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Selective *Lactococcus* Enumeration in Raw Milk*

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ABSTRACT

The *Lactococcus* diversity in cow and goat raw milk was investigated. To do so, a protocol had to be established for the specific enumeration of lactococci. Eight agar media and one control medium were analysed to compare their proficiency in evaluating the *Lactococcus* population in raw milk: M17 Nal, Elliker, modified Elliker, PCA + milk, modified KCA, modified Chalmers, Turner, FSDA. The M17 medium was used as reference. Eighteen pure strains were tested on these media for their selectivity towards lactococci: six *Lactococcus* species or subspecies, three *Leuconostoc*, three *Enterococcus*, two *Lactobacillus*, one *Streptococcus thermophilus*, one *Pseudomonas fluorescens*, one *Escherichia coli* and one *Staphylococcus aureus.* All these bacteria were chosen for their regular presence in raw milk. The KCA medium proved to be the most selective towards lactococci, on condition that 1) we discriminated the colonies using the catalase test and 2) we subtracted the *Enterococcus* population counted on BEA. However, it was not possible to separate the *Streptococcus* from the *Lactococcus* colonies on KCA. The "*Lactococcus*-like" population including these two genera was estimated at a mean level of 3.18 log(cfu)/mL and 4.14 log(cfu)/mL in cow and goat raw milk respectively. This is consistent with the data already published.

Keywords: Lactococcus; Culture Media; Raw Milk; LAB; Modified KCA

1. Introduction

Raw milk include different microflora traditionally grouped into three categories: positive, negative and neutral. Technological microflora is considered as positive. Among them, lactic acid bacteria (LAB), *i.e. Lactococcus*, *Leuconostoc*, homo and heterofermentative *Lactobacilli*, *Pediococcus*, and cheese surface bacteria—*Micrococcus*, non-pathogenic *Staphylococcus*, yeasts and moulds—are generally referred to. Spoiling bacteria—negative microflora—include for instance the *Pseudomonas* and *Escherichia* genera. Neutral bacteria, among them many *Archaebacteria*, are considered to have no effect on milk quality [1,2]. Raw milk LAB are known to contribute positively to cheese making. Despite being at a much lower level compared with the starter bacteria (3 log(cfu)/mL vs 6 to 7 log(cfu)/mL), wild LAB are able to participate in the acidification step and in the ripening [3,4] reaching levels as high as 7 - 8 log(cfu)/g [5]. Their enzymes help to modify the physico-chemical and biochemical environment of the cheese which allows the aromatic balance to develop [6]. In light of these observations, cheese makers are divided over the necessity to control pathogens, on the one hand, by lowering the total bacterial count and their will, on the other, to favour positive microflora [7,8]. This position which has proven to be antinomic until now can be justified by two explanations. The microbial flux within the farm which leads to the microbial enrichment of the milk is still unknown. A plate medium designed for the specific enumeration of positive microflora is lacking. This is particularly true for the *Lactococcus* population.

Lactococci, Qualified Presumption of Safety (QPS) microorganisms, are a technological microflora of importance. Wild lactococcal strains rapidly grow during the first steps of cheese making due to their proteolytic enzymes. Through the production of lactic acid, they participate in curd formation and contribute positively to the taste and texture characteristics of the cheeses [9]. In spite of these interesting technological abilities, the level of lactococci in raw milk is still unknown. No specific medium in which to count them is currently available because of the complex nutritional requirements of these bacteria. Consequently, rich non-selective media are used to study them. But these agar plates allow many other bacteria to form colonies exhibiting the same morpho-

*Diversity of the *Lactococcus* type population in cow and goat's raw milks.

type as that of lactococci.

Among the media mentioned in the literature to cultivate lactococci, M17 is frequently put forward as plate count agar and broth [10]. But the reducing power of lactococci linked with organic acid production is also tested on Turner agar and modified KCA [11,12]. Proteolytic and non-proteolytic strains are separated on FSDA agar (Fast Slow Differential Agar) and PCA (Plate Count Agar) supplemented with milk (1%) [13]. Inhibitors of gram negative bacteria (*i.e.* sodium azide) and acidity indicator (*i.e.* bromocresol purple) are sometimes added to PCA or M17 agar in order to improve their efficiency in detecting LAB and especially lactococci [14]. Modified Elliker agar and modified Chalmers agar are selective media which bring to light the acidifying bacteria [15,16].

