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Involvement of Heterofermentative Lactobacilli in Development of Open Texture in Cheeses

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ABSTRACT

Samples of Canadian Cheddar and Oka cheeses which exhibited gas formation and fissure defects were examined microbiologically. Analyses revealed that lactobacilli, especially heterofermentative types, as well as organisms capable of using citrate were more numerous in defective cheeses than in high quality products. Higher numbers of viable lactobacilli were obtained in assays where APT or MRS media were used than when MRS adjusted to pH 5.5 or Rogosa agar were used, especially when younger cheeses were sampled. The number of lactic streptococci did not differ between good quality Cheddar or rejected aged cheese. Coliforms, staphylococci, yeasts, molds and clostridia appeared to have no relationship with the formation of gas in cheeses late in the maturation process.

Conventional manufacture of commercial Cheddar involves addition of streptococci starter cultures (usually *Streptococcus cremoris* and *S. lactis*) to accomplish controlled acidulation of cheese milk *(5,26)*. The viability of such streptococci decreases rapidly following salt addition to the curd and bacterial cell lysis is believed to release intracellular dipeptidase activity important in the later development of desirable cheese flavor *(18)*. The numbers of viable streptococci increase in Cheddar aged longer than 7 months but lactobacilli from the largest proportion of the bacterial population present as early as 2 weeks following cheese manufacture and these levels are maintained throughout storage to 10 months of age *(23,26)*. Lactobacilli arise adventitiously from cells introduced with the starter culture, some survive pasteurization treatment of the milk or they may gain access by being transferred from dairy equipment and utensils used in manufacture. It is possible that these organisms are largely responsible for the final ripening process *(27)*. Thus complete control over the ripening of Cheddar aged more than 3 months has not yet been achieved, and normal ripening is largely dependent upon the nature and characteristics of lactobacilli present in the cheese milk purely by accident.

Franklin and Sharpe *(8)* have demonstrated that increasing the heat treatment of the milk beyond 63°C reduces the organoleptic quality of the cheese. Fryer *(10)* has shown that it is mainly lactobacilli and pediococci that multiply in cheeses while gram-negative rods (including coliforms), staphylococci, micrococci, group D streptococci and thermo-resistant corynebacteria diminish in number. Studies of the evolution of the total microflora during Cheddar maturation show, on the other hand, that flavor depends largely on the increase in the number of lipolytic bacteria as well as many other non-lactic acid bacteria. Several studies have examined the interactions between lactobacilli and other components of the microflora during cheddar ripening *(1,9,21,26)*.

The purpose of this study was to evaluate the microbiological status of two grades of "old" Cheddar cheese (grade 1 and rejected) to determine whether differences in cheese quality could be correlated with the appearance of an unusual group of microorganisms *(7)*. Specifically, our attention was directed toward the identification of causative agents responsible for the late gas defect in aged cheese. The late gas defect was seen as openness of cheese texture or "blown wrappers" without texture change in cheeses aged longer than 3 months. It was also of interest to examine similar defects in young Oka, Gouda and Saint-Paulin cheeses.

MATERIALS AND METHODS

Sample treatment

Twenty samples of grade 1 "old" Canadian Cheddar cheese were obtained from Agropur (Coopérative Agro-Alimentaire, Granby, Qc). The samples came from 280-kg blocks aged more than 12 months and were graded by Agriculture Canada inspectors. The graders scored the blocks of cheese for flavor, taste and texture according to the "Grading Manual of Dairy Products" published by Agriculture Canada. They were packaged in gas-impermeable laminated polyethylene film in 227-g sizes

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which were prepared for retail sale under a national brand name. Other lots of Saint-Paulin, Gouda, Oka and Cheddar cheeses, all manifesting an open texture defect of various degrees were also obtained from the manufacturer, Agropur. The cheeses were stored under standard conditions (10°C) until microbiological analyses were complete.

