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Full Length Research paper

Microbiological quality of high-pressure (HP) treated fresh cheese of bovine milk

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High pressure (HP) treatment at ambient temperature (25°C) was applied to freshly-prepared rennet-coagulated cheese of bovine (pasteurized) milk and the microbiological quality was examined for up to Day 31 using a Central Composite Design (CCD) of experiment. Bacterial counts were significantly ($p < 0.05$) reduced at the highest applied pressure. Estimated shelf-life was 17 days for control samples but approached 31 days at HP treatments of 150 MPa at 1 and 15 min. Treatments above 150 MPa with treatment time up to 29 min caused highly significant reductions in bacterial counts ($p < 0.01$ to $p < 0.001$). Gram-positive cocci and rods as well as Gram-negative rods were found during spoilage of the fresh cheese.

Key words: Response surface methodology (RSM), microbial quality, cheese, spoilage, shelf-life.

INTRODUCTION

High Pressure Processing (HPP) has made substantial progress in recent times and many workers have demonstrated its ability to enhance food safety for the food industry (Patterson, 2005). Cruz-Romero et al. (2008) reported this technology to be advancing considerably towards improving quality of food production. Apart from preserving the functional properties of foods, HPP can reduce and possibly destroy microorganisms present in food (Capellas et. al., 2000). However, industrial scale application of HPP to fresh cheese is yet to be realized (Okpala et al., in-pres).

High pressure (HP) treatment can be applied to dairy products with the aim of inactivating spoilage organisms (Daryaei et al., 2008). Fresh cheese can be contaminated during processing and shelf-life, followed by complex and serious deteriorations. Microorganisms such as *Listeria monocytogenes* and *Escherichia coli* are among the pathogenic microorganisms reported in fresh cheeses (Trujillo et al., 2000). High pressures in the range of 400 to 500 MPa for 5 to 15 min at 2, 10 and 25°C were applied to pasteurized goat's milk, prior to the manufacture of cheese, which had been inoculated with *E. coli* at the level of 10^8 CFU/g. No surviving *E. coli* was found in the HP treated fresh cheese after refrigerated storage for 1 day, apart from those treated with pressures of 400 and 450 MPa for 5 min at 25°C.

Also, there were no surviving colonies detected in any of the HP treated samples after 15, 30 or 60 days of refrigerated storage (Capellas et al., 1996).

Pressure resistance as demonstrated by some microorganisms such as *Bacillus* and *Staphylococcus* spp. has been well reported (Capellas et al., 2000). HP treatment using pressures up to 500 MPa for 10 min at 20°C showed significant ($p < 0.05$) reductions of the level of *L. monocytogenes* in raw milk. It was through this process that a safer but non-thermally processed camembert-like soft cheese was obtained (Linton et al., 2008). A near 100-fold reduction in starter bacteria numbers followed by delayed growth of non-starter lactic acid bacteria (NSLAB) were obtained in a 1 day-old full-fat cheese when HP treatment (400 MPa for 10 min at room temperature) was applied (Rynne et al., 2008).

Microbial reduction by HP treatment can result in the

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extension of food shelf-life (Carlez et al., 1994). A fresh lactic curd cheese, typical of Australian cheese makers was found to obtain limited refrigerated shelf-life of 3 weeks only. It was subjected to HP treatment using from 200 to 600 MPa of pressures for 5 min and the effect on its starter bacteria numbers and spoilage yeasts was determined. At 200 MPa, the growth of yeasts obtained in the fresh cheese samples was not significantly ($p > 0.05$) prevented, however, at pressures ≥ 300 MPa, it was effectively controlled for 6 to 8 weeks (Daryaei et al., 2008).

Response Surface Methodology (RSM), an experimental modeling technique used to estimate the link between a set of controllable experimental factors and observed results, is presently the most popular optimization techniques in the area of food science and this is as a result of its wide-ranging theory, high efficiency and practicability as well as its unsophisticated approach. The most frequently-used design of experiment associated with RSM is the Central Composite Design (CCD). Here, data predictability remains equi-directional from the center point. RSM has been used to study the effect of food constituents on *Staphylococcus aureus* inactivation by high pressure and mild heat (Gao et al., 2006).

