# The effect of cold storage, time and the population of *Pseudomonas* species on milk lipolysis

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**SUMMARY:** The aim of this study was to evaluate the lipolytic index (LI) of *Pseudomonas fluorescens* and *Pseudomonas putida* (2, 5, 6 log CFU/mL) in milk during 96 h by the Lipo R method. The strains were isolated from refrigerated raw milk (30 °C, 48 h), and species were confirmed by PCR, inoculated in reconstituted whole milk, and stored at 2 °C, 4 °C, and 8 °C. The storage time (ST) and temperature were associated with LI of *P. putida*. The interaction among lipolysis, temperature, and ST occurs even with a low population of *P. putida* and these variables combined together contributed to about 77% of the free fatty acids (FFA) in milk. The ST, temperature, and population of *P. fluorescens* showed a significant effect on its LI, and the variables contributed to about 43% of FFA. LI was about 224% higher in milk with *P. fluorescens* than with *P. putida.* The reduction in ST and milk temperature resulted in a decrease in lipid lysis and a lower index of FFA by *P. putida* and *P. fluorescens*, with *P. fluorescens* showing a higher lipolytic capacity.

**KEYWORDS**: *Enzymatic activity; Lipase; Milk lipolysis;* Pseudomonas fluorescens; Pseudomonas putida

**RESUMEN:** ***Efecto del almacenamiento en frío, el tiempo y la población de especies de* Pseudomonas *sobre la lipolisis de la leche.*** El objetivo fue evaluar el índice lipolítico (LI) (por el método Lipo R) de *Pseudomonas fluorescens* y *Pseudomonas putidas* (2, 5, 6 log CFU / mL) en leche durante 96 h. Las cepas se aislaron de leche cruda refrigerada (30 °C, 48 h), las especies se confirmaron por PCR, se inocularon en leche entera reconstituida y se almacenaron a 2, 4 y 8 °C. El tiempo de almacenamiento (ST) y la temperatura se asociaron con LI de *P. putida*. La interacción entre lipólisis, temperatura y ST ocurre incluso con una población baja de *P. putida* y estas variables combinadas contribuyeron a aproximadamente el 77% de los ácidos grasos libres (FFA) en la leche. El ST, la temperatura y la población de *P. fluorescens* mostraron un efecto significativo en su LI, y las variables contribuyeron a aproximadamente el 43% de FFA. LI fue aproximadamente un 224% mayor en leche con *P. fluorescens* que con *P. putida*. La reducción de la temperatura de ST y de la leche dio como resultado una disminución en la lisis lipídica y un índice más bajo de FFA por *P. putida* y *P. fluorescens*, esta última mostrando una mayor capacidad lipolítica.

*PALABRAS CLAVE: Actividad enzimática; Lipasa; Lipólisis de la leche;* Pseudomonas fluorescens; Pseudomonas putida

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## 1. INTRODUCTION

The preservation of raw milk by cold storage without adequate sanitary-hygienic measures can allow the growth of psychrotrophic micro-organisms, the major deteriorating agents in fluid milk and dairy products (Sorhaung and Stepaniak, 1997). *Pseudomonas* is considered the psychrotrophic predominant genus (Fagundes *et al.*, 2006; Pinto *et al.*, 2006; Arcuri *et al.*, 2008; Neubeck *et al.*, 2015; Xin *et al.*, 2017), producing heat resistant enzymes that hydrolyze fat and milk proteinswhich cause off-flavor and economic loss (Sorhaung and Stepaniak, 1997; Mu *et al.*, 2009).

*Pseudomonas fluorescens* and *Pseudomonas putida* are species of great relevance among the psychrotrophic microorganisms and stand out due to their short generation time at 0 °C to 7 °C (Sorhaung and Stepaniak, 1997), and more specifically at 4 °C. Therefore, they naturally become the predominant microbiota in milk stored in this temperature range (Dogan and Boor, 2003).

