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Suitability of a Moisture Distribution Assay to Assess the Microbial Stability of Butter Produced at a Small Scale

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Abstract: In this study, the suitability of a moisture indicator paper technique was investigated as a fast and readily performable tool to determine the stability of farm butter. Moisture distribution patterns along with Enterobacteriaceae and fungal counts were monitored during storage of butter produced from raw cream at a dairy farm. The same was done for butter produced from pasteurized cream under very hygienic conditions at a research institute. In dairy science and industry it is generally recognised that a fine and homogeneous distribution of moisture in butter limits microbial growth during storage. It was demonstrated, however, that the relation between moisture patterns and microbial counts during storage is not unequivocal for butter produced at a small scale. It was concluded that the hygiene conditions prevailing during butter production interfere with a relation which is usually considered as a universal principle.

Key words: Farm butter, microbial stability, moisture distribution

INTRODUCTION

In dairy farms, typically a small portion of the milk produced is processed into products such as yoghurt, soft cheeses, ice cream and/or butter. Although production volumes are limited, the products have to meet the prevailing (legal) microbiological criteria, ensuring the microbial quality and safety of the foods. In small scale production, however, these criteria are more difficult to achieve than at an industrial level. An important reason is that less possibilities for (on line) measurement and control of process parameters are available at farm level than at large scale. Most farmers rely on their practical experience to judge the process, rather than on objective measurements. For instance, the duration of the kneading step in butter manufacturing is not standardized but determined by visual inspection of the butter mass. This practice causes a higher batch-to-batch variation of the end product quality compared to industrially produced butter. Furthermore, the general hygiene level in farm production can be expected to be inferior to that of industrial production, where cleaning-in-place and stainless steel equipment is common (Van Zijl and Klapwijk, 2000). Therefore, the potential for microbial growth is larger in farm butter compared to that for industrial butter. Large scale butter production facilities may be able to perform microbiology analyses in the in house quality laboratory to assess the microbiological quality of their product. This is also in contrast to local producers, who usually only obtain microbiological data from a few external analysis per year which are legally obligatory. Moreover, since they lack exhaustive information on the microbial status of their butter, farmers decide on the expiration date based on their experience or by comparing with other farmers. The estimated microbiological stability of their product may therefore be unreasonably long or unnecessarily short.

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Butter should contain at least 80% milk fat and maximum 16% water and 2% dry non-fat milk material (Council Regulation (EC) No. 2991/1994). During the butter production process, fat granules are obtained which are kneaded into a uniform water-in-oil emulsion (Frede and Buchheim, 1994; Muir and Banks, 2003). Rahn and Boysen (1928) and Knudsen and Jensen (1933) have observed that during the kneading phase of the butter manufacturing process- also referred to as the working phase- the moisture droplets decrease in size and become distributed in a more uniform way. In addition, they demonstrated that longer working results in less microbial activity, i.e., acid formation and catalase activity, during storage of the product for several weeks. Although no evidence based on true microbial counts exists, nowadays it is generally recognized that a fine and even distribution of the moisture is essential for microbial stability of butter (Van Zijl and Klapwijk, 2000; Muir and Banks, 2003). In this respect, much effort has been paid in developing techniques to visualize moisture distribution. Methods based on light microscopy have been described, but in recent years more detailed information has been obtained with techniques such as electron microscopy, Confocal Scanning Laser Microscopy (CSLM) and pulsed field gradient nuclear magnetic resonance (pfg-NMR) (Rashevskaya *et al*., 2002; Van Lent *et al*., 2008). Whereas these techniques may be highly valuable for butter production at industrial scale, it is obvious that they will not be adopted by dairy farmers who locally produce and sell farm butter.

An easy and rapid way to visualize moisture distribution in butter employing indicator paper has been proposed by Knudsen and Sörensen (1938) and is laid down in the German standard DIN 10311 (1985). The aim of the present study was to evaluate this test as a fast tool to monitor the progress of the kneading process as well as to assess storage stability. We wanted to elucidate to what extent moisture patterns in butter coincide with actual microbial counts. The usefulness of the method was considered particularly in the context of small scale production, such as dairy farms, because in this type of production environment there is a high need for fast and reliable testing possibilities.

