Microbiological Research **165** (2010) 61—65

Available online at www.sciencedirect.com

ScienceDirect

Microbiological Research

www.elsevier.de/micres

SHORT COMMUNICATION

Exclusion of phospholipases (PLs)-producing bacteria in raw milk flushed with nitrogen gas (N_2)

Patricia Munsch-Alatossava[a,*], Oguz Gursoy[b], Tapani Alatossava[a]

[a]*Department of Food Technology, University of Helsinki, Viikki Campus, P.O. Box 66, FIN-00014 Helsinki, Finland*
[b]*Department of Food Engineering, Pamukkale University, TR-20020 Kinikli, Denizli, Turkey*

Received 20 June 2008; accepted 5 July 2008

KEYWORDS
Raw milk;
N_2 gas;
Psychrotrophs;
Bacterial phospholipases (PLs)

Summary

Prolonged cold storage of raw milks favors the growth of psychrotrophs, which produce heat-resistant exoenzymes of considerable spoilage potential; the bacterial proteases and lipases affect raw milk quality; among them phospholipases (PLs) may target the milk fat globule. More importantly, bacterial PLs are key virulence factors for numerous species.

Two studies examined the use of nitrogen (N_2) gas and examined its effect on psychrotrophs, proteases and lipase producers when the milk was stored in closed vessels; however, the effect on PLs producers is unknown. Here we show that by considering an open system the PLs producers were sooner or later excluded in raw milk (whereas the PLs producers in the non-treated controls culminated at 10^8 CFU/ml), by effective gas treatments that bring oxygen (O_2) levels in milk lower than 0.1 ppm. No increase of the PLs producers among the anaerobes was noticed during the course of the experiments. In the experiments performed at 6.0 °C, the delay after which the PLs producers were no longer detectable seemed independent of the initial level of PLs producers in raw milk (lower than 10^3 CFU/ml).

We anticipate that flushing pure N_2 gas in raw milk tanks, considered as open systems, along the cold chain of raw milk storage and transportation, may be an additional technique to control psychrotrophs, and may also constitute an interesting perspective for limiting their spoilage and pathogenic potential in food materials in general.

Introduction

Low bacterial counts ensure both quality and safety of raw milk. Recent practices of transporting and collecting raw milk are lengthening the distances between farms and dairy plant silos;

*Corresponding author. Fax: +358 9 191 58460.
E-mail address: patricia.munsch@helsinki.fi (P. Munsch-Alatossava).

0944-5013/$ - see front matter © 2008 Elsevier GmbH. All rights reserved.
doi:10.1016/j.micres.2008.07.001

consequently, and due to the presence of psychrotrophs able to grow below 7 °C, the total bacterial counts are increasing (Shah, 1994; Hayes and Boor, 2001; Mac Phee and Griffiths, 2002). Raw milk psychrotrophs comprise pathogenic bacteria like *Listeria monocytogenes*, or *Bacillus cereus* strains, although usually with low incidence; the most commonly occurring psychrotrophs remain the Gram-negative rods. Recently, we isolated representatives of *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, *Burkholderia* genera, which exhibited rather frequently antibiotic multiresistant features (Munsch-Alatossava and Alatossava, 2006, 2007).

The raw milk psychrotrophs are known for producing exoenzymes, like proteases and lipases, that withstand heat treatments; the hydrolytic products of milk proteins and lipids decrease the technological and sensory qualities of milk products, and hence cause significant economic losses for the dairy industry (Shah, 1994; Hayes and Boor, 2001; Mac Phee and Griffiths, 2002).