Recently, RSM was employed to examine the influence of HP treatment on the physico-chemical properties of fresh cheese. In that study, HP treatment influenced significantly ($p < 0.05$) the colour, fat, moisture, lipid oxidation, hardness and adhesiveness of the fresh cheese (Okpala et al., in-press). Since RSM was used to evaluate successfully the effects of HP treatment on physico-chemical properties of fresh cheese, we believed that applying it to evaluate the microbiological quality of HP treated fresh cheese would be useful. It is from such an investigation that the actual effect of HP treatment on the microbiological quality of the fresh cheese can be appreciated. No study of this kind appears to have been reported previously.

The aim of this study was to examine the effects of HP treatment at ambient temperature (25°C) on the microbiological quality of rennet-coagulated soft cheese of bovine (pasteurized) milk using a full 2-factor Central Composite Design (CCD) of experiment. Specific objectives were: (1) to determine the shelf-life of the HP treated fresh cheese compared with the control; and (2) to study the influence of HP treatment on the microbial population present in the fresh cheese using response surface methodology.

MATERIALS AND METHODS

Preparation of samples

One batch of freshly prepared soft cheese was obtained from a local Scottish dairy manufacturer. The milk used for the cheese preparation undergoes both pasteurisation (72 ± 3°C for 30 s) as well as rennet coagulation before the soft cheese is prepared. The cheese used for the study was chosen as a representative sample of rennet-coagulated soft cheese found in Scotland. Also, the dairy is representative of the industry and procedures employed are consistent in the making of the rennet coagulated soft cheese. The cheese samples were packed into clean screw-capped 1.5 ml polypropylene microcentrifuge tubes (Fisher Scientific UK Ltd, Leicestershire LE11 5RG, UK) after which they were refrigerated at 4 – 7°C prior to processing. Excluding the non-treated fresh cheese samples (controls), all experimental fresh cheese samples were high-pressure (HP) treated within 48 h of packaging.

High-pressure (HP) treatment

High pressure equipment was a stainless steel pressure vessel with a 50 ml working capacity and maximum design operating pressure of 600 MPa (High Pressure Equipment Company, Erie, Pennsylvania 16505, USA). Operating temperature in the chamber was regulated by immersion of vessel in a water bath (Grant Instruments [Cambridge] Ltd., Cambridgeshire SG8 6GB, UK). The pressure vessel was preheated to the required temperature to eliminate thermal lag time. After cheese samples were processed in the pressure chamber, the vessel was depressurized rapidly and the samples were recovered and refrigerated (4 – 7°C) until required for microbial studies.

Microbiological analyses

Media preparation

Taking into account that packaging of fresh cheese samples into microcentrifuge tubes for HP treatment was done manually, microbial analysis targeted microorganisms (Gram positive [+] or negative [–] bacteria) which had particular relevance to spoilage, health or had been reported in previous studies. Media employed included: *Bacillus cereus* Selective Agar (BSA), MacConkey Agar (MCA), and Mannitol Salt Agar (MSA) (Oxoid Ltd., Basingstoke, Hampshire, Hants RG24 8PW, UK). BSA, MCA and MSA are culture media sufficiently selective for the isolation and enumeration of Gram (+) and (–) rods as well as Gram (–) cocci bacteria, respectively. These media were employed in an attempt to separate the bacteria likely to emerge in the cheese samples during the spoilage period.

Media preparation was as follows: BSA (CM0617) used 20.5 g of agar solid suspended in 475 ml distilled water; MCA (pH 7.4 ± 0.2 at 25°C) used 52 g suspended in 1 L of distilled water; and MSA (CM0085) used 55.5 g suspended in 500 ml distilled water. Oxoid Polymyxin B (1 vial) supplement (SR0099E) as well as Sterile Egg Yolk Emulsion (SR0047) (Oxoid Ltd.) were prepared and added as directed by manufacturers to the BSA in order to suppress the growth of other microorganisms and contaminants. Nutrient Agar (NA) (CM0003; Oxoid Ltd.) used 28 g in 1 L of distilled water. Gentle boiling was applied to all media until complete solution was attained before autoclaving (121°C for 15 min) and followed by cooling.