MATERIALS AND METHODS

Butter Manufacturing

The experiments were conducted in July and August 2006 and October and November 2007. Three batches of butter produced at a Belgian dairy farm were monitored and one batch of butter from the pilot scale production plant present at the researchers institute. At the dairy farm, raw full fat milk was used which was obtained at the farm only. The fat content of the milk used for the first, second and third batch of butter was 38.9, 41.5 and 41.7 g L^{-1}, respectively and the protein content was 33.7, 34.5 and 34.7 g L^{-1} (data obtained from the Milk Control Centre Flanders, Lier, Belgium). The milk was skimmed at 37°C (freshly milked) in a disk centrifuge (Westfalia Separator, Oelde, Germany). The cream was collected in 30 L metal milk cans and soured by addition of a lactic acid starter culture. The frozen culture consisted of a *Leuconostoc* species and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* (Nizostar BK2, De Block, Edegem, Belgium). The inoculum was prepared by the farmer by slightly thawing the whole culture as purchased and dividing the mass with a clean knife in small portions of a few grams. The portions were kept frozen individually (-18°C) and for each batch of butter produced, one portion was used. The cream was incubated for approximately 24 h at 20 to 22°C. The cream was allowed to cool to 13 to 14°C by placing it in a refrigerator and then it was transferred into a vertical wooden churn (max 100 L, Tomega, Arville, Belgium). The cream was churned immediately by the high speed rotation of the wooden stirrer, located at the bottom of the vessel, until butter grains were formed. The buttermilk was drained off and the grains were washed by adding tap water, stirring and draining the washing water. This step was repeated once or twice until the washing water was clear. When the weather was warm at the time of butter making (first and second batch investigated), ice was added to the washing water in order to cool it. The washed butter grains were worked into a

uniform mass by the slow rotation of the stirrer in the churn. The churn was placed in a tilted position to drain the water. Working was stopped when no more water left the churn and the butter mass had a good consistency, as judged by visual inspection by the farmer. The butter was transferred by hand into a bowl and aliquots of 250 to 260 g were weighed. Each portion was brought into a rectangular shape using two wooden grooved spatulas and wrapped in waxed paper.

The butter making process in the institute was also a batch process. Raw full fat milk, obtained from a different farm and having a fat content of 45.9 g L^{-1} and a protein content of 37.5 g L^{-1}, was used (data obtained from the Milk Control Centre Flanders, Lier, Belgium). The milk was preheated to 40°C using a batch pasteurizer (Rademaker, Culemborg, The Netherlands) and centrifuged in a disk centrifuge (Rademaker, Culemborg, The Netherlands). The cream was pasteurized (15 sec at 74°C) in a plate pasteuriser (Packo Inox NV, Zedelgem, Belgium) and cooled to 22°C in the same equipment. The appropriate amount of a freeze-dried starter culture (FD-DVS Flora Danica, Chr. Hansen, Horsholm, Denmark, 20-30 Units 100 L^{-1}) was weighed and added. The culture contained *Lactococcus lactis* subsp. *lactis* var. *diacetylactis*, *Lactococcus lactis* subsp. *cremoris* and *Leuconostoc mesenteroides* subsp. *cremoris*. The cream was incubated for 24 h at 22°C and subsequently stored for 40 h at 14°C. Afterwards, it had a pH of 4.5 as indicated by a pH test strip (range 3.8-5.5, Brouwland, Beverlo, Belgium). Then the cream was transferred into a stainless steel churn (Rademaker, Culemborg, The Netherlands) and churned for 15 min at 14°C by the high speed rotation of the stainless steel stirrer present in the centre of the vessel. Buttermilk was drained off and the grains were washed three times by adding tap water, manually blending it through the mass with two wooden grooved spatulas and draining it. Then the butter was kneaded manually in the churn using the spatulas, until no more droplets appeared on the butter surface. The butter was transferred into a bowl using the spatulas. Aliquots of 250 g were weighed, shaped with the spatulas and wrapped in waxed paper.

Sampling

For the farm-made butter as well as for the institute butter, samples were collected (i) at the start of the working phase, (ii) during working when the process step was estimated by the farmer or production supervisor to be halfway and (iii) at the end of the working phase. In this way, butter samples containing moisture droplets of different sizes were obtained. Samples were also collected from the packaged butter. Aliquots of about 100 g were taken in an aseptic way, transported under refrigerated conditions and stored in the dark at 7°C. The butter was analysed for microbial counts and moisture distribution immediately after sampling (hereafter referred to as zero weeks of storage) and at two, four, six and eight weeks of storage.

