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Short Communication

Improvement of the raw milk microbiological quality by ozone treatment

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Abstract

This work studied the ozone gas bubbling effects in the quality of raw milk. Samples of raw milk were collected before and after ozone gas bubbling at 1.5 mg. L^{-1} for 5, 10 and 15 minutes. The ozone efficacy was evaluated by microbial reductions of *Enterobactereaceae*, total mesophilic aerobic (TMA), psychrotrophic, molds and yeast and *Staphylococcus* sp counts. The assays were carried out by using an experimental design fully randomized and results were statistically evaluated by Tukey test ($p < 0.05$). For milk without ozone sanitation (control sample), the counts of the *Enterobactereaceae*, mesophilic aerobic, psychrotrophic, molds and yeast and *Staphylococcus* sp were 2.39; 4.18; 3.01; 2.70; 2.16 log10 CFU.mL^{-1}, respectively. The ozonation for 5 minutes was not able to reduce the milk microbial counts. In contrast, treatment with ozone for 10 minutes caused significant reduction ($p < 0.05$) of *Enterobacteriaceae* (0.59 log), molds and yeast (0.25 log) and *Staphylococcus* sp (0.59 log). After 15 minutes of treatment, microbial inactivation were observed for the 6 groups of microorganisms evaluated, with reductions of 0.96; 0.60; 0.13; 0.48 and 1.02 log cycles for *Enterobacteriaceae*, mesophilic aerobic, psychrotrophic, molds and yeast and *Staphylococcus* sp, respectively. Therefore, the use of ozone gas bubbling can be adopted as a milk pre-process aiming to obtain a reduction of the microbial count in raw milk; however, this treatment did not eliminate the milk thermal process requirement.

Introduction

Cow milk is a food with high nutrient contents, high water activity and neutral pH. These characteristics makes the milk an ideal medium for microbial growth (Dahmer, 2006), resulting in fast developments of pathogens and spoilage microorganisms and consequent reduction of the milk shelf-life.

Mesophilic aerobic microorganisms and coliforms are commonly found at high counts in raw milk (Moraes *et al.*, 2009) and are indicators of the milking and storage quality (Oliveira *et al*., 2011). Some of these microorganisms are pathogens; but, the majority of them are just spoilage microorganisms, able to produces enzymes that seriously compromise the milk quality during its storage and reduces the quality of dairy products (Pedras *et al*., 2012).

Due to the distance between milk producers and processors in Brazil, milk needs to be refrigerated just after the milking process. The refrigerated storage and transportation of milk allows the development of psychrotrophic microorganisms. These microorganisms are associated to insufficient sanitary conditions in the milk production and storage and can rapidly grow at uncontrolled refrigeration temperature. Psychrotrophic are the main producers of proteolytic and lipolytic enzymes that affect milk stability and reduces it shelf-life (Hanamant and Bansilal, 2010; Corrêa *et al*., 2011).

The thermal treatment is the conventional method applied to reduce the microbial load in milk (Pedras *et al.*, 2012). It is very simple and effective, but results in several sensory and nutritional loss in the product (Pedras *et al*., 2012), especially when thermal treatment is carried out in low quality milk, which presents high microbial load.

An alternative to reduce the milk microbial load is using substances that inactivate microorganisms without affect milk safe and quality (e.g. ozone, peroxide and CO_2). The use of these substances can reduces the binomial of time and temperature applied in milk pasteurization due the lower counts of microorganisms in milk. Additionally, these substances can limit the enzyme production by microorganisms during its storage before thermal treatment, improving the stability of milk protein during its shelf-life.

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Ozone is highlighted by its antimicrobial properties and to be totally degradable in oxygen, with no waste/toxic products (Tiwari *et al*., 2008; Tiwari *et al.*, 2010) being Generally Recognized as Safe (GRAS) (Guzel-Seydim, Greene and Seydim, 2004, Dhillon *et al.*, 2009, Tiwari *et al*., 2010). An additional advantageous is the ozone lower equipment costs (Guzel-Seydim, Greene and Seydim, 2004).

