

The microflora of natural whey cultures utilized as starters in the manufacture of Mozzarella cheese from water-buffalo milk

S. COPPOLA, E. PARENTE, S. DUMONTET,
Antonella LA PECCERELLA

*Istituto di Microbiologia agraria e
Stazione di Microbiologia industriale
Università degli studi di Napoli - 80055 Portici, Italy*

Summary

The composition of 16 natural whey cultures from 3 different Mozzarella cheese plants was investigated. They consisted mainly of lactic acid bacteria, coliform bacteria and yeasts. Micrococci, butyric and propionic acid bacteria only occurred occasionally. *Lactobacillus lactis* was the most common species of *Lactobacillus* while *Streptococcus lactis* and *Str. thermophilus* were the most common species of *Streptococcus*. Enteropathogenic *Escherichia coli* were always present. Different species of *Leuconostoc* and yeasts belonging to the genera *Candida*, *Kluyveromyces*, *Debaryomyces* and *Brettanomyces* were also isolated. Acidifying and proteolytic capacity of the strains showed that these activities were widely affected by temperature and type of milk (cow or water-buffalo milk). Streptococci were the most active acid producers at the cheese vat temperature (37 °C).

Keys words : Water-buffalo Mozzarella cheese - Natural whey cultures.

Résumé

*La microflore naturelle du sérum utilisé comme levain
dans la fabrication de la Mozzarella de bufflonne*

On a étudié la composition de 16 cultures naturelles en sérum utilisées comme levain pour la production du fromage « Mozzarella ». Ces levains sont formés surtout des bactéries lactiques, des coliformes et des levures.

Les bactéries butyriques, propioniques et les microcoques ont été trouvés épisodiquement et à des niveaux inférieurs.

Les espèces les plus représentées sont *Lactobacillus lactis* parmi les lactobacilles, *Streptococcus lactis* et *Str. thermophilus* parmi les streptocoques, entérocoques inclus.

Parmi les coliformes, *Escherichia coli* est l'espèce la plus importante du point de vue quantitatif, mais aussi bien pour la présence de sérotypes entéro-pathogènes.

Quelques espèces de *Leuconostoc* et de levures, ces dernières appartenant aux genres *Candida*, *Kluyveromyces*, *Debaryomyces* et *Brettanomyces* ont été isolées.

Les caractéristiques acidifiantes et protéolytiques des souches isolées sont fort affectées par la température et la nature du milieu utilisé (lait écrémé de vache ou de bufflonne).

Les souches de streptocoques étaient les plus actives dans les essais effectués à la température utilisée en fromagerie (37 °C).

Mots clés : Mozzarella de bufflonne - Cultures naturelles en sérum.

Introduction

Mozzarella cheese made from water-buffalo milk is one of the most highly valued unripened « pasta filata » cheese in Italy.

At present, this type of cheese is still manufactured in the traditional way to ensure its particular organoleptic characteristics : raw whole water-buffalo milk is inoculated with a natural starter, consisting of the whey from the previous manufacture, bringing the acidity of the mixture to 10 °SH (Soxhlet-Henkel). The milk is then heated to 36-37 °C and sufficient rennet is added to obtain coagulation in 20 minutes. After 40 minutes the curd is cut to pieces of about 16 cm³ and left undisturbed, covered by the whey, until curd stretching is possible. This generally occurs 4 hours after the addition of the rennet. During this phase, usually referred to as « curd ripening », the natural microflora, which comes from both the raw milk and the whey culture, carries out complex biochemical activities, which have been only partially characterized (ADDEO and COPPOLA, 1983 ; COPPOLA *et al.*, 1985). Among these activities, acid development is the most important since only when the pH is low can enough dicalcium paracasein be converted, during stretching and mixing with hot water (85-90 °C), to monocalcium paracasein (CHAPMAN and SHARPE, 1981). Then the curd is milled, stretched and moulded. The whey which is removed from the cheese vat is stored at room temperature (18-22 °C) until used as a starter on the following day.

This manufacturing procedure has at least two consequences : variability in product quality and the risk that pathogenic microorganisms will survive in the finished cheese. In fact, it has previously been shown (ADDEO and COPPOLA, 1983) that the microbiological quality of water-buffalo milk is extremely variable and usually unsatisfactory ; no particularly selective incubation practice is used on the whey starter during incubation ; acid production and antagonisms by lactic acid bacteria during curd ripening do not ensure the complete disappearance of pathogenic microorganisms ; the heat treatment of the curd during stretching is neither even nor completely effective in killing pathogens.

The use of defined starter cultures has been recommended to aid the normal course of acid development and ensure constant quality standards. The Italian Ministry of Health (Ministero della sanità della Repubblica italiana, 1978), when requiring a heat treatment for milk used in unripened cheese manufacture, pointed out the opportunity of using starter cultures composed of

Streptococcus thermophilus and a small amount of *Lactobacillus bulgaricus* or *Lb. helveticus* to ensure acid production for curd ripening. According to FORMISANO and MINCIONE (1982) starters composed of *Str. thermophilus* or *Str. lactis* and *Lb. bulgaricus* are suitable, while VEDAMUTHU and WASHAM (1983) suggest *Lb. bulgaricus*, *Lb. helveticus* and *Str. thermophilus*, and LAW (1982) only *Str. thermophilus* and *Lb. bulgaricus*. All the authors cited exclude the participation of other microbial species as a secondary or associated microflora.

Actually, these suggestions have not found any practical application in the manufacture of Mozzarella cheese from water-buffalo milk in Italy. According to the cheese manufacturers, the natural starters cannot be replaced by commercial ones because of the significant loss in the organoleptic qualities of the cheese made with the commercial cultures, compared with the product obtained by the activity of the complex microflora of natural whey cultures.

