Physicochemical and microbiological evaluation of corrientes artisanal cheese during ripening

Avaliação físico-química e microbiológica de queijo artesanal corrientes durante a maturação

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Abstract

The aim of this study was to evaluate some physical and chemical parameters (total solids, pH, acidity, fat, acid degree value of fat, salt, protein and nitrogen fractions) and their effects on the beneficial (lactic acid bacteria: LAB) and undesirable microbial populations (coliforms, *Escherichia coli*, *Staphylococcus aureus*, moulds, and yeast) during ripening of Artisanal Corrientes Cheese, an Argentinian cow's milk variety, to determine whether a longer ripening period than usual improve its hygienic-sanitary quality. The protein content was much higher than that of other cow's milk cheeses with similar values of fat. The larger peptides showed values three times higher in the 30 day-old cheese than those obtained in the beginning of the process. *Staphylococcus aureus* and *Escherichia coli* were detected (3.04 ± 1.48 $\log_{10}$ cfu/g of cheese, 2.21 ± 0.84 $\log_{10}$ MPN/g of cheese) even at 15 and 30 days of ripening, respectively. The distribution of three hundred LAB strains classified to the genus level (lactococci:lactobacilli:leuconostocs) was maintained during the ripening period. The high number of LAB in rennet may have contributed to the fermentation as a natural whey starter, unknown source of LAB for this specific cheese so far. The physicochemical changes that occur during ripening were not big enough to inhibit the growth of undesirable microorganisms.
Keywords: *artisanal cheese; raw cow milk; ripening changes.*

Resumo

O objetivo deste trabalho foi avaliar alguns parâmetros físicos e químicos (sólidos totais, pH, acidez, gordura, grau de acidez da gordura, sal, proteínas e frações de nitrogênio) e seus efeitos sobre as populações microbianas benéficas (bactérias lácticas: LAB) e indesejáveis (coliformes, *Escherichia coli*, *Staphylococcus aureus*, fungos e leveduras) durante a maturação do Queijo Artesanal de Corrientes, uma variedade argentina do leite cru da vaca, para determinar se um tempo de maturação mais longo do que o atual melhora as condições higiênico-sanitárias do queijo. O teor de proteína foi muito maior que de outros queijos o leite de vaca com valores semelhantes de gordura. Os péptidos maiores apresentaram valores, no queijo de 30 dias, três vezes mais altos que os do início do processo. *Staphylococcus aureus* e *Escherichia coli* foram detectadas (3.04 ± 1.48 $\log_{10}$ ufc/g de queijo, 2.21 ± 0.84 $\log_{10}$ NMP/g de queijo) ainda aos 15 e 30 dias de maturação, respectivamente. A distribuição das trezentas estirpes de LAB classificadas ao gênero (lactococci:lactobacilli:leuconostocs), foi mantida durante toda a maturação. O número elevado de LAB em coalho pode ter contribuído na fermentação como um soro-fermento natural, fonte desconhecida de LAB para este queijo específico até agora. As mudanças físico-químicas que ocorreram durante a maturação não geram condições fortemente inibidoras para o crescimento de micro-organismos indesejáveis.
Palavras-chave: *queijo artesanal; leite cru da vaca; mudanças durante maturação.*

1 Introduction

The province of Corrientes (Northeastern Argentina) has a very long tradition of "Corrientes Artisanal Cheese" making. Some informal unpublished data fall between the years 1650 and 1700, the beginning of the cheesemaker art as a historical and cultural heritage in this region (VASEK; FUSCO; CARDOZO, 2011). This product does not have a Protected Designation of Origin status, but it plays an important role in dairy farmers' economy, especially for women. This production could be increased by controlling the fermentation process, preserving the artisanal characteristics of manufacturing.

Although no statistics are currently available, the volume of production of Corrientes Artisanal Cheese is very important. A survey conducted between 1994-1998 indicated the presence of 73 active cheese-makers in 29 different places distributed over the province extension area of 88.900 km^2, with an individual production of about 5-6 pieces of cheese/day during spring and summer. The demand for these cheeses is high, and they are intended mainly for the preparation of popular foods such as *"mbaypú"*, *"chipá"*, *"typyhú"*, *"kiveve"*, and *"borí-borí"* among others (VASEK; FUSCO; CARDOZO, 2011).

This cheese is a fatty blocked shape semi-soft product with a slightly acid sour flavor weighing 1.00-1.50 kg per piece. Its production, using raw cow's milk and homemade bovine milk coagulant, is based on spontaneous fermentation due to the

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development of the microbiota naturally present in the raw material. These cheeses are sold at different stages of ripening, generally fresh in the first week of production. However, over the last 4 years some cheese-makers have begun to produce cheeses with 1 month of ripening time, mainly at the end of the period of the highest level of cheese production, spring and summer, time when the cows are producing their highest milk yield.