Fat in milk is mainly present as globules (the milk fat globule, MFG), which are enveloped by a layer of surface-active material (MFG membrane) that may be the target of bacterial phospholipases (PLs; Shah, 1994; Fox, 2002). The disruption of the MFG results in an unstable fat emulsion (flocs on the surface of milk or cream); the triacylglycerols are then easy targets for lipases. PLs are produced by both bacteria and their eucaryotic hosts; for numerous species, bacterial PLs are key virulence factors, since many bacterial PLs are implicated in pathogenesis (Schmiel and Miller, 1999). As one example, two PLs C are made by *Pseudomonas aeruginosa* (Berka and Vasil, 1982; Sitkiewicz et al., 2006); many Gram-negative bacteria also possess outer membrane PL A activity (Sitkiewicz et al., 2006; Istivan and Coloe, 2006).

Consequently, any technique that would minimize the bacterial counts/reduce the spoilage or pathogenic potential along the cold storage chain of raw milk would be of value, for both technological and human health aspects. Different studies have examined the possibility of extending the shelf life of raw milk by treatments with carbon dioxide (CO_2) and/or nitrogen (N_2) gases (Ma et al., 2003; Rajagopal et al., 2005; Murray et al., 1983; Dechemi et al., 2005). Contrarily to the previous studies that considered "closed systems", we evaluated the possibility of flushing raw milk with pure N_2 gas by considering an "open system". The present study describes the effect of pure N_2, flushed into test bottles containing raw milk, on bacteria that produce PLs.

Materials and methods

The nitrogen flow through the system

The experimental system consisted of 250 ml flasks connected via 0.22 μm sterile filters (Schleicher-Schuell GmbH, Dassel, Germany) to flow meters (Brooks Instruments, BV, Veenendaal, Netherlands); 120 ml of bulk milk (provided by Valio Ltd., Finland) was flushed with N_2 gas (of 99.999% purity, type 5.0, AGA Riihimäki, Finland) at the rates of 120 ml/min (N1) or 40 ml/min (N2), or not flushed (C, the control). The system is called "open" since another sterile filter disk, placed on top of the outlet tubes (for each flask), allowed gas exchanges. The O_2 levels in milk, measured 30 min following the start of the flow with an OXI 300 Oxygen Meter (Macherey-Nagel, Dürer, Germany), were 10.5 ppm for C, less than 0.1 and 1.1 ppm for N1 and N2, respectively. The milk samples were mixed at 220 rpm on a multiplace magnetic stirrer; all raw milk test bottles were kept inside a refrigerated water bath, at 6.0 ± 0.1 or 12.0 ± 0.1 °C, during the course of the studies.

Microbiological analyses

The raw milk samples were obtained from the lorry tanks of Valio Ltd. (Finland); for the microbiological analyses, the N_2 flow was shortly interrupted; a sample of 0.5 ml of raw milk was removed, serially diluted in saline solution (0.85% NaCl); 20 μl droplets (from several dilutions) were laid onto plate count agar (PCA) plates (Chambers, 2002; Munsch-Alatossava et al., 2007) for determination of the total counts; 40 μl (of the same dilutions) was spread on the plates for enumeration of the PLs producers and colonies that displayed β-hemolysis. The PLs producers were enumerated on PCA supplemented with 10% egg yolk emulsion (Labema Oy, Kerava, Finland), after 2–3 d incubation at 30 °C. For the strict anaerobes, the plates were incubated, for 3–6 d, in anaerobic jars containing GenBox generators (BioMérieux, Marcy l' Etoile, France). The observation of a whitish opaque ring was considered as indicative of PL production (Esselmann and Liu, 1961; Dogan and Boor, 2003). The colonies that exhibited β-hemolysis on Trypticase soy agar supplemented with 5% sheep blood (BioMérieux) were recorded after 3–7 d incubation at 30 °C.

Results and discussion

Pure N_2 gas inhibited the bacterial growth in raw milk stored in an "open system" at 6.0 and 12.0 °C;

the bacterial counts constantly increased in all experiments for C, reached intermediate levels for N2 and were the lowest for N1 (Table 1). These results meet previous conclusions obtained with raw milk stored at 4 and 7 °C in closed vessels (Murray et al., 1983; Dechemi et al., 2005). Less β-hemolytic colonies were detected at 6.0 °C with N1 and to some lesser extent with N2 when compared to C (Table 1). No hemolytic colonies were detected at 12.0 °C with N1 (Table 1, Experiment 4).

