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Use of Bioluminescence in the Assessment of the Degree of Cleanliness of Milk Tanks in Goat Milk Processing Plants

Anwendung der Biolumineszenzmethode zur Bewertung des Sauberkeitsgrades der Sammeltanks in Ziegenmilchmolkereien

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Summary

The aim of the study was to assess the degree of cleanliness of milk storage tanks in 3 plants processing goat milk. The analysis included surfaces of austenitic steel types 304 and 316 with The different roughness levels of Ra 0.6 – 4.0 µm. The assessment was based on measurement results of bioluminescence in relative light units (RLU) and the conventional microbiological swabbing method with determination of colony forming units (CFU). Microbial counts classifying an examined surface as clean, acceptably clean and unacceptable were the basis for the predictions of the RLU ranges. High levels of proportional correlations were obtained for these methods. Regression lines and coefficients of determination are the basis for the high correlation scores ($r \geq 0.96$). The Different results of the measurements of the bioluminescence were recorded within each of the three ranges of hygienic cleanliness from 40 to 9650 RLU/100 cm^2. The greater the surface roughness of examined tanks, the significantly lower was the proportion of samples classified as clean (ΔRLU=35 %). Contaminated surfaces of *>8–0.44xSd* in cfu/cm^2 corresponded to values from 3.2 RLU/cm^2 (objects 2 and 3) and from 8.1 RLU/cm^2 (object 1). This proves the necessity to individually determine the ranges of the RLU values for each of the tested objects in terms of the surface structure and the specific character of the bacterial biofilm composition.

Keywords: milk storage tanks, surface, RLU, microbial counts

Zusammenfassung

Das Ziel der Untersuchungen war die Bewertung des Sauberkeitsgrades der Milchsammeltanks in drei Ziegenmilch verarbeitenden Molkereien. Untersucht wurden Oberflächen aus Austenitstahl Typ 304 und 316 mit verschiedenen Rauheitsniveaus von 0.6 Ra bis 4.0 µm. Die Bewertung wurde mit der Biolumineszenzmethode in Relative Light Units (RLU), sowie anhand des konventionellen mikrobiologischen Tupferverfahrens mittels der Bestimmung Koloniebildender Einheiten (KbE) durchgeführt. Die Keimzahl, anhand der die untersuchten Oberflächen als sauber, akzeptabel und unakzeptabel eingestuft wurden, war Basis für RLU-Klassifikation. Unter Beachtung der Erstellung von Regressionsgeraden und Korrelationskoeffizienten wurde ein hohes Niveau der proportionalen Korrelationen zwischen beiden Methoden festgestellt ($r \geq 0.96$). Es wurden unterschiedliche Ergebnisse der Biolumineszenzmessungen innerhalb der drei Sauberkeitsgrade von 40 bis 9650 RLU/100 cm^2 festgestellt. Bei Zunahme des Rauheitsniveaus der Tankoberfläche nahm der Anteil der Proben aus der ersten Klasse (sauber) signifikant ab (ΔRLU=35 %). Den verunreinigten Oberflächen *> 8–0.44xSd* in cfu/cm^2 entsprachen Werte ab 3.2 RLU/cm^2 (Objekte 2 und 3) und 8.1 RLU/cm^2 (Objekt 1). Die Tankoberflächenstruktur und spezifische Zusammensetzung des bakteriellen Biofilms weist auf die Notwendigkeit der individuellen Bestimmung der RLU-Bereiche für jedes der untersuchten Objekte hin.