The ozone is a high oxidative compound and has a broad antimicrobial spectrum, being able to inactivate vegetative and sporulated cells, yeast, molds and viruses, additionally is able to kill pests of grain storage (insects) and to degrade mycotoxins (Tiwari *et al*., 2010). Ozone destroys microorganisms by the progressive oxidation of vital cellular components. The cell wall is the first target of ozonation, with the oxidation of the polyunsaturated fatty acids and consequent loss of selective permeability and cell disruption. Additionally ozone causes the oxidation of sulfhydryl groups and amino acids of enzymes, peptides and proteins, including nucleic acids and vital enzymes (Khadre, Yousef and Kim, 2001, Guzel-Seydim, Greene and Seydim, 2004). In spores, ozone degrading the coat layers component thus exposing the cortex and the core to oxidation by ozone (Khadre and Yousef, 2001; Tiwari *et al.*, 2010).

Choi *et al.* (2012) obtained effective foodborne pathogens inactivation in apple juice by ozonation. Patil *et al*. (2010) also observed *E. coli* inactivation on apple juice by ozonation, reaching a 5 log reduction after 4 minutes (pH 3.0) and 18 minutes (pH 5.0). These results indicated that low pH improves the ozone efficacy, probably presenting a synergistic effect. However, results of Kim and Hung (2012) showed reductions of *E. coli* O157:H7 lower than 1 log cycle after ozonation of blueberries.

Considering the potential of ozone application, this work aimed to evaluate the efficacy of microbial inactivation by ozone bubbling in milk.

Materials and Methods

Milk samples

The raw milk was obtained from 24 healthy cows with < 500,000 somatic cells.mL^{-1}. To avoid wrong results, selected cows had not been treated with antibiotics during the last 7 days prior to milking. Before the milking step, the cow's teats were sanitized with chlorexidin solution at 2%. After milking, 20 liters of milk was added by 0.1% polysorbate 80 (Tween 80®) - to improve the contact with ozone (Chen *et al*., 1993; Goff and Jordan, 1989) - and then refrigerated at 4ºC. The tensoactive was used since preliminary assays showed no microbial reduction on milk directly sanitized with ozone (data no showed). The ozonation process was performed immediately after milk reaches the refrigerated temperature. Control samples for microbiological and physicochemical analysis were collect before and after the addition of the polysorbate, to evaluate if it had some effect on microbial inactivation.

Ozone Generation

The ozone was produced from pure oxygen using an ozone generator from Interozone (model 485 RT). At the equipment outlet, a hose with a porous plate made of ore sand was linked (aerate volume: 0.6 L. min^{-1}; size: 12 x 20 mm). This system was used to distribute the generated ozone as micro-bubbles in milk, improving the ozone mass transfer and homogeneity. The ozonation was carried out in a rectangular tank of 0.5 m long by 0.3 m wide by 0.5 m height).

Ozonation process

The 20 L of milk was stirred at approximately 20 rpm in the tank. The ozone generation system was turned on and the ozone concentration was monitored to reach 1.5 mg. L^{-1}. This concentration was the maximum ozone concentration able to be incorporate in milk, lower concentration was not evaluated due to the low efficacy of the process. Ozonated milk was collected after 5, 10 and 15 minutes of contact. These processes were carried out in duplicate. The collected samples were evaluated according microbiological and physicochemical parameters in triplicate.

Microbiological assays

The samples were evaluated by counts of total mesophilic aerobic (TMA), psychrotrophic, *Enterobacteriaceae*, yeasts and molds (YM) and search of *Staphylococcus* sp. and *Salmonella* sp. immediately after the ozonation. Table 1 shows the growth media and incubation time and temperature used for each evaluated microorganism (except for *Salmonella* sp).

For evaluation of *Salmonella* sp., it was used the Lactosa enrichment broth (DIFCO®) incubated at 35ºC/ 48 h followed by incubation on the Rapapport Selective broth (DIFCO®) at 45ºC/24 h. For positive samples, the cultures were isolated using the Brilliant green lactose bile agar (DIFCO®) and Salmonella-Shigella agar (DIFCO®) incubated at 35ºC/ 24 h (APHA, 1992). If a sample had been positive, the confirmatory biochemical tests have been performed.

The number of decimal reduction (NDR) was calculated considering the initial count (control

sample) and the count's obtained after the ozonation process, using the follow equation:

$$NDR\ (Log_{reduction}) = log_{(initial\ count)} - log_{(count\ after\ treatment)}.$$

Physicochemical assays

The fat, protein, acidity and density of the milk were measured using the equipment Ekomilk M, model Milkama Kam 98-2A. The nonfat dry extract (NFDE) was calculated using the data of density and fat content. These analyses were performed to evaluate if the produced milk was in accordance with Brazilian law.