For the BSA, MCA and MSA media, thorough mixing carried out before solution was poured out onto sterile Petri dishes (Scientific Laboratory Supplies Ltd., Cambridgeshire SG8 6GB, UK.) and left to solidify (18 – 24 h). Plates were labeled according to numbering of treatment: on one plate, treatments 1 to 8 in a clockwise manner circularly by sectors and on the other, treatment 9 and control (giving control ample space for complete streaking). NA medium was used to prepare slants and was employed for pour-plate analysis where bacteria numbers were determined.

Isolation of microorganisms and plate counting

At Days 3, 10, 17, 24 and 31 of storage period, inoculum from all

Table 1. Design matrix and variable combinations in experimental samples.

Runs	Levels	
	Pressure(MPa)	Time(min)
1	150	29
2	250	5
3	50	5
4	250	25
5	50	25
6	150	1
7	291	15
8	150	15
9	9	15

processed treatments and controls were surface spread on BSA, MCA and MSA media plates in duplicates. BSA, MCA and MSA media were incubated at 30°C from 18 to 24 h. Soon after the respective media reacted with the spread HP treated and non-treated fresh cheese samples, the isolates from BSA, MCA and MSA media were selected for further microbial analyses. Sub-cultures of isolates were prepared and staining carried out for Gram reaction with corresponding cell morphology noted. Catalase test employed 3% H_2O_2 (Sigma-Aldrich Ltd., Gillingham SP8 4XT, UK) on NA slants containing isolates. Oxidase activity employed oxidase strips (Merck KGaA, 64293 Darmstadt, Germany) on isolates of respective media.

The numbers of viable microorganisms (Log CFU g^{-1} units) of both HP treated and non-treated (control) fresh cheese samples were sought for at Days 3, 10, 17, 24 and 31. Under aseptic conditions, cheese samples (weighed 1 g sample) were homogenized by vortex mixing (2 min) with 9 ml autoclaved (121°C for 15 min) sterile distilled water to make up a 10^{-1} dilution. Serial dilutions up to 10^{-6} were prepared and pour-plate method was used to prepare colony counts. Aliquots of equal amounts (1 ml) were pipetted onto clean Petri dishes and equal volume of molten agar (sterile NA, pre-cooled at 45 - 50°C) was added and mixed 3 - 5 times, gently, clockwise and anticlockwise. All were carried out under aseptic conditions. When agar solidified, plates were incubated in an inverted position (30°C for 24 ± 3 h). Direct counts of colony numbers (CFU g^{-1}) were carried out immediately after plates were removed from incubator. Plates which obtained 30 to 300 colonies were recorded.

Experimental design and statistical analysis

A central composite experimental design with a single centre point and two experimental variables of pressure and time (Table 1) was used. Unless otherwise stated, data were collected in triplicate and expressed as means ± standard deviation (SD). Statistical analysis was performed by applying one –way analysis of variance (ANOVA) and mean comparisons were carried out between treatments (pressures and holding times) and control in terms of Log CFU $g^{-1}$of bacteria numbers using Fisher's least significant difference (LSD) test at confidence level of 95%.

Mean data of treatments (Log CFU g^{-1}) from Days 17, 24 and 31 were analyzed by response surface to determine influence of pressure and treatment time on the microbial population. Minitab 15 (Minitab Ltd., Coventry CV3 2TE, UK) was used for experimental design and data analysis. In all regression and response surface models, terms were retained only if their coefficients were significant at $p < 0.05$, and lack of fit was non-significant ($p > 0.05$).