Microbial Counts and Statistical Analysis

All microbiology analysis were carried out according to the relevant ISO Standards for microbiological analysis of dairy foods, as compiled by Dijk *et al*. (2007). Basically, a weighed amount of butter was melted in a water bath at 45°C for 10 min. Serial dilutions were prepared from the water phase using sterile physiological water (0.9% NaCl). Enterobacteriaceae were counted on Violet Red Bile Glucose Agar (VRBG, Oxoid, Ghent, Belgium) using the pour plate technique with double layer. Plates were incubated at 37°C for 48 h. Characteristic colonies having a purple to dark red colour, a diameter of more than 0.5 mm and sometimes a pink to purple precipitation halo, were counted as presumptive Enterobacteriaceae. Plates containing between 30 and 300 colonies were considered as countable. The average number of colony forming units (CFU) from duplicate plates were expressed per gram butter and transformed to log counts. Confirmation tests were performed with at least five suspect colonies per plate. After streak plating on Nutrient Agar (NA, Oxoid, Ghent, Belgium, 24 h at 37°C), oxidase tests were performed using Oxidase Identification Sticks (Oxoid, Ghent, Belgium)

according to the manufacturers prescriptions. Positive colonies were investigated for glucose fermentation test by picking them from the NA plates and stabbing into Glucose Agar tubes (Biokar Diagnostics, Allonne, France, 24 h at 37°C). A yellow colour throughout the whole tube indicated a positive reaction. Yeasts and moulds were counted on Oxytetracycline Glucose Yeast Extract Agar (OGYE, Oxoid, Ghent, Belgium) using the pour plate technique. Plates were incubated at 25°C for 5 days. Plates containing between 10 and 100 colonies were considered as countable. The number of yeasts and moulds per gram butter was calculated and expressed as described above.

In the three experiments with farm butter, Analysis of Variance (ANOVA, General Linear Model, GLM) was performed on the microbial counts to investigate whether the data could be pooled. Enterobacteriaceae and fungal counts were analysed separately. For both microbiological groups, the model contained the factors experiment (referring to the three batches of butter monitored), storage time (referring to storage periods of zero, two, four, six and eight weeks) and sampling time (referring to sampling before, during and after working and after packaging), as well as the interaction effects between factors. Multiple comparison of means was accomplished with Tukey HSD as well as with Duncan test (alpha = 0.05). For all statistical analysis SPSS Version 15.0 (USA) was employed.

Moisture Distribution

The moisture distribution in butter samples was determined according to the indicator paper method described in the German standard DIN 10311 (1985). Butter samples were stored at 7°C for at least 1 h before analysis. At the surface of the butter, a layer of about 1 cm was removed using a knife in order to examine the interior of the sample. Then, another layer was slowly cut off using a wire cutter consisting of a stainless steel wire fixed in a clamp (Rollschnitt Cheese Slicer, Westmark, Lennestadt, Germany). In this way, a cross section of the sample with intact moisture droplets was obtained. Without touching the surface, it was covered immediately with an indicator paper (Wator, Macherey-Nagel, Düren, Germany) and the paper was pressed slightly and equally on the butter surface using a clean knife. The paper was left for exactly 30 seconds and then removed. Dark blue to purple spots appeared on the paper where moisture was located on the surface. For the first batch of butter produced at the farm, moisture distribution was only determined at zero weeks of storage. For the second and third batch of butter produced at the farm and for the butter produced at the institute, all samples taken at all storage times were analysed.

RESULTS AND DISCUSSION

Microbial Counts and Statistical Analysis

The microbiological results obtained for the first, second and third batch of farm butter, which was produced from raw milk, are presented in Fig. 1-3, respectively. For the three batches, the *Enterobacteriaceae* counts as well as the yeast and mould counts in freshly prepared butter were situated between 3 and 5 log cfu g^{-1} product. In all three experiments, the Enterobacteriaceae population declined during the first two weeks of cold storage with two to three log cycles. From then on, a steady rise in their number was observed, reaching 2 to 4 log cfu g^{-1} after eight weeks. Apparently, only psychrotrophic and psychrophilic organisms survive during the first weeks of cold storage and start to grow afterwards. At zero and two weeks of storage, numbers of Enterobacteriaceae were very similar for samples taken at different stages of processing, i.e., for samples with moisture droplets of different sizes. From four weeks onwards, however, the numbers for different sample types diverged to some extent and in several cases counts tended to be lower as the sample was taken further during processing. The fungal counts remained more or less constant in the first two weeks of storage, rather than showing a remarkable decline as found for the Enterobacteriaceae. Later on during storage, an increase in counts was noticed particularly in butter samples taken before and during