Lipase is a glycoprotein that can hydrolyze long and short chain triglycerides, esters, monoglycerides and phospholipids, releasing fatty acids and glycerol molecules. The breakdown of fat globules leads to an increase in the fraction of short-chain fatty acids (C-4 to C-8), giving a rancid taste and odor to dairy products. The soapy taste and odor are produced by the hydrolysis of fatty acids with higher molecular weight (C-10 to C-12), whereas the metallic or oxidized taste is the result of the oxidation of unsaturated fatty acids to ketones and aldehydes (Chen *et al.*, 2003). According to Deeth and Fitz-Gerald (2006), although milk lipolytic degradation and it effects were not as intense as the proteolytic degradation, defects such as rancid, soapy, or bitter taste, as a result of lipase activity, are the first off-flavor changes that were detectable.

Therefore, the aim of this study was to evaluate the effect of cold storage, time and the *Pseudomonas fluorescens* and *Pseudomonas putida* populations on the lipolytic index of milk.

## 2. MATERIALS AND METHODS

### 2.1. Materials, reagents, and milk samples

The *Pseudomonas* spp. used in this study were isolated from cold raw milk samples from five dairy farms, with counts which ranged from $2.3 \times 10^5$ to $1.7 \times 10^6$ CFUmL$^{-1}$ (Almeida *et al.*, 2017). The milk samples were stored in cooling tanks, collected under aseptic conditions, and were kept in a Styrofoam box with reusable ice packs, until analysis. For *Pseudomonas* spp. isolation, CFC agar-base was used (30 °C for 48 h) (Almeida *et al.*, 2017) and the isolated strains were stored at −20 °C in BHI broth containing 40% glycerol.

The reagents and materials used in this study are described below. (a) CFC agar-base (Himedia, Mumbai, India), Tributyrin Agar (Himedia, Mumbai, India), Brain Heart Infusion (BHI) broth (Himedia, Mumbai, India), and skimmed milk powder (Molico, Nestlé, São Paulo, Brazil) were used for the determination of *P. fluorescens* and *P. putida* populations. (b) For PCR, the following reagents were used: (b1) Wizard Genomic DNA Extraction Kit (Promega Corporation, Madison, USA), buffer 10X (Invitrogen, CA, USA), MgCl$_2$ (Invitrogen, CA, USA), dNTPs set kit (Invitrogen, CA, USA), oligonucleotides PA-GS-F, PA-GS-R, 16 SPSER, 16 SPSEfluF, P734, P1455r (Invitrogen, CA, USA), GoTaq DNA polymerase (Invitrogen, CA, USA). (b2) For the positive control in PCR, ATCC 27853, ATCC 13525, and ATCC 31483 (Coleção de Culturas Tropical – Fundação André Tosello, São Paulo, Brazil) were used. (b3) Ultrapure agarose, ultrapure tris, EDTA, and Sybr Safe (Invitrogen, CA, USA) were used for electrophoresis. (c) isopropanol (Syth, Diadema, Brazil) petroleum ether (Syth, Diadema, Brazil), sulfuric acid (Syth, Diadema, Brazil), thymol blue (Sigma, Sao Paulo, Brazil), butanol (Syth, Diadema, Brazil), potassium hydroxide (Syth, Sao Paulo, Brazil) and ethanol (Syth, Diadema, Brazil) for the Lipo R methods.

### 2.2. *Pseudomonas* spp. isolation, confirmation of *P. fluorescens* and *P. putida*, and lipolytic indexes

#### 2.2.1. PCR

Bacterial genomic DNA was extracted using the Wizard Genomic DNA Extraction Kit following the manufacturer's instructions. The isolated DNA was stored at −80 °C. The extracted genetic material was subjected to PCR for the identification of the gender *Pseudomonas* spp. with forward PA-GS-F (5'-GACGGGTGAGTAATGCCTA-3') and reverse PA-GS-R (5'-CACTGGTGTTCCTTCCTATA-3') primers (Spilker *et al.*, 2004), which amplified the 16S rRNA (618 bp) gene region. For the identification of the species *P. fluorescens*, the bacterial isolates were subjected to PCR reactions with oligonucleotides 16 SPSEfluF (5'-TGCATTCAAAACTGACTG-3') and 16SPSER (5'-AATCACACCGTGGTAACCG-3') (Scarpellini *et al.*, 2004) for the 16S rRNA gene, which is characteristic of *P. fluorescens*. For the identification of *P. putida*, were followed the protocol with the oligonucleotides P734 (5'-CAA CTCGGGCGTTGGCATTCTGCT-3') and P1455r (5'-CAAGATCGCCTGGGTACGACGGTT-3') was followed, for amplifying a fragment of 744 bp from the gyrB gene (Yamamoto and Harayama, 1995) which is of *P. putida*. Ultrapure water was used as the negative control and DNA from the strains *P. fluorescens* (ATCC 13525) and *P. putida* (ATCC 31483) were used as the positive control.

