Table 4. Typical colonies of coagulase-positive *Staphylococcus* were found until the 14th day of ripening, albeit negative by the coagulase test. The presence of coagulase-negative *Staphylococcus* may be due to meat batter transformation during the ripening process since the bacterium resists low water activity and high salt concentrations. In addition, starter culture may be inhibited by competition and other microorganisms sensitive to pH and water activity reduction.

Table 4. Microbiological contaminants in fermented sausages during ripening process.

Time (days)	*Staphylococcus* coagulase-positive (CFU g⁻¹)	Total coliform (NMP g⁻¹)	Thermotolerant coliform (NMP g⁻¹)	*E. coli* (-/ +)	*Salmonella* sp. (-/ +)
0	< 100	7	75	(+)	(-)
7	< 100	4	9	(-)	(-)
14	< 100	< 3	< 3	(-)	(-)
21	< 100	< 3	< 3	(-)	(-)
28	< 100	< 3	< 3	(-)	(-)

Further, the presence of the same species of coagulase-negative *Staphylococccus* in meat products has been cited by Drosinos et al. (2005) and Cirolini et al. (2010) as desirable since the microorganisms cause color stabilization, rancidity prevention and enhancement of aromatic compounds.

As has been observed in current study, Campagnol, Fries, Terra, Santos, and Furtado (2007) verified that coagulase-positive *Staphylococccus* and *Staphylococcus* sp. decrease during the sausage´s ripening process with *Lactobacillus plantarum* as starter culture.

According to Brazilian legislation (Brasil, 2001), *Salmonella* sp. was absent in all sausage samples evaluated, during the ripening process. On the other hand, Hoffmann, Garcia-Cruz, Vinturim, and Carmello (1997) found *Salmonella* sp. in 13.3% of commercial fermented sausage samples evaluated.

Total coliforms in food products indicate hygienic conditions and their decrease during the ripening process has been reported in current analysis. According to Table 4, total and thermotolerant coliforms were found only on the first and seventh ripening days and *E. coli* was confirmed only in the initial period.

Similar results were found by Terra, Fries, and Terra (2004) who evaluated two fermented sausages samples, one using starter culture and another by spontaneous fermentation of raw material microorganisms. The authors reported coliform inhibition after 7 ripening days only in fermented sausages produced with starter culture.

On the other hand, Coloretti et al. (2014) registered increase in Enterobacteriacea from 2.6 on first day to 3.8 CFU g⁻¹ after 30 ripening days in fermented sausage using *Pediococcus pentosaceus* and *Staphylococcus xylosus* as starter cultures.

As observed in current study, microorganisms of the Enterobacteriaceae family in raw material could be derived from contaminated animal tissues during slaughter, since several Enterobacteriaceae species have been naturally found in animal gastrointestinal tract (Fernández-López, Sendra, Sayas-Barberá, Navarro, & Pérez-Alvarez, 2008).

Barbosa, Borges, and Teixeira (2014) and Bouton, Buchin, Pochet, and Beuvier (2009) highlighted the benefits of *Enterococcus faecium* use in fermented foods due to its ability to inhibit food-borne pathogens.

Enterococcus faecium viability during ripening process

The initial concentration of *Enterococcus faecium* in fermented sausage was 10.93 ± 0.03 log CFU g⁻¹ and decreased to 10.22 ± 0.07; 9.68 ± 0.02; 8.61 ± 0.19 and 8.47 ± 0.21 in 7, 14, and 28 ripening days, respectively (Figure 4). Statistical analysis showed significant differences ($p < 0.05$) from the seventh until the last ripening day.
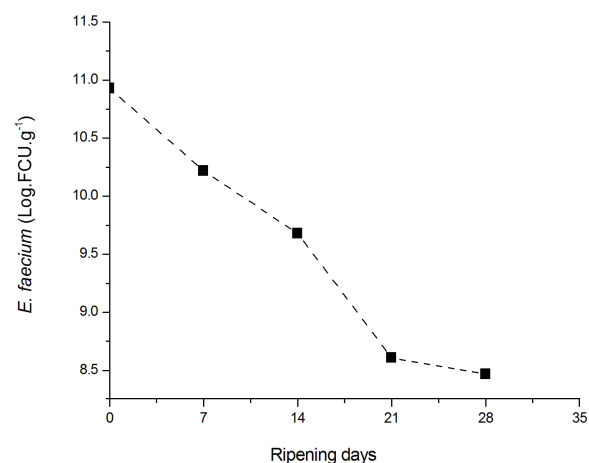
Figure 4. *Enterococcus faecium* viability in fermented sausage during ripening process.