RESULTS AND DISCUSSION

No viable count was obtained on Day 3 for both non-treated (control) and HP treated fresh cheese samples because plate count fell below the 30 CFU. However, the control, as well as treatments 9 MPa at 15 min and 50 MPa at 5 min obtained viable counts for Day 10 because they fell within range of 30 to 300 CFU counts at 10^{-1} dilution of cell suspensions only. Bacteria numbers of viable counts of HP treated fresh cheese for treatments 150 MPa/1 min, 150 MPa/15 min, 150 MPa/29 min, 250 MPa/5 min, 250 MPa/25 min and 291 MPa/15 min obtained reductions of 1.75, 1.81, 2.29, 2.42, 3.59 and 3.48 Log CFU g^{-1} at Day 17; 2.74, 2.81, 3.18, 3.42, 3.82, 3.88 Log CFU g^{-1} at Day 24; as well as 1.88, 2.37, 3.34, 3.85, 3.93 and 3.61 Log CFU g^{-1} at Day 31, over the control, respectively (Table 2). Our study tends to agree with the results of Daryaei and co-workers wherein viable count of *Lactococcus* reductions of 2 to 5 Log CFU g^{-1} in the fresh cheeses were obtained at pressures between 200 to 300 MPa under aerobic incubation conditions (Daryaei et al., 2008). Surprisingly, it was found that log unit reductions obtained for treatment 250 MPa for 25 min at Days 17 and 31 (3.59 and 3.93 Log CFU g^{-1} respectively) remained above treatment 291 MPa for 15 min (3.48 and 3.61 Log CFU g^{-1} respectively) at Days 17 and 31, but lower at Day 24, that is, 250 MPa for 25 min obtained 3.82 Log CFU g^{-1} while 291 MPa for 15 min obtained 3.88 Log CFU g^{-1}respectively. Capellas et al. (1996) showed that HP treatment of 400 to 500 MPa for 5 - 15 min (2 - 25°C) obtained 7 Log CFU g^{-1} reductions of microbial populations previously inoculated on fresh (Mató) cheese. They further observed that, where the microbial cells survived HP treatment, the microbial population remained at non-detectable levels after a week of storage of 4°C (Capellas et al., 1996).

Trujillo et al. (2000), after obtaining some level of control of microbial populations of HP-treated (400 MPa/2 – 25°C/5 min) cheese for 2 months in refrigerated storage at 4°C, further studied shelf-life extension of cheeses in which milk used for the cheese manufacture was not inoculated with target organisms. In those experiments, they considered that cheese became altered when counts approached 6 Log CFU g^{-1}, and this was in accordance with typical counts of cheese made without addition of preservatives (Trujillo et al., 2000). Similarly, since the present investigation likened latter studies of Trujillo et al. (2000), 6 Log CFU g^{-1} was taken as an indicator of likely spoilage. As a result, shelf-life of non-processed fresh cheese was found to attain Day 17 approximately relative to the advertised shelf-life of 14 days of the cheese product. For the HP treated fresh cheese samples, only treatments 150 MPa at 1 and 15 min obtained shelf-life to approach Day 30. However, we were unable to establish spoilage for treatments 150 MPa at 29 min, 250 MPa at 5 and 25 min as well as 291 MPa at 15 min because these treatments did not reach 6 Log CFU g^{-1}. Therefore, HP treatments (150 MPa at 29 min, 250 MPa at 5 and 25 min

Table 2. Viable microbial count (Log CFU g^{-1}) of treated and non-treated fresh (control) cheese samples at Days 10, 17, 24 and 31.

Pressure (MPa)/ time (min)	Days of storage[a]			
	10	17	24	31
Control	2.50±0.04	6.18±1.23	7.27±0.69	8.35±0.01
9/15	2.54±0.04	5.55±1.46	7.22±0.67	8.33±0.01
50/5	2.51±0.04	5.50±1.41	7.19±0.67	7.87±0.45
50/25	*	4.95±1.16	7.16±0.67	7.69±0.40
150/1	*	4.43±0.91	4.53±0.83	6.47±0.84
150/15	*	4.37±0.92	4.46±0.82	5.98±0.62
150/29	*	3.89±0.69	4.09±0.64	5.01±0.54
250/5	*	3.76±0.66	3.85±0.56	4.50±0.86
250/25	*	2.59±0.02	3.45±0.30	4.42±0.90
291/15	*	2.70±0.04	3.39±0.28	4.74±0.67