2. Materials and Methods

2.1. Reference Strains, Culture Maintenance and Reference Media

Eighteen known strains were used. *Lactococcus garvieae* CIP 102507, *Lc plantarum* CIP 102506, *Enterococcus faecalis* CIP103631, *Ec faecium* CIP106742, *Leuconostoc mesenteroides* subsp. *cremoris* CIP103009 (referred to hereafter as *Ln cremoris*), *Ln mesenteroides* subsp. *dextranicum* CIP102423 (*Ln dextranicum*), *Ln mesenteroides* subsp. *mesenteroides* CIP54178 (*Ln mesenteroides*), *Staphylococcus aureus* CIP103429, *Escherichia coli* CIP7624 were purchased from the "Collection de l'Institut Pasteur". *Ec durans*, *Lc lactis* subsp. *lactis* (*Lc lactis*), *Lc lactis* subsp. *cremoris* (*Lc cremoris*), *Lc lactis* subsp. *hordniae* (*Lc hordniae*), *Lc lactis* subsp. *lactis* biovar. *diacetylactis* (*Lc diacetylactis*), three *Lc plantarum* strains, *Streptococcus thermophilus*, *Lactobacillus paracasei*, *Lb plantarum* and *Pseudomonas fluorescens* originated from our laboratory collection. These strains had been previously isolated from raw milk samples and carefully characterized and identified [17]. All the strains were kept frozen at −80˚C in a mixture of the culture medium and glycerol 30% (Sigma-Aldrich).

After thawing, the strains were cultured in their specific maintenance broth, that is:

- *Lactococcus*, *Enterococcus* and *St thermophilus*: M17 broth (Biokar) for 24 h at 30˚C, 30˚C and 44˚C respectively;
- *Leuconostoc* and *Lactobacillus*: MRS broth (Biokar) for 24 h at 30˚C and 37˚C respectively;
- *Ps fluorescens*, *S aureus* and *E. coli*: BHI broth (Biokar) for 24 h at 30˚C, 37˚C and 37˚C respectively.

Purity tests were carried out in aerobic conditions at the same temperatures and on the same media, but in Petri dishes. Incubation lasted 24 h, except for *Leuconostoc* strains which were incubated for 72 h and for *Lactobacillus* strains, which were incubated for 48 h in anaerobic conditions.

2.2. Agar Media and Culture Conditions

Nine agar culture media were tested. Two elective media were dedicated to the enumeration of the mesophilic aerobic lactic acid bacteria, PCA (Biokar) with 1 g/L skim milk powder (Oxoid) and Elliker (Biokar). Three selective media were used: basic M17 agar (Biokar) was amended with 0.04 g/L nalidixic acid (Sigma-Aldrich); modified Elliker agar contained 1 g/L thallium acetate (Merk Group) and 25 mg/L bromocresol purple (Chimie-Plus) to underline acidifying strains; Chalmers media included polymixin-β-sulfate (100 iu·mL^{-1}, Sigma-Aldrich) and a redox indicator (TTC, triphenyl tetrazolium chloride, Sigma-Aldrich). Three media were chosen to highlight specific biochemical features: the proteolytic activity was checked on FSDA and the reducing power of strains on Turner and KCA agar. These three media and the Chalmers medium were all prepared in the lab. Basic M17 agar was employed as control. The characteristics of each medium are displayed on **Table 1**. The nine media were incubated for 48 h at 30˚C aerobically. These conditions are supposed to be optimal for the growth of the *Lactococcus* population.

2.3. Analytical Design

The nine media quoted above were checked for their ability to favour the growth of the different *Lactococcus* species and for their selectivity when in the presence of other bacterial populations in pure and mixed cultures. On the basis of these two experiments, the most suitable medium was then kept for further use on raw milk. Some presumed lactococcal isolates were collected for phenoltypic and genomic identification.

2.3.1. Microbiological Analysis

1) Tests on Reference Strains

The eighteen strains were cultured in optimal conditions. As soon as optical density corresponded to the growth phase, a sample was removed for enumeration on the nine media listed above. The results obtained were compared with one another and with the control medium (M17). Each experiment was duplicated.

2) Test on Re-Seeded Model Milk (RMM)

Re-seeded model milks (RMM) were performed as described by Dalmasso *et al.* [17]: pasteurized milk was seeded with the eighteen strains quoted above so as to mimic the habitual levels found in raw milk (**Table 2**). The RMM allowed us to test the proficiency of the media in evaluating the *Lactococcus* population in a mix of bacteria.

3) Milk Sampling

Milk samples were taken from the milk tank just after

Table 1. Characteristics and composition of nine media dedicated to the enumeration of the *Lactococcus* population.