All the PCR products were subjected to agarose gel (1.5%) electrophoresis, and the gel was stained with Sybr Safe, followed by photo-documentation under ultraviolet light.

### 2.2.2. Determination of P. *fluorescens* and P. *putida* populations

From eight and six strains confirmed to be *P. fluorescens* and *P. putida*, respectively, one strain of each bacterium was used for the experiment. The strains stored at −20 °C were inoculated in 200 mL of 12% milk reconstituted from skimmed milk powder and incubated at 21 °C for 48 h. The lipolytic capacity of the strains was measured by surface plating on Tributyrin Agar (21 °C, 72 h), considering the strains which showed a transparent halo (Frank *et al.*, 1992) as positive.

Subsequently, the number of viable cells of *Pseudomonas* was determined by surface plating on CFC (cefaloridine, fusidic acid, cetrimide) agar-base (Himedia, Mumbai, India), at 30 °C for 48 h (Dogan and Boor, 2003). After reaching the required bacterial cell count, decimal dilutions were made in 0.85% saline solution to obtain final concentrations of 2 log, 5 log, and 6 log CFU/mL. Each selected dilution was immediately used to set up cultures.

### 2.2.3. Inoculation with *Pseudomonas* and measurement of the lipolytic index

For the research, aliquots of 200 mL of 12% reconstituted milk from whole milk powder (sterilized at 121 °C, 15 min) (Ninho, Nestlé, Brazil) were used. Separately, the milk samples were inoculated with 2 mL of *Pseudomonas* inocula previously prepared (2, 5, and 6 log CFU/mL) and incubated at 2 °C, 4 °C, and 8 °C for 96 h. The temperature of 4 °C was selected to simulate the temperature stipulated by Brazilian regulations for raw milk storage at dairy farms. The storage of raw milk at 2 °C and 8 °C were determined to be common in the Brazilian dairy farm (Almeida *et al.*, 2017) and lipolytic activity, even in the presence of a small number of psychrotrophs, was detected at these two temperatures (Wiking *et al.*, 2003).

The levels of free fatty acids (FFA) (mEq/L) in the milk samples with *Pseudomonas* spp. inoculum were quantified by the Lipo R method (MAHIEU, 1984) in three steps. In an initial extraction step, milk (4mL) was added to 16 mL of Lipo R reagent (441 mL of isopropanol, 447.75 mL of petroleum ether, and 11.25 mL of 4 N sulfuric acid) and 5 mL of distilled water into test tubes with screw threads. The tubes were shaken by inversion 15 times, followed by resting for 5 min and the supernatant (supernatant-1) was obtained. Then the rinsing process was carried out by transferring 8 mL of the supernatant-1 to test tubes, followed by the addition

of 4 mL of rinsing solution (sulfuric acid at 0.05 % in aqueous medium) and the mixture was shaken (by inversion) 15 times. After resting for 5 min, the supernatant-2 was obtained. Finally, 4 mL of the supernatant-2 were mixed with 5 drops of thymol blue indicator (1 g of thymol blue in 1.6 L of Butanol-2), and titrated with potassium hydroxide in an ethanolic solution 0.002 N.

The final results were obtained using the formula: FFA mEq/L = (X − B). Fc

where X is the volume of base required to neutralize the supernatant-2; B is the volume of supernatant-2, and Fc is the correction factor for potassium hydroxide.

The *Pseudomonas* spp. enumeration (CFU/mL) and quantification of FFA levels (mEq/L) were carried out every 24 h in duplicate and triplicate, respectively, and time zero was considered the negative control. Two independent experiments were carried out with each of the strains.