Means ± standard deviation (SD) from triplicate measurements.
A = Measurements recorded in Log CFU g^{-1}.
Media used: Sterile NA, pre-cooled at 45 - 50°C.
*Counts outside the range of 30 to 300 CFU for serial dilutions of cell suspensions.
Storage temperature of 4 –7 °C

Table 3. ANOVA summary of *F*-ratios followed by corresponding R^2 values of viable microbial count of processed (treatments) versus non-processed fresh cheese samples during the experimental period[a].

	High pressure treatments (Pressure [MPa]/Time[min])					
	150/29	250/5	250/25	150/1	291/15	150/15
F-value	31.48***	41.37***	35.18***	10.89**	29.20***	18.03***
R^2 (%)	35	42	41	13	38	22

Significant *F*-ratios at **p < 0.01, ***p < 0.001.
[a]99% confidence interval for mean Log CFU g^{-1} based on pooled standard deviation (SD).

as well as 291 MPa at 15 min) showed the capacity to extend the shelf life of the HP treated fresh cheese beyond Day 31 at 4 - 7°C of refrigerated storage.

There was no significant difference (p > 0.05) obtained statistically for treatments 9 MPa at 15 min, as well as 50 MPa at 5 and 25 min compared to control throughout the storage period. However, at 99% confidence interval for mean Log CFU g^{-1} based on pooled standard deviation (SD), only treatment 150 MPa at 1 min was significantly different (p < 0.01), while treatments 150 MPa at 15 and 29 min, 250 MPa at 5 and 25 min, as well as 291 MPa at 15 min, were highly significant (p < 0.001) compared to control. Also, treatment 250 MPa at 5 min obtained highest *F*-value with corresponding R^2 value to explain variance while treatment 150 MPa at 1 min obtained the least value (Table 3).

The influence of pressure and treatment times on the microbial population (Log CFU g^{-1}) was determined at Days 17, 24 and 31. Response surface models were generated for the mean bacteria numbers obtained at these days. At Day 17, both pressure and treatment times was found to influence microbial population of HPP fresh cheese. Only linear effects for pressure (p = 0.000) and treatment time (p = 0.005) were significant (R^2 = 0.96):

Log CFU g^{-1} (Day 17) = 6.18644 – 0.01017P – 0.03110T…… (1)

The increase in pressure showed a rapid and steady decrease in bacterial numbers with a minimal effect of treatment time (Figure 1).

The data for Day 24 was not able to explain adequately the variations obtained for the relationship of HP treatments and the microbial population (R^2 = 0.92, *F*-ratio of 10.92). The model was not therefore used for any further analysis. In contrast, the model for Day 31 was influenced only by pressure (R^2 = 0.88, *F*-ratio of 1.09) which was significant (p = 0.000):

In this case, the applied pressure resulted in a rapid

Log CFU g^{-1} (Day 31) = 8.30895 – 0.01464P……..(2)

reduction of surviving microbial numbers (Figure 2). Yuste et al. (2001) previously indicated that to a high degree there are many variations in view of the barome-

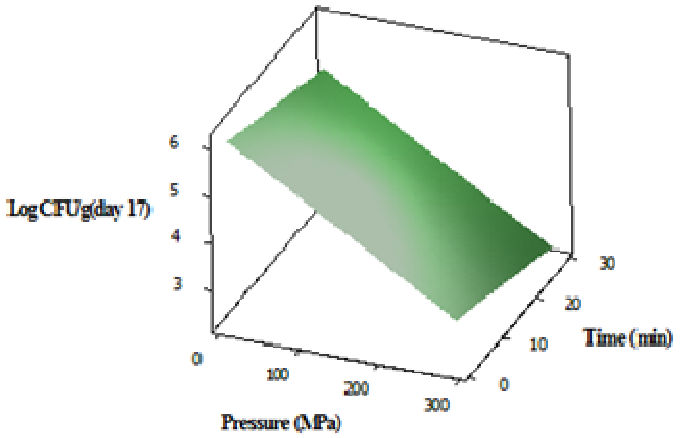

Figure 1. Changes in Log (CFU g^{-1}) of HPP fresh cheese with pressure and time at Day 17. For SDs of observations see Table 2.

