Lait 81 (2001) 365–379

Original article

New media for the numeration of cheese surface bacteria

Catherine DENIS[a], Micheline GUEGUEN[b]*, Eric HENRY[b], Delphine LEVERT[a]

[a] Adria Normandie, boulevard du 13 juin 1944, 14310 Villers-Bocage, France
[b] Laboratoire de Microbiologie Alimentaire, sous contrat INRA, Université de Caen, Basse-Normandie, Esplanade de la Paix, 14032 Caen Cedex, France

(Received 29 May 2000; accepted 1 August 2000)

Abstract — The characterisation of naturally occurring bacterial species present in cheese made from raw milk and/or the study of the equilibrium of the floras present during the ripening process necessitates the use of reliable culture media. Currently, there are relatively few methods available for specifically counting these microorganisms in the dairy environment. Three media were developed. These media were compared with Chapman and TSA/NaCl media for their ability to select and to allow the growth of the researched germs in 25 samples of different cheeses (Camembert, Pont l'Évêque, Livarot, Comté, Saint-Nectaire, Salers), as well as in 4 samples of raw milk. The CRBM medium (Cheese Ripening Bacteria Medium) permitted the recuperation of all ripening bacteria; this medium supplemented with bacitracine selected for the staphylococci, while supplemented with furazolidone, it permitted the numeration of corynebacteria present including the *Micrococcaceae*. These media were more selective against undesirable floras (enterobacteria, *Bacillus* spp., streptococci, yeasts and molds) and allowed a better growth of the studied bacteria. For all the types of cheese studied, the specificity of the media was satisfactory. The use of CRBM media therefore appears interesting in view of the microbiological characterisation of milk or cheeses made from raw milk.

corynebacteria / *Micrococcaceae* / cheese / ripening / numeration

Résumé — Nouveaux milieux pour le dénombrement de la flore de surface des fromages. Le recensement des espèces naturellement présentes dans les fromages au lait cru et/ou l'étude de l'équilibre des flores au cours de l'affinage nécessitent de disposer de milieux fiables. Or, il existe relativement peu de milieux de dénombrement spécifiques pour ces micro-organismes dans le domaine laitier. Trois milieux ont été développés. La performance de ces milieux, en terme de sélectivité et d'électivité, a été évaluée pour 25 échantillons de fromages (Camembert, Pont l'Évêque, Livarot,

* Correspondence and reprints
Tel.: (33) 2 31 56 56 21; fax: (33) 2 31 56 61 79; e-mail: gueguen@ibba.unicaen.fr

Comté, Saint-Nectaire, Salers) et pour 4 échantillons de lait cru, en comparaison avec les milieux Chapman et TSA/NaCl. Le milieu CRBM permettait la récupération des bactéries d'affinage dans leur ensemble ; supplémenté en bacitracine, il sélectionnait les staphylocoques tandis que la furazolidone permettait de dénombrer les bactéries corynéformes, y compris les *Micrococcaceae*. Le milieu CRBM et le milieu CRBM modifié par addition d'antibiotiques étaient plus sélectifs vis-à-vis de flores indésirables (entérobactéries, *Bacillus*, streptocoques, levures-moisissures). Ils présentaient par ailleurs une meilleure électivité vis-à-vis des bactéries d'affinage, la diversité des espèces recensées étant plus importante. Pour tous les fromages étudiés, la spécificité des milieux était satisfaisante. L'utilisation des milieux CRBM apparaît intéressante pour la caractérisation microbiologique des laits ou des fromages au lait cru.

bactérie corynéforme / *Micrococcaceae* / fromage / affinage / dénombrement

1. INTRODUCTION

One of the major concerns of industrial cheese producers is the insufficient taste of the products. Now, the organoleptic characteristics of cheeses are largely linked to the development of the ripening flora.

In comparison to lactic acid bacteria, which have been the subject of numerous studies (for example [6–7, 17]), there currently exists relatively few studies towards the ecology of the ripening bacteria present in milk and in their evolution on cheese surfaces. This type of research requires reliable analytical methods for counting, identifying and selecting the microorganisms being studied. This is even more complex as this group is not well defined from a taxonomical point of view and is undergoing constant changes. For example, the *Micrococcus* group has been divided into four genera: *Micrococcus*, *Kocuria*, *Dermacoccus* and *Nesterenkonia* [21]. Recently, it has been proposed to remove the staphylococci from the *Micrococcaceae* family. This family has integrated other genera and has been linked to the corynebacteria [22].

The media described in the literature are especially oriented towards the differentiation of pathogenic staphylococci [3, 5], of clinically originating corynebacteria (potassium tellurite AGAR) or of phytopathogens.

In order to make up for the lack of culture media adapted for cheese originating species, researchers have used media initially directed towards numerating the aerobic mesophilic flora in the food industry (PCA, PCA with milk or modified nutritive AGAR according to Lenoir [10]), or media used in the medical domain dealing with demanding bacteria (brain heart AGAR [16]; Mueller-Hinton [11]; Colombia AGAR). Sometimes these media have added blood or are further enriched in amino acids, in vitamin complexes or with Tween 80 [20] in order to respond to the nutritional needs of the corynebacteria.

Although these very rich media allow a better recuperation of corynebacteria in comparison with the PCA medium [15], they are lacking in selectivity. The addition of antibiotics generally prevents the growth of yeasts and of Gram-negative flora; however, the undesirable Gram-positive flora (*Streptococcaceae*, *Lactobacillaceae*...) remains, which is a problem especially as certain surface bacteria will grow slower.