Therefore a study was carried out to achieve a better knowledge of the microbiological composition of this culture since the rare reports available on natural whey cultures refer to hard cheeses (BOTTAZZI, 1962 ; ACCOLAS and AUCLAIR, 1964 ; MATTEUZZI, 1967 ; VALLES and MOCQUOT, 1972 ; BOTTAZZI and VESCOVO, 1973 ; BOTTAZZI *et al.*, 1977) whose manufacturing technologies are completely different from those of unripened « pasta filata » cheeses.

I. Materials and methods

Natural whey cultures used in cheese factories of the Province of Caserta, which is the most typical production area, were examined. A total of 16 samples were taken in January, April, September and December at three cheese factories (8 samples in two years from one, 4 samples each in one year from the other two) that utilized exclusively water-buffalo milk for cheese production. The pH of the samples ranged from 4.17 to 4.23.

Decimal dilutions of samples were performed in sterile quarter-strength Ringer's solution.

Mesophilic and thermophilic lactic acid bacteria were enumerated in skim milk (Oxoid), using the most probable number (MPN) technique. Test tubes were incubated for 48 h at 22 °C and 45 °C for mesophilic and thermophilic lactic acid bacteria respectively and coagulation of the milk was used as end point. Mesophilic and thermophilic lactobacilli were counted and isolated on Acetate Agar (HARRIGAN and McCANCE, 1976) after anaerobic incubation (BBL Gaspak System) for 48 h at 22 °C and 45 °C respectively. Mesophilic and thermophilic streptococci on Yeast Glucose Lemco Agar (NAYLOR and SHARPE, 1957) after anaerobic incubation (BBL Gaspak system) at 22 °C and 45 °C respectively. Since the media used are not selective for lactobacilli or streptococci, enumeration was confirmed by microscopic examination. A plate with about 10² colonies was selected for each medium/incubation temperature combination and a number of colonies equal to the square root of the total was picked at random using the Harrison disc described by HARRIGAN and McCANCE (1976). The lactobacilli were maintained as deep cultures respectively in semisolid (0.45 % agar) MRS Agar (DE MAN *et al.*, 1960) and the

streptococci in semisolid (0.45 % agar) Yeast-Glucose-Lemco-Agar supplemented with 0.6 % CaCO₃; in both media glucose was replaced by lactose.

Enterococci were enumerated in Enterococci Presumptive Broth (Difco), using the MPN technique; positive tubes were confirmed according to the manufacturer's instructions. Strains belonging to this group were also isolated on Yeast-Glucose-Lemco Agar and Barnes Agar (BARNES, 1956), and maintained like the other streptococci.

For the enumeration and isolation of *Leuconostoc spp.*, high-sucrose Mayeux Agar (MAYEUX *et al.*, 1962), incubated at 30 °C for 48 h, was used. Only large mucoid colonies were counted and isolated. Strains belonging to this genus were also isolated on plates of Yeast-Glucose-Citrate Agar (GARVIE, 1967) incubated anaerobically at 30 °C for 48 h. They were maintained on MRS Agar slants.

Because of the lack of suitable selective media, *Pediococci* were tentatively enriched in Elliker broth (ELLIKER *et al.*, 1956), incubated at 37 °C for 48 h and streaked on WL Differential Agar (Difco) plates, which were incubated anaerobically 37 °C for 48 h, as indicated by BOURGEOIS and LEVEAU (1980).

The most probable number of *Micrococcaceae* was determined in Tryptic Soy Broth (Difco) with 10 % NaCl, incubated for 48 h at 37 °C. Aliquots of tubes showing growth were streaked on plates of Mannitol Salt Agar (Difco) and Baird-Parker Agar (Oxoid). Colonies showing the morphology characteristic of *Staphylococcus aureus* were isolated and maintained on Nutrient Agar (Difco) slants. They were examined for morphology, Gram-reaction, catalase and, after subculturing in Brain Heart Infusion (Difco), for coagulase activity using rabbit plasma (BioMérieux).

Coliform bacteria were enumerated on Violet Red Bile Agar (Oxoid) after 24 h at 37 °C. McConkey Agar (Difco) and DCLS Agar (BBL) were also used for the isolation of *Enterobacteriaceae*. The isolates were maintained on Tryptone Glucose Yeast Extract Agar (Difco) slants and identified by means of the API System 20E. A preliminary serological typing was carried out on strains identified as *Escherichia coli* using Difco and Behring Institute antisera.

Butyric acid bacteria were enumerated using the technique described by ANNIBALDI (1969), incubating the parafin sealed tubes for 5 d. at 30 °C. Propionic acid bacteria were enumerated on the medium of Politi, modified according to GALLI *et al.* (1984). The plates were incubated anaerobically at 30 °C for 5 days.

Yeasts were enumerated using Davis' Salt Agar (DAVIS, 1958) adjusted to pH 3.5 with sterile 10 % citric acid solution. Plates were incubated at 26 °C for 5 days and colonies showing different morphologies were isolated. Yeast isolates were maintained on Malt Agar (Difco), and characterized according to LODDER (1970) and BARNETT *et al.* (1983).

For each microbial group all the colonies on a plate of 25-50 were picked for isolation and identification.

