Keywords: Pilot scale; Gas generator; Nitrogen gas (N2); Raw milk; Cold storage; Food safety

Introduction

Numerous bacterial genera have successfully settled down along the cold chain of raw milk storage and transportation; the ability of these bacteria to produce heat-stable exoenzymes disturb dairy processes, affecting yield and quality of dairy products [1,2,4]. To the exception of some human pathogens like Bacillus cereus or Listeria monocytogenes, psychrophilic bacteria are mainly considered as spoilage bacteria. Recent studies also brought to evidence that raw milk psychrotrrophs, a majority were Gram negative representatives, carry multiple antibiotic resistance features [6]. Control systems that could reinforce the cold chain, wherever it exists, or could improve the raw milk’s quality and safety wherever the cold chain fails would be of value. Several studies have reported an extension of shelf life of milk after the addition of carbon dioxide gas (CO2) [5,10,11]. Two studies have investigated the treatment of raw milk with nitrogen gas (N2) applied to a close system [3,9]. Our previous studies that considered the application of a pure N2 (99.999 %) gas flow through system (an open system) to raw milk [3,9]. The results obtained at a pilot plant scale demonstrate the potential of this technology, that could be applied in order to improve quality and safety of raw milk, during prolonged storage along the cold chain.

Materials and Methods

The pilot scale N2 gas flushing treatment unit

The pilot scale N2 gas flushing unit consisted of a N2 gas generator unit (Figure 1A), two milk tanks and a cooling unit (Figure 1B). Two milk tanks (of 170 L total capacity) were connected to the cooling unit, that produced cold water (≤ 3°C) which constantly circulated between the jackets of the tanks and the cooling unit. The temperature of the raw milk was kept at 5.5 ± 0.4°C in both tanks throughout each experiment. In each experiment, both tanks contained 110 L water (for the preliminary tests), or raw milk of the same stock; the volume of the head space of the tank was 60 L (35 % of the tank volume). The outlet (diameter 16 mm) in both tanks was kept open to the air (enabling gas exchanges with the environment). The inlet of the control tank (tank 2, no N2 gas treatment) was closed. The inlet of the tank 1, that received N2 treatment, was connected by a tube (4 mm inner diameter); the other end of the tube was connected to a flow meter, connected itself to the control (no N2 gas treatment). The treatments also reduced phospholipases (PLs)-producing and Bacillus cereus type bacteria. The results obtained at a pilot plant scale demonstrate the potential of this technology, that could be applied in order to improve quality and safety of raw milk, during prolonged storage along the cold chain.
Oxygen and temperature measurements

The O2 (oxygen) content of the gas produced by the Nitrox N2 gas generator unit was measured directly from the gas flow, released from the gas reservoir tank, at the end of the tube by using a headspace analyser: PBI DANSENSER CHECKMATE 9900 (RINGSTED, DANEMARK). The temperature and the dissolved O2 content of the raw milk in the tanks were measured with a THERMO SCIENTIFIC ORION 4 STAR RDO portable meter (THERMOFISHER SCIENTIFIC INC. USA).

Source of raw milk and microbiological analyses of raw milk samples

Typically in late evening preceding the day of the start of an experiment, raw milk for each experiment was pumped by a truck driver from the dairy company Valio Ltd. (Helsinki, Finland) from the truck tank (that contained raw milk from farms) to the storage raw milk tank (of 1000 L capacity) of the pilot plant unit. The raw milk was stored at 3.5 ± 0.5ºC overnight. The following morning the cold raw milk was distributed into the two milk tanks (110 L per each tank) of the pilot scale unit (Figure 1B). For the microbiological analyses, about 20 mL of raw milk were collected from the tanks of the pilot scale unit, and serially diluted; the numbers of colonies of total aerobic bacteria, of phospholipases (PLs)-producers and of Bacillus cereus type bacteria (pink colony, PL-positive) were determined on Plate Count Agar (PCA, Lab M), PCA supplemented with 10% egg yolk emulsion (Sigma), and Mannitol Egg Yolk Polymyxin Agar (MYP, Oxoid), respectively, after 2-3 days incubation at 30ºC. In addition for each raw milk sample, a pH measurement was done at the beginning and at the end of each experiment, and both odour and appearance of the milks were recorded, too. The counts of total aerobic bacteria are detailed in Figures 3A-B for two experiments.