### 2.2.4. Statistical analysis

The influence of storage time, temperature, and *Pseudomonas* spp. population over the lipolytic index was assessed through a multiple regression analysis with three explanatory variables: storage timeat four levels (24, 48, 72, and 96 h); temperature, at three levels (2 °C, 4 °C, and 8 °C) and bacterial population, with three levels (2, 5, and 6 log CFU/mL). A total of 72 experimental runs were conducted, since plating was performed in duplicate. The response data was plotted as a response surface to obtain a better understanding of the underlying mechanism behind the lipolytic ability of *Pseudomonas*. The data were analyzed using the software Statistica, release 13.2 (Quest Software Inc., California, USA).

We also accounted for the existing differences in the lipolytic index between the strains of *P. fluorescens* and *P. putida*. Thus, differences were evaluated by the Mann-Whitney U test and the significance level was set at α = 0.05.

## 3. RESULTS AND DISCUSSION

The storage time and temperature were positively associated ($p < 0.05$) with the lipolytic index of *P. putida* according to the multiple regression analysis. In this way, the higher the storage time and/or temperature, the higher the level of free fatty acids in milk was, although without a causal association between these variables. Enzyme production by *Pseudomonas* spp. strains occurred mainly at the end of the log phase of cell growth and in a suboptimal growth environment (Mahieu, 1991).

The lipolysis over storage time and incubation temperature occurred even with a low population of *P. putida* (2 log CFU/mL), since the *P. putida* concentration was not statically associated ($p = 0.07$)

with the lipolytic index. However, due to the marginally significant effect of the *P. putida* population on the lipolytic index, this variable was kept in the model. The production of enzymes such as proteases, lipases, and phospholipases by psychotropic micro-organisms is affected by temperature, oxygen availability, environmental factors, pH, and substrate concentration, as well as the growth stage of the microorganism (Nuñez and Nuñez, 1983).

Despite the fact that storage time, temperature and *P. putida* population do not depend on each other to influence lipolysis, when combined, these variables contributed to about 77% ($r^2 = 0.77$) of the free fatty acids in milk reported herein, which means that the model fits and lipolysis can be predicted by the equation presented in Figure 1.

With regards to *P. fluorescens*, it was found that the storage time ($p < 0.01$), temperature ($p < 0.01$), and *P. fluorescens* population ($p < 0.01$) showed a significant effect on its lipolytic index. Consequently, the higher the storage time, temperature, and *P. fluorescens* population, the higher the levels of free fatty acids in milk were, a non-causal association that highlights the importance of good practices, lower storage temperatures, and processing milk in the shortest time in order to reduce off-flavors in dairy products. The storage time was the strongest factor influencing the lipolysis, followed by temperature and finally, by population size. These variables contributed to about 43% ($r^2 = 0.43$) of the free fatty acids in milk, and the lipolysis by *P. fluorescens* can be predicted by the equation presented on Figure 2. In the study on the isolation of psychrotrophic micro-organisms from refrigerated raw milk, it was observed that all the *P. fluorescens* strains had lipolytic capacity at 4 °C, 7 °C, 10 °C, and 21 °C (Arcuri *et al.*, 2008).

Regardless of the storage time or temperature, the lipolytic index was about 224% higher in the milk samples inoculated with *P. fluorescens* than in the samples with *P. putida* (Table 1). Research performed in the past to observe the genetic diversity and the production of extracellular enzymes (protease, lipase, and lecithinase) by *Pseudomonas* spp. in the samples of raw and pasteurized milk demonstrated that the majority of the isolates were *P. fluorescens* and *P. putida*. Moreover, 69% of the *P. fluorescens* strains were positive for all enzymatic activities, while the majority of *P. putida* strains (87.5%) were negative for all activities tested (Dogan and Boor, 2003).