Media have been made selective against this undesirable Gram-positive flora by the addition of NaCl on account of the halotolerant character of ripening bacteria present on the surface of cheeses. In this way, the PCA medium enriched with 6.5% NaCl has been used by Accolas et al. [1]

for Camembert, while Seiler [19] chose PCA with only 3% NaCl for Cheddar. The Chapman medium, initially used for staphylococci (NaCl content of 7.5%), has also often been used [10] with, in certain cases, the addition of antifungicide like fongizone [12, 13].

Due to the sensitivity of corynebacteria to the acidification of the medium resulting from the development of staphylococci and remaining floras such as lactic acid bacteria and enterococci, numerous authors have buffered the media with calcium carbonate [23, 25] or with phosphate [25].

Other authors have searched for a way to come closer to the physical and chemical conditions present in curdled milk by replacing the carbon source (in general glucose) by sodium lactate [8, 9]. This compound corresponds, in cheese, to the substrate for growth of ripening flora; furthermore, it has the advantage of limiting the acidification of the medium.

The use of these different media shows that it is difficult to have a relatively complete recuperation of the surface flora as well as to analyse the counts, in the frame of ecological microbial studies, as the undesirable flora can sometimes be numerous.

This research has therefore been aimed at optimising three new counting media adapted to cheese: the first being for all surface bacteria, the second for coryneform bacteria (including *Micrococcus* and related genera) and the third for staphylococci.

2. MATERIALS AND METHODS

2.1. Microorganisms and culture conditions

Ten collection strains were used: 6 *Micrococcaceae* (*Micrococcus luteus* UCMA 741AL, *Kocuria rosea* IP 7115, *Kocuria varians* IP 8173, *Staphylococcus caseolyticus* ATCC 13548, *Staphylococcus equorum* IP 103502, *Staphylococcus xylosus* UCMA 852) and 5 coryneform bacteria (*Arthrobacter nicotianae* WS 2231, *Brevibacterium linens* ATCC 9175, *Corynebacterium variabilis* ATCC 33010, *Corynebacterium species* WS 2220, *Microbacterium lacticum* ATCC 8180). Approximately 100 strains of ripening bacteria isolated from milk and diverse types of cheeses were also tested.

Twelve members of the undesirable flora were selected: *Enterococcus faecalis* IP 5855, *Streptococcus bovis* UCMA 5623, *Bacillus subtilis* UCMA 1, *Bacillus cereus* UCMA A30, *Leuconostoc mesenteroides* UCMA 32, *Weissella paramesenteroides* UCMA 235, *Listeria monocytogenes* UCMA 205. Wild strains isolated from dairy products were also studied: *Hafnia alvei*, *E. coli*, *Pseudomonas fluorescens*, *Lactobacillus plantarum*, and *Lactobacillus casei*.

The strains were stored at –80 °C in cryovials in TSBY medium with 15% glycerol.

For each experiment, 2 successive precultures were made in TSBY broth and incubated under agitation for 24–48 h (the cultures were used during the stationary phase).

2.2. Development of the CRBM media (Cheese Ripening Bacteria Media)

For the development of the base medium, the following components were tested on all or part of the microorganisms: carbon source: D-L sodium lactate (Sigma, St-Quentin Fallavier, France) (60% solution) at 1.5% or 2% with or without the addition of glucose (Prolabo, Fontenay-sous-bois, France) (0.5%); nitrogen source: casein tryptopeptone (BBL distributed by AES, Combourg, France) at 1% with or without the addition of yeast extract (BBL)

(0.7%); Tween 80 (Sigma) at 0.1%; NaCl (Sigma) (0% to 7%); buffers: 50 mmol·L^{-1} Tris (Sigma), 50 mmol·L^{-1} Tris maleate (Sigma), 50 and 200 mmol·L^{-1}, calcium carbonate, 15 and 29 mmol·L^{-1} dipotassium phosphate, 15 mmol·L^{-1} dipotassium phosphate (Panreac, Touzart & Matignon, Vitry-sur-Seine, France) +0.2% sodium glycerophosphate (Merck, Fontenay-sous-bois, France), 23 mmol·L^{-1} dipotassium phosphate +0.25% sodium glycerophosphate; vitamin complex from Kao and Michayluk (Sigma) incorporated at 1% just before use of the medium; $MgSO_4$ $7H_2O$ (Prolabo) at 0.2% and 0.5%; calcium chloride (Prolabo) $CaCl_2$ $2H_2O$ (0.5 to 2 g for 100 g) or calcium carbonate $CaCO_3$ (0.5 to 0.75 g for 100 g); 0.01% choline (Prolabo).

For selectivity of the base medium: natamycine (Delvocid Gist-Brocades, Seclin, France) at 9 and 18 mg·L^{-1}; nalidixic acid Sigma at 40 and 60 mg·L^{-1}; lithium chloride at 0.5% and 1.5%.

For the development of media for the differentiation of cheese ripening bacteria: bacitracine (Sigma) 5 and 40 mg·L^{-1} or furazolidone (Sigma) 1 and 20 mg·L^{-1} (the furazolidone solution is prepared in acetone and conserved away from light).

Once optimised, the CRBM base medium was composed of the following compounds (g·L^{-1}), in order of incorporation: dipotassium phosphate, 5; sodium lactate (60% solution), 33.4; $MgSO_4$ $7H_2O$, 2; tryptone, 10; yeast extract, 7; NaCl, 50; Tween 80, 1; agar agar, 15; adjusted to pH 7.3 and autoclaved at 120 °C, 15 min.

Two antibiotics were added at the moment of usage of the medium: natamycine 9 mg·L^{-1} and nalidixic acid 40 mg·L^{-1}, for the elimination of the fungal flora, Gram-negative bacteria and *Bacillus* respectively.

Incubation was carried out in aerobic conditions at 25 °C for 5 d, then at room temperature and daylight for 4 to 15 d to allow for maximum development of colonies and pigmentation. Given the incubation conditions, 20 mL of media was put into 90 mm diameter Petri dishes.