Constituents ($g \cdot L^{-1}$, except steril whey)	M17[a] [10]	M17 Nal[a]	PCA[b] + milk 1% [14]	Elliker[b] [18]	Modified Elliker[b] [15]	FSDA[b] [13]	Modified Chalmers[b] [16]	Modified KCA[b] [12]	Turner[b] [11]
Tryptone	2.5	2.5	5	20	20	-	-	17	5
Polypeptone	2.5	2.5	-	-	-	-	3	-	-
Soytone	5	5	-	-	-	-		-	-
Yeast extract	2.5	2.5	2.5	5	5	-	3	4.25	5
Meat extract	5	5	-	-	-	-	3	-	-
Gelatine	-	-	-	2.5	2.5	-	-	2.1	-
Milk[c]	-	-	10	-	-	100	-	-	-
Glucose	-	-	1	5	5	-	20	4.25	0.5
Lactose	5	5	-	5	5	-	20	4.25	-
Saccharose	-	-	-	5	5	-	-	-	-
Ascorbic acid	0.5	0.5	-	0.5	0.5	-	-	-	-
NaCl	-	-	-	4	4	-	-	3.4	-
$MgSO_4$	0.25	0.25	-	-	-	-	-	-	-
β-glycerophosphate disodium	19	19	-	-	-	19	-	-	-
K_2HPO_4	-	-	-	-	-	-	-	-	2
Calcium lactate	-	-	-	-	-	-	-	6.8	-
Sodium citrate	-	-	-	-	-	-	-	1.7	-
Calcium citrate[d]	-	-	-	-	-	-	-	5	-
Whey (v/v)[e]	-	-	-	-	-	-	-	0.1	-
L-arginin	-	-	-	-	-	-	-	-	2
Bromocresol purple	-	-	-	-	0.025	-	-	-	-
TTC[f]	-	-	-	-	-	-	-	0.1	0.05
Litmus	-	-	-	-	-	1	-	-	-
Neutral red	-	-	-	-	-	-	0.005	-	-
$CaCO_3$	-	-	-	-	-	-	20	-	-
Thallium acetate	-	-	-		1	-	-	-	-
Nalidixic acid	-	0.04	-	-	-	-	-	-	-
Sodium acetate	-	-	-	1.5	1.5	-	-	-	-
Polymixin-β-sulfate[f]	-	-	-	-	-	-	100 i.u./mL	-	-
Agar	15	15	12	15	15	10	15	12.8	15
Buffered pH	7.1 - 7.2	7.1 - 7.2	-	-	-	-	6	-	-

M17, PCA, Elliker are ready to use (Biokar), the other media are laboratory-made. [a]Media sterilised at 115˚C, 20 min; [b]Media sterilised at 121˚C, 15 min; [c]Re-formed skim milk powder separately sterilised at 110˚C, 10 min; [d]Calcium citrate separately sterilised at 121˚C, 15 min; [e]Whey separately sterilised at 110˚C, 10 min; [f]TTC (triphényl tetrazolium chloride) and Polymixin-β-sulfate sterilised by filtration (0.45 µm).

milking. Eight milk samples were collected on eight farms from the Rhône-Alpes Region (France): Cf1, Cf2, Cf3, Cf4, Cf5, Cf6, Gf1 and Gf2 ("C", "G" and "f" refer respectively to cow, goat and fresh milk); three raw cow milk samples came from three farms in the Massif Central Region: Cf7, Cf8 and Cf9. Six raw cow milk and two

Table 2. Theoretical bacterial concentrations in the re-seeded milk samples. Data expressed in log(cfu)/mL.

	Lc lactis	*Lc cremoris*	*Lc hordniae*	*Lc plantarum*	*Lc garvieae*	*Lc diacetylactis*	*Ln mesenteroides*	*Ln dextranicum*	*Ln cremoris*	*Ec faecalis*	*Ec faecalis*	*Ec durans*	*Lb plantarum*	*Lb paracasei*	*St thermophilus*	*E coli*	*Ps fluorescens*	*S aureus*	*Total population*
Level	2.3	2.3	1.4	1.4	1.4	1.4	2.2	2.2	2.2	1.5	1.5	1.5	1.7	1.7	2.7	2.7	2.0	2.3	3.4

raw goat milk samples were collected from eight farms in the Franche-Comté Region: Ct1, Ct2, Ct3, Ct4, Ct5, Ct6, Gf3 and Gf4 ("t" means that the milk was thawed before use).

Thawed samples were kept at −80˚C until analysis (within one week). Fresh samples were stored at 0/+4˚C and analysed within 12 hours after milk sampling.