It is well known that the differences in sensory quality between raw and pasteurized milk cheeses depend (PELÁEZ; REQUENA, 2005) mainly on the diversity and complexity of the microbial communities present in raw milk and on the factory environment. This biodiversity generates particular flavors in these cheeses contributing to the increase in global demand over the last few years (WOUTERS et al., 2002; SMIT; SMIT; ENGELS, 2005). Therefore, the wild strains of lactic acid bacteria (LAB) with particular technological characteristics are really required for new collections for industrial applications.

Accordingly Corrientes region is a singular niche of wild LAB due to the absence of dairy industries. Cheese production is artisanal and small-scale with spontaneous fermentation and the use of artisanal rennet; this is the reason why this natural ecosystem remains free from commercial strains of LAB.

Since there are not local governmental programs to protect this traditional product and its biodiversity, the cheese-markers established an organization ("Association of Fruits and Vegetable Producers, Farmers of non-traditional production and related of Corrientes") for the defense of their artisanal production in order to maintain the typical characteristics of this traditional cheese.

The scientific knowledge of Corrientes Artisanal Cheese is very limited. Pereira, Vasek and Fusco (1995) and Vasek et al. (2004) carried out studies on its hygienic-sanitary conditions and their relationship to its acceptance under the present laws (MERCADO…, 1993), and Vasek et al. (2008) carried out the microbiological and physicochemical characterization of this cheese at points of sales. These cheeses showed high levels of contamination with high probability of causing foodborne diseases although the occurrence of illnesses associated with consumption of these cheeses is unknown in this region.

Traditionally, these cheeses are sold fresh, but when the season of high production declines, some cheese-makers produce cheeses that were aged for one month for economic reasons. There are no data on the biochemical and microbiological changes that occur throughout the manufacture and ripening process.

The aim of this study was to determine the microbial load and evaluate some physical and chemical parameters and their effect on the microbial populations, both beneficial and undesirable, during ripening of cheeses to determine whether a longer ripening period than usual improve the hygienic-sanitary quality of these cheeses.

2 Materials and methods

2.1 Cheese making and sampling

Four batches of six cheeses were carried out in a farm-house in the Province of Corrientes (Argentina) region. This farm-house was chosen for its long-standing tradition of cheese making. The cheeses were produced in the spring and summer, seasons of high production, which coincides with the most abundant rains and the highest environmental temperatures.

The cheeses were manufactured by the cheese-maker following the traditional steps: fresh whole raw cow's milk was added to 1.0-1.5% (v/v) homemade bovine rennet. The artisanal rennet was prepared by immersing a strip of dried and salted bovine abomasum (1 g NaCl + 2 g raw material) in raw milk for 24 hours at a proportion of 60 g/L of milk. The whey resulted was transferred to a clean glass bottle and kept under refrigeration until use. No starter culture was added.

After 2 hours at 30-35 °C, the enzymatic curd was cut to the size of rice grains. The whey was drained off partially, and the curd grains were transferred to a cotton cloth to complete the drainage of the whey; next, a small amount of solid salt (1.8-2.0%, w/v) was gently mixed with the curd.

The cotton cloth with the fresh salty curd was put into a plastic mould and slightly pressed manually for 10 min and left to drain over night at 15 °C. The cheeses were ripened on the farm for 30 days under cold conditions (8 °C) and were turned over periodically.

Milk, rennet, curd, and 1, 5, 7, 15, and 30 day-old cheese samples were collected from each batch, according to the standards of the International Dairy Federation (INTERNATIONAL..., 1995); each batch consisted of six cheeses, and one whole cheese was taken each day of sampling. The samples were transported to the laboratory under refrigerated conditions (4-6 °C) and analyzed on the same day.

The cheese rind (2 cm, approximately) was discarded, and three sub-samples were extracted with a screw hold, grated, and prepared according to Ref. Nº 968.12 (ASSOCIATION..., 2006) for the chemical analyses and according to IDF (INTERNATIONAL..., 1996) for the microbiological analyses.

2.2 Physicochemical analysis

Total Solids (TS), Titratable Acidity (TA), Total Nitrogen (TN) (Kjeldahl method) and Fat (Gerber method) were determined in the milk and rennet samples according to AOAC (ASSOCIATION..., 2006) guidelines, 990.19-33-2-43, 920.124, 991.20-33.2.11, 2000.18, 33.2.27A, respectively. Titratable acidity was expressed as grams of lactic acid/100 mL of milk/rennet, and the percentage of protein was calculated using the conversion factor of 6.38.

