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Inactivation of *Pseudomonas fluorescens*, *Listeria innocua* and *Lactobacillus helveticus* in skimmed milk processed by high pressure homogenization

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Abstract

This study aimed to describe the inactivation kinetics of *Pseudomonas fluorescens, Listeria innocua* and *Lactobacillus helveticus* in skimmed milk processed by high pressure homogenization (HPH). The skimmed milk was inoculated by 10^7 CFU·mL^{-1} of each culture and subjected to HPH process (up to 300 MPa). The viable cells were enumerated after each process. Mathematical models were adjusted in the microbial count reductions to determine the inactivation kinetics. The microbial inactivation showed an exponential profile, requiring pressures of 200, 250 and 260 MPa for complete inactivation of *P. fluorescens L. innocua* and *L. helveticus*, respectively. Thus, it was concluded that the HPH process (above 260MPa) is effective to inactivate spoilage microorganisms in milk, being similar to thermal pasteurization process.

Introduction

High pressure homogenization (HPH) also known as dynamic high pressure is a non-thermal process that uses the same principle of a conventional homogenization, however works at pressures up to 400 MPa (Hayes and Kelly, 2003; Pinho *et al*., 2011). This process is an alternative treatment for heat-labile products, allowing maximum retention of nutrients and improving the sensory quality (Wuytack, Diels and Michiels, 2002; Tribst, Franchi and Cristianini, 2008).

In the HPH process, the treatment fluid is forced under high pressures to pass through a narrow gap (Pedras *et al.*, 2012). It creates a fast acceleration (200 m/s at 340 MPa) undergoing an extreme drop in pressure as the fluid exits the valve (Floury *et al.,* 2004). The physical consequences in the fluid are high turbulence, impact, shear and cavitation (Hayes and Kelly, 2003; Pinho *et al*., 2011). These effects is able to inactivates microorganisms in several foods, mainly due to the disruption of the cell membrane (Campos and Cristianini 2007; Tribst, Franchi and Cristianini, 2008; Tribst *et al.*, 2009; Pedras *et al*., 2012).

Pseudomonas, Listeria and *Lactobacillus* are microorganism genera important for the dairy industry. *Pseudomonas fluorescens* is highlighted as an important spoilage microrganism, able to produces proteases and lipases that cause technological problems, such as yield reduction and off-flavor development in cheeses (Cousin, 1982; Fairbairn and Law, 1986; Gervilla *et al*, 1997a). *Listeria innocua* is a specie of *Listeria* that have high phylogenetic affinity with *Listeria monocytogenes*, which is a pathogen able to growth in refrigerated milk, cheeses with a high content of NaCl (10%, 0.935 Aw) and fermented products with pH 4.4 (Gervilla *et al*.,1997b). Therefore, the use of *Listeria innocua* as an indicator of technological processes reduces the risks of contamination during laboratory test and, at same time, allows precisely prevising the effects on *Listeria monocytogenes. Lactobacillus helveticus* is recognized as the microorganism of *Lactobacillus genera* with higher resistance to high pressure processing (Capra *et al*., 2009). Additionally, this microorganism is found in many milk fermented products as starter culture.

Several studies described the efficacy of HPH in the inactivation of microorganisms present in milk (Pedras *et al*., 2012), including *Pseudomonas, Listeria* and *Lactobacillus genera* (Pedras *et al.*, 2012; Picart *et al*., 2006; Roig-sagues *et al*., 2009; Vannini *et al*., 2009; Hayes, Fox and Kelly, 2005). These studies established the level of microbial inactivation obtained for specific pressures; however, some

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works showed that it is not possible to generilize the correlation of microbial inactivation and pressure as linear relationship (Roig-Sagues *et al.*, 2009; Hayes, Fox and Kelly, 2005; Tribst *et al.*, 2009). Therefore, to access the microbial inactivation profile by HPH it is necessary to carry out the experiment at various pressures. Up to now, no information about the profile of *Pseudomonas fluorescens, Listeria innocua* and *Lactobacillus helveticus* inactivation by HPH was found. This is important to determine HPH process condition for applying in milk to obtain a product similar to the thermal pasteurized. Therefore, the aim of this work is to determine the kinetics inactivation of these microorganisms by HPH process.