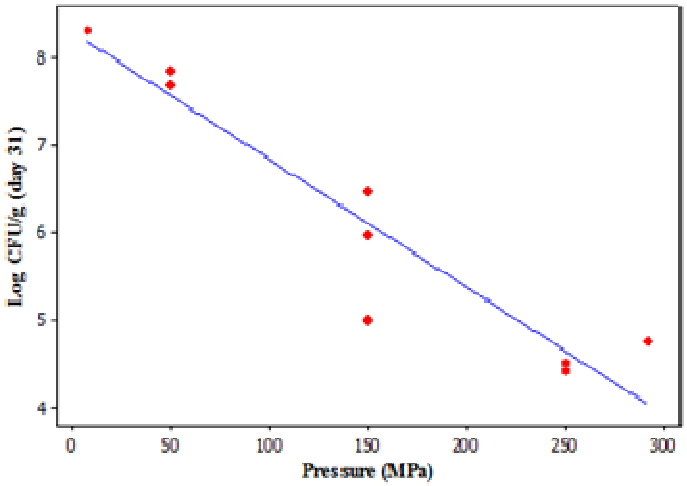

Figure 2. Changes in Log (CFU g^{-1}) of HPP fresh cheese with pressure at Day 31. For SDs of observations see Table 2.

tric sensitivity of microorganisms. The mechanism of inactivation of microorganisms by HP treatment is not yet fully understood. HP treatment is said to challenge the cell membranes of microorganisms and thus, result in damage, distortions as well as the cell becoming permeable in response. As a result of this occurrence, changes in morphology, cell membrane and wall, biochemical reactions and genetic mechanisms can result. In effect, HP treatment is suggested to affect the cytoplasmic membrane and initiates the crystallization of membrane phospholipids which lead to microbial inactivation (Yuste et al., 2001). Laboratory media have been used by some workers to show the mechanisms by which cell inactivity occurs during HP treatments (Chilton et al., 1997). In the present study, different results were obtained for microbial recovery among the BSA, MCA and MSA media. In some cases, the apparent observed colony density visua-

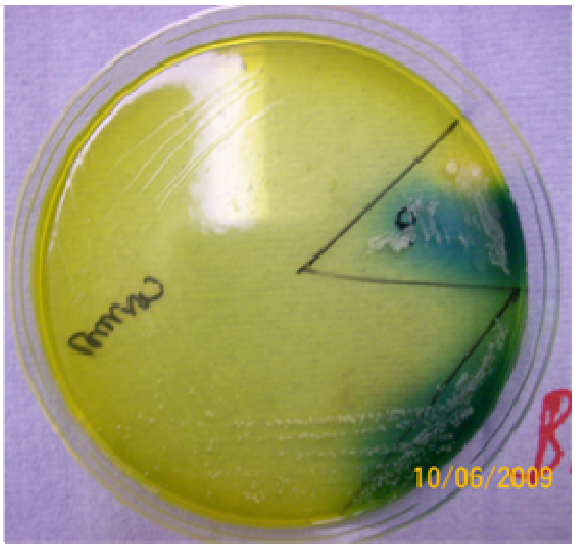

Figure 3. Blue color change of BSA medium for treatments 9 MPa at 15 min and control after Day 24.

lised on the media corresponded with the pressure treatment such that treatments of higher pressures showed reduced colony density. However, this was not consistent in all cases because the reverse was obtained in some media. In addition, in situations where high colony density was observed visually, it was expected that degree of colony density of microorganisms on the media would match up with the anticipated degree of colony-media reaction. Yet, this was not always the case.