Microbiological analysis

Samples of 50 ± 1 g were aseptically removed from the packages and placed in 450 ml of sterile 0.1% peptone and homogenized in a Stomacher 400 (A.J. Seward Lab., London, U.K.) for 1 min. Samples were serially diluted in peptone and examined for a variety of bacterial types. *(a) Total aerobic bacteria* were asessed with plate count agar (PCA; Difco) which was incubated at 30°C for 3-4 d. *(b) Lactobacilli* were enumerated using Rogosa SL Agar, Lactobacilli MRS broth plus 1.5% agar (Difco), all purpose tryptone agar (APT, Difco) MRS agar adjusted to pH 5.5 with 1 *N* lactic acid before autoclaving *(3)* and incubated in anaerobic jars (Gas-Pak system, BBL) at 30°C for 3 d. The Gas-Pak envelopes created a hydrogen: carbon dioxide atmosphere (90:10). *(c) Lactic streptococci* were enumerated on Neutral Red Chalk Lactose Agar *(15)* and incubated anaerobically at 30°C for 3 d. *(d) Spore-forming gas-producing anaerobes* (clostridia) were enumerated by a most probable numbers method (MPN) using the medium of Bryant and Burkey *(4)* with minor modifications. Ten ml of cheese homogenate was heated to 75°C for 15 min. Portions of 1.0, 0.1 and 0.01 were used to inoculate test tubes containing 10 ml of medium. The MPN was based on 5 tubes for each dilution. An agar plug, about 1.5-cm thick was carefully layered on the medium surface in each tube (2% agar). The tubes were examined for gas production after 3, 7 and 14 d of incubation at 37°C. *(e)* This same pasteurized (75°C, 15 min) cheese homogenate was used to determine *mesophilic and thermophilic spore-formers* on APT which was incubated at 37°C and 55°C, respectively, both aerobically and anaerobically (in jars with "Gas-Paks"). *(f) Citrate-utilizing organisms* were enumerated on the medium of Kempler and McKay *(17). (g) Yeasts and molds* were enumerated on Rose Bengal Chloramphenicol Agar (RBCA, Oxoid), a selective medium for eukaryotic microorganisms in food. Bacteria are inhibited by the antibiotic, yeasts appear pink because of the uptake of rose bengal, while molds appear white and filamentous and eventually become grey or black. These plates were incubated at 22°C for 3 to 4 d. *(h) Total presumptive coliforms* were enumerated on Violet Red Bile Agar (VRBA, Difco) incubated at 37°C for 24 h. *(i) Staphylococci* (coagulase-negative and -positive) were enumerated on Baird-Parker medium (Difco) incubated at 37°C for 48 h.

All plating was done in duplicate, adhering to the standard methods for the examination of dairy products, of the American Public Health Association *(2)*.

Chemical analysis

Moisture, salt and fat were analyzed according to the standard methods for the examination of dairy products, of the American Public Health Association *(2)*.

Statistical analysis

Bacteriological count data ($\log_{10}$) were analyzed using one-way variance analysis with the Duncan multiple range test *(29)*. Variance analysis in a "split plot" design was used to establish the difference among the various treatments.

RESULTS AND DISCUSSION

The number of lactic acid bacteria recovered on the test media used and a statistical evaluation are presented in Tables 1 and 2. Recoveries were significantly lower when Rogosa and MRS adjusted to pH 5.5 with 1 *N* lactic acid were used to examine Saint-Paulin, Gouda and 1-month-old Cheddar cheeses. Bacterial recoveries were only slightly lower on MRS (pH 5.5) and Rogosa agars when Oka samples were examined, but for Cheddar aged 12 months or more, bacterial numbers were similar on all four media. The analysis of variance of lactobacilli counts showed a significant difference ($P \leq 0.01$) between Cheddar and Oka cheeses, but no significant differences were obtained between Cheddar and Gouda, or Cheddar and St-Paulin. Use of different media during enumeration of lactic acid bacteria in cheeses should be considered as there was a significant difference ($P \leq 0.01$) among APT, MRS, $MRS_{5.5}$, and Rogosa agars (Table 2).

In Oka and Cheddar cheeses aged 12 months or longer, lactobacilli constituted the dominant portion of the micro-flora, as noted by Perry and Sharpe *(25)* and Prentice and Brown *(26)* for Cheddar. This may explain, in part, the almost identical results obtained with MRS and APT.