Kumaresan *et al.*, (2007) evaluated the deterioration of raw milk by psychrotrophic micro-organisms and their lipolytic activities. They concluded that 50% of the panelist respondents were able to identify the rancid taste when the levels of free fatty acids were between 0.18 and 0.20 mEq/kg; when it was above 0.25 mEq/kg, all the respondents were able to detect rancidity. In our study, if we consider
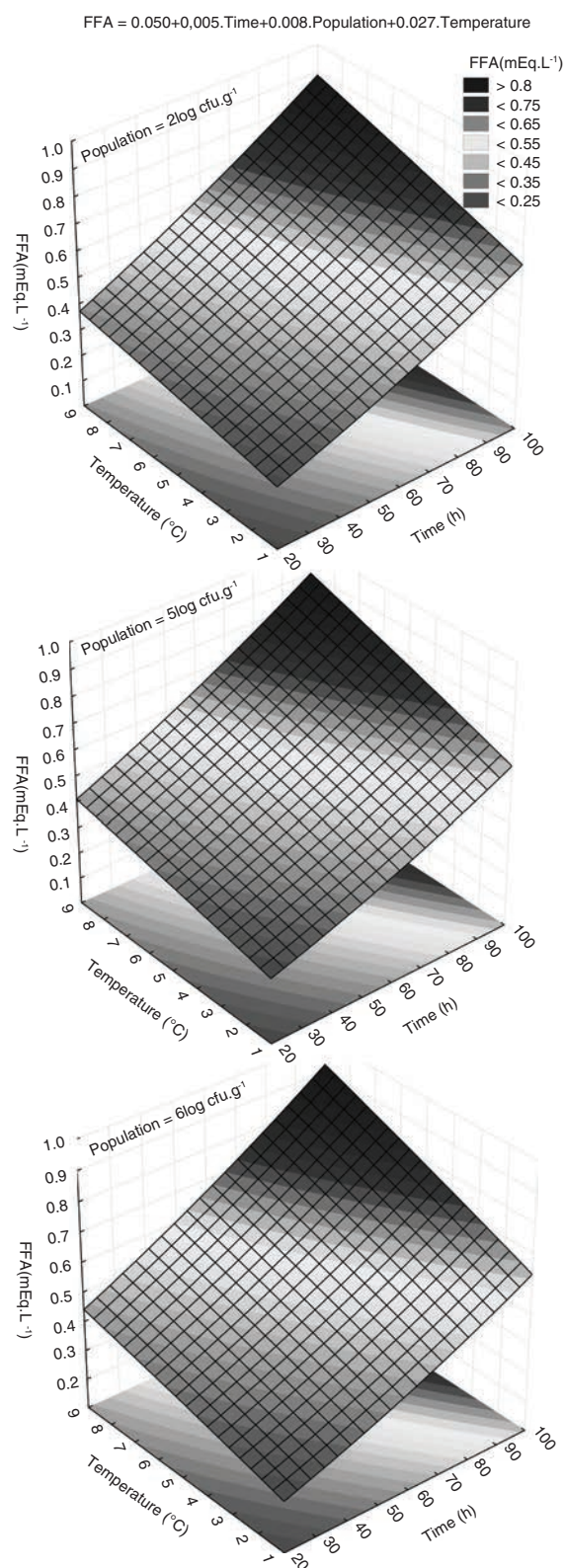


FIGURE 1.   Free fatty acids in reconstituted milk (mEq/L) according to storage time, temperature and *P. putida* population*.
*According to multiple regression analysis; two replicates performed

FFA = 0.7969+0,008.Time+0.049.Population+0.048.Temperature



TABLE 1.   Mean of free fat acids index (mEq/L) of reconstituted milk samples inoculated with *P. putida* or *P. fluorescens,* according to storage time and temperature.

| Storage time | Temperature | Free fat acids index (mEq/L)* | |
|---|---|---|---|
| | | *P. putida* | *P. fluorescens* |
| 24 h | 2 °C | 0.3216[a] | 1.4472[b] |
| | 4 °C | 0.3216[a] | 1.6080[b] |
| | 8 °C | 0.4288[a] | 1.6080[b] |
| 48 h | 2 °C | 0.4288[a] | 1.5008[b] |
| | 4 °C | 0.4288[a] | 1.5544[b] |
| | 8 °C | 0.6432[a] | 1.6080[b] |
| 72 h | 2 °C | 0.4824[a] | 1.7152[b] |
| | 4 °C | 0.5360[a] | 1.8224[b] |
| | 8 °C | 0.6432[a] | 1.8224[b] |
| 96 h | 2 °C | 0.6432[a] | 1.876[b] |
| | 4 °C | 0.7504[a] | 1.9296[b] |
| | 8 °C | 0.8576[a] | 2.5192[b] |