Materials and Methods

Preparation of inoculum

The culture of *Pseudomonas fluorescens* IB 2312 was acquired in the Biological Institute (Campinas, Brazil), the *Listeria innocua* LH 475 was obtained in the Laboratory of Hygiene of the School of Food Engineering (Department of Food Technology - UNICAMP, Campinas, Brazil) and the *Lactobacillus helveticus* CCT 3737 (ATCC 15009) was acquired in the Tropical Culture Collection (Campinas, Brazil). For the HPH assays, the microorganisms were pre incubated in TSB at 30ºC / 20 h *(Pseudomonas fluorescens)*, in TSBYE broth 0.6 % at 37ºC/ 18 h *(Listeria innocua)* and in MRS broth at 37ºC/24 h. The pre incubation was standardized to obtain cultures in the end of the growth phase, which affects the sensibility of cells culture to HPH processing. The cultures were separately added to milk at concentration of about 10^7 CFU.mL^{-1}.

High pressure homogenization

The high-pressure treatments were performed in a Stansted homogenizer, model FPG 7400H:350 (STANSTED Fluid Power LTD®. Essex, UK) at pressures from 0 to 300 MPa, with a flow rate of approximately 270 mL.min^{-1}. A shell and tube heat exchanger (2"-3/4" of diameter, 6"-1/4" of length and 1.25 sq. ft. of heat transfer surface - SPIREC®) for cooling was connected to the homogenizer, to reduce the temperature of the fluid exiting the homogenizer valve. The heat exchanger outlet was connected to an aseptic collection system. The temperature of the milk during the process was monitored by thermocouples fixed at the sample input (T1), the outlet of the homogenization valve (T2) and at the exit of the heat exchanger (T3).

Firstly, the partially skimmed milk (0.51 ± 0.02 % of fat content) was pre inoculated and subjected to high pressure homogenization at pressures of 100, 150, 200, 250 and 300 MPa. Based on the results obtained, small intervals of pressure were set to evaluate the inactivation of each microorganism, in order to obtain mathematical models for describe the inactivation caused by HPH. The pressures evaluated were 100, 150, 170, 190 and 200 MPa to *P. fluorescens*, 100, 150, 200, 220, 240 and 250 MPa to *Listeria innocua* and 100, 150, 200, 220, 230, 250 and 260 MPa to *Lactobacillus helveticus*.

The Number of Decimal Reduction (NDR) reached after each process was determined following the equation 1.

$$NDR = \log N_o - \log N_f \qquad \text{(Eq. 1)}$$

Where, N_o is the initial count of the sample (CFU.mL^{-1}) and N_f is the count reached after the HPH process (CFU.mL^{-1}).

Microbiological analysis

The counts of *Listeria innocua* and *Pseudomonas fluorescens* were performed using spread-plating technique on TSA medium (Oxoid, USA), incubated at 30 °C for 24 hours and TSAYE 0.6% medium (Oxoid, USA) incubated at 37 °C for 24 hours, respectively. The count of *Lactobacillus helveticus* was performed by pour-plating with overlay using MRS medium (Oxoid, USA) and incubation at 37 °C for 72 hours (Haun, 2004; Gervilla *et al*., 1997a,b; Gervilla *et al*., 1999).

Results and Discussion

The temperature is an important parameter to be controlled in HPH processing, since it affects the process effects on microorganisms, protein denaturation, enzyme inactivation and reduction of fat globules size in milk (Datta *et al*., 2005; Deeth, Datta and Versteeg, 2013). The initial temperature was set at 23.0 ± 1.0 °C for all sample, in order to standardize the process.

The Figure 1 shows that temperature increased immediately after the homogenization valve (T2) and that exist a linear relationship between the applied pressure (P) and temperature (T) of the fluid after the homogenization valve ($T = 0.17 \cdot P + 28.58$, $R^2 = 0.99$), with an increase of 17.2 °C / 100 MPa.