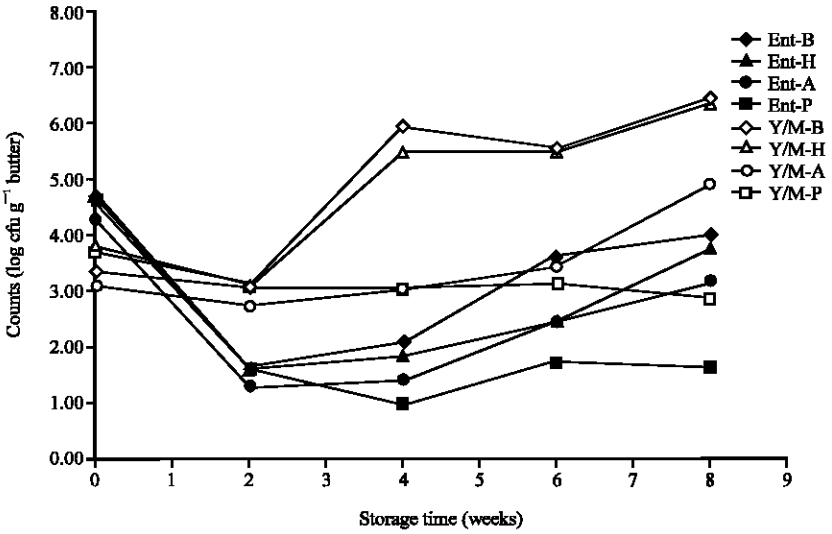

Fig. 1: Numbers of Enterobacteriaceae (Ent) and Yeasts and Moulds (Y/M) during storage of butter samples taken at different stages of processing (before working B, approximately halfway working H, after working A and after packaging P) of the first batch monitored at a local dairy farm

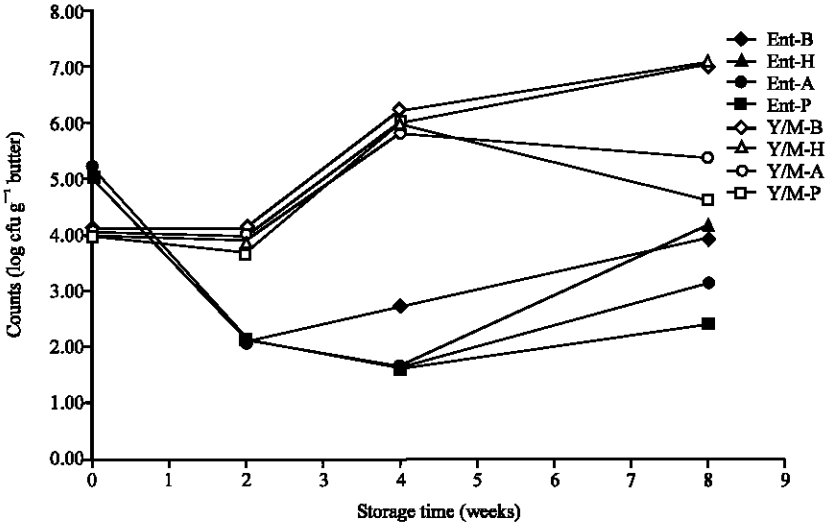

Fig. 2: Numbers of Enterobacteriaceae (Ent) and Yeasts and Moulds (Y/M) during storage of butter samples taken at different stages of processing (before working B, approximately halfway working H, after working A and after packaging P) of the second batch monitored at a local dairy farm. No analyses were performed at six weeks of storage

working. In completely worked and in packaged butter, the number of fungi increased hardly or not at all, except in the second experiment. At four weeks and later on in the first and third experiment, fungal counts diverged. They were usually lower for samples with smaller droplets taken further during processing, comparably to the Enterobacteriaceae.