[a,b]Means followed by different letters in lines differ by the Mann-Whitney test (P < 0.05);*means of two replicates

the sensory threshold of 0.25 mEq/kg (Kumaresan *et al.*, 2007), all milk samples with *P. putida* and *P. fluorescens* would have a rancid taste (Table 1), which means sensorial quality defects in milk and dairy products.

## 4. CONCLUSIONS

Lower temperature and storage time are important factors that can reduce lipid lysis in milk caused by *P. putida* and *P. fluorescens*, and avoid the effects of this enzyme in dairy products. Good cow milking to control *P. fluorescens* populations, which was more lipolytic than *P. putida*, will have a direct effect on the reduction of lipolysis in milk.

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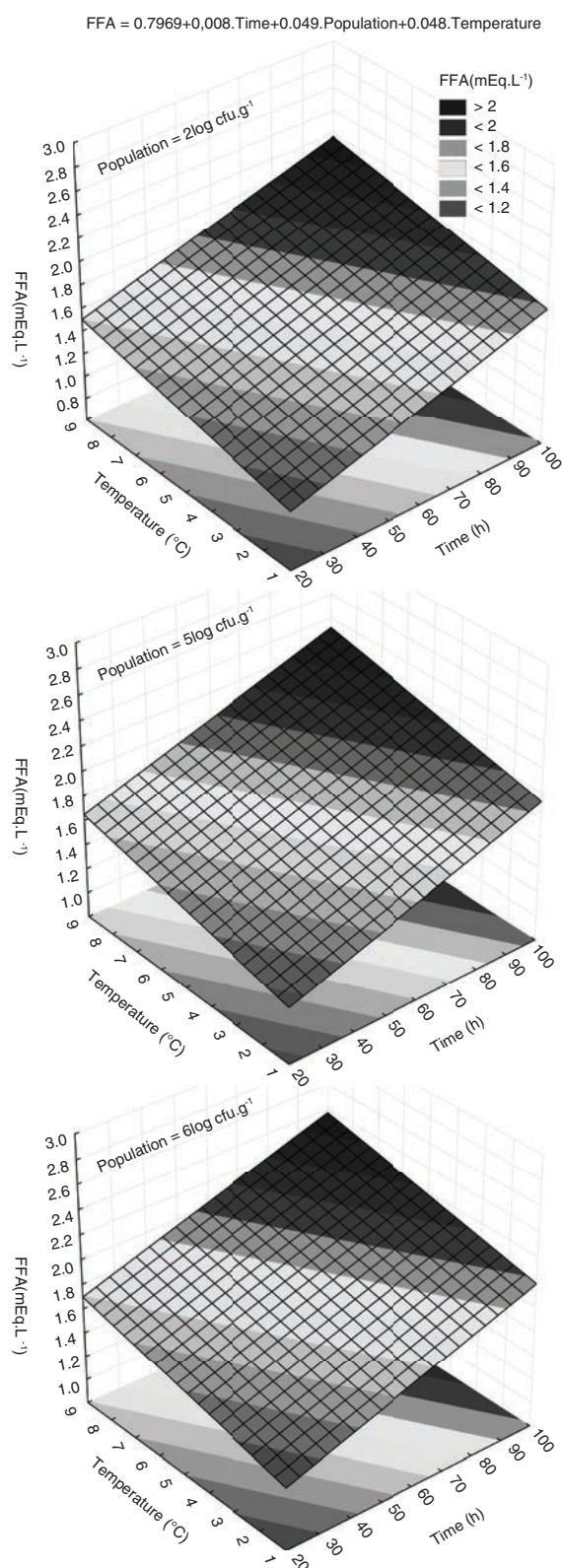
FIGURE 2.   Free fatty acids in reconstituted milk (mEq/L) according to storage time, temperature and *P. fluorescens* population*
*According to multiple regression analysis; two replicates performed.

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