TS, TA, TN, and NaCl, were determined in the curd and cheese samples according to AOAC (ASSOCIATION..., 2000) standards 926.08, 920.124-33.7.14, 920.123-33.7.12, 935.43-33.7.10, respectively. Titratable acidity was expressed as grams of lactic acid/100 g of cheese/curd, and the percentage of protein was calculated using the conversion factor of 6.38. Fat quantification was performed according to the IDF standard (INTERNATIONAL..., 1997).

The pH was measured with a microprocessor pH meter HI 8520 (HANNA INSTRUMENTS, USA) in liquid samples and in a 50% (w/v) slurry of curd or cheese with distilled water.

All determinations were performed three times.

2.3 Nitrogen fraction analysis

The total nitrogen (TN) content in the cheese samples was determined by the Kjeldhal method following the AOAC standard (ASSOCIATION..., 2000) procedure according to Ref. Nº 920.123-33.7.12.

The Jin and Park (1995) procedure was followed for the extraction of soluble fraction in water (W-SF). Grated cheese (10 g) mixed with 40 mL of water was processed for 2 minutes using a GLAS-COL k4424 (TERRE HAUTE IN, USA) homogenizer. The homogenate was held at 40 °C for 1 hour, and then it was centrifuged at 3000 g for 30 minutes. After centrifugation, the suspension was cooled at –5 °C for 10 minutes, the upper layer of fat removed, and the supernatant filtered through Whatmann Nº 1 filter paper (CLIFTON, NY). The Kuchroo and Fox (1982) method was used to extract the soluble fraction in trichloroacetic acid (12%): TCA-SF and the Aston et al. (1983) method for soluble fraction in (5%) phosphotungstic acid (PTA-SF).

The nitrogen content in 10 mL of each fraction (W-SF, TCA-SF, and PTA-SF) was determined in triplicate by the Kjeldhal method for quantification of W-NS, TCA-SN and PTA-SN, respectively, and expressed as %TN.

All determinations were performed three times.

The values of three secondary variables were calculated from the primary nitrogen data according to Ardö (1999): 'Casein N' by TN minus W-SN, 'larger peptides N' by W-SN minus TCA-SN, and 'smaller peptides N' by TCA-SN minus PTA-SN.

2.4 Microbiological analysis

Appropriate further decimal dilutions of homogenates (curd and cheese) and liquid samples (milk and rennet) were made in a peptone-saline solution (0.1-0.85%, w/v) for the enumeration of different microbial groups. Coliforms to 30 °C and *Escherichia coli* (*E. coli*) were determined in Mc Conkey broth (Merck) and BRILA broth (Merck), according to the Most Probable Number method (FENG et al., 2002). *Staphylococcus aureus* was determined on Baird-Parker agar (Merck) according to IDF (INTERNATIONAL..., 1990a) and confirmed by a positive coagulase test; moulds and yeasts were determined on acidified potato-dextrose agar (INTERNATIONAL..., 1990b). In addition, different media cultures were used to enumerate the lactic acid bacteria (LAB): Elliker agar (Biokar Diagnostic), for total LAB at 30 °C for 72 hours; M17 agar (Merck), for presumptive lactococci at 30 °C for 48 hours; MRS agar (Merck), for presumptive lactobacilli at 30 °C for 72 hours under anaerobiosis; and MSE agar (Merck), for presumptive leuconostocs at 25 °C for 5 days.

2.5 Identification of the lactic acid bacteria to genus level

At least five colonies were taken randomly from each plate, purified by streaking three times on Elliker agar plates, and stored at –20 °C in a cryoprotective medium: 10% reconstituted skim milk powder containing 0.5% (w/v) yeast extract, 1.0% (w/v) glucose, and 10% (v/v) glycerol. The working strains were transferred to Elliker broth medium and stored at 4 °C. Prior to the identification tests, the isolates were sub-cultured twice in the same broth for activation.

The identification tests were mainly performed on LAB to genus level according to Axelsson (1998). Three hundred isolates were examined for morphology, Gram staining, catalase activity, spore formation, CO_2 production from glucose under anaerobic conditions, aerobic ability to grow at 10 and 45 °C, ability to grow at initial pH 9.6, and aerobic growth in 6.5% NaCl broth.

Additional tests for presumptive lactobacilli were used: the ability to grow in acetate agar after 3 days at 30 °C and the inability to grow at pH 9.0.