2.3. Reference media

Two media were compared: Chapman medium (BK O30, Biokar, Pantin, France) with or without a supplement of 2.5% of yeast extract (AEB 171 106, AES, Combourg, France) and TSA (Tryptone Soja Agar) medium (AEB 152 852, AES, Combourg, France) enriched with NaCl (5%) and with natamycine (9 mg·L^{-1}).

The Chapman medium with a concentration of 7.5% NaCl is usually used for the numeration of the halotolerant flora in dairy products. This medium has been tested with or without a supplement of yeast extract because of the nutritional requirements of corynebacteria. For the same reasons, TSA medium has been preferred to PCA medium. TSA medium is enriched with 5% NaCl as for the CRBM medium to allow a better recovery of surface bacteria which are less salt resistant. Natamycine (9 mg·L^{-1}) is also added to TSA/NaCl medium.

Incubation conditions were identical to those used for the CRBM media.

2.4. Origin and preparation of samples

For the comparison between the CRBM and Chapman media: 4 raw milk samples from Basse-Normandie with a quality level super A (< 50 000 germs per mL and < 25 000 somatic cells per mL) and 8 samples of ripened cheeses made from raw milk (Comté, Pont l'Évêque, Livarot, Camembert) obtained from different sellers were tested.

For the comparison between the CRBM and TSA/NaCl media: 17 samples of either industrial or farm cheeses (Comté, Pont

l'Évêque, Livarot, Camembert, Salers and Saint-Nectaire) were collected at different stages of the ripening process at the production sites.

The analyses were carried out on 10 mL of milk diluted to 1/10 in tryptone-salt or on 10 g of cheese rind diluted to 1/10 in a solution of dipotassium phosphate.

2.5. Microbial numeration

The cultures were plated on the surface of the media using a spiral plater. After the incubation period, the numeration was performed on Petri dishes showing a good separation of colonies (between 30 and 300 colonies per plate). The diverse types of colonies that appeared were counted according to their speed of appearance and their traits (morphology, pigmentation, opaqueness).

2.6. Phenotypic identification test

The square root corresponding to the number of each type of colony was taken in each type of medium. A primary purification was done by two successive isolations on TSA medium.

The identification of more than 900 isolates was carried out by following these criteria:

– mobility and morphology on fresh culture (24 to 48 h) and old culture (1 week) in a way to observe an eventual change (Mulder and Antheunisse, 1963; cited by Richard and Zadi [14]);
– Gram coloration on young culture;
– for the Gram-positives: (i) if catalase positive cocci, a differentiation test using antibiotics was used (furazolidone 20 $\mu g \cdot mL^{-1}$ + bacitracine 0.04 UI according to Bergère and Tourneur [4] and inoculation of an API 20 or 32 Staph gallery (Biomérieux, Lyon, France), (ii) if catalase negative cocci, they were assumed to be enterococci or streptococci, (iii) if bacilli, a catalase test was first used to differentiate between lactobacilli and coryneforms, then the coryneforms were studied using the API Coryne system (Biomérieux, Lyon, France).

2.7. Statistical analysis

To compare the numerations on the different media, a mean comparison test (statgraphics plus) has been done. Twelve samples have been analysed for the comparison between CRBM and Chapman, and 17 samples for the comparison between CRBM and TSA/NaCl with 2 repetitions for each sample.

3. RESULTS

3.1. Validation of CRBM medium in milk and cheese: comparison between the Chapman and TSA/NaCl media

3.1.1. Quantitative aspect: numeration

Numerations on Chapman and CRBM media showed that the bacterial population was between 7.9×10^2 and 6.4×10^3 $CFU \cdot mL^{-1}$ for the 4 raw milks, between 3.5×10^9 and 3.3×10^{10} $CFU \cdot g^{-1}$ for the samples of ripened Pont l'Évêque, Livarot and Comté and a bit less high for Camembert.

Numerations on TSA/NaCl and CRBM media showed that the population was between 2.4×10^8 and 8.3×10^{10} $CFU \cdot g^{-1}$, but variations were observable according to the cheese studied and its ripening level (Tab. I).

No significant difference at a threshold of $P = 0.05$ was observed between CRBM medium and the two reference media. Moreover, the CRBM medium showed a

Table I. Comparison of counts of cheese surface bacteria on CRBM and TSA/NaCl media.

Cheese	Origin	CRBM	TSA/NaCl
Pont l'Évêque	industrial / ripened	7.7×10^8	7.3×10^8
	industrial / half-ripened	5.9×10^9	9.3×10^9
	farm / ripened	2.4×10^8	5.5×10^8
	farm / half-ripened	1.2×10^{10}	1.3×10^{10}
Livarot	industrial / ripened	7.1×10^9	1.2×10^{10}
	industrial / half-ripened	1.1×10^{10}	1.5×10^{10}
	farm / ripened	4×10^{10}	8.3×10^{10}
	farm / half-ripened	2.4×10^{10}	2.6×10^{10}
Camembert	industrial / ripened	2.6×10^9	8.5×10^9
	industrial / half-ripened	3.4×10^9	5.9×10^9
	farm / ripened	4.9×10^8	7.1×10^8
	farm / half-ripened	1.3×10^9	2.4×10^9
Comté	4 months ripening	5.9×10^9	7.7×10^9
	6 months ripening	5.3×10^9	7.3×10^9
	13 months ripening	3.3×10^{10}	5.7×10^{10}
Saint-Nectaire	ripened	1.6×10^{10}	3.1×10^{10}
Salers	ripened	4.7×10^9	7.7×10^9

superior size of colonies, more marked pigmentation and a larger variety of morphologies.