The colonies from the six samples Cf1, Cf2, Cf3, Gf1, Ct1 and Ct2 were first counted. Some catalase negative colonies thought to belong to the *Lactococcus* genus were then purified on M17 agar in view of phenotypic and genotypic characterisation. Each isolate was stored at −80˚C in a mix of medium and 30% glycerol. The other samples were just checked for the enumeration of the *Lactococcus* population.

2.3.2. Phenotypic Characterization

Four tests were chosen for their aptness in identifying the *Lactococcus* genus. The absence of the catalase was attested by pouring a drop of H_2O_2 directly on each colony (no gas production). Microscopic observation after Gram stain indicated the shape (cocci), the arrangement (small chains) and the position of the bacteria (Gram+). The type of lactic acid isomer produced was determined using the D-/L-lactate enzymatic test (ENZYTEC, R-Biopharm). This test was performed on the supernatant of a 24 h culture of each bacterium. *Lactococcus*, *Streptococcus* and *Enterococcus* strains produce L-lactate whereas *Leuconostoc* strains produce D-lactate, and heterofermentative *Lactobacillus* bacteria produce D-, L- or a mix of D- and L-lactate. BEA (Biokar) was used to separate presumed lactococci from enterococci. Lactococci are generally unable to develop on BEA due to bile salt inhibition. BEA is dedicated to the specific count of enterococci (this was checked on the eighteen strains, although the results are not reproduced here).

2.3.3. Genotypic Identification

The isolates presumed to belong to the *Lactococcus* genus—catalase negative, producing L-lactate, unable to grow on BEA—were cultured at 30˚C for 24 h in 5 mL of M17 broth. The total DNA was extracted using the Nucleospinb tissue kit (Machery-Nagel). Pure DNA was stored at −20˚C until use. A multiplex PCR assay was performed as described by Pu *et al.* [19], using the following primers: 1RL, LacreR, LgR and PilpraR. PCR products were electrophoresed through 1% (w/v) agarose gel (Sigma-Aldrich) in TBE buffer (Tris—Borate—EDTA pH 8, Sigma-Aldrich) at 100V for 3 h. The DNA fragments were stained with ethidium bromide (Sigma-Aldrich), viewed under UV light (302 nm, Biorad) and photographed on a digital camera (Camedia C-5060). The band patterns were normalized and processed using the GelCompar 3.1 software (Applied Maths). The size of PCR products was determined with a DNA size marker (Sigma-Aldrich).

Some isolates were subjected to gene sequencing, having been chosen as representative strains of each cluster obtained from the multiplex PCR dendrogram. The partial 16 S rRNA gene sequence analysis was performed by the company BACT UP (France) and the complete 16 S rRNA gene sequence analysis was performed by Idymik Company (France).

2.4. Statistical Analysis

A repeatability test was performed with three *Lactococcus* strains. They were incubated at 30˚C in M17 broth. During their exponential growth phase, each of them was enumerated on ten M17 agar dishes. Standard errors were calculated with the Student number for a 5% threshold value for a (n − 1) degree of freedom. The standard-error was evaluated to ± 0.10 log(cfu)/mL for populations between 7 and 9 log(cfu)/mL. Thus, two results were considered as significantly different if the variation was superior to 0.10 log(cfu)/mL. Statistics—variance analysis, means, etc.—were performed by means of the XLSTAT software (2011, version 5.01).

3. Results

3.1. Growth Test of Reference Strains

3.1.1. Medium Specificity towards Each Strain

None of the four *Lc plantarum* strains grew on modified Elliker (**Table 3**). *Lactococcus* colonies were difficult to count on FSDA, irrespective of the species. The enumeration of lactococci on PCA + milk, Elliker, M17 Nal (+ nalidixic acid), Turner, KCA, and Chalmers did not produce significantly different results from those obtained on the control medium (M17 agar), differences

Table 3. Results of growth tests of eighteen strains—*Lc lactis, Lc cremoris, Lc hordniae, Lc plantarum, Lc garvieae, Lc diacetylactis, Ln mesenteroides, Ln dextranicum, Ln cremoris, Ec durans, Lb plantarum, Lb paracasei, St thermophilus, E. coli, Ps fluorescens, S. aureus*—on eight media—FSDA, modified Elliker, M17 Nal, PCA + milk, Elliker, modified Chalmers, modified KCA, Turner—and on the control medium—M17.

