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Effect of high-pressure carbon dioxide processing on the inactivation of aerobic mesophilic bacteria and \textit{Escherichia coli} in human milk

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\textbf{ABSTRACT}

The effect of high-pressure carbon dioxide processing on inactivation of aerobic mesophilic bacteria and \textit{Escherichia coli} ATCC 25922 inoculated in human milk was investigated. The effect of the ratio between sample mass and CO$_2$ (1:0.2; 1:0.6 and 1:1 m/m); depressurization rate (1, 5.5 and 10 MPa/min); and pressure cycling (1, 3 and 5) were the process variables studied. The best reductions in aerobic mesophilic bacteria as well as in \textit{E. coli} (>6.0 and >5.0 log, respectively) were obtained with a ratio of 1:1, a depressurization rate of 10 MPa/min, and one cycle of pressurization/depressurization. The depressurization rate was found to be an important variable in the inactivation process. The results suggest that high-pressure carbon dioxide processing can be applied to human milk as a safe alternative to the pasteurization employed in human milk banks.

\textbf{1. Introduction}

It has been well established that human milk provides all the nutrients and immune factors necessary for the growth and development of newborns and babies (Ballard & Morrow, 2013; Souza, Delgadillo, & Saraiva, 2016). In normal situations, shortly after birth, newborns are fed directly from their mother’s breasts. However, many infants are fed with milk from human milk banks (HMBs) because sometimes preterm newborns do not have enough strength to suckle. In addition, some mothers show physiological and/or emotional problems, resulting in the reduction of milk production. Among the microorganisms isolated in the fecal coliform test, \textit{Escherichia coli} is the one that is most frequently detected, thus becoming the classic indicator of the possible presence of enteric pathogens in food, particularly in human milk (Novak & Almeida, 2002).

Novak, Almeida, Asensi, Moraes, and Rodrigues (2001) reported that infections that occur during the first year of life constitute one of the most important causes of elevated morbidity and mortality rates among infants. The frequency and severity of infections caused by \textit{E. coli} are influenced by the immaturity of the immune system observed for this age group. Therefore, human milk is required to undergo a strict quality control process conducted throughout the collection.
processing and distribution of the product in order to ensure its safety and quality (BRAZIL, 2008). However, De Oliveira et al. (2016) state that the low-temperature long-time pasteurization (62.5°C /30 min), usually applied in HMBs, modifies biological quality and microstructure of the human milk.

The high-pressure carbon dioxide (CO₂) processing is an emerging technology and has been studied as a promising alternative for the reduction of microorganisms in liquid whole egg (Garcia-Gonzalez et al., 2009), ham melon juice (Chen et al., 2010), milk (Ceni et al., 2016), apple juice (Porebska, Sokolowska, Skapska, & Rzoska, 2017) and mozzarella-type cheese (Sikin, Walkding-Ribeiro, & Risvi, 2016). CO₂ is a low-cost inert, non-toxic and non-flammable gas, and it has high density, low viscosity, it is available having high purity, it does not generate waste, it does not create any environmental problems, and also it can be used at temperatures below 50°C (Choi, Bae, Kim, & Rhee, 2009; Zhang et al., 2006). To our knowledge, the use of this technology to ensure the safety and microbiological quality of human milk has not been reported in the literature. In this study, the impact of high-pressure carbon dioxide processing on the microbial inactivation of aerobic mesophilic bacteria and E. coli ATCC 25922 inoculated in human milk was determined. Also, the effects of process variables, such as the ratio of CO₂ to sample mass ratio, depressurization rate and the number of compression/decompression cycles, were investigated.

2. Experimental

2.1. Selection of voluntary lactating donors

Eight human milk donors, aged 21–33 years, were invited to participate in the study when they sought the CIAM (Center for Breastfeeding Encouragement) at the Maternity Ward of the University Hospital at Federal University of Santa Catarina (UFSC, Florianópolis, Brazil) to relieve breast engorgement. The volunteers received clarification on the purpose of the research and signed an informed written consent. The research project was submitted to the Ethics Committee for Research with Human Beings at UFSC. It was approved on 11 January 2016 and was registered under number 1.386.799.

2.2. Samples

The human milk samples were collected manually into sterilized glass jars, following the hygiene guidelines outlined in the Operating Manual for HMB (BRAZIL, 2008) and subsequently grouped to create a pool of human milk. After collection, the samples were immediately frozen and stored in a freezer at −20°C until the time of the analyses, which were conducted within a maximum period of 15 days. For the processing procedures and the analysis of the human milk, the transportation of the samples to the laboratories was made in coolers containing recyclable ice.

2.3. Inoculum preparation

A surrogate E. coli ATCC 25922 stock culture was maintained on Brain–Heart Infusion (Acumedia, Lansing, Michigan, USA) slants at 4°C. The culture for experiment was subcultured twice in Nutrient broth (Oxoid, Basingstoke, Hampshire, England) incubated at 35°C for 18 h until reach the concentration of 10⁸ CFU/mL.