The temperature increase is related with the increment of the fluid energy due to exposure to to high turbulence, impact, shear and cavitation forces (Hayes and Kelly, 2003; Pinho *et al.*, 2011). This change on the temperature was expected and similar to the reported in literature by other authors, which found temperature increase ranging from 16 to 20 ºC/ 100 MPa (Hayes

Table 1. Number of decimal reductions caused by HPH (n = 3)

Pressure (MPa)	Number of decimal reductions (NDR)		
	Pseudomonas fluorescens	*Listeria innocua*	*Lactobacillus helveticus*
100	2,54	0,34	0,29
150	3,78	0,87	0,60
200	> 7,62 *	1,33	1,17
250	> 7,62 *	> 7,28 *	3,06
300	> 7,62 *	> 7,28 *	> 6,79 *

* No counts were obtained

and Kelly, 2003; Pereda *et al.*, 2007; Serra *et al.*, 2007). These variations in the temperature increment occurred due to: (1) initial milk temperature (major inlet temperatures results in lower temperature increment during HPH processing (Datta *et al.*, 2005; Hayes and Kelly, 2003; Hayes, Fox and Kelly, 2005; Pereda *et al.*, 2008), (2) different types of valves, that exert different rates of shear on the fluid (Donsì, Annunziata and Ferrari, 2013); and (3) the composition of the milk, since the solid content affect the number of collisions between particles, which is linked to the higher shear and impact forces (Hayes and Kelly, 2003; Roig-Sagués *et al.*, 2009).

A shell and tube heat exchanger was installed immediately after homogenizer valve, guaranteeing milk cooling to 28ºC after 0.7 s. Thus, the heating effect of the homogenization process on microbial inactivation in the milk was minimized.

Table 1 shows the number of decimal reductions obtained after HPH process. Results showed that *P. fluorescens*, *L. innocua* and *L. helveticus* required, respectively, pressures of 200, 250 and 300 MPa to reach complete inactivation of the initial load (107 CFU.mL^{-1}). Therefore, *L. helveticus* was the most resistant target evaluated. The mechanism of HPH inactivation has been attributed to the combined effects of turbulent flow, cavitation, impact of cells with solid surfaces at high velocity, and shear stress (Pinho *et al.*, 2011; Pathanibul, 2009), that causing the rupture of cell wall (Diels *et al.*, 2005; Pedras *et al.*, 2012).

It was observed that microbial inactivation did not follow a linear profile, being not possible to establish the microbial inactivation kinetics by these results. Thus, it was performed additional experiments to reduce the evaluated pressures intervals, aiming to adjust mathematical models able to describe the inactivation profile of each microorganism by HPH.

The Figure 2 shows the kinetics of microbial

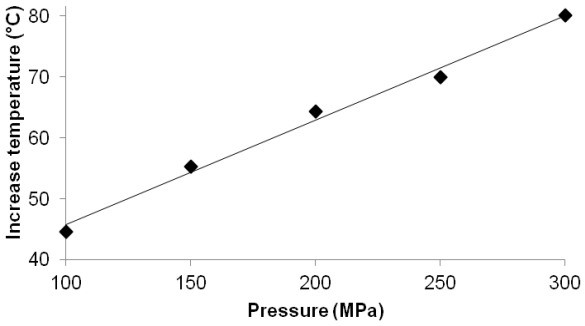

Figure 1. Temperature increases in skimmed milk samples processed by high pressure homogenization .Inlet temperature = 23.0 ± 1.0°C. Results shown are mean of triplicate trials on individual milk samples