Lactobacillaceae were identified according to ROGOSA (1974) while *Streptococcaceae* were identified according to DIEBEL and SEELEY (1974). The techniques utilized are described by HARRIGAN and McCANCE (1976). The optical isomer of lactic acid was determined according to KRUSH and LOMPE (1982) using Merck reagents and Boehringer enzyme preparations. In addition, the strains were examined for acid production and proteolytic activity in milk. For acid production, strains were subcultured consecutively in skim milk + 0.5 % yeast extract, skim milk + 0.2 % yeast extract and twice in skim milk, incubating each time for 12 h at 30 °C for the lactic streptococci and mesophilic lactobacilli, for 12 h at 45 °C for the thermophilic lactobacilli and streptococci and for 12 h at 37 °C for enterococci. The last subculture was used to inoculate skim milk and skimmed water-buffalo milk, which were incubated at the temperatures indicated above for 4 h and 6 h before pH measurement. A factorial design was used with only two replicates per treatment, because of the very large number of degrees of freedom available to estimate the error mean square. Multiple mean comparisons were carried out using Student-Newman-Keuls' Test. For proteolytic activity the technique of Hull (HULL, 1947) modified by CITTI *et al.* (1963) was used. A completely random design with three replicates per strain was used for the statistical evaluation of the data. Multiple mean comparisons were carried out using F-protected Least Significant Difference.

II. Results

The ranges of the microbial counts obtained in the different whey starters are summarized in table 1. No correlation between the time of sampling or between the dairy sampled and the microbial counts for any microbial group was found.

Table 2 reports the results of a statistical analysis carried out on three replicates from a single whey culture sample. Data were log transformed before the confidence limits were estimated.

Over 700 strains were isolated, purified and characterized. Homofermentative isolates from Mayeux agar and isolates from WL-Differential agar not producing DL-lactate were discarded because their biochemical characteristics did not correspond to those of *Leuconostoc spp.* or *Pediococcus spp.* respectively. That such strains were isolated on the media is a reflection of the lack of selectivity of Mayeux and WL-Differential agars. About 5 % of the strains isolated could not be identified because of various biochemical anomalies. A number of strains were lost before final identification. Finally, 316 strains were identified. *Lactobacillaceae*, *Streptococcaceae* and *Micrococcaceae* are reported in table 3. The last dilution from which a single species was isolated can be regarded as a rough indication of its relative abundance.

Among the lactobacilli, *Lb. lactis* was the most frequent species, followed by *Lb. leichmanii* and *Lb. salivarius*. *Lb. bulgaricus* was isolated from a single sample of whey. Its presence suggested the use of a commercial starter culture ; however information obtained from the dairy from which this sample was taken suggested that this did not occur. Twelve strains of *Lb. salivarius*

TABLE 1

*Microbial counts in natural whey cultures utilized as starters in Mozzarella cheese manufacture from water-buffalo milk.
(LOG of MPN/ml or CFU/ml)^a*

*Dénombrements microbiens dans 16 cultures naturelles en sérum utilisées comme levain dans la production du fromage « Mozzarella ».
(LOG du NPP/ml ou UFC/ml)*

Microbial group	Range	Most frequent count
<i>Lactic acid bacteria :</i>		
<i>Thermophilic</i>	7.3 - 8.3	8.3
<i>Mesophilic</i>	7.6 - 7.9	7.9
<i>Thermophilic lactobacilli</i>	7.0 - 9.2	8.3
<i>Mesophilic lactobacilli</i>	6.6 - 8.0	7.2
<i>Thermophilic streptococci</i>	7.3 - 9.3	8.0
<i>Mesophilic streptococci</i>	7.3 - 8.7	8.3
<i>Enterococci</i>	6.9 - 8.0	7.5
<i>Leuconostocs</i>	4.6 - 6.7	5.2
<i>Micrococci</i>	0.6 - 3.2	0.6
<i>Coliform bacteria</i>	4.6 - 6.0	5.9
<i>Propionic acid bacteria</i>	0 - 3.0	0
<i>Butyric acid bacteria</i>	- 0.8 - 0.01	- 0.2
<i>Yeasts</i>	4.1 - 7.4	5.9

^a The number of the cultures examined was 16.

TABLE 2

Statistical analysis of 3 replicates from a single natural whey culture utilized as starter in Mozzarella cheese manufacture from water-buffalo milk

*Analyse statistique des 3 répétitions d'un dénombrement microbien dans 1 culture naturelle en sérum utilisée comme levain dans la production du fromage « Mozzarella »
(LOG du NPP/ml ou UFC/ml)*

Microbial group	Mean ^a	Conf. limits (p = 5 %) ^a
<i>Lactic acid bacteria :</i>		
<i>Thermophilic</i>	8.0	6.5-9.5
<i>Mesophilic</i>	7.6	6.9-8.4
<i>Thermophilic lactobacilli</i>	7.0	6.9-7.1
<i>Mesophilic lactobacilli</i>	6.6	6.6-6.7
<i>Thermophilic streptococci</i>	7.5	7.4-7.5
<i>Mesophilic streptococci</i>	8.0	7.1-8.9
<i>Enterococci</i>	7.5	6.4-7.0
<i>Leuconostocs</i>	6.7	6.4-7.0
<i>Micrococci</i>	0.7	0.3-1.0
<i>Coliform bacteria</i>	6.0	5.9-6.2
<i>Propionic acid bacteria</i>	none	
<i>Butyric acid bacteria</i>	- 0.2	- 0.6-0.2
<i>Yeasts</i>	5.3	5.2-5.4

^a LOG MPN/ml or CFU/ml.