TABLE 1. *Lactobacillus counts*[1] *($\log_{10}$/g) of the defective cheeses on different media.*

Samples	APT	MRS	MRS (pH 5.5)	Rogosa agar
Saint Paulin (2 months)	$7.80^{a2} \pm 0.20$	$7.46^{a} \pm 0.10$	$6.20^{b} \pm 0.05$	$6.20^{b} \pm 0.01$
Gouda (2 months)	$8.15^{a} \pm 0.15$	$8.18^{a} \pm 0.03$	$5.48^{b} \pm 0.01$	$5.85^{b} \pm 0.05$
Oka (2 months)	$8.74^{a} \pm 0.26$	$8.36^{a} \pm 0.06$	$8.06^{a} \pm 0.04$	$8.26^{a} \pm 0.03$
Oka 24-2 (2 months)	$8.28^{a} \pm 0.02$	$8.33^{a} \pm 0.03$	$7.68^{b} \pm 0.12$	$7.53^{b} \pm 0.13$
Oka 29-2 (2 months)	$9.23^{b} \pm 0.07$	$9.09^{ab} \pm 0.01$	$8.58^{ab} \pm 0.10$	$8.47^{a} \pm 0.03$
Oka 1-3T (2 months)	$8.52^{ab} \pm 0.12$	$8.75^{b} \pm 0.05$	$8.06^{a} \pm 0.04$	$8.20^{a} \pm 0.05$
Oka-6 (2 months)	$8.70^{ab} \pm 0.10$	$9.07^{a} \pm 0.02$	$8.56^{a} \pm 0.06$	$8.45^{a} \pm 0.07$
Cheddar 81 (32 months)	$6.55^{a} \pm 0.15$	$6.73^{a} \pm 0.01$	$6.66^{a} \pm 0.56$	$6.56^{a} \pm 0.06$
Cheddar 82 (24 months)	$7.76^{a} \pm 0.04$	$8.03^{a} \pm 0.02$	$7.70^{a} \pm 0.05$	$7.64^{a} \pm 0.20$
Cheddar 82 (21 months)	$7.06^{a} \pm 0.14$	$7.17^{a} \pm 0.13$	$7.11^{a} \pm 0.24$	$6.92^{a} \pm 0.08$
Cheddar 84 (1 month)	$8.11^{a} \pm 0.11$	$8.26^{a} \pm 0.19$	$5.60^{b} \pm 0.25$	$5.48^{b} \pm 0.04$

[1] $\bar{X} \pm$ S.E. = mean ± standard error, n = 2.

[2] Means in the same row bearing a common letter do not differ ($p \leq 0.05$).

TABLE 2. *Variance analyses of data in Table 1.*

Source of variation	d.f.	Variance
Cheeses	10	
Cheddar 81 vs Oka	1	21.99**
Cheddar 82[a] vs Oka	1	11.93**
Cheddar 84 vs Oka	1	16.62**
Cheddar 81 vs Gouda	1	0.34
Cheddar 82 vs Gouda	1	1.35
Cheddar 84 vs Gouda	1	0.01
Cheddar 84 vs St-Paulin	1	0.32
Cheddar 82 vs St-Paulin	1	1.38
Cheddar 84 vs St-Paulin	1	0.01
Cheddar 81 vs Cheddar 82	1	3.38(*)
Media	3	
Rogosa vs MRS	1	7.71**
Rogosa vs APT	1	8.28**
MRS vs MRS 5.5	1	8.62**

(*) Significance; $p \leq 0.10$.
* Significance; $p \leq 0.05$.
** Significance; $p \leq 0.01$.
[a] Cheddar 82 (24 months).

Nonetheless, it should be noted that neither of these latter media are selective and will support growth of a variety of organisms in addition to lactobacilli *(3)*; with micrococci, staphylococci, pediococci and even salmonellae capable of microaerophilic or anaerobic growth on MRS but not *Brochothrix*, many streptococci and leuconostocs (Holley, unpublished). Use of acidified MRS and Rogosa Agars reduced the spectrum of organisms capable of growth due to lactic acid, whereas Rogosa Agar also excluded some lactobacilli *(6)*.