3.1.2. Qualitative aspect: medium selectivity and diversity of the collected species

3.1.2.1. Comparison between Chapman medium supplemented or not with yeast extract and CRBM medium

The comparison between Chapman and CRBM media was carried out, on one hand, by studying the proportions between micrococci/staphylococci, coryneform bacteria (rods) and undesirable floras and, on the other hand, by studying the intensity of colony pigmentation.

The proportions between floras differed from one medium to another (Figs. 1 and 2).

Overall, the CRBM medium was much more selective than the Chapman media was.

The nature of the counted undesirable germs differed according to the medium (lactic acid bacteria and enterococci on CRBM with small colonies easy to spot; yeasts and *Bacillus* spp. essentially on Chapman medium).

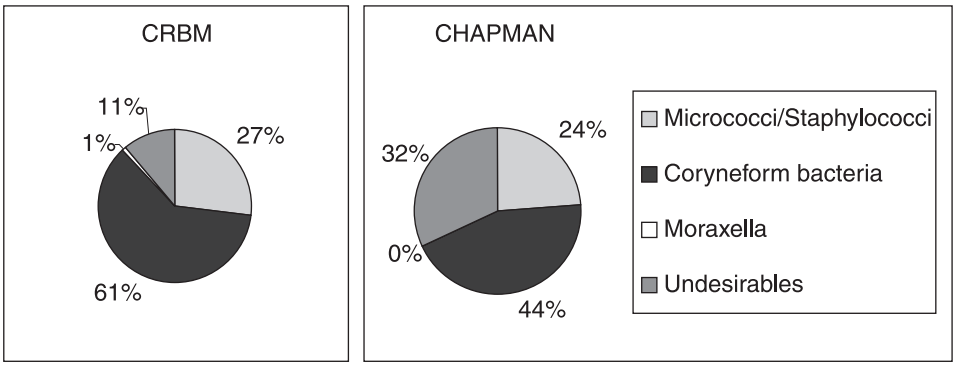

Figure 1. Distribution of the counted floras from raw milk on CRBM and Chapman media (average of the results obtained for 4 samples of milk obtained from 4 different farms).

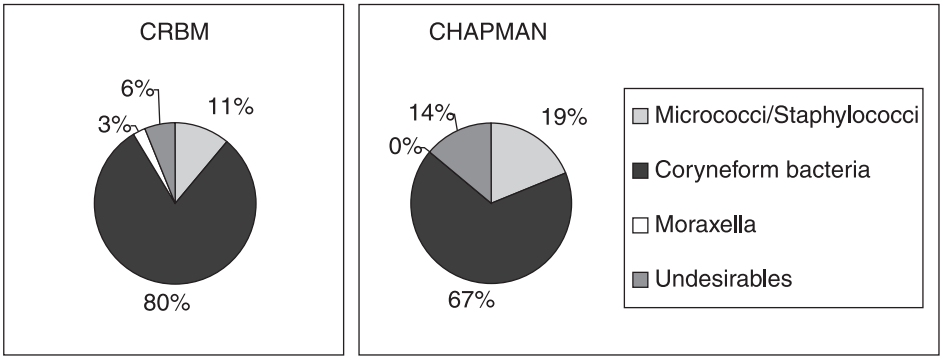

Figure 2. Distribution of the counted floras from cheeses on CRBM and Chapman media (average of the results obtained for 8 cheeses: Pont l'Évêque, Livarot, Camembert, Comté).

Concerning the surface bacteria, whose pigmentation on CRBM medium was more pronounced, the coryneforms were predominant. Furthermore, the *Staphylococcus* spp. were more frequent than the *Micrococcus* spp.

The phenotypic characterisation of more than 200 isolates systematically permitted us to take an inventory of more biotypes on CRBM medium (7 to 18) than on Chapman medium (2 to 11) (Tab. II). From the 200 isolates, 84 strains representative of the different biotypes were frozen at –80 °C and were used in a growth test in the presence of NaCl (5 to 7.5%). The majority of the isolates resisted at 7.5% NaCl but 4 of them isolated from CRBM medium were sensitive to levels greater than 6% of salt and therefore would not have been detected on Chapman medium.

CRBM medium gave a more complete listing of the different species present due to its ability to have a better bacterial growth and diversity.

Table II. Identification of the surface bacteria isolated from raw milk and cheeses; ( ) number of different biotypes.

Origin	Chapman	CRBM
Raw milk	*Brevibacterium* (1) *Corynebacterium* (6) *Staphylococcus lentus* (1) *St. saprophyticus* (2) *St. xylosus* (1)	*Brevibacterium* (3) *Corynebacterium* (7) *Staphylococcus lentus* (1) *St. hyicus* (2) *St. sciuri* (2) *Micrococcus* sp.(2) *Moraxella* (1)
Comté	*Brevibacterium* (4) *Corynebacterium* (2)	*Brevibacterium* (4) *Corynebacterium* (1) *Staphylococcus capitis* (1) *Kocuria varians* (1) *Moraxella* (1)
Pont l'Évêque	*Brevibacterium* (1) *Corynebacterium* (1) *Staphylococcus capitis* (1) *St. sciuri* (1) *St. xylosus* (1)	*Brevibacterium* (2) *Corynebacterium* (1) *Staphylococcus sciuri* (1) *St. xylosus* (1) *Kocuria varians* (2) *Kocuria rosea* (1)
Livarot	*Brevibacterium* (2) *Corynebacterium* (1) *Staphylococcus capitis* (1) *St. saprophyticus* (2)	*Brevibacterium* (6) *Corynebacterium* (6) *Kocuria varians* (7)
Camembert	*Corynebacterium* (1) *Staphylococcus capitis* (1)	*Brevibacterium* (2) *Corynebacterium* (1) *Staphylococcus* sp. (1) *St. xylosus* (2) *St. sciuri* (1) *St. capitis* (1) *Moraxella* (1)