2.4. Contamination of the milk samples

The thawed human milk samples were vigorously mixed and divided into two portions. The first portion (10 mL) was inoculated at a concentration of 8 log CFU/mL of E. coli culture, which reaches about 6 log CFU/mL of E. coli in the human milk samples. The second portion (control sample) was not contaminated, and it was used for calculating the difference of the artificial contamination compared to the natural contamination of the milk.

2.5. Microbiological analyses

In order to confirm the concentration of the inoculum, decimal dilutions of the E. coli suspension were carried out and then were plated in Petrifilm® EC (E. coli/Coliform Count Plate) and incubated at 35°C for 48 h. The determination of aerobic mesophilic bacteria and E. coli was performed in raw human milk with no inoculation (control sample), in raw human milk inoculated with E. coli and after each inactivation processes using Petrifilm® plates AC (Aerobic Count Plate) and EC, according to Association of Official Analytical Chemists International (2002). Replicate counts were expressed as averaged log CFU/mL and the reductions after process as log orders CFU/mL.

2.6. Experimental design and apparatus

Table 1 shows the central composite design used to evaluate the effects of the ratio between sample mass and CO₂ (1:0.2; 1:0.6 and 1:1 m/m), pressure cycling (1, 3 and 5) and depressurization rate (1, 5.5 and 10 MPa/min) on the total count of viable aerobic mesophilic bacteria and E. coli. The initial working pressure was 8 MPa, at this point the system was kept for a short period to allow stabilization of the system, and then the pressure was increased to 20 MPa (rate of 10 MPa/min), and kept constant up to experiment completion. After the procedure, the system pressure was reduced to 8 MPa at the rate of depressurization pre-established by the experimental design, which also established the number of pressure cycles of each experiment. At the end of this process, the pressure was manually reduced from 8 MPa to atmosphere pressure. For all experiments, the time was set at 120 min and the temperature maintained at 33°C.

A static-synthetic method was used in a variable-volume high-pressure cell to perform the inactivation experiments. This method was previously described by Soares, Lerin, Cansian, Oliveira, and Mazutti (2013). Figure 1 shows an overall view of the apparatus which was used. The experimental set-up, briefly, consisted of: a variable-volume view cell (maximum internal volume = 27 mL); two sapphire windows for visual observation; an absolute pressure transducer (Smar LD 301), with a precision around 0.03 MPa; a portable programmer (Smar, HT 201) for the pressure data acquisition; and a syringe pump (ISCO 260D). The inactiva-

| Variables                  | Levels   |
|----------------------------|----------|
| Ratio m/m (human milk/CO₂) | 1:0.2    |
| Pressure cycles            | 1 3 5    |
| Depressurization rate (R)  | 1.0 5.5 10.0 |
After inoculation, and after high-pressure carbon dioxide processing using different process conditions. In the raw human milk no E. coli was detected (data not shown). The initial aerobic mesophilic bacteria count was about 4 log CFU/mL in human milk and about 7 log CFU/mL in milk inoculated with E. coli. It can be observed a decrease in both aerobic mesophilic bacteria and E. coli after applying the pressurized carbon dioxide using different process conditions.

The best results were obtained in experiments 5, 6, 7 and 8, with log reductions ranging from 3.6 to >6.0 in relation to mesophilic bacteria, and from 3.7 to >5.0 in relation to E. coli (Table 2). The greatest aerobic mesophilic bacteria and E. coli reductions were observed for the depressurization rate of 10 MPa/min, thus suggesting that an increase in depressurization rate favors the inactivation of E. coli in human milk.

Experiments 7 and 8 (Table 2) showed that the number of cycles had a positive effect only when associated with a higher depressurization rate, for both aerobic mesophilic bacteria and E. coli. Silva et al. (2013) using supercritical carbon dioxide reported that the depressurization rate caused a positive effect on inactivation of E. coli in broth and the number of cycles was an important factor in the inactivation. However, the number of cycles was associated with a longer time of experiment (five cycles, depressurization rate = 11 MPa/min, 120 min), leading to a complete inactivation in exposure times of over 120 min. On the other hand, higher depressurization rates were associated with short time experiments (one cycle, depressurization rate = 11 MPa/min, 20 min and 30 min). In the present study, besides different exposure times were not performed (the experimental time was adjusted to 120 min), our results indicated that the depressurization rate exerts a more positive effect on microbial inactivation than the number of cycles when compared with the results obtained by Silva et al. (2013).

Figures 3 and 4 show the effects of the processing variables on Pareto chart. This is a simple and practical tool, widely used in statistical analysis, to provide information about the effects of main and cross interaction (variables), whether they are positive or negative, as well as a magnitude of the effect. This tool can be obtained directly from the statistical treatment of experimental data of any available commercial software. Given a level of confidence of 95% (p < 0.05), it was possible to observe that the depressurization rate was a significant variable (p < 0.15) in the inactivation of both aerobic mesophilic bacteria and E. coli, while the number of cycles and ratio between milk and CO2 had no significant effect on inactivation of both aerobic mesophilic bacteria and E. coli. The positive effects of a higher depressurization rate were similar when five depressurization cycles were applied, as can be noted in experiments 7 and 8 compared to experiments 3 and 4, for both aerobic mesophilic bacteria and E. coli, as observed in Table 2. The best inactivation rates were obtained in experiments 5 and 6 (5.5 and >6.0 for aerobic mesophilic bacteria, respectively; and 4.8 and >5.0 for E. coli, respectively), where higher depressurization rates (10 MPa/min) were used regardless of the amount of CO2. The depressurization causes a sudden expansion of CO2, which has been solubilized within the microbial cells, leading to their rupture and consequent inactivation of microorganisms (Karaman & Erkmen, 2001).