Statistical analysis

For microbiological evaluation, the experiments were fully randomized with 5 treatment (control – with no ozonation -, milk added by tween - with no ozonation -, and ozonated samples for 5, 10 and 15 minutes). Each treatment was evaluated in triplicate. For physicochemical evaluation, the same samples were evaluated and analyses were performed with 10 repetitions.

Results and Discussion

The counts of total mesophilic aerobic (TMA), psychrotrophic, *Enterobacteriaceae*, *Staphylococcus* sp., *Salmonella* sp. and yeasts and molds, before and after ozonation are shown in Table 2.

According to Table 2, the addition of tensoactive caused no significant reduction in any evaluated microorganism, being the reductions observed attributed only to the ozone effects. Additionally, the ozone bubbling during 5 minutes was not enough to cause significant microbial inactivation. On the other hand, 10 minutes of ozonation reduced the counts of *Enterobacteriaceae* (0.58 log cycle), *Staphylococcus* sp. (0.52 log cycle) and yeasts and molds (0.25 log cycle). The ozonation for 15 minutes results on reductions of TMA, *Enterobacteriaceae*, psychrotrophics, *Staphylococcus* sp. and yeasts and molds of 0.60, 0.96, 0.13, 1.02 and 0.48, respectively (Table 3).

Therefore, the microbiological results highlighted that ozone was effective to reduce the native flora of the milk. Although smaller (between 0.5 and 1 log cycle), these reductions is possibly enough to improve the quality of raw milk, increasing its shelf life before thermal process. Moreover, the reduction of the initial microbial load on milk allows the application of milder heat treatment. On the other hand, the results showed that ozonation applied isolated cannot guarantee the milk safety.

Table 1. Growth media and incubation time and temperature used for each evaluated microorganism

Microorganism	Growth media	Time and temperature of incubation	Reference
TMA counts	PCA	35°C/48 hours	(APHA, 1992)
Psychrotrophics	PCA	7°C/10 days	(APHA, 1992)
YM counts	PDA	22°C/48 hours	(APHA, 1992)
Enterobacteriaceae	VRBA	35°C/48 hours	(APHA, 1992)
Staphylococcus sp	BP	35°C/48 hours	(APHA, 1992)

PCA (plate count agar - DIFCO®), MRS (Lactobacillus MRS agar - HIMEDIA®) PDA (potato dextrose agar - DIFCO®), VRBA (violet red bile dextrose agar - DIFCO®), BP (Baird Parker agar - DIFCO®)

Table 2. Microbial counts (Log10 CFU.mL^{-1}) in milk before and after ozonation

Microorganisms	1	2	3	4	5
TMA	$4.18^{a} \pm 0.03$	$3.78^{a} \pm 0.58$	$3.48^{a} \pm 0.58$	$3.60^{a} \pm 0.02$	$3.58^{a} \pm 0.02$
Enterobacteriaceae	$2.39^{a} \pm 0.08$	$2.41^{a} \pm 0.10$	$2.26^{a} \pm 0.05$	$1.81^{b} \pm 0.04$	$1.43^{c} \pm 0.10$
Psychrotrophic	$3.01^{a} \pm 0.03$	$3.01^{a} \pm 0.03$	$2.93^{ab} \pm 0.06$	$2.93^{ab} \pm 0.02$	$2.88^{b} \pm 0.03$
Staphylococcus sp	$2.16^{a} \pm 0.09$	$2.10^{a} \pm 0.04$	$2.00^{a} \pm 0.09$	$1.64^{b} \pm 0.05$	$1.14^{c} \pm 0.16$
Yeasts and Molds	$2.70^{a} \pm 0.04$	$2.68^{a} \pm 0.03$	$2.63^{a} \pm 0.02$	$2.45^{b} \pm 0.04$	$2.22^{c} \pm 0.08$
Salmonella sp.	n.f.*	n.f.	n.f.	n.f.	n.f.

1: control without Tween®; 2: control with Tween®; 3: ozonated sample during 5 minutes; 4: ozonated sample during 10 minutes; 5: ozonated sample during 15 minutes. Results showed as average ± standard deviation. Different letters indicates significant differences at $p < 0.05$. n.f. not found.