3.1.2.2. Comparison between TSA/NaCl and CRBM media

The comparison between TSA/NaCl and CRBM media was based on colony pigmentation (once again more prominent on CRBM medium), the selectivity against the undesirable floras and on the proportions of *Micrococcus* spp. and corynebacteria as well as *Staphylococcus* spp. (Fig. 3). For 2 cheeses, Salers and Saint-Nectaire, no undesirable flora was detected on the medium. For the other cheeses, Pont l'Évêque, Livarot, Camembert and Comté, the undesirable flora level was lower on CRBM medium (20%, 1%, 26% and 18% respectively) than on TSA/NaCl medium (34%, 14%, 42% and 34% respectively). The nature of the detected undesirable flora is described in Table III. The CRBM medium was more selective than the TSA/NaCl medium. For the TSA/NaCl medium, the

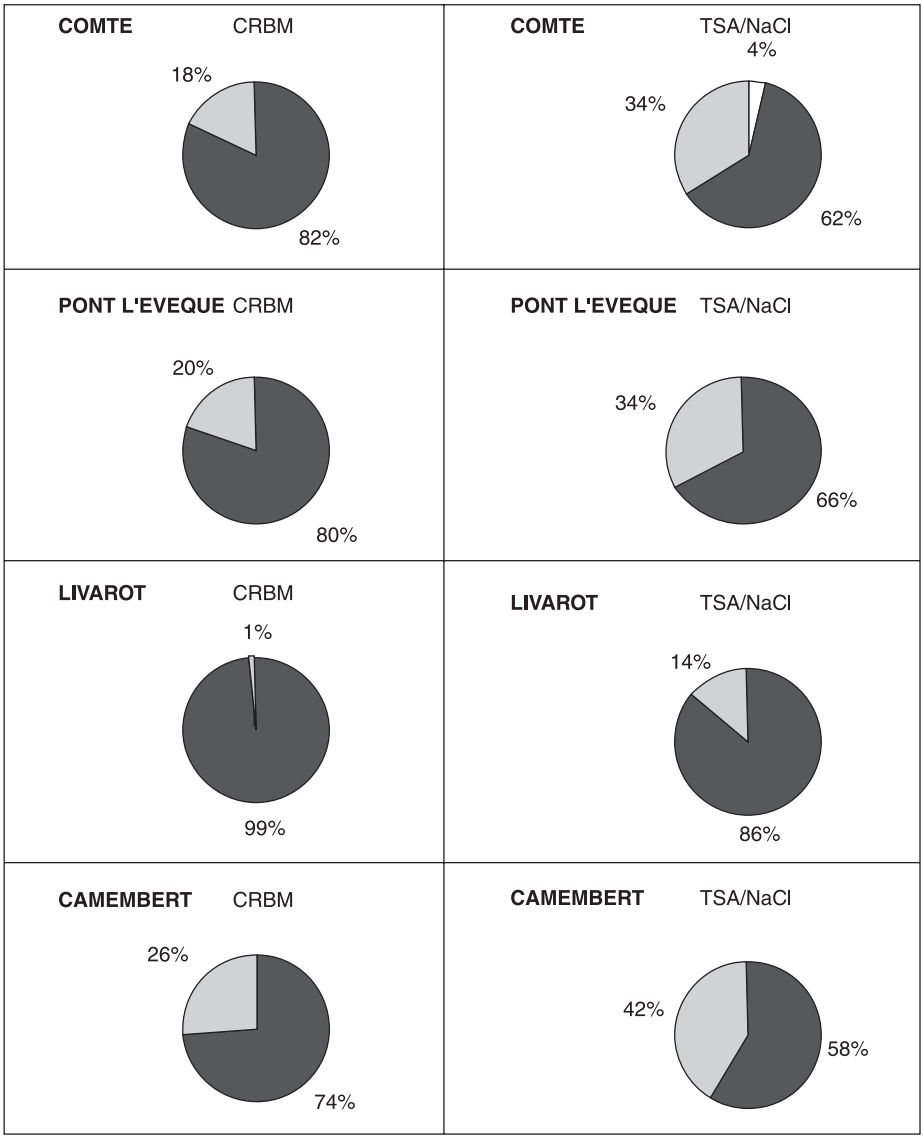

Figure 3. Distribution of the counted floras from cheeses on CRBM and TSA/NaCl media (Comté: average of the results obtained for 3 cheeses; Pont l'Évêque, Livarot, Camembert: average result from 4 cheeses). ■ Corynebacteria / *Micrococcus*, □ *Staphylococcus*, ▭ Undesirable flora.

Table III. Distribution of the undesirable floras (percentage of growing microorganisms); B, *Bacillus*, EB, enterobacteria, EC, enterococci, L, lactobacilli, M, Molds, Y, Yeast.

Cheese (1)	Medium (2)			
	TSA/NaCl	CRBM	CRBM/furazolidone	CRBM/bacitracine
Pont l'Évêque	B 2 EB 18 EC 6 Y 9	 EC 1 Y 19	 Y 24	B 8 EB 28 Y 15
Livarot	 EB 9 L 5	 EB < 1 Y < 1	 	B 15 EB 74 Y 11
Camembert	 EB 12 EC 9 L 12 Y 9	 EB < 1 EC 2 L 23 Y 1	 L 5 Y 5 / M 8	B < 1 EB 33 EC 22 M 2
Comté	 L 32 Y 2	 L16 Y 2	 L 25	EB 19 L 28
Saint-Nectaire	no undesirable floras			
Salers	no undesirable floras			

(1) Pont l'Évêque, Livarot and Camembert (average of the results obtained for 4 samples), Comté (average of the results obtained for 3 samples), Saint-Nectaire and Salers (one sample).
(2) CRBM with nalidixic acid (40 mg·L^{-1}) and natamycine (9 mg·L^{-1}); CRBM/furazolidone (10 mg·L^{-1}) with natamycine (9 mg·L^{-1}); CRBM/bacitracine (30 mg·L^{-1}) with natamycine (9 mg·L^{-1}) for Pont l'Évêque, Livarot and Camembert and with nalidixic acid (40 mg·L^{-1}) and natamycine (9 mg·L^{-1}) for Comté, Salers and St-Nectaire.

undesirable flora was principally represented by lactobacilli, enterobacteria and yeasts while with the CRBM medium, it was essentially lactobacilli and yeasts but in lower numbers.