Table 3 shows the results of microbiological analysis of fissured and unfissured Oka cheese. The affected products contained 20 times the number of lactobacilli found in the unaffected product. The same was true for citrate-utilizing organisms. Staphylococci were almost four times as numerous in defective cheese, whereas coliforms were rare both in freshly made and defective cheeses and therefore were not likely responsible for the condition of the affected cheese. Spore formers, including clostridia, were half as numerous in rejected cheeses and probably did not cause the swollen condition either, especially since spore forming anaerobes were found in greater number in normal Cheddar cheese by Elliot et al. *(7)* and Prentice and Brown *(26)*.

To determine whether the lactobacilli were predominantly homofermentative or heterofermentative, 30 gram-positive, catalase-negative colonies (lactobacilli) were isolated from fissured and unfissured Oka cheese. Among the isolates from fissured cheese, 12 (40%) were heterofermentative by the test of Gibson and Abd-el-Malek *(13)* and produced NH_3 from arginine, while only one isolate (3.3%) from the unfissured cheese was heterofermentative. The large numbers of lactobacilli in fissured cheeses, plus the relatively high proportion of heterofermentative types among these suggest that they may be involved in fissure formation by gas generation.

Table 4 gives the results of microbiological analysis of grade 1 versus rejected Cheddar cheeses. Lactobacilli were close to 100 times more numerous in rejected cheeses (10^9 cells/g) than in grade 1 cheeses (10^7 cells/g). There was no significant difference ($P \leq 0.05$) between the counts obtained on Rogosa Agar and MRS agar, confirming the observations from Table 1 for Cheddar aged 21 months or more. The counts were somewhat higher than those obtained by Prentice and Brown *(26)*, but approximated those reported for defective Cheddar by Elliot et al. *(7)*. Our results differed from those of Hunter *(16)* in that lactobacilli in rejected "old" Cheddar are not at a numerical disadvantage with respect to other microorganisms. Other studies, including that of Naylor and Sharpe *(22)* who used a more selective medium (acetate agar) showed that lactobacilli grew from the beginning of maturation (of Cheddar) and reached a maximum during the initial 2-6 months ripening period. Among other work, maximum lactobacilli counts varied considerably: 6.9×10^8/g after 21 d *(28)*; 9×10^8/g after 120 d *(20)*; 3×10^6 after 180 d *(23)*. In the latter instance, the lactobacillus content of the milk used was less than 1/ml.

TABLE 3. *Microbiological analyses (log_{10}/g)[a] of Oka cheese (open vs non-open).*

Microorganisms	Open	Non-Open
Lactobacilli	8.48 (12:30)[b]	7.28 (1:30)[c]
Lactic streptococci	9.18	9.26
Citrate-negative bacteria	8.79	9.09
Citrate-positive bacteria	8.57	7.30
Total coliforms	1.00	1.60
Staphylococci (coagulase-negative and -positive)	4.83	4.28
Yeasts	1.00	1.00
Molds	4.11	4.48
Aerobic mesophilic spores	3.26	3.15
Anaerobic mesophilic spores	2.51	2.92
Aerobic thermophilic spores	2.70	3.00
Anaerobic thermophilic spores	2.48	3.04
clostridia	2.65	2.89

[a]Mean of two analyses in duplicate of 4 month aged Oka cheeses.
[b](12:30): 12 heterofermentative lactobacilli isolated from among 30 colonies per plate.
[c](1:30: 1 heterofermentative *Lactobacilus* isolated from among 30 colonies per plate.

TABLE 4. *Microbiological analyses (log_{10}/g)[1] of 12-month aged grade 1, and late-gassing Cheddar cheeses.*

Analysis	Grade 1	Late-gassing
Lactobacilli (MRS Agar)	7.33 ± 0.05[a2]	8.93 ± 0.18[b]
Lactobacilli (Rogosa Agar)	7.37 ± 0.07[a]	8.83 ± 0.19[b]
Citrate positive bacteria	6.40 ± 0.10[a]	8.09 ± 0.24[c]
Citrate negative bacteria	7.42 ± 0.03[b]	7.54 ± 0.28[bc]
Lactic streptococci	7.25 ± 0.05[a]	7.55 ± 0.22[a]
Total bacteria	7.23 ± 0.07[a]	8.74 ± 0.20[b]

[1]X ± S.E. = means ± standard error, n ±20.
[2]Two means in the same row bearing a common letter do not differ ($p \leq 0.05$).