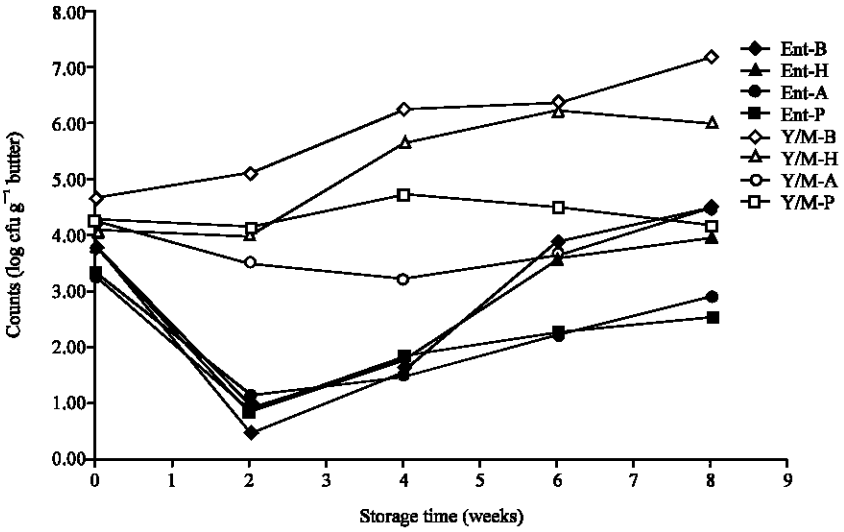

Fig. 3: Numbers of Enterobacteriaceae (Ent) and Yeasts and Moulds (Y/M) during storage of butter samples taken at different stages of processing (before working B, approximately halfway working H, after working A and after packaging P) of the third batch monitored at a local dairy farm

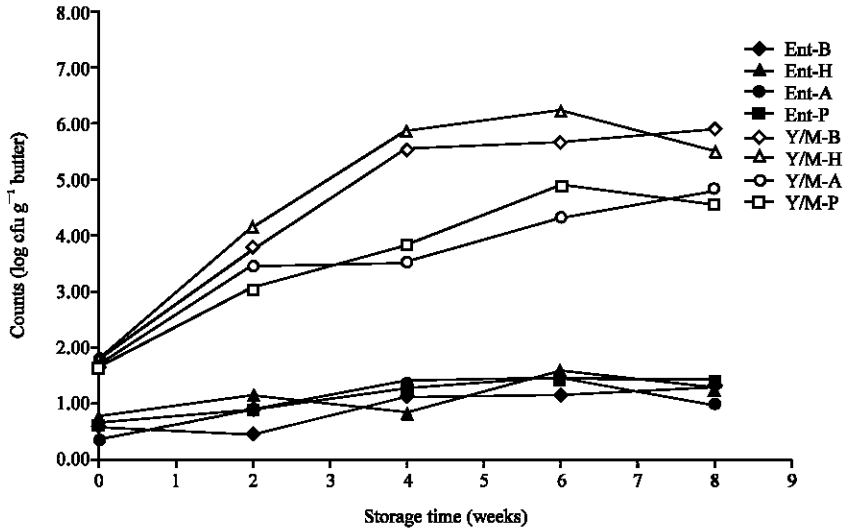

Fig. 4: Numbers of Enterobacteriaceae (Ent) and Yeasts and Moulds (Y/M) during storage of butter samples taken at different stages of processing (before working B, approximately halfway working H, after working A and after packaging P) of the batch which was manufactured in the pilot scale production unit present in the authors research facility

The institute butter yielded completely other findings with respect to the initial microbial load as well as to its evolution during storage (Fig. 4). Numbers of Enterobacteriaceae were very low in the fresh butter. There was barely any growth of this population when the butter was stored and consequently counts were similar for the diverse sample types. In contrast and surprisingly, yeasts

and moulds, which were initially present in a substantially lower number than in fresh farm butter, grew with three to four log cycles. Such strong growth was not observed in any batch of the farm butter. At two, four and six weeks, however, the numbers appeared to be related to sampling times or droplet sizes, just as observed in stored farm butter. The fact that the initial microbial load in the institute butter was considerably lower than the farm butter can be explained by the pasteurisation of the cream and the higher hygiene level during subsequent processing in the institute.

To investigate whether it was possible to pool the data obtained for the three batches of farm butter, a regression analysis was performed. The high R-squared values (Table 1) indicated a good fit for the model for Enterobacteriaceae as well as that for fungi. The analysis further revealed that the main effect of the factor experiment was highly significant, both for Enterobacteriaceae as well as for fungi ($p<0.05$). Therefore, the three tests with farm butter were to be considered as different experiments and data could not be pooled. In addition, the two other main effects of storage time and sampling time were highly significant, indicating that these factors were relevant in the model and influenced the data in a statistically significant way. For Enterobacteriaceae, no interaction was found between the factor experiment and the factor sampling time ($p>0.05$). This means that the influence of sampling time or droplet size on the Enterobacteriaceae count was independent of the experiment. For all other combinations of factors, relating to Enterobacteriaceae as well as to fungi, interactions were found ($p<0.05$), meaning that the effect of one factor on the microbial counts was influenced by each other factor.