	Lc lactis	*Lc cremoris*	*Lc hordniae*	*Lc plantarum*	*Lc garvieae*	*Lc diacetylactis*	*Ln mesenteroides*	*Ln dextranicum*	*Ln cremoris*	*Ec faecalis*	*Ec faecium*	*Ec durans*	*Lb plantarum*	*Lb paracasei*	*St thermophilus*	*E. coli*	*Ps fluorescens*	*S aureus*
FSDA	A	A	A	A	A	A												
Modified elliker				A										A	A	A	A	
M17																		
M17 Nal																A		A
PCA + milk																		
Elliker																		
Modified chalmers									A				A	A		A		
Modified KCA							A	A	A			A	A	A	A			
Turner							A	A	A				A	A	A			A

A: absence of growth. Gray-coloured: growth.

being less than or equal to 0.10 log(cfu)/mL ($p < 0.05$). Therefore, modified Elliker and FSDA media were set aside at this step of the study.

The eighteen strains tested were all able to develop on M17, PCA + milk and Elliker media. *E. coli* and *Ps fluorescens* were inhibited on M17 Nal. On these four media, the colony's morphotype was identical—small, circular, smooth, shiny and creamy, whatever the genus or the species. Therefore, these media did not appear suitable for the specific enumeration of lactococci. On modified Chalmers, if we consider the "non *Lactococcus*" strains, eight strains out of twelve were able to form colonies, their morphotype being identical to the one of lactococci. Consequently, this media was also set aside. On Turner and KCA, only four strains out of the twelve "non *Lactococcus*" strains were able to form pink or red *Lactococcus* type colonies: *Ec faecium*, *Ec faecalis*, *E. coli* and *Ps fluorescens*. *Ec durans* was only observed on Turner and *S aureus* on KCA.

To sum up, growth on KCA and Turner gave the most convincing results for the selective enumeration of presumed *Lactococcus* and *Enterococcus* genera. These two media were kept for further analysis whereas the others were set aside. Since enterococci were able to develop on these two media, we proposed to specifically count enterococci on BEA medium and to subtract the enterococcal count from the Turner or KCA count.

3.1.2. Species Recovery from RMM

A sample of RMM was plated on BEA agar, Turner and KCA. Catalase negative colonies with a pink or a red colour were enumerated specifically on these last two media. The result obtained corresponded to the level of presumed lactococci + enterococci. The count result obtained on BEA was then subtracted from the KCA and the Turner results. This gave an estimation of the presumed lactococcal microflora.

The microbial results gathered from three RMM are displayed on **Table 4**. The "KCA protocol" led to a fairly accurate estimation of the *Lactococcus* population, the discrepancy between objective and estimated data being inferior to 0.21 ± 0.09 log(cfu)/mL. A variance analysis was performed on these data. No statistical difference was observed whatever the milk sample or strain considered. On the contrary, the Turner medium systematically gave unusable results, enterococci and lactococci both being underestimated. Consequently this medium was not retained for the following analyses.

3.2. Protocol Design for Estimating the *Lactococcus* Population

The microbial results obtained on six raw cow milks—Cf1, Cf2, Cf3, Gf1, Ct1, Ct2—are shown on **Table 5**. Between 50% and 100% of the catalase negative isolates were picked up from KCA Petri dishes. The phenotypic and genotypic characterisation of these isolates allowed presumed *Lactococcus* colonies to be separated from other contaminants—namely enterococci. All the *Lactococcus* isolates were Gram + cocci producing L-lactate and were inhibited by bile salts. On the basis of these

Table 4. Estimation of the *Lactococcus* population in re-seeded model milk (RMM) using the KCA and turner media. The *Enterococcus* population (enumerated on BEA) has been subtracted from the results obtained on these two media. Results are expressed in log(cfu)/mL.

	Lc species	Control (M17)	KCA	Turner	KCA (difference with the control)	Turner (difference with the control)
RMM 1	*Lc cremoris*	3.31	3.44	A	0.13	A
	Lc garvieae	3.41	ND	ND	ND	ND
	Lc hordniae	3.60	3.81	2.88	0.21	0.7
	Lc lactis	3.82	3.93	2.24	0.11	1.6
	Lc plantarum	3.82	3.91	1.82	0.09	2.0
RMM 2	*Lc cremoris*	4.69	4.79	A	0.10	A
	Lc garvieae	4.39	4.38	A	0.01	A
	Lc hordniae	4.38	4.42	A	0.04	A
	Lc lactis	4.69	4.80	A	0.11	A
	Lc plantarum	ND	ND	ND	ND	A
RMM 3	*Lc cremoris*	4.69	4.60	A	0.09	A
	Lc garvieae	4.39	4.20	A	0.19	A
	Lc hordniae	4.38	4.50	A	0.12	A
	Lc lactis	4.69	4.80	A	0.11	A
	Lc plantarum	ND	ND	ND	ND	ND

ND: not determined; A: absence of growth.

