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Calorimetric assessment of microbial growth in milk as affected by different conditions

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Abstract

Milk quality assessment is of paramount importance for dairy facilities. Standard microbiological techniques are time consuming and demand for faster, accurate methods is increasing. On the other hand, inefficient temperature control to handle raw milk, due to lack of appropriate facilities, requires the search for strategies to maximize quality from the microbiological standpoint. Activation of the lactoperoxidase system (LPS) has been established as an easy and safe procedure to attain such results. Good quality milk samples were obtained in sterile containers right after milking. A test temperature of 30 °C was chosen based upon preliminary tests. Raw unpasteurized milk was incubated at 30 °C for 16 h. Microbial growth was assessed at 4 h intervals and the maximum value attained was 4.59×10^6 CFU ml^{-1} after 8 h. Simultaneously, metabolic heat rate from the same samples was measured under isothermal conditions at 30 °C. A regression equation was obtained to relate microbial growth and heat output, with a coefficient of determination of 0.99. Milk samples were divided in three equal aliquots for (a) pasteurization at 72 °C for 15 s; (b) activation of LPS, and (c) untreated control. LPS activated milk maintained good quality during 8 h, while pasteurized milk showed an initial increase in microbial population after 8 h followed by a decline possibly due to residual activity of its LPS.

Keywords: Milk; Microbial growth; Isothermal calorimetry; Metabolic heat; Pasteurization; Lactoperoxidase system

1. Introduction

Milk quality assessment is a permanent need in any dairy facility. Microbial contamination may start within the cow itself and significantly increase with further handling. In addition to human pathogens, other microorganisms may decrease milk quality through enzymatic reactions [1,2]. Different tests have been devised to assess milk quality and are widely used by industry, nonetheless, an important need remains for rapid and accurate methods to determine microbial load at any given time.

Furthermore, developing countries frequently lack appropriate facilities for milk cooling and storage. The latter becomes a problem since microbial contaminants are capable of rapid growth at room temperatures. While pasteurization has been the universal method to eliminate most pathogens from milk, an

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alternative method is presently being used in many countries fostered by the Lactoperoxidase World Program from FAO. This method consists on the activation of the lactoperoxidase system (LPS), a naturally occurring antimicrobial system found in milk, with thiocyanate and hydrogen peroxide as its components [3]. The LPS system generates antimicrobial compounds [4] such as ascorbate radicals, whose formation is related to the ascorbate peroxidase activity of LP in cow's milk [5].

Activation of the LPS is among the most cost-effective approaches to extend shelf life of pasteurized and raw milk [6], and it has been recognized as an easy and safe procedure to maintain milk quality from the microbiological standpoint [7]. LPS activation may be attained by the proper addition of thiocyanate and hydrogen peroxide to milk [8]. The addition of immobilized lactase and glucose oxidase further enhances the LPS antimicrobial effects [9]. More recently, a spectroscopic method was devised for determination of LP [10]. No significant changes in lactose and protein content have been observed after LPS activation [11].

Modern calorimetric techniques represent a substantial advantage as compared to traditional microbiological methods, due to ease for continuous monitoring, simple performance requirements and rapid results. Additionally, the LPS sensitivity is similar to that of other bioassays [12]. Applications of calorimetry to microbiological sciences have ranged from the evaluation of bacteriological quality of seafood [13] to microbial degradation of lead in contaminated soils [14], characterization of *Saccharomyces* from rainbow trout intestines [15], and fusion of Sendai virus with vesicles of oligomerizable lipids [16]. Also, it was recently used to test the antimicrobial efficacy of plant extracts on *Streptococcus* mutants, responsible for dental caries [17]. In an early report, microcalorimetry was also used to monitor the growth of bacteria in milk [18].

The objectives of this study were to develop a time-saving and accurate calorimetric method to assess microbial populations in milk samples to be used as an alternative to traditional microbiological techniques. Additionally, to quantify the effect of pasteurization and activation of LPS on microbial growth and their metabolic heat rate.

2. Experimental

2.1. Sample description and quality assessment

Milk samples were obtained from a commercial dairy, directly poured from the automatic milking machine into a sterile glass container and transported to the laboratory in less than 15 min. Each sample was separated in four equal aliquots and assigned to three experimental treatments: (a) pasteurization at 72 °C for 15 s [3]; (b) activation of LPS by addition of 0.173 mM sodium thiocyanate and 0.19 mM sodium percarbonate [19], and (c) control. The fourth aliquot was used for quality determinations.