Previous results of TMA inactivation in pepper were similar to the obtained in the present work (Ketteringham *et al.*, 2006), but 2-5 decimal reductions were obtained using ozonated water at similar concentration in apples (Achen and Yousef, 2001), lettuce (Cavalcante, 2007), meat (Novak and Yuan, 2004) and tomatoes (Fan *et al.*, 2012). These different ozone efficacy may is related to differences in ozonation system, microbial target and characteristics of each food.

Table 4 presents the average results of the physicochemical parameters evaluated on milk before and after ozonation. These results were evaluated according to Brazilian law, which determines that raw milk must have minimum fat, nonfat dry extract (NFDE) and protein content of 3.0, 8.4 and 2.9%, respectively; relative density between 1.028 and 1.034 (15/15°C, g. mL^{-1}) and acidity between 14 and 18% (Brasil, 2002).

The physicochemical results showed that all evaluated samples were in accordance to Brazilian law (Brasil, 2002); additionally, it was observed the tensoative addition did not altered the milk physicochemical parameters ($p > 0.05$). The ozonation up to 15 minutes did not change the evaluated parameters. These results were expected, since the process would not change the milk

Table 3. Decimal reductions in microbial counts in milk after ozonation

Microorganisms	1	2	3	4	5
TMA	-	0.40 ± 0.60	0.70 ± 0.61	0.58 ± 0.05	0.60 ± 0.04
Enterobacteriaceae	-	-0.02 ± 0.03	0.13 ± 0.12	0.58 ± 0.11	0.96 ± 0.14
Psychrotrophic	-	0.00 ± 0.05	0.08 ± 0.07	0.08 ± 0.05	0.13 ± 0.06
Staphylococcus sp	-	0.06 ± 0.05	0.16 ± 0.18	0.52 ± 0.14	1.02 ± 0.09
Yeasts and Molds	-	0.02 ± 0.04	0.07 ± 0.06	0.25 ± 0.08	0.48 ± 0.09
Salmonella sp.	n.f.*	n.f.	n.f.	n.f.	n.f.

1: control without Tween®; 2: control with Tween®; 3: ozonated sample during 5 minutes; 4: ozonated sample during 10 minutes; 5: ozonated sample during 15 minutes. Results showed as average ± standard deviation. n.f. not found.

Table 4. Milk physicochemical parameters of milk before and after ozonation

Physicochemical parameter	1	2	3	4	5
Protein (%)	$3.37^a \pm 0.02$	$3.39^a \pm 0.03$	$3.39^a \pm 0.03$	$3.38^a \pm 0.04$	$3.35^a \pm 0.04$
FAT (%)	$3.67^a \pm 0.14$	$3.64^a \pm 0.10$	$3.64^a \pm 0.10$	$3.66^a \pm 0.13$	$3.62^a \pm 0.06$
Density ($g.mL^{-1}$)	$1.0329^a \pm 0.00$	$1.0324^a \pm 0.01$	$1.0324^a \pm 0.00$	$1.0321^a \pm 0.00$	$1.0329^a \pm 0.00$
NFDE (%)	$8.69^a \pm 0.08$	$8.70^a \pm 0.07$	$8.70^a \pm 0.07$	$8.77^a \pm 0.09$	$8.72^a \pm 0.05$
Acidity (°D)	$15.87^a \pm 0.76$	$16.10^a \pm 0.80$	$16.10^a \pm 0.80$	$15.90^a \pm 0.74$	$15.8^a \pm 0,71$

1: control without Tween®; 2: control with Tween®; 3: ozonated sample during 5 minutes; 4: ozonated sample during 10 minutes; 5: ozonated sample during 15 minutes. Results showed as average ± standard deviation. Different letters indicates significant differences at $p < 0.05$.

centesimal composition. However, these analyses were performed since they are mandatory in Brazil.

Although changes on physicochemical parameters of food be unusual, previous results obtained by Oliveira *et al.* (2008) showed a significant change in the centesimal composition of fish fillet due to increase of fish dripping after ozonation.

Conclusion

The raw milk studied showed an adequate microbiological quality and the ozone bubbling for 15 minutes was able to causes 0.5 to 1 decimal reductions in the milk native flora. Complementary, no changes were observed in the physicochemical parameters of the milk. Thus, the results highlight the ozonation bubbling as an alternative to reduce the microbial load in raw milk, improving its quality and increasing the milk shelf-life before thermal processing.

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