Concerning the surface flora, the examined samples of Pont L'Évêque, Livarot and Camembert showed a large dominance of *Micrococcus* spp. and corynebacteria over the staphylococci on either TSA/NaCl or CRBM medium. For the Comté, Saint-Nectaire and Salers samples, the staphylococci, although in a minority, represented 0.3% to 16% of the cheese ripening bacterial population (Fig. 4).

3.2. Differentiation within the surface floras: validation of the CRBM/furazolidone and CRBM/bacitracine media

The sensitivity of the collection strains and of the cheese originating strains towards the two antibiotics was studied with the intention of differentiating the micro-

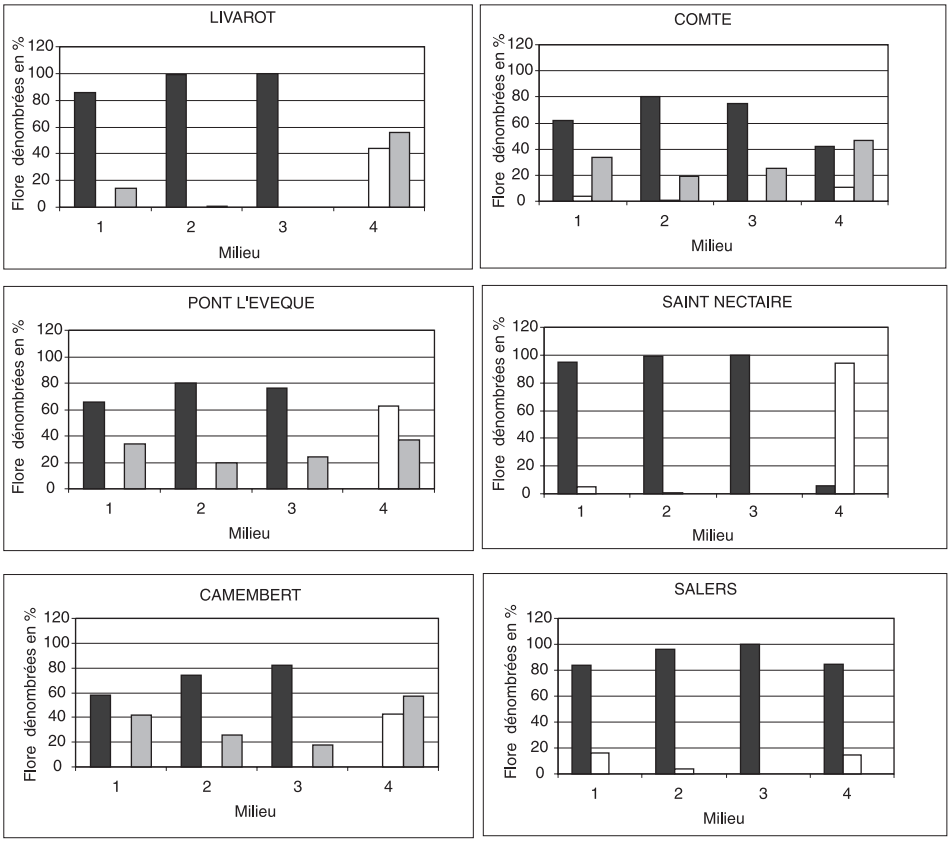

Figure 4. Distribution of the counted floras from the cheeses on CRBM, CRBM modified and TSA / NaCl media. Livarot, Pont l'Évêque and Camembert: average of the results obtained for 4 samples of cheese (2 different sites and 2 degrees of ripening: half-ripened and ripened); Comté: average of the results obtained for one site of production and 3 durations of ripening; Saint-Nectaire and Salers: one sample of each ripened cheese. ■ Corynebacteria / *Micrococcus*, □ *Staphylococcus*, ▭ Undesirable flora. 1 = TSA + NaCl (5%) with natamycine, 2 = CRBM with nalidixic acid and natamycine, 3 = CRBM / furazolidone with natamycine, 4 = CRBM / bacitracine with natamycine.

bial groups composing the bacterial flora present on the surface of cheeses.

3.2.1. Validation of CRBM + furazolidone medium

Tested with either collection or cheese collected pure strains, the CRBM medium with furazolidone (10 mg·L^{-1}) inhibited all staphylococci strains while recuperation levels of *Micrococcus* / *Kocuria* spp. and corynebacteria were 80% to 100% (Tab. IV). A decrease in concentration of furazolidone (5 mg·L^{-1}) did not improve the performance of the medium; rather it allowed a growth of 40% of the staphylococci strains.

A furazolidone concentration of 10 mg·L^{-1} was kept for the validation of the medium for cheese. Taking into account the

Table IV. Behaviour of *Staphylococcus* and corynebacterial strains (including *Micrococcaceae*) in the presence of furazolidone or bacitracine.