After a 28-day ripening process, *Enterococcus faecium* remained viable in fermented sausages (8.47 ± 0.21 CFU g⁻¹), confirmed by electronic scanning microscopy (Figure 5B) showing high cell concentration in the meat matrix after 28 days and fat globules typical of fermented sausages. Figure 5A shows raw material structure containing the same bacterial cells, fat globules and fibrous areas typical of meat muscle structure.

Enterococcus faecium growth during ripening process may be influenced by many factors, such as processing conditions, time, temperature, relative humidity (Sanz, Flores, Toldra, & Feria, 1997), amount of sugar and salt used (Ibañez, Quintanilla, Cid, Astiasaran, & Bello, 1996, González-Fernández, Santos, Jaime, & Rovira, 2003), and meat fat content. Fermentation and ripening conditions in current study were adequate by *E. faecium* viability.

Several studies have demonstrated starter culture viability in fermented sausages, such as *Pediococus pentosaceus* and *Staphylococcus xylosus* (Coloretti et al., 2014), *L. sakei* and *S. equorum* (Simion et al., 2014)*, Lactobacillus gasseri* (Arihara et al., 1998),

Lactobacillus paracasei, Lactobacillus casei and *Lactobacillus rhamnosus* (Macedo, Pflanzer, Terra, & Freitas, 2008).
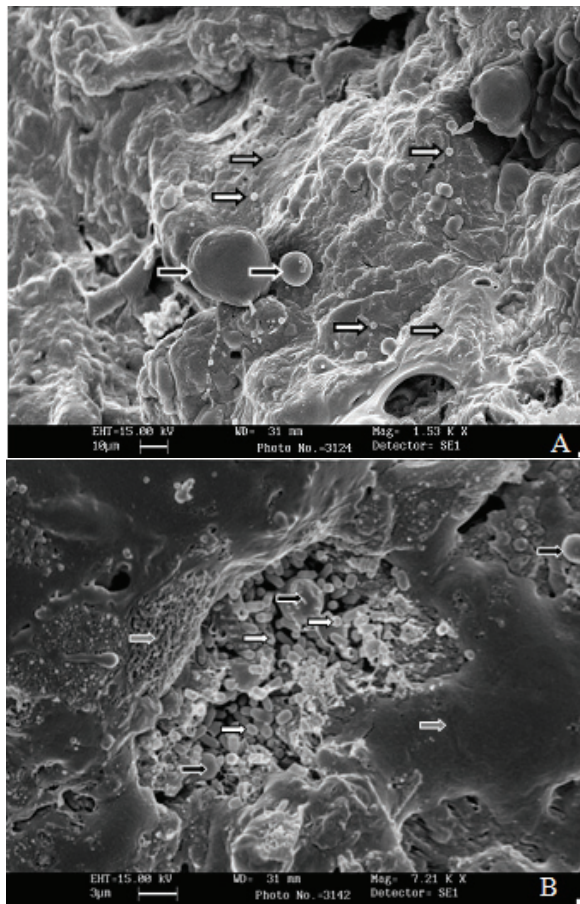

Figure 5. SEM of meat batter (A) and fermented sausage produced with *E. faecium* as starter culture after ripening process (B). White arrow: same bacterial cells in raw material (A) and high microbial density adhered to fermented sausage matrix (B), grey arrow: meat structure of raw material (A) and fermented sausage matrix (B); black arrow: fat globules present in raw material (A) fermented sausage matrix (B).