During spoilage period of the HP treated fresh cheese, colonies isolated from BSA medium obtained Gram-positive rods which were spore forming under microscopy (x100) and were oxidase positive. Subcultures spread on NA slants reacted positively to H_2O_2 (catalase test). Treatments of higher pressures obtained reduced colony density on the BSA media. This situation was clearly demonstrated in treatments 150 MPa, 250 MPa and 291 MPa at Days 17 and 24. However, the degree of turquoise to peacock blue coloration of BSA medium was not consistent with colony densities recovered because low colony density showed colour change in some cases while high colony density did not. It was treatment 9 MPa for 15 min as well as the control after Day 24 that showed high colony density which exhibited a turquoise to peacock blue coloration especially at the initial streaking point on the medium and upwards (Figure 3).

Similarly as time progressed, the blue color change was found to occur in all treatments (Figure 4). In the MSA media, no reaction was observed on colony spread of HPP fresh cheese up to Day 10. At Day 24, the fermentation of the mannitol was obtained at treatments 9 MPa at 15 min, 50 MPa at 5 and 25 min but with decreasing colony density in 150 MPa at 1, 15 and 25 min. Similarly, as time progressed after Day 24, fermentation of mannitol became obvious as bright yellow zones oc-

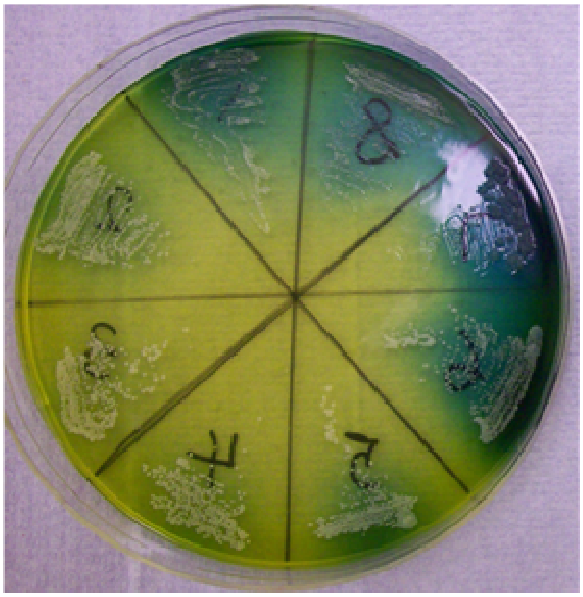

Figure 4. Blue color change of BSA medium after Day 24 for all treatments (1 - 8).

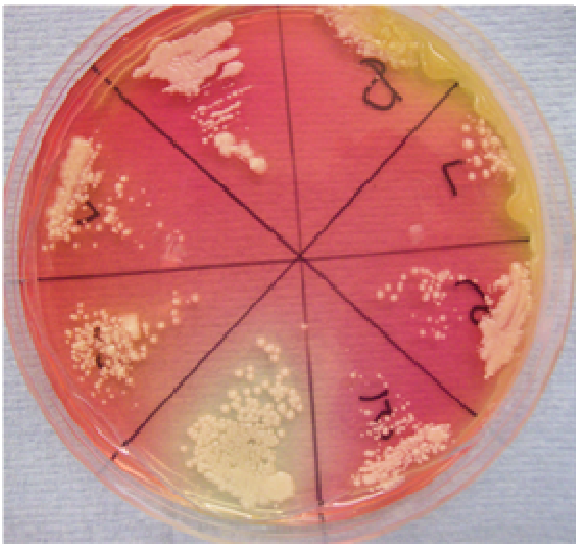

Figure 5. Bright yellow zones at initial streaks of treatments 7 and 8 on MSA medium as time progressed after Day 24.

curred at the initial streaks of sectors 7 and 8 (Figure 5). Microscopy showed isolates from MSA plates as Gram-positive cocci at Day 24 and subcultures spread on NA slants reacted negatively to catalase tests. In MCA media, microscopy showed Gram-negative rods at Day 24. Colonies featured red pink colorations at treatments of low pressures on one hand (sector 3 [50 MPa, 5 min]) and of less holding time even though at higher pressure (sector 8[150 MPa, 15 min]). Furthermore, the colonies which were recovered showed pale pink coloration on the