Schlüsselwörter: Milchsammeltank, Oberfläche, RLU, Keimzahl

Archiv für Lebensmittelhygiene 61, Heft 2 (2010), Seiten 37–80

Introduction

Surface cleanliness in the case of machines, technological line elements and facilities involved in the production process, is most frequently examined visually, also using the sense of smell or touch. The application of these methods daily each time after washing and disinfection does not reflect the actual cleanliness level. This pertains especially to examined surfaces which are made from materials differing in roughness and thus also exhibiting differences in maintaining the bacterial biofilm (Julien et al., 2002; Nedeljkovic & Horvat, 2007; Rosmaninho et al., 2007; Oulahal et al., 2008). Reliable results of a cleanliness examination are obtained when using microbiological methods. However, traditional microbiological testing methods such as hygiene swabbing, wipe-rinse, and direct or blotting methods are time-consuming and at the same time laborious. A result of traditional testing methods obtained 48 or 72 h after swabbing makes it impossible to undertake any corrective action, as in practice the production cycle has long been in progress. Safety may be guaranteed thanks to the application of state-of-the-art testing methods in the monitoring of cleanliness of examined objects, using physico-chemical properties of microorganisms, advances in genetics and cell biochemistry (Griffiths, 1996; Hawronskyj & Holah, 1999; Cho & Yoon, 2007). The remarkable advantages of these methods include prompt results and easy performance (Aycicek et al., 2006). However, the key issue for the appropriate application of these methods is the ability to interpret the recorded results (Lappalainen et al., 2000; Larson et al., 2003). The determination of a correlation between traditional microbiological methods and results of modern methods, will facilitate an adequate utilization of rapid tests. These are tests assessing the cleanliness of examined objects as effective tools monitoring their hygienic status (Bautista et al., 1992; Griffiths, 1995; Cooper et al., 2007, Cais and Pikul, 2008).

The aim of the study was to assess the degree of cleanliness of goat milk storage tanks in three different processing plants. The assessment was based on results of bioluminescence measurements and traditional microbiological assays of the examined surfaces. The results of the measurements and assays were used to determine the range of cleanliness for each of the analyzed objects. The cleanliness range was defined as: good, acceptably clean and unacceptable.

TABLE 1: *Material and finish of steel objects.*

Object sampling	Material		Types of plate surface	Surface treatment		Surface roughness Ra (µm)
	AISI	DIN		DIN	AISI	
1	304	X5CrNi 18 10	Hot-rolled sheet, annealed, pickled sheet, lusterless surface, rough	IIa	1	4.0
2	304	X5CrNi 18 10	Cold-rolled sheet. annealed, pickled sheet, smooth without luster	IIIb	2D	0.6
3	316L	X2CrNiMo 17 13 2	Cold-rolled sheet, annealed, pickled sheet, smooth without luster	IIIb	2D	0.8

DIN = Deutsche Industrie Norm; AISI = American Iron and Steel Institute

Material and Methods

Collection of samples

The experiment was conducted in 3 goat milk processing plants. The surfaces of bulk tanks used to store milk after pasteurization were analyzed. Examined surfaces varied in terms of their structure and finish (Table 1). Two of the examined objects were manufactured from chromium-nickel austenitic steel type 304 with low contents of 18-8 carbon (objects 1 and 2). The other object was made from high-alloy austenitic steel type 316L (object 3). It is stainless chromium-nickel steel, heat resistant and extremely corrosion-resistant.

The cleanliness status of adjacent surfaces with a total area of 200 cm^2 was investigated using the traditional swabbing method (100 cm^2) and by bioluminescence (100 cm^2). Swabs were collected from visually clean and dry surfaces at least 2 h and not later than 4 h after the completion of washing and disinfection procedures. Swabs were collected from an area limited by a 10 cm x 10 cm frame by moving a sterile swab 5 times parallel to one of the frame sides and next perpendicular, tilting it at a 45° angle.

Microbiological analysis

Microbiological contamination of surfaces was determined by the traditional swabbing method. This consisted of the following stages: wiping of the area limited by the frame with a swab moistened with a dilution fluid, rinsing of the swab, preparing dilutions, submerging cultures of 1 cm^3 each onto two dishes, incubation in a microbiological thermostat WTB Binder (Tuttlingen, Germany) and recording of microbial counts per 1 cm^2. Collected swabs were analyzed within 2 h after sampling. Standard diluents and microbial media were used in the experiment (ISO 6610, 1992; EN ISO 8261, 2001).

Bioluminescence method

The Cleanliness status by bioluminescence was assessed based on results of ATP measurements with a luminometer from FireFly Charm Sciences Inc. (Malden, USA) and swabs from PocketSwab Plus Charm Science Inc. (Lawrence, USA). The measurement procedure was performed following the meter and swab manufacturers' instructions. The total testing time including the reading did not exceed 45 s. The result was given in relative light units (RLU).