TABLE 3

Lactobacillaceae, Streptococcaceae and Micrococcaceae isolated from natural whey cultures utilized for Mozzarella cheese manufacture from water-buffalo milk

Lactobacillaceae, Streptococcaceae et Micrococcaceae isolées de cultures naturelles en sérum utilisées comme levain dans la production du fromage « Mozzarella »

Species	Last positive sample dilution	Number of strains
<i>Lb. lactis</i>	10 ⁻⁷	25
<i>Lb. leichmanii</i>	10 ⁻⁷	10
<i>Lb. salivarius</i>	10 ⁻⁶	13
<i>Lb. helveticus</i>	10 ⁻⁷	4
<i>Lb. bulgaricus</i>	10 ⁻⁷	1
<i>Lb. fermentum</i>	10 ⁰	3
<i>Lb. viridescens</i>	10 ⁻⁵	1
<i>Lb. casei</i>	10 ⁻⁵	1
<i>Lb. casei</i> subsp. <i>pseudoplanarum</i>	10 ⁻⁷	1
<i>Lb. plantarum</i>	10 ⁻⁷	7
<i>Str. lactis</i> subsp. <i>lactis</i>	10 ⁻⁸	81
<i>Str. lactis</i> subsp. <i>cremoris</i>	10 ⁻⁷	2
<i>Str. lactis</i> subsp. <i>diacetylactis</i>	10 ⁻⁷	8
<i>Str. faecium</i>	10 ⁻⁶	2
<i>Str. faecalis</i>	10 ⁻³	3
<i>Str. salivarius</i> subsp. <i>thermophilus</i>	10 ⁻⁸	14
<i>Leuc. dextranicum</i>	10 ⁻⁵	7
<i>Leuc. lactis</i>	10 ⁻⁴	1
<i>Leuc. cremoris</i>	10 ⁻⁵	1
<i>Staph. aureus</i>	10 ⁻³	7

did not produce acid from sorbitol. A strain which did not ferment trehalose was identified as *Lb. lactis* and one which did not ferment amygdalin as *Lb. leichmanii*. These biochemical anomalies were less frequent among the streptobacteria: a strain that did not produce acid from melizitose was identified as *Lb. casei* and six strains were tentatively identified as *Lb. plantarum*. Thermophilic lactobacilli were identified more frequently than mesophilic ones.

Identification of streptococcal strains showed, without doubt, that *Streptococcus lactis* subsp. *lactis* and *Str. salivarius* subsp. *thermophilus* were the most common species; yet, strains belonging to *Str. lactis* subsp. *diacetylactis* and *Str. lactis* subsp. *cremoris* were isolated from the higher dilutions of the whey cultures examined. Non-hemolytic streptococci, identified as *Str. faecium* and *Str. faecalis* were isolated from lower dilutions. However, enterococci were found in all the samples studied.

Leuc. dextranicum was the species of *Leuconostoc* most easily isolated, probably because of the « selectivity » of the high sucrose medium used. *Leuc. cremoris* and *Leuc. lactis* were occasionally isolated.

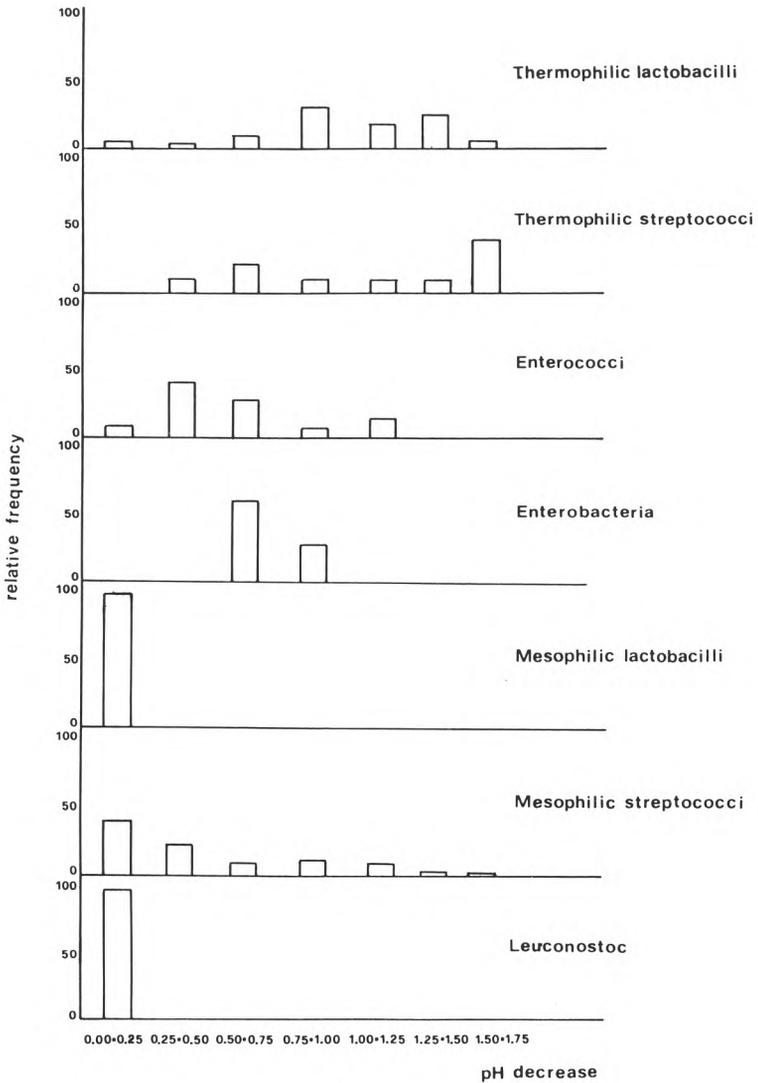


Fig. 1

Acid-producing ability in bovine skim milk (see the text for temperature of incubation) of the strains isolated from natural whey cultures utilized as starters in the manufacture of water-buffalo Mozzarella cheese.

Production d'acide dans le lait écrémé de vache par des souches isolées de cultures naturelles en sérum utilisées comme levains dans la production du fromage « Mozzarella ».

All isolates from Mannitol Salt and Baird-Parker media were coagulase positive and were presumptively identified as *Staphylococcus aureus*.