Another factor which undoubtedly influenced the maximum lactobacillus count achieved was the bacterial composition of the starter culture. Some decomposition products in the culture may specifically favor growth of lactobacilli *(14,19,22,23)*.

While evidence is largely circumstantial, the larger number of lactic acid bacteria, specifically citrate-positive types, in defective Cheddar (Table 4) implies that they may be at least, in part, responsible for the gas defect observed in these cheeses *(11)*. Citrate-positive organisms include many heterofermentative lactobacilli, some homofermentative lactobacilli, streptococci including *S. lactis* ss. *diacetylactis,* and *Leuconostoc* species. Some ambiguity of results obtained using Kempler and McKay *(17)* medium was seen, and this is consistent with an early report on similar techniques *(12)*. These latter workers suggested that many citrate-utilizers simply do not produce dark blue color (Prussian blue), especially species of *Leuconostoc*. Lactic streptococcus counts in cheeses were very high and no difference was observed between grade 1 and rejected cheese. Nonetheless, it should be mentioned that the medium, Neutral Red Chalk Lactose Agar *(15)*, used for the enumeration of lactic streptococci is not selective and will support growth of lactobacilli (Laleye, unpublished) at this stage of cheese ripening. These lactic streptococci were probably not, therefore responsible for gas-swelling of cheese. Moreover, there is a good chance that these are the organisms deliberately introduced as the starter culture, i.e., *S. cremoris* and *S. lactis,* which predominate at the beginning of maturation and whose growth establish the conditions necessary for lactobacillus growth *(10)*. Their survival is largely a strain characteristic *(5)*.

No distinction was made between lactobacilli and pediococci growing on MRS but it is almost impossible that pediococci could be responsible for gas defects in experimentally observed cheeses as they are non-gas-forming homofermentative strains *(24)*. The total count of bacteria was significantly higher in rejected cheese than in grade 1 cheese, and this was largely due to lactobacilli as revealed by microscopic examinations. Other organisms were insignificant: yeasts and molds, approximated 10 CFU/g in grade 1 and were $\leq$ 4 CFU/g and 17 CFU/g in grade 1 and rejected cheese, respectively. These organisms did not likely have an important role in late-gassing of Cheddar cheese.

Examination of the chemical composition of Cheddar cheese revealed that moisture: 34.9 $\pm$ 0.8% and 34.8 $\pm$ 0.8, salt in moisture: 4.8 $\pm$ 0.3% and 4.8 $\pm$ 0.4, moisture in non-fat substance: 51.4 $\pm$ 1.2% and 51.9 $\pm$ 1.4 for grade 1 and rejected cheeses, respectively, were normal. There was no significant difference ($P \leq 0.05$) between these values for grade 1 and rejected cheeses. Additionally, the standard deviations of analyzed parameters for the 20 blocks of cheese were negligible. It appears that in this study, the compositional quality of grade 1 and late-gassing cheeses was not detectably different.

CONCLUSION

For young cheeses, lactic acid bacteria counts using Rogosa Agar and MRS at pH 5.5 were lower than those obtained using APT and MRS media, whereas in older cheeses these differences were not obvious. This indicated that as the cheese aged, lactobacilli increased to become a major part of the dominant microflora of these cheeses. In rejected (swollen and fissured) cheeses, lactobacilli were very numerous and included a significant proportion of heterofermentative types which may be responsible for gas defects in these cheeses. Lactic streptococci, coliforms, yeasts and molds, and spore-formers had little or no involvement in the production of gas.

More detailed characterization of the *Lactobacillus* species in grade 1 versus late gassing Cheddar cheese may assist in development of effective methods for control of the cheese ripening process.

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that were conditionally virulent in the adult mice (A. L. Reyes, J. T. Peeler, C. H. Johnson, P. L. Spaulding, and G. N. Stelma, Jr., Abstract, 86th Annual Meeting of the American Society for Microbiology, P2, p. 275) is still questionable. They were not infective by the oral route (Table 1), but they were invasive. It is possible that these strains are unable to survive in the stomach and would not cause septicemia in any host. However, it is also possible that infant mice are not sufficiently immunocompromised to allow these strains to cause systemic infections. In that case, these strains might still be a hazard to severely compromised human hosts via the oral route.

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