The following standard techniques for milk quality assessment were used: pH was measured with a Corning 450 pH meter (Corning Inc., Corning NY), alcohol 68%, reductase time, and total microbial counting by plating on standard plate count agar [20].

2.2. Calorimetric studies

Isothermal calorimetry was performed using a CSC 4100 model DSC (Calorimetry Science Corporation, Spanish Fork, Utah). This equipment has four hermetic hastealloy cells of 1 cm^3, sensitivity of $\pm 1\,\mu W$ and a scanning capacity ranging from −30 to 130 °C. Each of the first three cells contained one treatment sample and the fourth ampoule was kept empty as a reference. Chamber temperature was kept constant with a circulating water bath (PolyScience, Niles, II) at 15 °C. Milk aliquots of 750 μl were placed in each ampoule under aseptic conditions and after measurements DSC cells were cleaned with absolute methanol and rinsed with tridistilled water to avoid milk fat accumulation. Data were baseline corrected and normalized to a 1 ml volume.

In an exploratory work, microbial heat production was estimated by isothermal calorimetry at 5 °C intervals between 5 and 40 °C. Samples were kept at 4 °C before exposing them for 1 h at each temperature tested.

In a second experiment, the same treatments were established and kept at 30 °C in an incubator (Lab-line Instruments Incorporation, Melrose Park, Ill) for up to 24 h. Isothermal measurements at 30 °C were performed to follow heat production and microbial development. Controls were measured every 4 h, while

Table 1
Initial quality of milk samples used

Test	Value	Comment
pH	6.8	Acceptable range
Acidity	14°D	Acceptable range
Alcohol 68%	Negative	No acidity developed
Peroxide presence	Negative	No presence of preservatives
Reductase time	10 h	Good microbiological quality
Total plate count	14,000 CFU ml^{-1}	Good microbiological quality

pasteurized and LPS activated samples were measured every 8 h. First, microbial heat production was measured and individual cell samples were serially diluted and plated on PCAA to determine their total plate count. Statistical analysis was performed on a completely randomized design with a factorial arrangement for milk treatments and incubation temperatures. Data were analyzed by analysis of variance (ANOVA) and mean separation, when appropriate, was done by Tukey ($P \leq 0.05$). Regression analyses were done to correlate heat production and microbial development [21].

3. Results and discussion

3.1. Milk initial quality assessment

Parameters shown in Table 1 correspond to a milk sample of good quality i.e. microbial count of 14,000 CFU ml^{-1}, a reductase time of up to 10 h, low acidity and absence of chemical preservatives [20].

3.2. Exploratory calorimetric assay

The effect of temperature on the outcome of pasteurization and LPS activation on raw milk was determined based on the heat produced by growing microorganisms. Table 2 shows that treatment response was very similar at 5 °C. However, higher incubation temperatures measured at 5 °C intervals yielded significant differences among treatments. While raw milk responded by a quick increase in microbial heat production, pasteurized and LPS activation developed a close, but significantly different, evolution. Considering that all samples were maintained at 4 °C before being transferred to the DSC cells, where incubation at the testing temperature took place for 1 h, the amount of heat recorded equaled the amount of heat produced by growing microorganisms during such period. Thus, this highly significant interaction between milk treatment and incubation temperature demonstrates that such treatment responses will depend on the temperature at which the product is handled [2,22].

3.3. Relationship between heat production and microbial growth

Highly significant differences in microbial growth in raw milk were found during an incubation period of 16 h at 30 °C (see Fig. 1). From an initial population of 0.015×10^6 CFU ml^{-1}, after 8 h, growth reached 4.6×10^6 CFU ml^{-1}. Increasing acidity, substrate and oxygen depletion [23] may have caused later reductions to 3.1 and 1.4×10^6 CFU ml^{-1}, at 12 and 16 h. Accordingly, metabolic heat rate followed a similar pattern with 9, 13, 123, 109 and 51 μW ml^{-1} at 0, 4, 8, 12 and 16 h, respectively. Third order polynomials fitted these data with determination coefficients of 0.95 and 0.88 for microbial growth and metabolic heat rate [24].

Data from individual samples were pooled to relate metabolic heat rate measured by isothermal calorimetry with their corresponding total plate counts (see Fig. 2). Regression analysis defined a third order

Table 2
Effect of incubation temperature (°C) and incubation time on metabolic activity produced by microbial development in milk

Treatment	Metabolic activity q (μW ml^{-1})							
	5 °C	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C	40 °C
Control (raw, unpasteurized)	5.0	12.1	12.6	20.2	23.9	26.9	28.0	38.8
Pasteurized	4.1	9.2	10.8	12.1	15.0	17.3	20.3	23.0
LPS activation	4.1	10.9	12.3	13.2	16.1	20.3	23.0	24.0

S.E.M. for the difference between two means, 0.29 μW ml^{-1}.