The majority of the strains belonging to the family *Enterobacteriaceae* was identified as *Escherichia coli*. A preliminary screening with antisera against enteropathogenic and enterotoxigenic strains of this species showed that 4 were agglutinated by Difco *E. coli* OK antiserum Poly-A (10^{-4}), 7 by Poly-B (10^{-4}), 3 by Poly-C (10^{-5}), 17 by Poly-D (10^{-5}), 2 by Poly-E (10^{-4}), 2 by both Poly-A and -B (10^{-4}), 1 by both Poly-B and -C (10^{-4}), 1 by Poly-A, -B and -C (10^{-4}), 1 by both Poly-C and -D (10^{-4}), 4 by both Poly-D and -E (10^{-4}) and 50 were not agglutinated. 4 strains were identified as *Enterobacter agglomerans* (10^{-5}) and 10 strains as *Klebsiella pneumoniae* (10^{-6}). The figure in brackets is the dilution of whey starter from which such strains were isolated. 18 strains showed atypical biochemical profiles and were not identifiable with API System 20E.

In general identification of yeast strains was limited to the genus. 1 strain was identified as *Debaryomyces hansenii* (highest dilution from which this species was isolated : 10^{-6}), it fermented glucose and galactose ; 2 strains were identified as *Kluyveromyces marxianus* (10^{-6}), fermenting glucose, galactose and lactose ; 3 strains were identified as *Brettanomyces* (10^{-6}) two of which fermented glucose, galactose and lactose ; 30 strains were identified as *Candida* (10^{-6}) : 27 of them fermented glucose and galactose, 3 strains did not.

Figure 1 shows the acid-producing ability in bovine skim milk of the different groups isolated. Thermobacteria and mesophilic and thermophilic streptococci produced the greatest amounts of acid.

All the *Leuconostoc* strains and the mesophilic lactobacilli showed very low acid-producing ability and the enterobacteria showed an intermediate one. Differences among strains of all groups were highly significant, as demonstrated by analysis of variance (data not shown).

Figure 2 summarizes some central tendency and dispersion statistics of the distribution of acid-producing ability of the groups mentioned above. Horizontal and vertical bars show the range of acid-producing ability in skimmed bovine milk and water-buffalo milk respectively and intersect at medians. 25th and 75th percentiles are shown as ticks on the bars.

Analysis of variance (data not shown) showed that for the mesophilic lactobacilli, acid production in bovine skim milk was significantly higher ($p = 1\%$) than that in water-buffalo skimmed milk, without interaction between the sources of variability (bacterial strain and milk type). On the contrary, for the thermophilic lactobacilli and the *Leuconostoc spp.* interaction is highly significant. The majority of the strains of these groups had higher acid production in bovine skim milk, but some strains showed the reverse pattern. In contrast, the enterococci and thermophilic streptococci had higher acid-producing ability in water-buffalo skimmed milk ; the interaction between strain of bacteria and species of milk was highly significant. Mesophilic streptococci did not show differences between species of milk nor bacterial strain-species of milk interaction. Enterobacteria produced almost twice as much acid in bovine skim milk as in water-buffalo skimmed milk (the diffe-

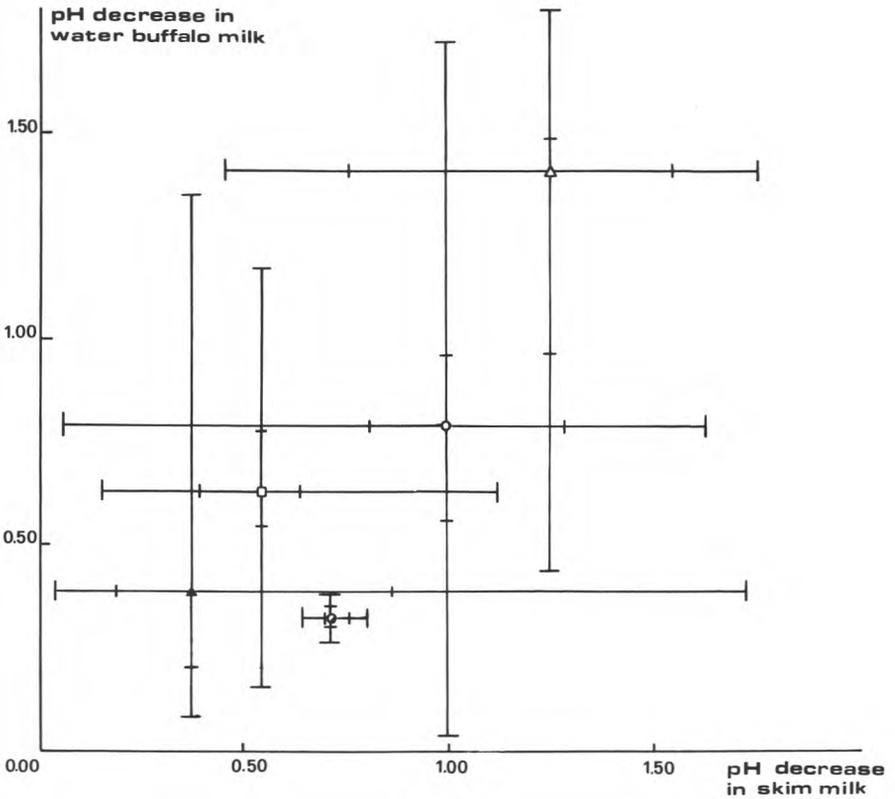


Fig. 2

Central tendency and dispersion statistics on the distribution of acid-producing ability in the taxonomical groups of strains isolated from natural whey cultures utilized as starters in the manufacture of water-buffalo Mozzarella cheese. Horizontal and vertical bars show the range of acid producing ability after 6 h in skim milk and skimmed water buffalo milk respectively and intersect at medians. 25th and 75th percentiles are shown as tics on the bars.

Tendance centrale et statistiques de dispersion pour la distribution de la capacité acidifiante dans les groupes taxonomiques des souches isolées de cultures naturelles en sérum utilisées comme levain dans la production du fromage « Mozzarella ». Les lignes horizontales et verticales définissent la fourchette de production d'acide après 6 h dans le lait écrémé de vache et le lait écrémé de bufflonne respectivement. Les lignes s'entrecoupent en correspondance de la médiane. Le 25^e et le 75^e percentiles sont individualisés par des traits sur les lignes.