Table 5. Analysis of raw cow milk samples from the Rhône-Alpes region (Cf1, Cf2, Cf3, Cf4, Cf5, Cf6), from the Massif Central region (Cf7, Cf8, Cf9), and from the Franche-Comté region (Ct1, Ct2, Ct3, Ct4, Ct5, Ct6) and raw goat milk samples from the Rhône-Alpes region (Gf1, Gf2), and from the Franche-Comté region (Gf3, Gf4). "C", "G", "f" and "t" refer respectively to cow, goat, fresh and thawed milks. Results are expressed in log(cfu)/mL of raw milk.

Medium and *microflora enumerated*	Cf1	Cf2	Cf3	Gf1	Ct1	Ct2	Ct3	Ct4	Ct5	Ct6	Cf4	Cf5	Cf6	Gf2	Cf7	Cf8	Cf9	Gf3	Gf4
Level on KCA	3.15	4.33	4.37	3.73	2.46	2.79	1.95	2.30	3.32	3.33	3.19	-	-	-	-	-	-	-	-
Level of the catalase negative population on KCA	2.61	3.87	2.26	3.19	2.29	2.64	1.90	1.78	2.49	2.30	2.87	3.64	3.40	4.15	3.12	2.54	2.41	3.28	4.60
Level of the *Enterococcus* population on BEA	2.11	2.11	2.68	2.89	2.04	2.71	1.00	2.28	2.46	2.36	2.00	2.18	1.9	3.03	2.75	1.7	1.00	1.00	2.51
Level of the presumed *Lc*-like phenotype population	2.45	3.87	*	2.89	1.92	*	1.85	*	1.30	*	2.81	3.63	3.38	4.11	2.88	2.48	2.40	3.28	4.60
% of catalase-isolates picked up on KCA	50%	100%	-	100%	100%	67%		-	-	-	-		-	-	-	-	-	-	-
Level of *Lc*-like phenotype estimed with genotypic identification	2.30	3.86	-	2.41	1.89	1.74		-	-	-	-		-	-	-	-	-	-	-

*Inferior to detection threshold.

tests, the level of the *Lactococcus* population was estimated at between 1.74 to 3.86 log(cfu)/mL.

These results were compared with those obtained by subtracting the enumeration on BEA (*Enterococcus* population) to the catalase negative microflora count obtained on KCA. The difference between the two estimations of the *Lactococcus* population was inferior or equal to 0.48 log(cfu)/mL. No *Lactococci* were detected in milk Cf3. This result was consistent with the methodology used for estimating the lactococci because this latter population was inferior to the detection threshold. The enterococcal count on BEA agar was superior to the catalase negative colony count obtained on KCA.

3.3. *Lactococcus* Diversity

The diversity of the lactococcal isolates was investigated by multiplex PCR (**Figure 1**). The 46 isolates picked from the five samples—Cf1, Cf2, Gf1, Ct1, Ct2—were

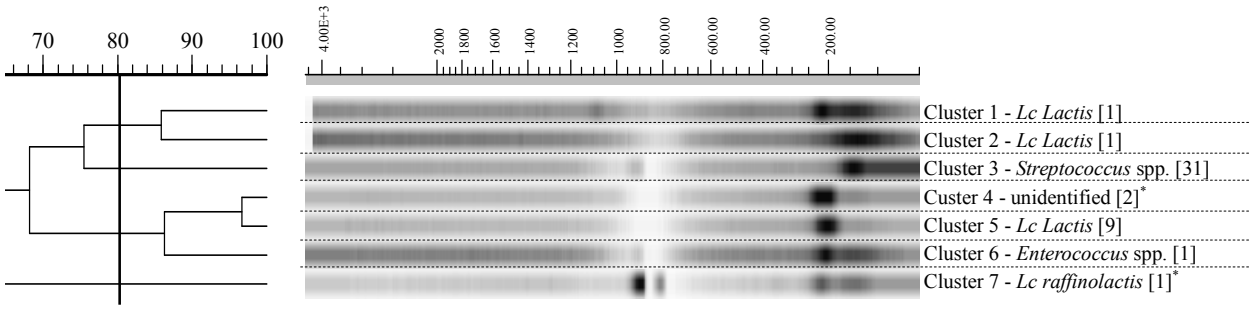

Figure 1. Dendrogram drawn by UPGMA of correlation value of normalized multiplex-PCR patterns from lactococci. Each pattern is identified by a cluster number and by a number between brackets referring to the number of strains which displayed this profile. The seven clusters from 1 to 7 are defined at a coefficient of similarity of 80% materialized by a bold vertical line. Strains coming from goat's raw milk are identified by a *.