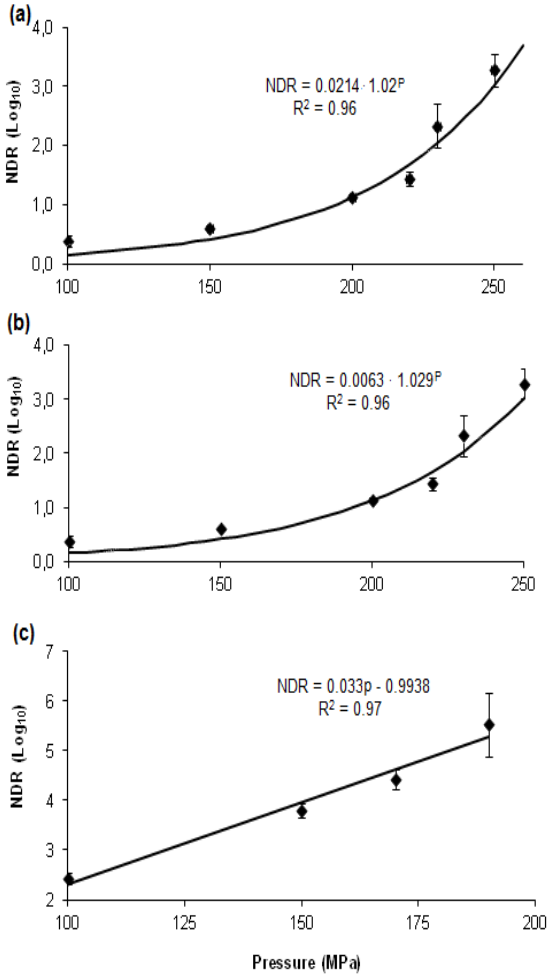

Figure 2. Inactivation kinetics of *Lactobacillus helveticus* (a), *Listeria innocua* (b) and *Pseudomonas fluorecens* (c) inoculated in skimmed milk and processed by high pressure homogenization (n = 3)

inactivation in skimmed milk processed by HPH. The results were modelled according to Power Modificated equation ($y = a \cdot b^x$) or linear model ($y = ax+b$), with R^2 values varying from 0.92 to 0.96.

Among the microorganisms studied, P. fluorescens was the lesser resistant to the HPH

process, with reduction of 2.43 log cycles at 100 MPa and complete inactivation (7.31 log cicles) at 200 MPa. Other authors have reported inactivation of P. fluorescens similar to the obtained in this work (Wuytack, Diels and Michiels, 2002; Hayes, Fox and Kelly, 2005).

L. innocua and *L. helveticus* were more resistant to 200 MPa than *P. fluorescens* ($p < 0.05$), reaching, respectively, inactivation of 1.58 and 1.14 log cycles at this pressure. It is generally established that Gram positive microorganisms have cell wall more resistant to HPH than Gram negative ones (Wuytack *et al.*, 2002) and the results obtained in this work corroborates this statement. At pressures between 200 and 260 MPa, it was observed an exponential increase in the inactivation of *L. innocua* and *L. helveticus*, being the *L. innocua* less resistant to HPH, reaching complete inactivation at 250 MPa (7.31 log cycles). To the contrary, the *L. helveticus* reduced just 3.28 log cycles in this same pressure. The complete inactivation of *L. helveticus* (6.8 log cycles) occurred at 260 MPa, thus, the increased of the 10 MPa of the pressure process (250 MPa to 260 MPa) reduced almost 3.5 log cycles.

These results differ from those found by Wuytack *et al*. (2002), who found that pressures of 230 MPa were able reduces only 1 log cycle of *P. fluorescens*. This difference can be explained by different equipment and shape of head impact, food matrix and strain of culture studied. No previous studies had evaluated the effect of HPH in *L. helveticus*. However, Dosualdo (2003) and Campos and Cristianini (2007) reported that 250 MPa were able to inactivate 7 log cycles of *Lactobacillus fructivorans* in coconut water and *Lactobacillus plantarum* in orange juice. In carrot and apple juice Pathanibul *et al*. (2009) showed that the pressure at 350 MPa reduced 5 log cycles of *L. innocua*.

A linear model could be adapted to *P. fluorescens* inactivation data. For the other two microorganisms, the model that better fitted in the data was the Power Modificated equation, due to the rapid increase of the inactivation at pressures between 200 and 260 MPa. These models can be used in future works to predict the inactivation of these microorganisms by HPH and also to helps the dairy industry to establish pressures of homogenization to guarantee adequate levels of microbial inactivation, considering the NDR desired.

Conclusions

The results showed that the microbial inactivation by HPH (100 to 300 MPa) presents an exponential or linear kinetics, being dependent of the type of the microorganism. Among the studied bacteria, the *L. monocytogenes* showed less resistance of the pressure, reaching total inactivation (7.31 log cicles) at 200 MPa. The *L. innocua* and *L. helveticus* showed high resistance up to 200 MPa, but above this pressure, there was an exponential increase of microbial inactivation in a narrow pressure range (200-260 MPa), reaching total inactivation load (~7 log cycles) after 250 *(L. innocua)* or 260 MPa *(L. helveticus)*.

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