- thermophilic lactobacilli △ thermophilic streptococci
 ▲ mesophilic streptococci □ enterococci
 ● enterobacteria

rence was significant at the 1 % level); interaction was not statistically significant.

Figure 3 shows the distribution of thermobacteria, mesophilic and thermophilic streptococci and enterococci on the basis of pH decreases in bovine skim milk after 6 hours of incubation at cheese vat temperature (36-37 °C). At this temperature the majority of thermophilic lactobacilli showed a lower acid-producing ability, and only caused the pH of skim milk to decrease of 0.5-1 units. The most active acid-producers were the thermophilic streptococci.

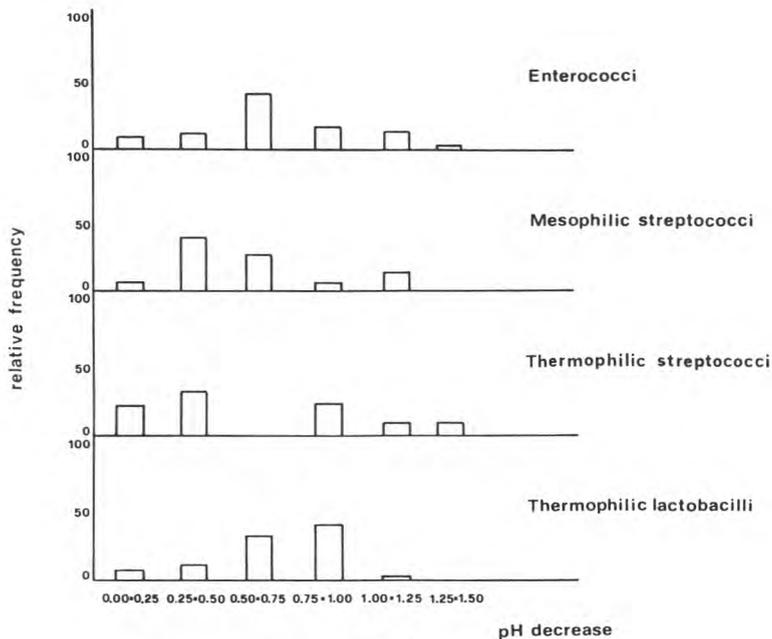


Fig. 3

Acid-producing ability in bovine skim milk at 37 °C of the strains isolated from natural whey cultures utilized as starters in the manufacture of water-buffalo Mozzarella cheese.

Production d'acide dans le lait écrémé de vache à 37 °C par des souches isolées de cultures naturelles en sérum utilisées comme levain dans la production du fromage « Mozzarella ».

Moreover, a fair number of enterococcal strains (about 13 %) were able to reduce the pH of skim milk by 1-1.25 units at this temperature. The mesophilic streptococci (mainly *Str. lactis*) showed a surprisingly high acid-producing ability at this temperature. Under the same experimental conditions, natural whey cultures caused a pH decrease of 0.85-0.88 units in skim milk and 0.90-1.13 units in water-buffalo skimmed milk. Figure 4 shows the distribution of the groups on the basis of their proteolytic activities in bovine skim milk. Enterococci had the highest proteolytic activity; some were able to release

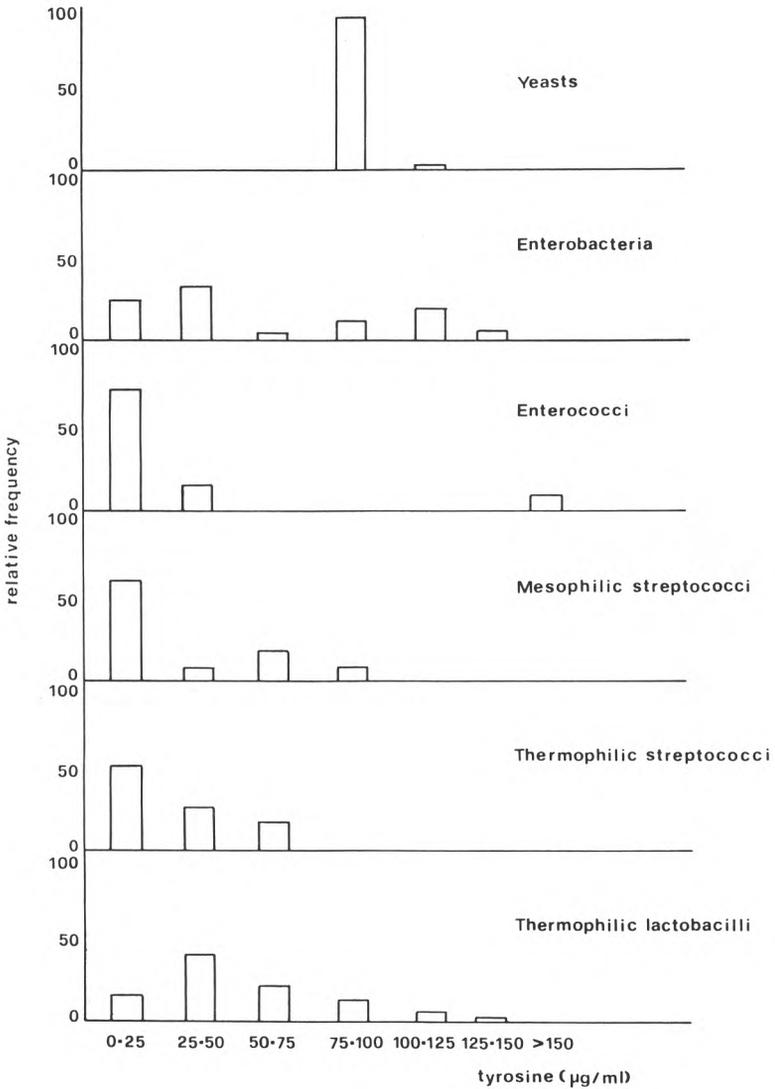


Fig. 4

Proteolytic activity in bovine skim milk (see the text for temperature of incubation) of the strains isolated from natural whey cultures utilized as starters in the manufacture of water-buffalo Mozzarella cheese.