Genus	Origin	N [1]	Addition to CRBM:			
			5 mg·L^{-1} furazolidone	10 mg·L^{-1} furazolidone	30 mg·L^{-1} bacitracine	40 mg·L^{-1} bacitracine
Staphylococcus	Collection	8	0	0	8[2] (100 [3])	8[2] (100[3])
	Cheese	24	13[2] (54[3])	0	19[2] (79[3])	17[2] (71[3])
	Total	32	13[2] (40[3])	0	27[2] (84[3])	25[2] (78[3])
Micrococcus	Collection	3	3 (100)	3 (100)	0	0
and related	Cheese	19	15 (79)	15 (79)	0	0
genera	Total	22	18 (82)	18 (82)	0	0
B. linens	Collection	1	1 (100)	1 (100)	0	0
	Cheese	14	14 (100)	14 (100)	1 (7)	1 (7)
	Total	15	15 (100)	15 (100)	1 (7)	1 (7)
Corynebacteria	Collection	5	4 (80)	4 (80)	0	0
	Milk/Cheese	52	44 (85)	44 (85)	2 (4)	2 (4)
	Total	57	48 (84)	48 (84)	2 (3)	2 (3)

[1] Number of strains tested; [2] number of growing strains; [3] percentage of growing strains.

inhibitory character of the furazolidone towards certain undesirable bacteria, natamycine was added in the base medium but not nalidixic acid.

The growth of enterobacteria, *Bacillus* spp. and *Staphylococcus* spp. was inhibited. The medium was therefore specific towards the *Micrococcus/Kocuria* and corynebacteria genera (Fig. 4). Lactobacilli sometimes interfered on the medium but the colonies (small and translucid) were easily detected. On the other hand, yeasts were able to develop in spite of the use of natamycine.

3.2.2. Validation of CRBM + bacitracine medium

On the pure cultures, the CRBM medium with bacitracine (30 mg·L^{-1}) allowed the elimination of all micrococci and related genera strains as well as 96% of the corynebacteria strains; the recuperation level of the staphylococci strains was 80% to 100% (Tab. IV). A lower concentration in bacitracine (25 mg·L^{-1}) did not increase this rate, but instead 20% of micrococci strains and 14% of corynebacteria strains developed (results not shown). An increase of the bacitracine concentration (40 mg·L^{-1}) gave a larger inhibition of the corynebacteria but also further limited the recuperation of the staphylococci (Tab. IV).

With the cheeses, the CRBM medium containing bacitracine (30 mg·L^{-1}) was first tested without nalidixic acid. Under these conditions, the medium lacked selectivity against the undesirable floras. Contrary to the furazolidone, the bacitracine was not able on its own to eliminate Gram-negative bacteria and *Bacillus* spp. The comparison between the CRBM/baci-

tracine medium with and without nalidixic acid was carried out for 3 different cheeses (results not presented) and confirmed that the incorporation of nalidixic acid in this medium is recommended.

The efficiency of the bacitracine supplementation for recuperating the staphylococci is illustrated by Figure 4. Being the minority in all the cheese samples analysed, they represented up to 95% of the counted flora on CRBM/bacitracine.

4. DISCUSSION

The performance of the CRBM medium was compared to that of two other media currently used for the numeration of cheese surface bacteria. The Chapman medium was relatively selective on account of the high level of NaCl (75 $g \cdot L^{-1}$) but it did not include antibiotics; the composition of the TSA/NaCl medium was closer to that of CRBM in terms of selective compounds (NaCl 50 $g \cdot L^{-1}$ and addition of natamycine 9 $mg \cdot L^{-1}$).

Compared to these two media, the CRBM medium composition was much closer to that of a cheese and allowed a better growth and diversity of the ripening bacteria. Moreover, it was much more selective against the undesirable floras, which were inhibited or gave only small and easily detectable colonies (lactic acid bacteria). However, the yeasts, in some cases, interfered in an important way on the medium even though there was added natamycine. This problem may however be partially avoided by the utilisation of increased concentrations of added natamycine or of other antibiotics (for example amphotericine).

The resistance towards furazolidone (20 $\mu g \cdot mL^{-1}$) and bacitracine (0.04 UI) constituted a discrimination test for the *Micrococcus* and *Staphylococcus* genera [18]. A peptone agar with 20 µg furazolidone$\cdot mL^{-1}$ is recommended for the separation of staphylococci from micrococci [24]. For this reason, these two antibiotics were chosen for the differentiation of the surface bacteria. The results obtained for the collection and wild types pure strains as well as for the cheeses showed that the addition of bacitracine (30 $mg \cdot L^{-1}$) favoured the recuperation of *Staphylococcus* spp. while the furazolidone (10 $mg \cdot L^{-1}$) selected for *Micrococcus* spp. and related genera like the coryneform bacteria. These results were in agreement with the recent evolution in bacterial taxonomy that tends to show that the *Micrococcus* spp. are much more closely related to the coryneform bacteria than to the staphylococci [2, 22].

The population of species present in the different media showed that the coryneform bacteria were majorly present followed by *Staphylococcus* spp. and then *Micrococcus*/*Kocuria* spp.

For the soft cheeses (Pont l'Évêque, Livarot, Camembert), the counted surface bacteria on TSA, CRBM and CRBM/furazolidone were essentially coryneform bacteria (including *Micrococcus* and related genera). The staphylococci were only detected on CRBM/bacitracine medium while no coryneform bacteria were isolated from this medium. Compared to the reference media, the CRBM/bacitracine medium was the only one allowing for the recuperation of a large number of staphylococci and that also gave a total inhibition of the corynebacteria from the soft cheeses tested. For the hard cheeses, this medium performed equally well; however, the presence of coryneform bacteria was sometimes noticed in large quantities. An anaerobic incubation could allow an increase of the specificity of this medium.

The CRBM/furazolidone medium inhibited the *Staphylococcus* spp. In this medium, the suppression of nalidixic acid allowed better colony formation and did not affect its selectivity; therefore the CRBM/furazolidone may be used without nalidixic acid.