Irrespective of the raw milk storage temperatures, the PLs producers proliferated for C during the course of the experiments, and increased but reached lower levels than C for N2 (Figure 1). Most interestingly, after 3, 7 or 10 d, the PLs producers disappeared under N1 at 6.0 °C storage (Figure 1a–c), and similarly at 12.0 °C (Figure 1d). Irrespective of the rate, the nitrogen flow did not favour anaerobic PLs producers (data not shown).

To the best of our knowledge, no study has so far considered the PL activity exhibited by bacteria present in raw milk kept under modified atmospheres in general, and N_2 gas in particular. The present observations indicate an additional advantage of flushing raw milk with N_2; besides lowering the total counts (Murray et al., 1983; Dechemi et al., 2005; Table 1), and having an impact on protease and lipase producers (which are lowered in parallel with the total counts; Murray et al., 1983; Dechemi et al., 2005), it seems that the higher rate N1, sooner or later, also excluded the PLs producers in raw milk. Interestingly, the kinetics of the collapse seems to be less dependant on the initial level (around 10^3 CFU/ml for all experiments) than on the initial microflora, which seems to be the major determinant of the decay of PLs producers. Considering the bacteria that displayed β-hemolysis, the drop in numbers or attainment of non-detectable levels under N1 condition is also of interest; however, whether it may be only the consequence of the disappearance of bacterial types that produce hemolytic PLs needs further investigations.

The observation that PLs producers were excluded in raw milk by pure nitrogen gas (N_2), flushed into the headspace of the test bottles containing raw milk, is of major technological importance, hence meaningful for the raw milk quality, as the integrity of the fat globule membrane may be preserved. More importantly, considering the point that different types of PLs could be pathogenic determinants (Schmiel and Miller, 1999; Sitkiewicz et al., 2006; Istivan and Coloe, 2006) and since the exclusion seemed not to be "Gram specific", therefore this result may be particularly meaningful for the raw milk safety. Indeed, DNA-based methods are requested in order to clarify the identity of the PLs producers initially present in the raw milk samples.

It appears that flushing pure N_2 into bottles containing raw milk could constitute a complementary procedure to storage at low temperatures, of arresting bacterial growth and hence retarding the spoilage of raw milk; this technique may be especially valuable in warm climates or in economical contexts that lack the appropriate technology to guarantee both quality and safety of raw milk. We anticipate that flushing pure N_2 gas in raw milk tanks, considered as open systems, along the cold

Table 1. Increase of CFU/ml, in log scale ($\triangle$ log CFU/ml), compared to the initial levels, considering the total counts (on PCA[a]), the β-hemolytic colonies (on RBA[b]), when the raw milk was flushed with pure N_2; the treatments N1 (120 ml/min) and N2 (40 ml/min) were compared to the respective controls (C)

Raw milk storage temperature (°C)	Experiment no./ length in days	Medium	Initial total counts log CFU/ml	Δ log CFU/ml		
				C	N1	N2
6.0	Exp. 1/6	PCA	3.9	4.9	0.4	2.3
		RBA		5.2	1.6	3.0
6.0	Exp. 2/7	PCA	3.9	4.9	1.1	3.7
6.0	Exp. 3/10	PCA	3.8	5.0	3.3[c]	3.7
12.0	Exp. 4/4	PCA	4.2	5.6	3.9[d]	4.6
		RBA		6.1	n.d[e]	4.0

[a]PCA, plate count agar.
[b]RBA, red blood agar.
[c]Rupture of the connecting tube of flask N1, between days 6 and 7.
[d]The N_2 flush was most efficient after 2 and 3 d, as 3-log units separated the total counts recorded for C and N1; the difference fell to 1.7-log units after 4 d.
[e]n.d., not detected; no case of hemolysis on a total of 97 colonies.