**2.7. Statistical analysis**

All the results were analyzed using Statistica® 12.0 (Statsoft Inc., Tulsa, OK, USA) considering a significance level of 95% (p < 0.05). Pareto chart was used to better evaluate the effects of each factor, considering a significance level of 95% (p < 0.05).

**3. Results and discussion**

Table 2 shows the total count of viable aerobic mesophilic bacteria in human milk with no inoculation (control sample), total count of viable aerobic mesophilic bacteria and E. coli after inoculation, and after high-pressure carbon dioxide processing using different process conditions. In the raw human milk no E. coli was detected (data not shown). The initial aerobic mesophilic bacteria count was about 4 log CFU/mL in human milk and about 7 log CFU/mL in milk inoculated with E. coli. It can be observed a decrease in both aerobic mesophilic bacteria and E. coli after applying the pressurized carbon dioxide using different process conditions.

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Soares et al. (2013) reported a higher inactivation rate of a culture of *Listeria monocytogenes* using a depressurization rate of 10 MPa/min while Matos (2013) reported the inactivation of aerobic mesophilic bacteria, *Vibrio* spp. and *Vibrio parahaemolyticus* in oysters using one processing cycle, small amounts of CO₂ (1:0.2) and a low depressurization rate (1 MPa/min). However, when the amount of CO₂ was higher (1:0.8), the highest inactivation rate occurred at the highest depressurization rate (10 MPa/min).

Erkmen (1997) achieved total inactivation of aerobic bacteria and *Staphylococcus aureus* artificially inoculated in whole cow’s milk treated at 14.6 MPa for 5 h, and in skimmed milk treated at 9 MPa for 2 h, both at 25°C. The same author evaluated the inactivation of *E. coli* in whole and in skimmed milk, with a reduction of 6.42 and 7.24 log, respectively. In both studies, the milk fat may have increased the resistance to the penetration of CO₂ into the microbial cells. It may also have influenced on the microbial inactivation in the present study since the fat content in human milk ranges between 3.2 and 3.6 g/mL (Ballard & Morrow, 2013).

Werner and Hotchkiss (2006) evaluated the effects of subcritical and supercritical CO₂ in a continuous flow system on the reduction of naturally occurring psychrotrophic microorganisms and of bacterial spores and *Pseudomonas fluorescens* that were artificially inoculated in skimmed milk. These authors noted a higher mortality rate when the milk was treated at supercritical CO₂ conditions. For the total microbial population, maximum reductions of 5.36 and 5.02 log were noted for *P. fluorescens*, at 35°C and 20.7 MPa, and an average reduction of 3.81 and 2.93 log, respectively, at 30°C, with pressures between 10.3 and 20.7 MPa. Liao, Kui, Xiaojun, and Xiaosong (2014) evaluated the inactivation of microorganisms naturally present in raw cow’s milk and obtained a maximum decrease of 4.96 log in aerobic bacteria after a period of 70 min of exposure, at 25 MPa and 50°C. Yeast and mold were completely inactivated after 70 min of exposure, at 40°C and 25 MPa, or 50 min at 50°C and 25 MPa. The coliform bacteria were completely inactivated by the treatments at 25 MPa for 20, 30, 50 and 70 min at 50, 40, 30 and 20°C, respectively.
In this study, a significant decrease in the microbial count of aerobic mesophilic bacteria and of E. coli was observed (Table 2). Ceni et al. (2016) reported a sharp decrease in the microbial count when a residence time of more than 20 min was applied. The greater microbial reduction obtained was a result of high temperature, pressure and exposure time applied. Besides, the amount of pressurized gas, in turns, implies in an increased interaction with the microorganisms, thus being able to promote the inactivation of the existing microbiota (Matos, 2013). Although different parameters were used to evaluate the inactivation of the same microorganism in the present study, it is possible to suggest that the time of treatment (maintained at 120 min) and the pressure (20 MPa) that were applied have a positive effect on microbial inactivation.

4. Conclusions

The best reductions in the counts of both aerobic mesophilic bacteria and E. coli were obtained when the following parameters were used: carbon dioxide: sample mass ratio of 1:1, depressurization rate of 10 MPa/min and one pressurization/depressurization cycle. Results obtained in this work indicated that high-pressure carbon dioxide processing is a promising alternative for replacing the current human milk pasteurization process using low temperatures. The depressurization rate was an important variable in the inactivation of E. coli in human milk. Further studies on high pressure carbon dioxide processing should be conducted towards assessing the effects of even faster depressurization rates and also kinetic inactivation profile on the natural contamination of human milk as well as on its nutrients, to ensure their safety and quality.

Disclosure statement

No potential conflict of interest was reported by the authors.

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