On the contrary, concerning the CRBM/bacitracine medium, the addition of nalidixic acid is recommended. This difference in behaviour is not to be linked to the action of the 2 antibiotics which have no effect with Gram-negative bacteria. It is likely that this was due to competition events: the staphylococci being the minority, the undesirable floras were favoured.

In conclusion, the CRBM media appeared to be an interesting tool for studying the microbial ecology of surface bacteria present on cheeses. The CRBM media were not only more selective against undesirable floras than the Chapman and TSA/NaCl media but they also gave a better colony pigmentation thus allowing a better inventory of the population diversity.

In raw milk, the use of CRBM media allowed the recuperation of ripening bacteria even though they were clearly less dominant than in the food products. This can be of great interest for the study of milk biodiversity.

ACKNOWLEDGEMENTS

This research was accomplished in the frame of the ACTIA program (Association de Coordination Technique pour l'Industrie Agroalimentaire, Paris, France). The authors are indebted to Arilait Recherches (Paris, France) for constructive scientific discussions and financial support.

REFERENCES

[1] Accolas J.P., Melcion D., Vassal L., Étude de la flore superficielle des fromages de Gruyère et de Beaufort, in: Compte rendu du 20e congrès International de Laiterie, Paris, 1978, pp. 773–774.

[2] Amiel C., Potentialités de la spectroscopie infrarouge à transformée de Fourier (IRTF) pour l'identification et la caractérisation de bactéries d'implication laitière, Thèse de doctorat de l'Université de Caen - Basse-Normandie, 2000, 140 p.

[3] Baird Parker A.C., An improved diagnostic and selective medium for isolating coagulase positive *Staphylococci*, J. Appl. Bacteriol. 25 (1962) 12–19.

[4] Bergère J.L., Tourneur C., Les bactéries de surface des fromages, in: Hermier J., Lenoir J., Weber F. (Eds.), Les groupes microbiens d'intérêt laitier, CEPIL, Paris, 1992, pp. 127–163.

[5] Chapman G.H., The significance of sodium chloride in studies of *Staphylococci*, J. Bacteriol. 50 (1945) 201–203.

[6] Corroler D., Mangin I., Desmasures N., Gueguen M., An ecological study of lactococci isolated from raw milk in Camembert cheese registered designation of origin area, Appl. Environ. Microbiol. 64 (1998) 4729–4735.

[7] Desmasures N., Mangin I., Corroler D., Gueguen M., Characterisation of lactococci isolated from raw milk produced in the Camembert region of Normandy, J. Appl. Microbiol. 85 (1998) 999–1005.

[8] Ferchichi M., Production de méthane-thiol à partir de L-méthionine par *Brevibacterium linens* CNRZ 918. Induction, caractérisation, nature des systèmes enzymatiques, Thèse de docteur ès sciences, Université de Paris VII, 1984, 96 p.

[9] Lecocq J., Interaction entre *Geotrichum candidum* et *B. linens*. Influence des facteurs intervenant en technologie fromagères, Thèse de docteur ès sciences, Université de Caen, 1991, 270 p.

[10] Lenoir J., La flore microbienne du Camembert et son évolution au cours de la maturation, Lait 43 (1963) 262–271.

[11] Mueller H.J., Hinton J., A protein free medium for primary isolation of *Gonococcus* and *Meningococcus*, Proc. Soc. Exp. Biol. Med. 48 (1941) 330–333.

[12] Piton C., Évolution de la flore microbienne de surface de gruyère de Comté au cours de l'affinage, Lait 68 (1988) 419–433.

[13] Richard J., Évolution de la flore microbienne à la surface des Camemberts fabriqués avec du lait cru, Lait 64 (1984) 496–520.

[14] Richard J., Zadi H., Inventaire de la flore bactérienne dominante des Camemberts fabriqués avec du lait cru, Lait 63 (1983) 25–42.

[15] Richard J., Moller A.H., Gratadoux J.J., Rapid method for selecting appropriate solid media for the counting of aerobic microorganisms, J. Appl. Bacteriol. 54 (1983) 329–334.

[16] Rosenow E.C., Studies on elective localization. Focal infection with special reference to oral septis, J. Dental Res. 1 (1919) 205–249.

[17] Salama S.M., Musafija-Jeknic T., Sandine W.E., Giovannoni S. J., An ecological study of lactic acid bacteria: isolation of new strains of *Lactococcus* including *Lc. lactis* subsp. *cremoris*, J. Dairy Sci. 78 (1995) 1004–1017.

[18] Schleifer K.H., Wesley E.K., A simple test system for the separation of *Staphylococci* from *Micrococci*, J. Clin. Microbiol. 1 (1975) 337–338.

[19] Seiler M., Identification of cheese smear coryneform bacteria, J. Dairy Res. 53 (1986) 439–449.

[20] Skerman T.M., Jayne-Williams D.J., Nutrition of coryneform bacteria from milk and dairy source, J. Appl. Bacteriol. 29 (1966) 167–178.

[21] Stackebrandt E., Koch C., Gvozdiak O., Schumann P., Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Dermatococcus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend., Int. J. Syst. Bacteriol. 45 (1995) 682–692.

[22] Stackebrandt E., Rainey F.A., Ward-Rainey N.L., Proposal for a new hierarchic classification system, *Actinobacteria* classis nov., Int. J. Syst. Bacteriol. 47 (1997) 479–491.

[23] Toolens H.P., Koning Theune W., A selective medium for the detection of *Brevibacterium linens* in cheese, Milchwissenschaft 25 (1970) 79–83.

[24] von Rheinbaben K.E., Hadlok R.M., Rapid distinction between micrococci and staphylococci, Antonie van Leeuwenhoek 47 (1981) 41–51.

[25] Zadi H., Étude de la flore bactérienne non lactique des camemberts fabriqués de façon traditionnelle, Thèse de doctorat de l'Université de Paris-Sud, 1981, 54 p.