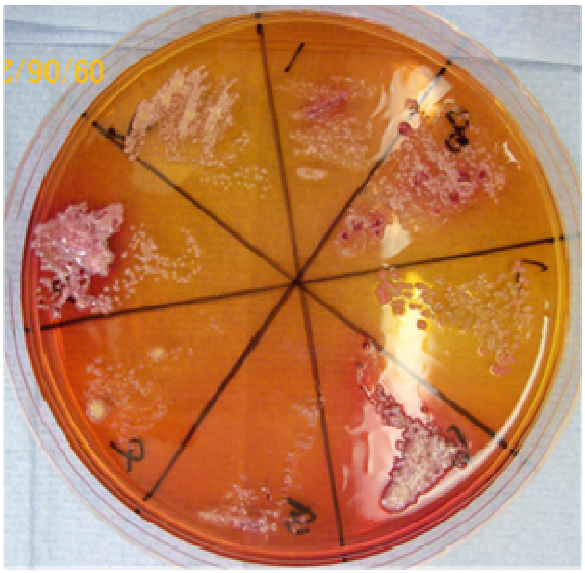

Figure 6. Typical red colonies and microbial reaction on MCA medium at Day 24.

MCA media (Figure 6) and negative reaction for both catalase and oxidase tests

As can be observed in this study, reduced colony density was found to occur at higher pressure treatments of 150, 250 and 291 MPa levels as shown by the microbial spreads on the BSA, MCA and MSA media. Also, there was the absence to the slow nature of the build-up of colony and media reactions observed visually at the initial spoilage periods as revealed on these media. These results and results from other workers (Capellas et al., 2000; Daryaei et al., 2008; Rynne et al., 2008) showed that HP treatment inactivated microorganisms present in the HP treated fresh cheese.

However, the reduced colony density obtained at higher pressure treatments and the slow nature of the build-up of colony and media reactions both tend to suggest that HP treatment may have caused a partial inactivation of some of the microorganisms. HP treatment can weaken the microorganisms especially the cell structure which can hinder immediate bacterial growth and as a result time is needed for its recovery on the selective media (Yuste et al., 2001).

In contrast to the works of Capellas et al. (1996) and (2000), as well as Daryaei et al. (2008), the present study neither had target microorganisms inoculated on the milk used for the cheese making nor had the microorganisms enumerated on the fresh cheese as was used in the present study prior to the HP treatment. Also, the level of microbial contamination obtained in the HP treated fresh cheese may have occurred as a result of the manual handling of the cheese prior to HP treatment. It is equally possible that many of the bacteria, perhaps, may have been lactic acid types and were likely part of bacteria numbers counted. It was found that the BSA, MCA and

MSA culture media could not be used to separate easily the 'lactics' from the 'spoilers'. It is therefore very essential that both processing conditions as well as product quality are taken into account when assessing the influence of HP treatment on the viability of food microorganisms (Trujillo et al., 2002).

Conclusion

HP treatment between 150 and 291 MPa influenced the microbiological quality of fresh cheese and extended the product's estimated shelf-life beyond 31 days. Cell viability decreased significantly, compared with an untreated control, in the HP treated cheese ($p < 0.05$). Pressure treatment time had minimal effect 17 days after treatment and no effect 31 days after, whereas pressure had a linear effect. The extension of shelf-life which resulted after HP treatment because of inactivation of microorganisms present in the treated samples reiterates the potentials of HP treatment to enhance the safety of dairy products such as fresh cheese.

Further investigations into the inactivation kinetics of the microorganisms would help elucidate the effects of pressure and processing time. It would be worthwhile if further microbiological studies can be carried out to make out the specific microorganisms. Overall, information presented in this paper suggests that industrial scale HP treatment of dairy products such as fresh cheese is feasible and useful.

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