arranged in 7 clusters (80% similarity threshold). Only one profile is observed in each cluster. Cluster 6 is an example of an enterococcal profile. The isolate picked from a raw goat milk sample Cf2 was identified as *Enterococcus* spp by DNA sequencing. Nine isolates picked from samples Cf1, Ct1 and Ct2 form cluster 5. A 228 pb band is observed. This size is specific to the *Lactococcus lactis* species, a result confirmed by DNA partial sequencing of the 16 S rRNA genes of one isolate coming from the Ct1 sample. The remaining 36 isolates could not be identified by multiplex PCR. One representative of each cluster was thus identified by sequencing. With the exception of cluster 4, formed by 2 isolates collected from raw goat milk, all these strains were able to be identified. Clusters 1 and 2 were associated with *Lc lactis*. These two isolates were taken from the Cf1 sample. Cluster 7 included one strain identified as *Lc raffinolactis*. This isolate came from raw goat milk (sample Gf1). It is noteworthy that these three isolates did not present the typical DNA bands observed for *Lc lactis* (238 pb) and *Lc raffinolactis* (860 pb), although sequencing identification was doubtless (>97%). Cluster 3 included 31 isolates coming from the milk Cf2. Among them, one isolate was assigned to *Streptococcus* ssp. by partial 16S rRNA gene sequencing. This result was partially confirmed by a sequencing test made by another lab which established the genus assignment. This isolate was inhibited by bile salts, a characteristic of lactococci. The presence of streptococci on the surface of KCA, although not surprising, led us to limit the protocol here developed to the evaluation of the *Lactococcus* and the *Streptococcus* population. Thereafter in this article, this microflora will be designated as "*Lactococcus*-like" microflora, implying that the distinction between lactococci and streptococci by the KCA culture-dependant methodology is not possible.

3.4. *Lactococcus*-Like Microflora Levels in Raw Milk

The *Lactococcus*-like population ranged from 1.30 to 3.87 log(cfu)/mL for raw cow milk and from 2.89 to 4.60 log(cfu)/mL for raw goat milk. In four raw cow milk samples, this population was inferior to the detection threshold. In this case, the enterococcal microflora probably overwhelmed lactococci. No differences were observed considering the geographical origin of the raw milk.

4. Discussion

The bacteria from the *Lactococcus* genus are used as starters to ferment many different dairy products. They are also present at low levels in raw milk, coming from plants and biofilms present in the milking machine [17]. Lactococci have been studied extensively for many years and the yearly number of publications varies between less than 300 to more than 400 articles. Surprisingly, whereas the knowledge—genetic, metabolic, transcryptomic, etc.—of this microorganism has been greatly improved, no attempts have been made to develop media for its specific enumeration in a complex food matrix. Moreover, knowledge has specifically focused on *Lc lactis*, and little is known about the other species or subspecies. In this work, we tried to take stock of the level and the diversity of lactococci in raw milk. The preliminary step in reaching this objective involved developing a methodology that would allow the *Lactococcus* population to be estimated in raw milk, whatever the species or the subspecies present.

Many media have been proposed in the past for the enumeration of lactococci. In spite of substantial efforts, none of them proved to be selective. Among them, M17, PCA + milk and Elliker enable the growth of mesophilic aero tolerant LAB [10,20]. The addition of 0.04 $g \cdot L^{-1}$ nalidixic acid to these three media allows the Gram negative bacteria to be depressed. But other Gram positive bacteria are still able to grow, an observation also made by Corroler *et al.* [21]. The use of thallium acetate in the modified Elliker medium [19], another Gram