The fungal counts could not be analysed further statistically due to the occurrence of numerous interactions between factors. It was useful, however, to examine the Enterobacteriaceae counts for each storage period individually and to apply GLM (ANOVA) without interaction between experiment and sampling time (Table 2). The effect of the factor experiment was significant at zero and two weeks of storage ($p<0.05$), which implies that the numbers of Enterobacteriaceae in samples taken at the same processing time differed between batches, e.g., the counts of all samples before kneading of the three batches were different. At four, six and eight weeks of storage, the effect of the factor experiment was not significant ($p>0.05$). There was no significant indication for an effect of sampling time at zero to four weeks of storage, or, Enterobacteriaceae counts were not influenced by the sampling time.

Table 1: Significance of effects in the General Linear Model describing all microbiological data obtained for farm butter

	Significance	
Factors	Enterobacteriaceae[a]	Fungi[b]
Experiment[c]	0.000	0.000
Storage time[d]	0.000	0.000
Sampling time[e]	0.000	0.000
Experiment × Storage time	0.000	0.048
Experiment × Sampling time	0.695	0.026
Storage time × Sampling time	0.001	0.000

[a]R-Squared = 0.979; [b]R-Squared = 0.957; [c]Referring to the three batches of butter monitored; [d]Referring to storage periods of zero, two, four, six and eight weeks; [e]Referring to sampling before, during and after working and after packaging

Table 2: Significance of effects in the General Linear Models for the individual storage periods describing the Enterobacteriaceae counts obtained for farm butter

	Significance	
Storage time (weeks)	Experiment[f]	Sampling time[g]
0[a]	0.000	0.106
2[b]	0.000	0.819
4[c]	0.454	0.231
6[d]	0.241	0.065
8[e]	0.313	0.001

[a]R-Squared = 0.972; [b]R-Squared = 0.925; [c]R-Squared = 0.555; [d]R-Squared = 0.892; [e]R-Squared = 0.933; [f]Referring to the three batches of butter monitored; [g]Referring to sampling before, during and after working and after packaging

However, a weakly significant indication was observed (p = 0.065) at six weeks storage and at eight weeks the effect of sampling time appeared to be highly significant. The differences between the means obtained at eight weeks were subjected to a Tukey HSD as well as a Duncan test with an alpha level of 0.05. It could be concluded from both tests that only the counts before and during working were not different. After eight weeks of storage Enterobacteriaceae counts were significantly lower for butter which was subjected to a complete working process than for butter which was not or partly kneaded. Hence, the importance of proper kneading and thus of droplet size in restricting the growth of Enterobacteriaceae increased during storage.

Moisture Distribution and Relation to Microbial Counts

In Fig. 5, the indicator papers obtained from the second batch of farm butter are shown. The figure includes images from samples taken before, halfway and after working and after packaging, at zero and eight weeks of storage. These images demonstrate the evolution of moisture distribution in function of processing or sampling time and in function of storage time. The images obtained in the first and third experiment with farm butter were very similar (data not shown). For farm butter sampled at the time of production, the indicator papers showed clearly more and larger droplets in samples