Statistical analysis

Results of bioluminescence and those of the conventional microbiological method were compared following the division of object surfaces into those classified as clean, i.e. Pass ($\leq 5 - 0.44 \times Sd$ in cfu/cm^2), conditionally clean, i. e. Alert ($5 - 0.44 \times Sd <$ and $\leq 8 - 0.44 \times Sd$ in cfu/cm^2) or unacceptable, i. e. Fail ($> 8 - 0.44 \times Sd$ in cfu/cm^2) for the total number of object samples (n = 20). Pearson's linear correlation coefficients were calculated in order to determine the degree of proportional correlations that there were between values of the conventional microbiological method and those obtained by bioluminescence. On this basis regression lines were plotted and the correlation coefficient (r) were estimated. The significance of the correlation coefficient was determined in order to evaluate correlations between variables. The significance test for correlation

coefficients was based on the assumption of the normal distribution of remainder values of variable y and on the equality of variances of remainder values for all values of variable x. In order to eliminate departures from the linearity of Pearson's distribution, which might cause an increase in the sum squares of deviations from regression lines, scatter diagrams were analyzed for results recorded for each examined object. Statistical calculations were performed using the data analysis software system STATISTICA (version 8.0) by StatSoft, Inc. (2007).

Results and Discussion

Results of microbiological tests for the 3 objects found in the production line in the dairy ranged from 0.4 to 20.2 cfu/cm^2 at a mean of 4.6 cfu/cm^2. The significant variation in the results for individual objects made it possible to determine three levels of surface cleanliness for each of the objects. The proportion of samples for individual surfaces considered clean ranged from 5 % to 75 %. The proportion for those with the alert cleanliness level conditionally acceptable to initiate the production cycle ranged from 1 % to 6 %. The proportion of samples, for those unacceptably dirty was from 15 % to 45 %. The high variation in the cleanliness levels of the examined objects resulted from the different degrees of surface roughness of the objects. This roughness also varied the effectiveness of washing and disinfection in order to remove the formed bacterial biofilm. The proportion of samples classified as clean was markedly higher with a decrease in roughness of examined steel surfaces (from 5 % in object 1 – roughness of 4.0 µm, to 75 % for objects 2 – roughness of 0.6 µm). The formation of biological film on an abiotic surface starts with the moment the first cell is deposited. The mechanism of the attachment reaction is a specific response of bacteria to environmental conditions. The viability of bacteria on abiotic surfaces indicates a potential hazard (Scott & Bloomfield, 1990; Sharma & Anand, 2002a). Hilbert et al. (2003), when investigating adhesiveness of bacteria on different steel surfaces, detected from 6.19 to 7.17 cfu/cm^2. After the analyzed surfaces were washed the bacterial counts decreased markedly, amounting to 0.3–4.69 cfu/cm^2. The authors selected for analyses steel surfaces with a roughness ranging from 0.01 to 2.0 µm.

The probability of normal distribution was analyzed to assess the suitability of results of microbiological tests and results obtained using a luminometer, to determine their correlations (Fig. 1). About 99 % of the results were found within the covariance ellipsis defined by the covariance matrix. It was these points which corresponded to identical probability. Each object requires the determination of a separate correlation followed by the prediction process. This fact is evidenced by the significant difference between the mean value of correlation coefficients for all 6 objects and the correlation coefficient calculated for all samples jointly (n = 60) r = 0.62. Irrespective of the type of objects, no deviation was shown from linearity. Linearity, measures the dependence between log microbial count from 1 cm^2 analyzed object, and log number of relative light units