Results

Optimization of N2 gas treatment to minimize the O2 content in raw milk

In order to maximize the effectiveness of the N2 gas treatment in the pilot scale system, data on the kinetics of the reduction of the dissolved O2 content were required. For that purpose, preliminary series of experiments were done with 110 L water per tank kept at 5.5 ± 0.4°C; the gas produced by the N2 gas generator 1 unit was tested at a flow rate of 4 L/min. The treatment based on N2 gas flushing alone reduced the dissolved O2 content in cold water quite slowly: more than 24 hours were needed to reach the equilibrium state. Contrarily, by bubbling the N2 gas into the water, only about 3 hours were needed to reach the equilibrium state. A long-lasting bubbling step of raw milk with N2, however, produced such a large amount of foam that this treatment disqualified the milk for further uses (data not shown). A 6 h bubbling treatment did not produce that adverse effect, although it enabled to achieve a quicker drop of the O2 content; consequently this step was added in all further experiments. An optimal sequence of raw milk treatment with N2 gas comprised first 6 h gas bubbling, followed by continuous gas flushing in the head space until the end...
of the treatment. Among the gas flow rates that were tested, the most efficient seemed to be 4 L/min; the O2 content of the produced N2 gas was less than 0.014%. Figure 2 indicates the dissolved O2 content in raw milk, that had been treated by the bubbling/flushing procedure at a flow rate of 4 L per min, with the gas produced by the N2 gas generator unit; the dissolved O2 level of the raw milk was reduced within 8 h close to zero ppm; in the control raw milk tank, the reduction of dissolved O2 started only after about 90 hours, most probably, due to the higher bacterial metabolic activity.

Effect of N2 gas flushing on storage time of cold raw milk

The results of two experiments, where 110 L of raw milk kept in the cooling tank (5.5 ± 0.4°C) were treated for 6 and 7 days with N2 gas flushed through the head space after a bubbling period of 6 h are detailed in Figure 3 (Error bars = ± SD from triplicate platelings). The initial bacterial counts were of 6 and 10×10⁹ CFU/mL (Figures 3A-B). In the tank where no N2 gas was applied (the control raw milk), one log-unit (that is 10-fold) increase in total bacterial counts was observed after about 2 days storage; but with the N2 gas treatment, one log-unit (10-fold) increase required 4.5 days (Figure 3A) to 5.5 days (Figure 3B); the treatment enabled an about 2.5-fold longer storage time. The pH values of the N2 gas treated and control raw milks after 6 and 7 days were of 6.75 (Control tank) and 6.81 (N2 treated milk), of 6.74 (Control tank) and 6.75 (N2 treated milk), respectively. At the end of the experiments, the control tanks released a bad odor, typical of a spoiled milk, contrarily to the N2 treated milk where no undesirable odor was noticeable.

Effect of N2 gas flushing on the levels of phospholipases producers and Bacillus cereus type bacteria

Both total bacteria and PLs producers were enumerated on PCA plates supplemented with egg yolk (Table 1). Similarly, all colonies and Bacillus cereus type bacteria (pink colony, PL-positive) were recorded on MYP plates (Table 1). The N2 gas treatments in the pilot scale reduced the levels of PLs producers, in both experiments by 1.8 and 2.3 log-units, after 6 and 7 days storage, respectively. A similar observation was also made with B. cereus type bacteria; the amounts of B. cereus type bacteria (pink, PL positive colony) observed on MYP plates were 2.5 or 2 log-units lower in the N2 gas flushed raw milk, compared to the control milks after 5 or 6 days storage, respectively (Table 1), although, the total bacteria growing on MYP plates were not significantly different from the control after 6 days storage. The N2 gas significantly reduced the growth of total bacteria and excluded phospholipases (PLs) producers including Bacillus cereus-type from raw milk [7,8]. In the present study, the N2 gas treatment applied at pilot scale, still considered an open system with continuous N2 gas flushing through the head space of the tank that followed a 6 h bubbling period, was effective to extend the storage life of raw milk by about 2.5-fold at a temperature of 5.5°C (Figures 1-3). Noteworthy, the raw milk volumes treated in the pilot scale system (up to 110 L) were over 900-fold larger than the volume previously handled in the laboratory scale system [7, 8]. Furthermore, in the present study, we demonstrate that an extension of the storage life of raw milk can be achieved by using a N2 gas generator, that produced N2 gas from compressed air, which suggests that N2 gas flushing technology could be applied at industrial scale. The grade of N2 gas, produced from the compressed air by the gas generator, that still contained 0.014 % O2, was effective enough to produce a significant growth retarding effect (Figure 3), although this effect was lower than the effect obtained with high purity N2 gas (type 5.0) at high flushing flow rate at laboratory scale [8]. A 6 h period of N2 bubbling constituted a solution to reduce the amount of dissolved O2 quickly to an equilibrium state (Figure 2) without causing milk foam formation, which could pose a technological problem for further milk handling. Interestingly, phospholipases (PLs) producing bacteria, including Bacillus cereus-type, were showing special sensitivity to the N2 gas flushing treatment at a pilot scale, too (Table 1). These results corroborate previous observations made at laboratory scale [7,8]. We assume that the difference between partial and total exclusion of these bacterial types may be due to the lower purity of the gas and, to the kinetics of achieving a minimum dissolved O2 level: the dissolved O2 level was lower than 0.1 ppm after 30 minutes at laboratory scale whereas over 8 h were required to reach the lowest level of dissolved O2 at pilot scale (Figure 2). In our opinion, this technology could be used (i) to minimize the bacterial growth during cold chain storage of biological liquids like raw milk, (ii) to extend the storage life of biological liquids and/or (iii) to replace the absence of a cold chain and consequently improving raw milk quality and safety. A cold chain of raw milk storage and transportation can be considered as an open system that comprises a farm milk tank, a truck tank for transportation and a silo tank for storage before dairy processing. Immediately after cow milking, besides a minimal bacterial load, the raw milk does not contain yet any significant amount of dissolved O2; the fresh raw milk at the level of the farm milk tank could be directly treated with N2 gas flushing without any preliminary bubbling step. Providing that the initial dissolved O2 level in farm raw milk is lowest, the N2 flushing treatment could be further applied at truck and silo tanks. Future studies should focus on the demonstration of the technical feasibility together with the evaluation of economical aspects of continuous N2 gas flushing technology at an industrial scale along the cold chain of raw milk storage and transportation, from a farm tank until a dairy silo.