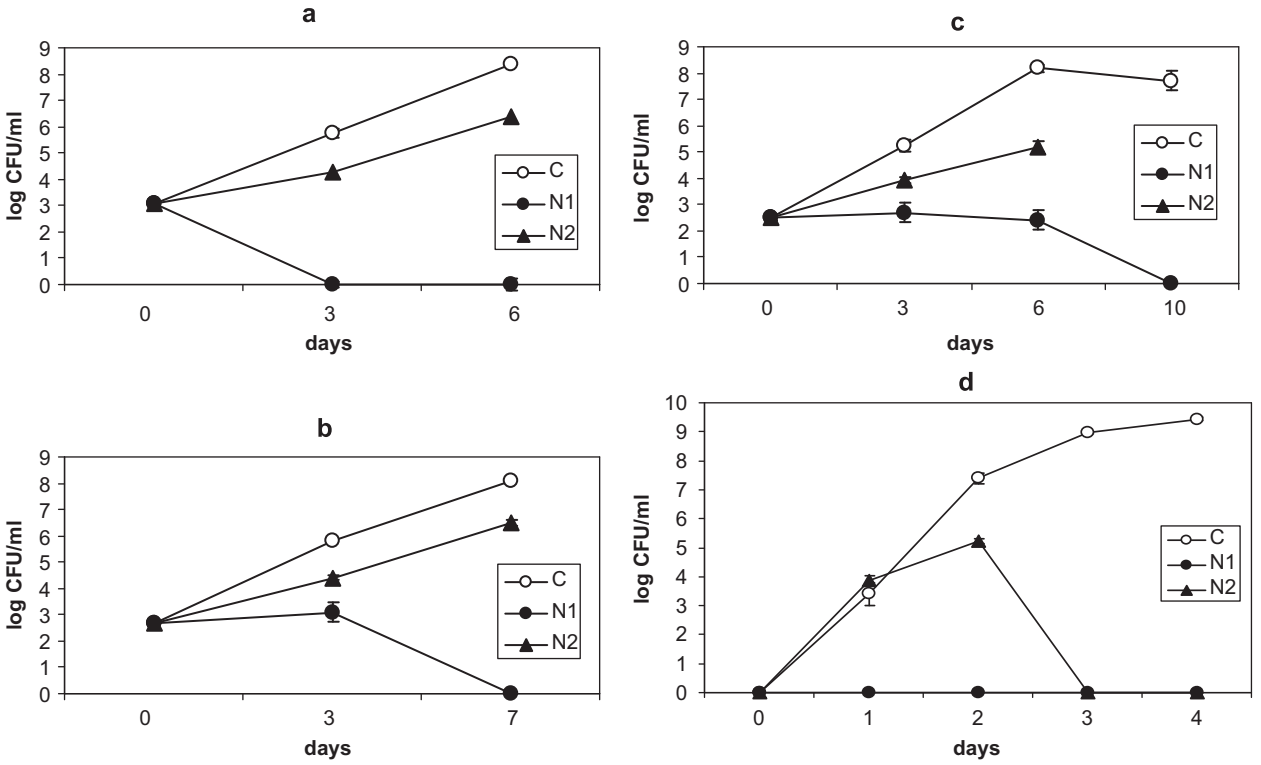

Figure 1. Effect of nitrogen gas (N_2) on phospholipase producers present in raw milk stored at 6.0 °C (a/Experiment 1, b/Experiment 2, c/Experiment 3) and 12.0 °C (d/Experiment 4). The treatments N1 (●, N_2 of 120 ml/min) and N2 (▲, N_2 of 40 ml/min) were compared to the respective controls (C, ○). Symbols represent mean values of duplicates (with the exception of the point C/3 days /Experiment 3); error bars indicate standard deviation.

chain of raw milk storage and transportation may constitute an interesting perspective for limiting the spoilage and pathogenic potential; this technique may be applied to food materials in general.