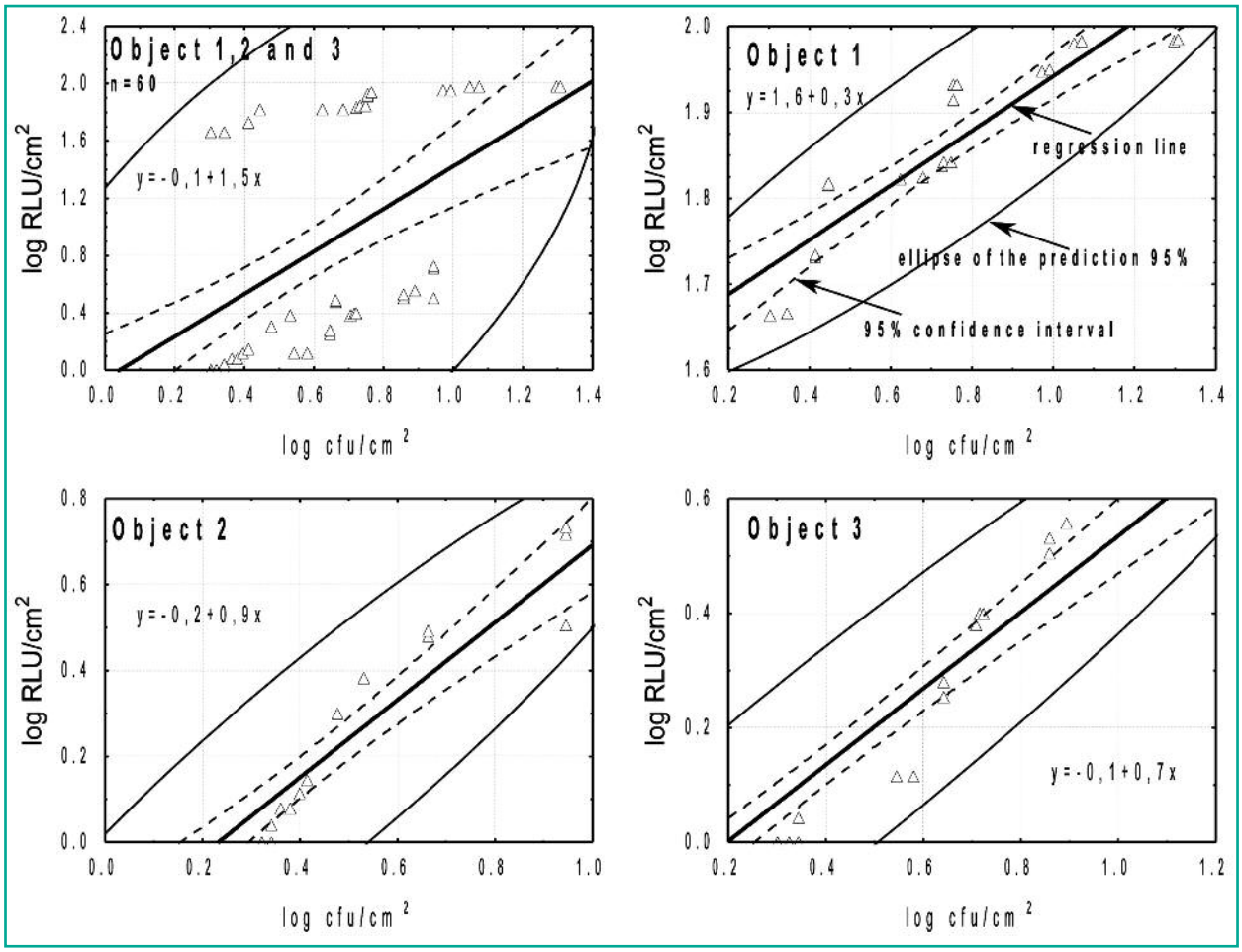

FIGURE 1: *Conditional probability of normal distribution for the dependence of cleanliness data of all objects.*

Archiv für Lebensmittelhygiene 61, Heft 2 (2010), Seiten 37–80

RLU\cm^2. For each object a regression line was plotted by the initial ordinate within a range from –0.2 for object 2 to 1.6 for object 1. In turn, the slope of the line fell within the range from 0.3 for object 1 to 0.9 for object 2. Up to 40 % of the samples were outside the adopted confidence interval. Cho & Yoon (2007) used in their model studies the high dependence of microbial counts and results of RLU measurements, to determine detection levels using a luminometer.

Such a high regularity of results was reflected in the values of correlation coefficients 0.92<r<0.96 between microbial counts assayed using the microbiological method and the number of relative light units measured with a luminometer (Fig. 1). The significance of the correlation between results recorded for each of the objects is confirmed by the high value of correlation for the analyzed variables. Its rational exponent is the coefficient of determination close to 1. A high correlation was also found for results of bioluminescence and the conventional method reported by Larson et al. (2003). When examining 219 surfaces of 4 cm x 4 cm those authors detected 2.97 log cfu and at the same time recorded on average 2.61 log RLU at r = 0.45. The value of measured RLU ranged from 0.8 to 4.6 log RLU, which corresponded to 15–44000 RLU. The correlation coefficient calculated by the authors of the study was r=0.82.

High coefficients of probability density ß, calculated for each object, show proportionally the relative contribution of the independent variable – the microbial count determined using the traditional microbiological method, to the prediction of the dependent variable, i. e. to the number of relative light units measured with a luminometer (Table 2).