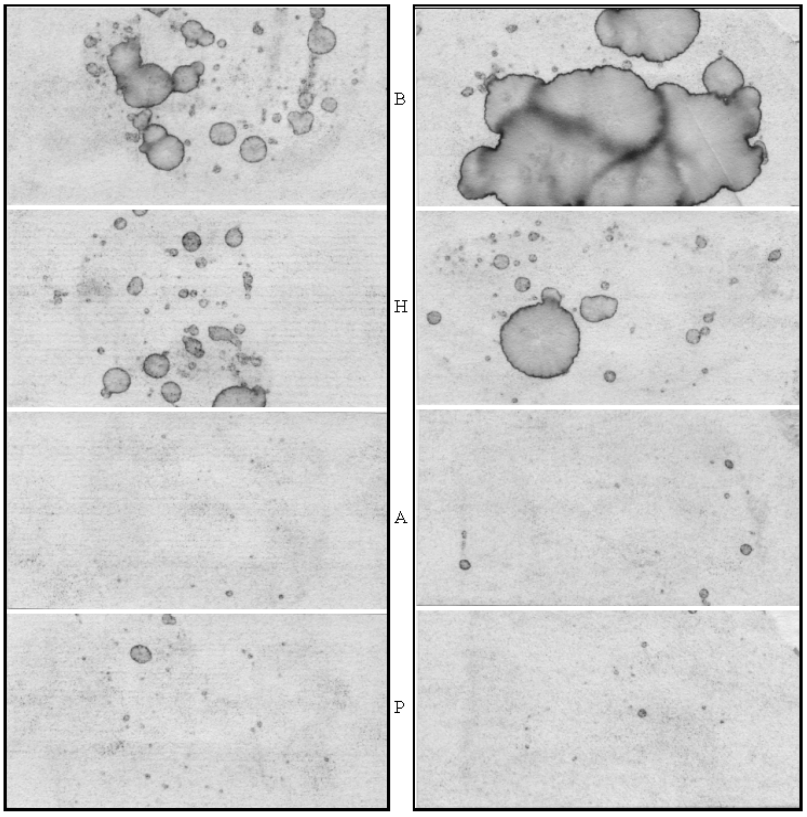

Fig. 5: Indicator papers from butter samples taken at different stages of processing (before working B, approximately halfway working H, after working A and after packaging P) of the second batch monitored at a local dairy farm (cfr. microbial counts in Fig. 2). Images presented in the left column were obtained at the start of the storage period (zero weeks) and images in the right column were obtained at eight weeks of storage

before and halfway kneading than for completely worked butter and packaged butter. In the latter two stages, droplets were generally small in number and size and appeared as dots or tiny spots on the indicator paper. At zero weeks, the difference observed between images obtained before and halfway working on the one hand and those acquired from completely worked and packaged butter on the other were not reflected in corresponding differences in microbial counts, as the respective counts for Enterobacteriaceae and fungi coincided for all sample types. At eight weeks, droplets appeared as large coloured areas on the paper in particular in butter which was not kneaded. Initial spots in worked and packaged butter did not substantially enlarge or rise in number. Therefore, at eight weeks of storage (and also at four and six weeks in this second experiment and at four, six and eight weeks in the third experiment, data not shown) the aforementioned divergence between moisture patterns before plus halfway working and completely worked plus packaged butter was larger than at zero weeks. This related more or less to the microbial counts, since at four, six and eight weeks of storage usually lower numbers were found in completely worked and packaged butter than in not or partly kneaded butter. For farm butter produced from raw cream, it can therefore be concluded that the presence of multiple large droplets supports microbial growth to a larger extent than (a limited number of) small droplets.

In the moisture distribution of the institute butter (Fig. 6), no clear distinction could be made between patterns for not and partly worked butter and for completely worked and packaged butter.