Discussion

The effects of N2 gas on the growth of psychrotrophic bacteria in raw milk or on the growth of a Pseudomonas fluorescens strain inoculated in sterile milk have been studied previously at laboratory scale using a closed system after a short O2 removal period by N2 gas flushing [3,9,12]. All those studies concerned volumes of raw milk of 250 mL or less. In our previous studies, we tested an open system for raw milk N2 gas treatment at laboratory scale (120 mL raw milk) by introducing a continuous high purity N2 gas flow (type 5.0, with a N2 content of 99.999%), from a gas bottle, through the headspace (about 130 mL) of a raw milk test bottle (of a total volume of 250 mL) at a constant flow rate and at temperatures of 6, 7 or 12°C. The results with this small scale open system indicated that the treatments with N2 gas significantly reduced the growth of total bacteria and excluded phospholipases (PLs) producers including Bacillus cereus-type from raw milk [7,8]. In the present study, the N2 gas treatment applied at pilot scale, still considered an open system with continuous N2 gas flushing through the head space of the tank that followed a 6 h bubbling period, was effective to extend the storage life of raw milk by about 2.5-fold at a temperature of 5.5°C (Figures 1-3). Noteworthy, the raw milk volumes treated in the pilot scale system (up to 110 L) were over 900-fold larger than the volume previously handled in the laboratory scale system [7, 8]. Furthermore, in the present study, we demonstrate that an extension of the storage life of raw milk can be achieved by using a N2 gas generator, that produced N2 gas from compressed air, which suggests that N2 gas flushing technology could be applied at industrial scale. The grade of N2 gas, produced from the compressed air by the gas generator, that still contained 0.014 % O2, was effective enough to produce a significant growth retarding effect (Figure 3), although this effect was lower than the effect obtained with high purity N2 gas (type 5.0) at high flushing flow rate at laboratory scale [8]. A 6 h period of N2 bubbling constituted a solution to reduce the amount of dissolved O2 quickly to an equilibrium state (Figure 2) without causing milk foam formation, which could pose a technological problem for further milk handling. Interestingly, phospholipases (PLs) producing bacteria, including Bacillus cereus-type, were showing special sensitivity to the N2 gas flushing treatment at a pilot scale, too (Table 1). These results corroborate previous observations made at laboratory scale [7,8]. We assume that the difference between partial and total exclusion of these bacterial types may be due to the lower purity of the gas and, to the kinetics of achieving a minimum dissolved O2 level: the dissolved O2 level was lower than 0.1 ppm after 30 minutes at laboratory scale whereas over 8 h were required to reach the lowest level of dissolved O2 at pilot scale (Figure 2). In our opinion, this technology could be used (i) to minimize the bacterial growth during cold chain storage of biological liquids like raw milk, (ii) to extend the storage life of biological liquids and/or (iii) to replace the absence of a cold chain and consequently improving raw milk quality and safety. A cold chain of raw milk storage and transportation can be considered as an open system that comprises a farm milk tank, a truck tank for transportation and a silo tank for storage before dairy processing. Immediately after cow milking, besides a minimal bacterial load, the raw milk does not contain yet any significant amount of dissolved O2; the fresh raw milk at the level of the farm milk tank could be directly treated with N2 gas flushing without any preliminary bubbling step. Providing that the initial dissolved O2 level in farm raw milk is lowest, the N2 flushing treatment could be further applied at truck and silo tanks. Future studies should focus on the demonstration of the technical feasibility together with the evaluation of economical aspects of continuous N2 gas flushing technology at an industrial scale along the cold chain of raw milk storage and transportation, from a farm tank until a dairy silo.

**Conclusion**

The N2 gas separated from compressed air (that contained still
about 0.014% O2) and continuously flushed in an open system, was an effective treatment to extend the storage life of up to 110 L raw milk by 2.5 fold. This result further extended the potential of N2 gas based treatments observed at bench scale, and constitutes somehow a good starting point for practical applications at dairy farming and industrial levels. Obviously other food raw materials or food products could be considered as potential targets for N2 gas flushing technology. The production of electricity by a generator, that enables the production of compressed air and subsequently the production of N2 gas, could constitute an alternative approach to store raw milk and other microbiologically sensitive food materials under conditions, where electricity production is restricted and accordingly where cold storage as a too high energy demanding technology is excluded.

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