Activité protéolytique à 37 °C dans le lait écrémé de vache de souches isolées de cultures naturelles en sérum utilisées comme levain dans la production du fromage « Mozzarella ».

more than 150 μg tyrosine/ml. Typically, the strains of this group could be divided into two classes one of which contained a few strains which were highly proteolytic ($> 150 \mu\text{g}$ tyrosine/ml) and the other, a larger number of strains capable of releasing not more than 50 μg tyrosine/ml. Thermobacteria and enterobacteria showed very variable proteolytic activity, while mesophilic streptococci had low activities. All yeast strains showed fairly good proteolytic activities.

Natural whey cultures can release 66-72 μg tyrosine/ml under the same experimental conditions.

III. Discussion and conclusions

The major aim of this study was to quantify the main microbial groups and to isolate the largest possible number of different species occurring in natural whey starters. The microbial contents were characterized by large variability; this is attributable in part to the difficulties in the enumeration of the different groups of microorganisms. No selective media are available for many of those groups and those that are of little value (e.g. Mayeux and WL agars for *Leuconostoc* and *Pediococci* respectively). Nevertheless the results show an obvious and complete predominance of lactic acid bacteria. Many species of the genera *Lactobacillus* and *Streptococcus* were isolated. The presence of both thermophilic and mesophilic species can be attributed to the incubation conditions and use of natural whey starters in the manufacture of Mozzarella cheese.

Lower counts of other microbial groups (enterococci, *Leuconostocs*, enterobacteria, yeasts) were obtained. Nonetheless they may be responsible for some biochemical activity in the manufacture of Mozzarella cheese from water-buffalo milk.

The numerical importance of the enterococci suggests that further research on their role in proteolysis during curd ripening should be carried out.

Leuconostocs, enterobacteria and yeasts reached counts in the range of $10^4/\text{ml}$ in some instances but more often $> 10^5$. Their biochemical activities should also be taken into account. The ability of *Leuconostoc* to produce flavour compounds and to synthesize large amounts of extracellular polysaccharides could be important in Mozzarella cheese manufacture.

The occurrence of enterobacteria (about 10^6 cfu/ml) is related to the use of raw milk and to the storage conditions of the whey culture, neither of which include any kind of precaution or control of contamination. Current manufacturing technologies of Mozzarella cheese relies, as in the past (SCASSELATI-SFORZOLINI *et al.*, 1956), on the heat treatment of the curd during stretching for the destruction of pathogenic or potentially pathogenic microorganisms. However, coliforms are often found in the product ready for consumption, because the curd stretching step includes an uneven temperature profile and because subsequent contamination occurs. The claim by HOWIE (1981), that *E. coli*, whatever its number, is not an indicator of the occurrence of enteric pathogens like *Salmonella* and *Shigella* in dairy products is widely

acceptable. The contribution of these microorganisms to the transformation of water-buffalo milk into Mozzarella cheese cannot be considered secondary, both because of their active metabolism (proteolysis, mixed sugar fermentation, etc.) and because of the size their population attains during curd ripening. However, the isolation of enteropathogenic serotypes infers that about 50 % of the strains of *E. coli* isolated could be agents of infant or adult diarrhoea. Therefore, the occurrence of these microorganisms represents a health risk and it is concluded that the rationalization of Mozzarella cheese manufacture must involve the pasteurization of cheese milk and the use of technologies that inhibit the growth and production of toxins by *E. coli*.

Finally, yeasts can reach high numbers in the whey culture. Previous studies (ADDEO and COPPOLA, 1983) showed that they do not multiply during curd ripening. The role they play in this biological system, in synergy with other microbial groups, is not understood. Further investigations may show a role for them; at present there is no experimental evidence to suggest that these microorganisms must be considered contaminants.

In conclusion the natural whey cultures currently used as starters in Mozzarella cheese manufacture consist of a large number of microorganisms and a great variety of microbial groups. The occurrence of potentially pathogenic microorganisms and the inconstant technological performances of the starter suggest that natural whey cultures should be replaced by selected starters of known composition. It is felt, though, that the range of biochemical activities attributable to the complex microflora of the natural starters described in this study cannot be reproduced using simple associations of lactobacilli and streptococci.

Reçu le 19 août 1986.

Accepté pour publication le 15 janvier 1988.

Acknowledgements

This research was supported by grants provided by Regione Campania and Ministero della Pubblica Istruzione, Rome.

References

- ACCOLAS J.P., AUCLAIR G., 1964. Etude bactériologique des levains utilisés dans la fabrication de Gruyère. *Ann. Technol. Agric. INRA*, 13, 85-96.
- ADDEO F., COPPOLA S., 1983. Aspetti tecnologici e microbiologici della trasformazione del latte di bufala in mozzarella. *Latte*, 8 (10), 706-723.
- ANNIBALDI S., 1969. Modificazione della prova di Weinzirl per la ricerca dei clostridi butirrici nel latte. *Sci. Tec. Latt.-Casearia*, 20 (4), 75-79.
- BARNES E.M., 1956. Methods for the isolation of faecal streptococci (Lancefield Group D) from bacon factories. *J. Appl. Bacteriol.*, 19 (2), 193-203.
- BARNETT J.A., PAYNE R.W., YARROW D., 1983. *Yeasts. Characteristics and identification*. Cambridge University Press, Cambridge, London and New York.
- BOTTAZZI V., 1962. Ricerche sulla microbiologia del formaggio Grana. Nota III. Studio della microflora del siero-fermento usato nella fabbricazione del formaggio Grana tipico. *Ann. Microbiol.*, 12 (1), 59-72.
- BOTTAZZI V., BERSANI C., SARRA P.G., MAGRI M., 1977. Caratteristiche dei batteri lattici presenti nelle colture naturali in siero. I. Tassonomia, morfologia, attività da beta-galattosidasi e da lattico-deidrogenasi. *Sci. Tec. Latt.-Casearia*, 28 (6), 430-442.