However, whether pure N_2 gas diverts the pathogenic arsenal of raw milk psychrotrophs needs to be further investigated. Future studies elucidating the destiny of PLs producers, when raw milk is flushed with N_2, are needed.

Acknowledgements

This work was supported by a research grant from the University of Helsinki, and by a personal grant from the Center for International Mobility (CIMO) to Dr. O. Gursoy. We thank Prof. P. Jelen for critical reading of the manuscript.

References

Berka RM, Vasil ML. Phospholipase C (heat-labile hemolysin) of *Pseudomonas aeruginosa*: purification and preliminary characterization. J Bacteriol 1982;152: 239–45.

Chambers JV. The microbiology of raw milk. In: Robinson RK, editor. Dairy microbiology handbook. 3rd ed. USA: Wiley-Interscience; 2002. p. 39–69.

Dechemi S, Benjelloun H, Lebeault JM. Effect of modified atmospheres on the growth and extracellular enzyme activities of psychrotrophs in raw milk. Eng Life Sci 2005;5:350–6.

Dogan B, Boor KJ. Genetic diversity and spoilage potentials among *Pseudomonas* spp. Isolated from fluid milk products and dairy processing plants. Appl Environ Microbiol 2003;69:130–8.

Esselmann MT, Liu PV. Lecithinase production by gram-negative bacteria. J Bacteriol 1961;81:939–45.

Fox PF. Fat globules in milk. In: Roginsky H, Fuquay JW, Fox PF, editors. Encyclopedia of dairy sciences. London: Academic Press; 2002. p. 1564–8.

Hayes MC, Boor KJ. Applied dairy microbiology. In: Marth EH, Steele JL, editors. Raw milk and fluid milk products. New York: Marcel Dekker; 2001. p. 59–76.

Istivan TS, Coloe PJ. Phospholipase A in Gram-negative bacteria and its role in pathogenesis. Microbiology 2006;152:1263–74.

Ma Y, Barbano DM, Santos M. Effect of CO_2 addition to raw milk on proteolysis and lipolysis at 4 °C. J Dairy Sci 2003;86:1616–31.

Mac Phee JD, Griffiths MW. Psychrotrophic bacteria; *Pseudomonas* spp. In: Roginsky H, Fuquay JW, Fox PF,

editors. Encyclopedia of dairy sciences. London: Academic Press; 2002. p. 2340–5.

Munsch-Alatossava P, Alatossava T. Phenotypic characterization of raw-milk associated psychrotrophic bacteria. Microbiol Res 2006;161:334–46.

Munsch-Alatossava P, Alatossava T. Antibiotic resistance of raw-milk-associated psychrotrophic bacteria. Microbiol Res 2007;162:115–23.

Munsch-Alatossava P, Rita H, Alatossava T. A faster and more economical alternative to the standard plate count (SPC) method for microbiological analyses of raw milks. In: Méndez-Vilas A, editor. Communicating current research and educational topics and trends in applied microbiology. Badajoz: Formatex; 2007. p. 495–9.

Murray SK, Kwan KKH, Skura BJ, Mac Kellar RC. Effect of nitrogen flushing on the production of proteinase by psychrotrophic bacteria in raw milk. J Food Sci 1983; 48:1166–9.

Rajagopal M, Werner BG, Hotchkiss JH. Low pressure CO_2 storage of raw milk: microbiological effects. J Dairy Sci 2005;88:3130–8.

Schmiel DH, Miller VL. Bacterial phospholipases and pathogenesis. Microbes Infect 1999;1:1103–12.

Shah NP. Psychrotrophs in milk: a review. Milchwissenschaft 1994;49:432–7.

Sitkiewicz I, Stockbauer KE, Musser JM. Secreted bacterial phospholipase A_2 enzymes: better living through phospholipolysis. Trends Microbiol 2006;15:63–9.