The number of RLU for each cleanliness level was defined based on the boundary values of microbial counts on the analyzed surface. This, determine the three ranges of cleanliness (Table 3). The established boundary values for cleanliness levels of examined surfaces included experimental data. Bautista et al. (1992), assessed the hygienic status of surfaces using both the conventional method and by bioluminescence. He, showed that in 74 % of the analyzed surfaces the results obtained by the traditional method and by bioluminescence were consistent. In 36 % of the surfaces the RLU results indicated that the surfaces were not sufficiently clean, although it was not confirmed using the conventional method. Prior to washing, the authors on 20 surfaces measured 4–2191 RLUon 20 surfaces, while after washing they detected 2–285 RLU. In turn, Aycicek et al. (2006) found that 97.5 % of examined surfaces could be considered clean on the basis of results recorded by both the conventional method and bioluminescence. The other 2.5 % of investigated objects turned out to be clean based on ATP-bioluminescence results. The microbial count assessed by the conventional method, however, did not show the objects to be clean. The percentage of objects, assessed as clean by the authors on the basis of bacterial counts and which turned out to be dirty based on the RLU data, was 74.6 %. At the same time when examining 14 different objects, e. g. steel and plastic, the authors showed a wide spectrum ranging from 1435 to 90959 measured RLU. The suitability of bioluminescence in the assessment of cleanliness status was shown by Cooper et al. (2007) to be within the range of 83 % to 100% prior to washing and 90 % to 100 % after surface washing. The authors decided that on the average 84 % of the surfaces they examined were clean based on the RLU data, but only 66 % were clean based on the conventional microbial count method. This proves the necessity to define detailed accurate RLU ranges for specific surfaces.

Conclusions

Assessment of the degree of cleanliness for surfaces of dairy production line facilities may be conducted based on bioluminescence measurement. Surface roughness and thus the deposited biofilm have a significant effect on the values of the RLU measurements (in this experiment ranging from 40 to 9650 RLU/100 cm^2). Results prove that it is necessary to first perform traditional microbiological assays in order to determine ranges of hygienic status and perform predictions. Swab results obtained with the use of a luminometer from a surface classified as clean in one of the dairies (objects 2 and 3) in another processing plant may be considered unacceptable (object 1).

TABLE 2: *Analysis of a dependence of bioluminescence results (RLU) on microbial counts (cfu), α = 0.05, df = 38.*

Object sampling	Mean log cfu/1cm^2 ± Sd	Mean log RLU/1 cm^2 ± Sd	t-Test	r	p
1	0.74 ± 0.29	0.86 ± 0.10	–1.66	0.96	0.104
2	0.44 ± 0.27	0.19 ± 0.27	2.94	0.96	0.005
3	0.42 ± 0.37	0.15 ± 0.26	2.63	0.98	0.01

df = degrees of freedom; Sd = standard deviation; t-test = value for the t-Student' test; r = correlation coefficient; p = statistical differences

TABLE 3: *The bioluminescence cleaning method compared to the microbiological method.*

Object	Cleanliness levels for object	Results of microbiological method (cfu/cm^2)	Bioluminescence results (RLU/cm^2) Experimental values	Calculated values*
1	Pass	≤ 2.66	≤ 5.4	≤ 6.3
	Alert	2.66 < x ≤ 5.66	6.5 ≤ x ≤ 6.9	6.3 < x ≤ 7.1
	Fail	> 5.66	≥ 8.2	> 7.1
2	Pass	≤ 3.90	≤ 2.0	≤ 2.1
	Alert	3.90 < x ≤ 6.90	3.0 ≤ x ≤ 3.1	2.1 < x ≤ 3.7
	Fail	> 6.90	≥ 4.3	> 3.7
3	Pass	≤ 3.98	≤ 1.3	≤ 1.8
	Alert	3.98 < x ≤ 6.98	1.8 ≤ x ≤ 2.5	1.8 < x ≤ 3.1
	Fail	> 6.98	≥ 3.2	> 3.1

*calculated on the base: Pass (≤ 5 – 0.44 x Sd in cfu/cm^2, Alert (5 – 0.44 x Sd < and ≤ 8 –0.44 x Sd in cfu/cm^2), Fail (> 8 – 0.44 x Sd in cfu/cm^2)

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