- BOTTAZZI V., VESCOVO M., 1973. Ricerche sulla microbiologia del formaggio Grana. IX parte : Caratteri e distribuzione dei biotipi di *L. helveticus* nelle colture naturali (sieri-fermenti). *Sci. Tec. Latt.-Casearia*, 24 (1), 23-39.
- BOURGOIS C.M., LEVEAU J.Y. (eds.), 1980. *Techniques d'analyse et de contrôle dans les industries agro-alimentaires*. Volume 3. *Le contrôle microbiologique*. APRIA, Paris, 122.
- CHAPMAN H.R., SHARPE M.E., 1981. Microbiology of cheese. In : *Dairy microbiology*. vol. 2, ed. Robinson R.K., Applied Sciences Publishers, London and New York, 157-243.
- CITTI J.E., SANDINE W.E., ELLIKER P.R., 1963. Some observations on the Hull method for measurement of proteolysis in milk. *J. Dairy Sci.*, 46, 337.
- COPPOLA S., PARENTE E., DUMONTET S., COPPOLA R., 1985. La fabbricazione della mozzarella di bufala : attività fermentative durante la maturazione della cagliata. *Ann. Microbiol.*, 35, 211-220.
- DAVIS J.G., 1958. A convenient semisynthetic medium for yeast and mould counts. *Lab. Pract.*, 7, 30.
- DEIBEL R.H., SEELEY H.W., 1974. *Streptococcaceae fam. nov.* In : *Bergey's Manual of Determinative Bacteriology*, ed. Buchanan R.E. and Gibson N.E., Gibbons R.E., Baltimore N.E., Williams and Wilkins.
- ELLIKER P.R., ANDERSON A.W., HANNESSON G., 1956. An agar culture medium for lactic streptococci and lactobacilli. *J. Dairy Sci.*, 39, 1611-1612.
- FORMISANO M., MINCIONE B., 1982. *Studio monografico sugli aspetti bio-tecnologici della lavorazione del latte di bufala*. Arti Grafiche Russo, Caserta.
- GALLI A., OTTOGALLI G., VOLONTERIO G., 1984. Metodo per la conta ed identificazione dei batteri propionici nei prodotti lattiero-caseari. *Ind. Latte*, 20 (1), 19-32.
- GARVIE E.I., 1967. *Leuconostoc oenos* sp. nov. *J. Gen. Microbiol.*, 48 (3), 431-438.
- HARRIGAN W.F., McCANCE M.E., 1976. *Laboratory methods in food and dairy microbiology*. Academic Press, London, 320-321.
- HOWIE J., 1981. Significance of *Escherichia coli* in cheese. *Lancet*, October 24, 939.
- HULL M.E., 1947. Studies on milk proteins. II. Colorimetric determination of the partial hydrolysis of the proteins of milk. *J. Dairy Sci.*, 30, 881.
- KRUSH VON U., LOMPE A., 1982. Schnelltest zum qualitativen Nachweis von L(+)- und D(-)-Milchsäure für die Bestimmung von Milchsäurebakterien. *Milchwissenschaft*, 37 (2), 65-68.
- LAW B.A., 1982. Cheeses. In : *Fermented foods*, ed. Rose A.H., Academic Press, London, 148-198.
- LODDER J., 1970. *The yeasts*. North-Holland Publ. Co., Amsterdam.
- DE MAN J.C., ROGOSA M., SHARPE M.E., 1960. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.*, 23 (2), 130-135.
- MATEUZZI D., 1967. Studi sulla microflora dei sieri-innesti del formaggio Parmigiano-Reggiano. Nota I. Identificazione delle specie più rappresentative. *Ann. Microbiol.*, 17 (2), 201-210.
- MAYEUX J.V., SANDINE W.E., ELLIKER P.R., 1962. A selected medium for detecting leuconostoc organisms in mixed strains starter cultures. *J. Dairy Sci.*, 45, 655-656.
- Ministero della sanità della Repubblica italiana, 1978. Circolare n° 88. December 15.
- NAYLOR J., SHARPE M.E., 1957. Lactobacilli in Cheddar cheese I. The use of selective media for isolation and of serological typing for identification. *J. Dairy Res.*, 25, 92-103.
- ROGOSA M., 1974. Genus I. *Lactobacillus*. In : *Bergey's Manual of Determinative Bacteriology*, ed. Buchanan R.E. and Gibson N.E., Gibbons R.E., Baltimore N.E., Williams and Wilkins.
- SCASSELATI-SFORZOLINI G., ROSSINI G., MARIOTTI G., 1956. Influenza della tecnica di lavorazione della mozzarella sul contenuto microbico del prodotto finito. *Nuovi Ann. Ig. Microbiol.*, 7 (2), 133-138.
- VALLES E., MOCQUOT G., 1972. Etude sur la technique de préparation de la présure utilisée dans les fabrications traditionnelles des fromages de gruyère de Comté et d'Emmental. *Lait*, 52 (515-516), 259-282.
- VEDAMUTHU E.R., WASHAM C., 1983. Cheese. In : *Biotechnology* vol. 5, ed. Rehm H.J., Reed G., Verlag-Chemie, Weinheim, 213-313.