negative inhibitor, proved to hinder the growth of the four *Lactococcus plantarum* strains we tested. Even if few references exist on this species, Demarigny *et al.* [22] regularly found this bacterium in natural whey starters. The observations we made on FSDA were identical to those of Grattepanche *et al.* [23]. If the protease positive colony morphotype—round, regular, smooth—was easily observed, protease negative colonies were never detected. Modified Chalmers did not enable us to clearly distinguish the different lactic acid bacteria genera on the basis of their morphotype. In fact, two media were found to be of interest, Turner agar and KCA. Among other components, these plate count agar include TTC, a redox potential indicator. Acid fermentation by lactococci generally leads to the concomitant decrease in the redox potential. Colonies then appear red/pink. In pure culture, KCA and Turner agar gave similar enumerations to those obtained with the reference M17 medium, whatever the *Lactococcus* species or subspecies. But in mixed-culture, the Turner agar underestimated the *Lactococcus* population. Among several hypotheses, we can propose as a possible explanation that microorganisms competed for nutrients. Indeed, *Lactococcus* is a nutritionally demanding bacterium. For instance for *Lc lactis*, the amount of amino acid auxotrophies varies between 6 to 8 [24]. The Turner composition is less rich than KCA (less nitrogen and glucidic sources). The resulting nutritional stress that can occur on Turner may explain the discrepancy between control (M17) and test (Turner) data. KCA proved, then, to be the most suitable medium. On KCA, a catalase test must be added to distinguish catalase negative colonies supposed to be assigned to *Lactococcus* or *Enterococcus*. At the same time, *Enterococcus* bacteria have to be counted on BEA. This result is then subtracted from the result obtain on KCA. This gives a rather good estimation of the *Lactococcus* population in pure or mixed cultures.

Six raw cow milk samples were used to evaluate the diversity of the *Lactococcus* species. The isolates collected from these raw milk samples were subjected to multiplex PCR [19] and partial 16 S rRNA gene sequence analysis. The multiplex PCR allowed 9 isolates out of 44 to be accurately identified. The relevance of this method when applied on wild strains appears here debatable. Indeed, the work of Pu *et al.* [19] only focused on selected starter strains. While the multiplex PCR gave pertinent results on these strains, our own observations would appear to cast doubt on the pertinence of this methodology when applied to wild bacteria. Although many reasons for this could be put forward, at this time, we have no satisfactory explanation to offer. The partial gene sequencing did, however, bring other information. *Lc lactis* was detected without any doubt in three raw cow milk samples and *Lc raffinolactis* in one raw goat milk sample. Two isolates picked from one raw goat milk could not be identified by DNA sequencing. Moreover, sequencing allowed us to discover *Streptococcus* strains which had first been assigned to *Lactococcus* on the basis of their phenotype.

Streptococcus has already been observed in raw cow milk. Thus, the presence of this genus is not surprising. Franciosi *et al.* [25] identified *St. dysgalactiae*, *St. parauberis*, *St. suis*, *St. macedonicus* in raw cow milk. Desmasures et Beuvier [26] also reported *Streptococcus* ssp. in raw cow and goat milk. Jans *et al.* [27] designated the *Streptococcus bovis/Streptococcus equinus* complex (SBSEC) "as the predominant LAB in spontaneously fermented African milk products, […] Mexican, Greek, and Italian cheese".

Some of the streptococcal strains show a lot of similitudes with lactococci, among them, the bile salt inhibition. In our methodology, it would seem to be impossible to distinguish *Lactococcus* from *Streptococcus* strains. This could lead us to question the actual identification of the enumerated microflora already published in the literature. Indeed, our results suggest that some *Streptococcus* strains may have been confused with the *Lactococcus* genus. The mean level of *Lactococcus*-like microflora in eleven raw cow milk samples was estimated at 3.18 log(cfu)/mL with a minimum of 1.30 log(cfu)/mL and a maximum of 3.38 log(cfu)/mL. In four raw goat milk samples, the mean was superior: 4.14 log(cfu)/mL with a minimum of 2.89 log(cfu)/mL and a maximum of 4.60 log(cfu)/mL. These results are consistent with those published on raw cow and goat milk in the literature [26]. No differences were observed between raw milk coming from the three different regions. Published data taking into account the geographical origin of milk samples is still lacking. Moreover, the number of samples analysed in this study was probably not sufficient to assess the possible influence of this factor.

The study of lactococcal diversity brings out, unsurprisingly, the presence of *Lc lactis* in the majority of raw cow milk [25,26,28]. On the other hand, *Lc raffinolactis*, found in raw goat milk, is reported to a lesser extent in the literature on raw goat milk [22,29].

5. Conclusion

Our methodology is getting close to being able to enumerate lactococci. The results obtained were congruent with those in the literature. However, it was not possible to separate streptococci, which are phenotypically similar to lactococci, unless the whole catalase negative isolates on KCA were to be gene sequenced. This is unsuitable in a dairy lab for routine analyses. Our results confirm the impossibility of developing a methodology dedicated to selectively enumerating the lactococcal microflora. However, it would be interesting to extend this work to en-

compass more raw milk samples and to examine in particular the streptococcal microflora.

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