Enterococcus faecium survival during simulation of gastrointestinal condition: *in vitro* assay

The assay evaluates *in vitro* microbial resistance at conditions simulating the transit time in the human intestinal tract. As shown in Table 5, after 28 days of ripening, *Enterococcus faecium* in sausage matrix survived until 360 hours in simulation gastrointestinal conditions, but there was a reduction in *E. faecium* population during gastric (30-120 min), enteric I (240 min) and enteric II (360 min) phases, with no statistically significant difference ($p > 0.05$) between enteric phases I and II (Figure 6).

Same letters on the same column do not differ statistically ($p < 0.05$) by Tukey´s test.

Table 5. Sensorial acceptance of *E. faecium* sausages compared to commercial sausage.

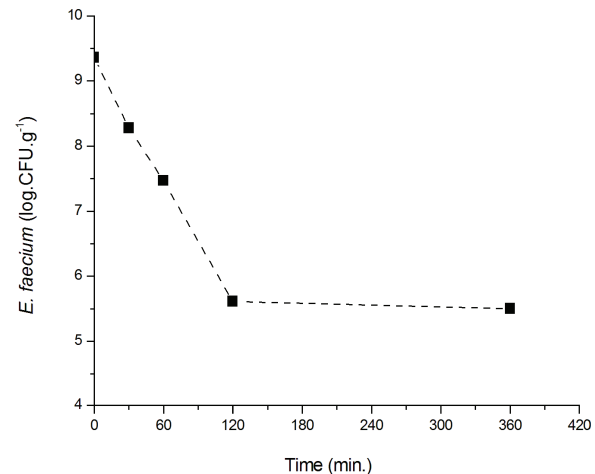|	Appearance		Aroma		Color	
	Acceptance rate					
		%		%		%
Commercial sausage	6.17[a]	68.56	6.63[a]	73.67	6.48[a]	72.00
E. faecium sausage	6.50[b]	71.22	6.68[a]	74.22	6.70[a]	74.44

Figure 6. Results of *in vitro Enterococcus faecium* resistance in simulated gastrointestinal conditions.

The above behavior was registered by Ji et al. (2013) using *Leuconostoc citreum* that showed viability of 2 log CFU mL^{-1} at enteric phase II, and by Buriti, Castro, and Saad (2010) who added *Lactobacillus acidophilus* in symbiotic cooled and frozen guava mousse. The authors reported best survival in presence of insulin as a prebiotic compound.

Results on *E. faecium* survival are very important for future uses of the strain as potentially probiotic bacteria, although specific and detailed tests should be undertaken to this end.

Sensorial acceptance

Table 5 shows results for sensory acceptance of sausages produced with *E. facium* and commercial sausages, with regard to appearance, aroma and color. According to Dutcosky (2007), a good acceptance range has to be higher or equal to 70%.

According to Simion et al. (2014), the sensorial profile of fermented products is a combination of physicochemical, biochemical and microbiological modifications.

Sausage formulated with *E. faecium* as starter culture presented an over 70% acceptance for all evaluated parameters, higher than that for commercial sausage, with statistical interference ($p < 0.05$) only for appearance (Table 5). Results indicate that sausages prepared with *E. faecium* as starter culture have been well received by tasters and thus have good commercialization potential.

Simion et al. (2014) verified better results for aroma when *Lactobacillus acidophilus* CECT903 and *Staphylococcus equorum* SA25 were used as starter culture for fermented sausage production of a traditional Romanian dry sausage.

Conclusion

Enterococcus faecium was efficient as starter culture for the production of fermented sausages, with resistance to curing salt and sodium chloride in fermented sausage and viability during ripening process. Fermented sausages showed adequate microbial parameters from the seventh to the last ripening day. The bacterium also revealed to be in *vitro* resistant for human digestive tract simulation of and higher sensorial acceptance than for commercial sample in relation to aroma, color and appearance.

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