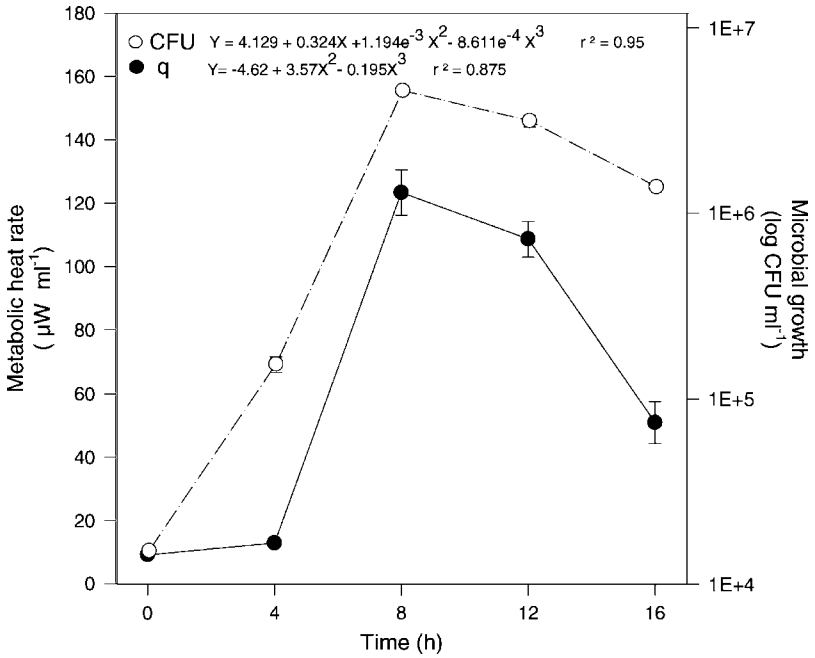

Fig. 1. Metabolic heat production and microbial development in raw unpasteurized cow milk kept at 30 °C. Highly significant differences ($P < 0.001$) were found for both variables. Vertical bars indicate S.D., when bars are not visible, their values are smaller than symbols.

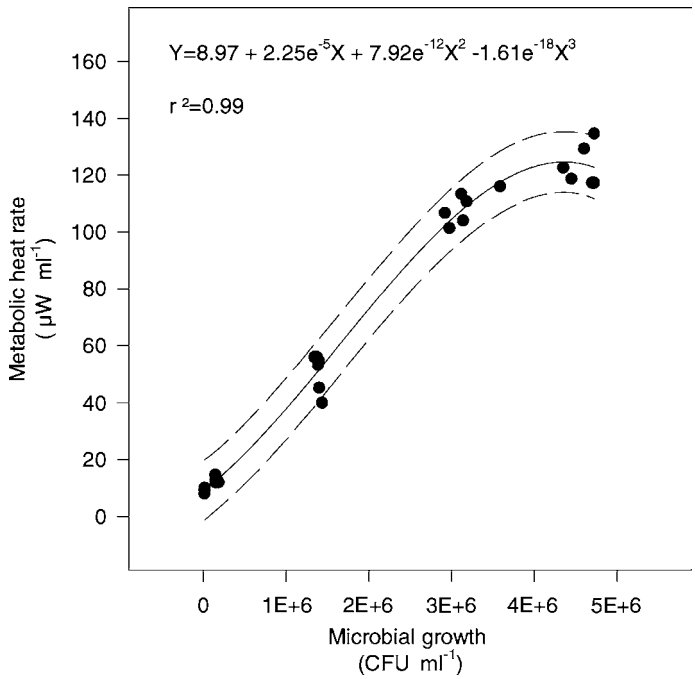

Fig. 2. Relationship between metabolic heat measured by isothermal calorimetry at 30 °C and its corresponding microbial count determined by total plate count from raw milk samples incubated at 30 °C. Parallel curves indicate a 95% confidence interval.

polynomial fitting those data with a 0.99 coefficient of determination. A typical sigmoidal pattern was clearly exhibited, likewise, heat production rate monitored by microcalorimetry has previously shown typical growth phases in *Saccharomyces* spp. [16]. Therefore, a very close prediction on milk microbial growth can be safely assumed based on metabolic heat rate readings. Considering that such a task takes only 30–40 min compared with the 48 h required for colonies to grow on plates and that each reading yields three replicates at the same time, it represents a very valuable timesaving strategy. Such results confirm previous experiences where seafood bacteriological quality was estimated by microcalorimetry [13].