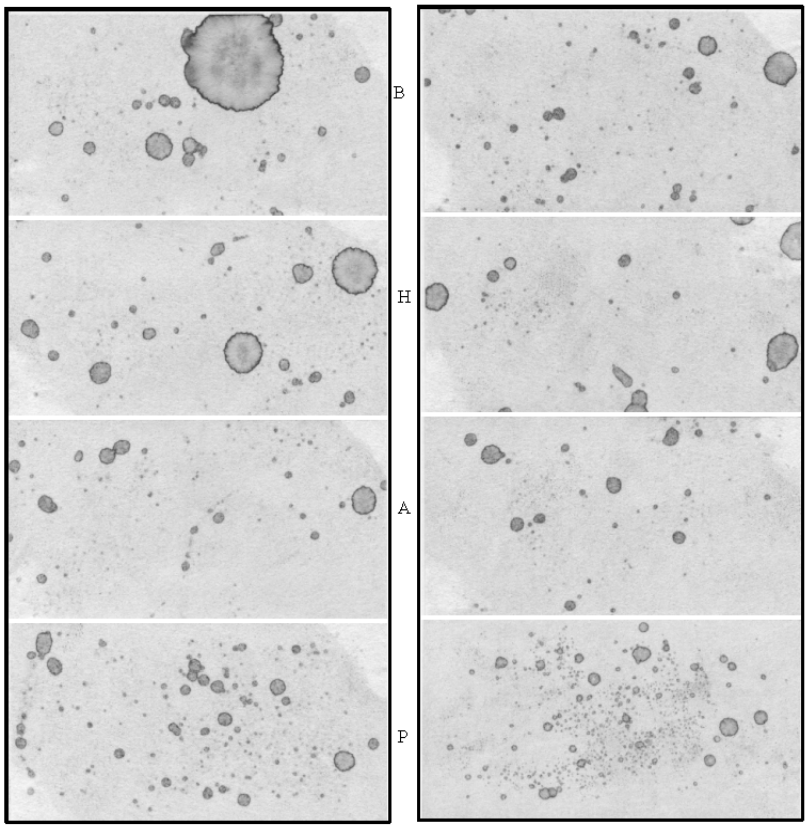

Fig. 6: Indicator papers from butter samples taken at different stages of processing (before working B, approximately halfway working H, after working A and after packaging P) of the batch manufactured in the pilot scale plant (cfr. microbial counts in Fig. 4). Images presented in the left column were obtained at the start of the storage period (zero weeks) and images in the right column were obtained at eight weeks of storage

In samples assessed at manufacturing, a considerable number of medium-sized spots appeared on all four sample types. In addition, a few rather large droplets appeared on not and partly worked butter surfaces. In contrast to farm butter, there was no enlargement of droplets during storage and images remained more or less equal. The constancy of the moisture distribution during storage corresponded to the Enterobacteriaceae counts which remained steady, but not to the extensive growth of yeasts and moulds. The discrepancy between the behaviour of bacteria and fungi in this experiment is striking. In literature, most information on growth of micro-organisms in butter, or more general in water-in-oil emulsions, is related to bacteria. It is stated that microbial growth in a droplet is limited by the interior area available and the quantity of nutrients present (Verrips and Zaalberg, 1980; Robins and Wilson, 1994; Delamarre and Batt, 1999). Rahn and Boysen (1929) suggested that their deductions were probably not true for moulds and they brought up that moulds have the ability to force their way from one droplet to another. Also Ter Steeg *et al*. (2001) claimed that mould growth in emulsions is not contained within droplets. Still they found that water containment and dispersion are key factors in mould spoilage. Moreover, the growth and activity of moulds can be of importance to that of bacteria and yeasts. Moulds generally can grow at lower water activities (a_w) than yeasts and bacteria (Jay *et al*., 2005). Water originating from mould metabolic activity, however, may lead to growth of organisms characterised by higher minimal a_w values. The data obtained in this study are in line with a study of Verrips and Zaalberg (1980). Yeasts were inoculated in a water-in-oil emulsion with a water phase of 40% and their growth depended on the size-distribution of the water droplets and the storage temperature. Initially about 1 log cfu g^{-1} yeasts were present. After 12 weeks, the yeast counts reached about 4 log cfu g^{-1} in the coarse emulsion stored at 15°C and about 3 log cfu g^{-1} in the coarse emulsion stored at 7.5°C.

Moisture droplets with microscopic dimensions can not be made clearly visible using the indicator paper method and for this reason the value of the method may be questioned. Rahn and Boysen (1929) suggested that not all droplets in butter are infected. They reasoned that during working, large infected moisture droplets are divided up into finer ones. When this process continues, the major part of the droplets becomes so small that they end up being sterile. Therefore it may be stated that the possibility to detect coalescence of droplets during keeping is more important than showing the finest microscopic droplets.

CONCLUSIONS

This study demonstrated that low numbers and sizes of spots on the indicator paper images of farm butter produced from raw cream do not imply that no microbial growth will occur during storage of the butter. On the other hand, in situations where the microbial load of butter is expected to be low, due to pasteurisation of milk and/or cream and proper hygienic conditions during manufacturing, the appearance of a few relatively large spots is not always dramatic for bacterial growth. Nevertheless, care must still be taken for fungal growth.

The test is relevant to be used during kneading of the butter to monitor the status of the moisture distribution, with large droplets indicating that longer kneading is necessary. The usage of the paper method to determine the end of the working phase can be useful in particular when kneading is done by hand. When working is performed in the churn and the amount of water leaving the churn is checked regularly, the paper method can serve as a confirmatory test. Images can also be taken from the packaged product. The detection of large droplets can urge the farmer to shorten the habitual shelf life period when no intensive hygiene programme is implemented in the production process. Although the indicator paper method is rapid and readily performable, users should be clearly informed on what to deduce (and what not) from the images in relation to the hygienic measures present or absent in their own production process.

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