3.4. Effect of pasteurizing and LPS activation on heat production and microbial growth on milk

Fig. 3a describes the microbial total count as affected by pasteurization, LPS activation and raw milk as control at 0, 8 and 16 h when kept at 30 °C. Controls showed an exponential increase reaching 4.59×10^6 CFU ml^{-1}after 8 h. This peak was followed by a sharp decline to 1.39×10^6 CFU ml^{-1} at 16 h. As previously described, such a decrease may be caused by increasing acidity, and substrate and oxygen depletion [23]. High initial counts in the pasteurized treatment were due to delays in processing because of logistic limitations, which caused an increase to 0.062×10^6 CFU ml^{-1} after 8 h at 30 °C. A

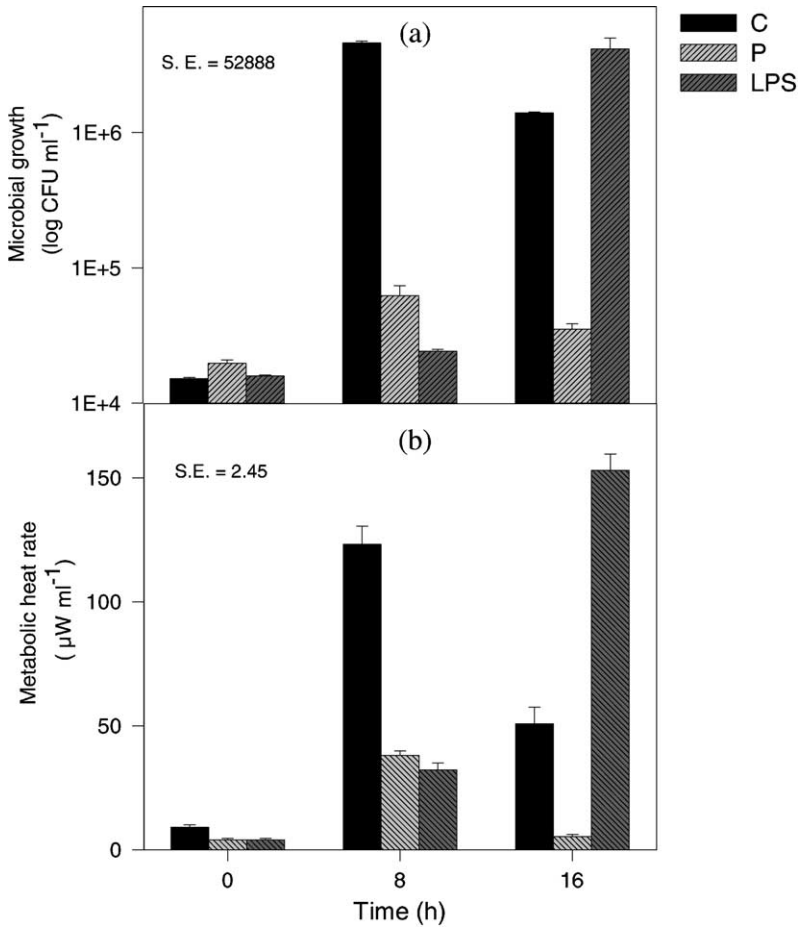

Fig. 3. Microbial growth (a) and metabolic heat production (b) from milk kept at 30 °C as affected by pasteurization (P), LPS activation and untreated raw milk (C). A highly significant interaction ($P < 0.001$) was found between milk treatment and incubation period.

former report demonstrated that keeping pasteurizing temperatures at 72 °C do not inactivate LPS, which maintain 70% of its activity [3]. This situation may be accountable for the further decline in microbial population observed after 16 h. LPS was capable of protecting milk from microbial deterioration for the first 8 h. This is in agreement with data formerly reported [19]. However, once such protective effect vanishes, microbes reassume their growth and after 16 h counts were as high as those found in control samples after 8 h. As far as heat production (Fig. 3b) treatments behaved in a similar fashion as microbial growth, confirming the direct relationship between the amount of respiring cells and their respective production of heat of metabolism. The pattern observed on both microbial growth and metabolic heat production represents a condition that clearly sets the limits for this tool, thus, its use should take into account the temperature at which the product will likely be exposed [25,26].

4. Conclusions

A mathematical relationship between heat rate production and microbial growth was found. Data demonstrated that isothermal calorimetry was a useful technique to quantify metabolic heat produced by microbes and that it is an accurate tool for estimating microbial populations in milk. Changes caused by pasteurization and activation of LPS were also detected.

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