2.6 Statistical analysis

The microbiological data were transformed into logarithms prior to statistical analysis. Analysis of variance was performed to determine the effect of ripening time on the components. Means with a significant difference ($p < 0.05$) were compared by the least squares difference (LSD) test; principal component analysis was performed using the correlation matrix of standardized data of the microbiological, chemical, and proteolysis results obtained during the manufacture and ripening of the cheeses. Statistical analysis and graphics were performed using the Infostat Software. (DI RIENZO et al., 2008).

3 Results and discussion

3.1 Physicochemical parameters during manufacture and ripening

Table 1 shows the physicochemical parameters (mean ± standard deviation) throughout the manufacture and ripening of Corrientes Artisanal Cheese.

Total solid content in the curd (26.72 ± 1.1) increased ($p > 0.05$) during the aging process reaching the final values of 58.56 ± 1.5% (w/w) in 30 day-old cheeses.

The high moisture values observed in these cheeses (57.1-60.1%, at 30 days of ripening) are probably due to the combination of two factors. The first is the tropical climate of this region, which is characterized as hot and humid from October to March (spring and summer), with maximum daily temperatures of 35-45 °C and 95-97% of atmospheric humidity. The second is that all of these family productions of cheeses use commercial refrigerators for ripening the cheeses without internal control of moisture, temperature, and aeration. The high ambient temperatures and relative humidity in addition to frequent opening of the refrigerator door result in a low probability of maintaining proper conditions of cheese ripening without packaging, thus generating ecosystems suitable for the

Table 1. Physicochemical composition during the manufacture and ripening of Corrientes Artisanal cheese (mean value ± standard deviation corresponds to three determinations in four cheeses).

	Milk	Rennet	Curd	Cheese ripening time (days)				
				1	5	7	15	30
TS[A]	10.52 ± 0.6[a]	10.25 ± 0.3[a]	26.72 ± 1.1[b]	51.52 ± 0.1[c]	52.24 ± 0.8[c]	52.81 ± 0.3[c, d]	53.94 ± 0.2[d]	58.56 ± 1.5[e]
pH	6.53 ± 0.1[a]	3.93 ± 0.2[b]	6.00 ± 0.5[c]	5.06 ± 0.1[d]	4.33 ± 0.0[e]	4.53 ± 0.0[e]	5.02 ± 0.0[d]	5.50 ± 0.0[f]
TA[B]	0.13 ± 0.1[a]	0.82 ± 0.2[b]	0.12 ± 0.2[a]	0.75 ± 0.1[b]	1.98 ± 0.3[c]	2.85 ± 0.2[d]	2.75 ± 0.2[d]	2.55 ± 0.7[d]
Protein (TN x 6.38)[C]	40.67 ± 2.9[a]	29.09 ± 0.1[b]	31.83 ± 1.9[b]	44.22 ± 2.5[a]	48.25 ± 3.0[c]	49.66 ± 2.0[c, d]	52.97 ± 1.6[d]	55.06 ± 1.6[e]
Fat[C]	42.19 ± 2.4[a]	ND	31.86 ± 1.7[b]	47.03 ± 1.2[c, d]	46.25 ± 1.1[c]	48.88 ± 1.2[d, e]	50.78 ± 1.9[e, f]	51.42 ± 0.8[f]
ADV	ND	ND	ND	1.03 ± 0.0[a]	1.32 ± 0.1[a, b]	1.63 ± 0.0[b, c]	1.96 ± 0.2[c]	3.76 ± 0.7[d]
NaCl[C]	ND	ND	ND	3.87 ± 0.4[a]	4.63 ± 0.3[b]	4.64 ± 0.4[b]	4.37 ± 0.4[a, b]	4.08 ± 0.3[a, b]

TS: total solids; TA: titratable acidity; TN: total nitrogen; ADV: acid degree value; ND: not determined. [A]the results are expressed as g 100 g^{-1} of milk, rennet, curd or cheese; [B]g of lactic acid 100 mL^{-1} of milk or rennet and 100 g^{-1} of curd or cheese; [C]the results are expressed as g 100 g^{-1} of TS. [a-f]Means in the same row with different superscripts differ significantly ($p < 0.05$).

proliferation of the adventitious microorganisms and a weighty spoilage factor in the period of highest cheese production.

The pH of milk, slightly acid (6.53 ± 0.1), decreased ($p < 0.05$) in the curd and underwent a rapid fall ($p < 0.05$) reaching the lowest values between the fifth and seventh days of ripening and then it increased ($p < 0.05$) to 5.50 at 30 days of ripening. The titratable acidity reached the maximum value at the 7th day of ripening (2.85 ± 0.2 g of lactic acid 100 g^{-1} of cheese), showing an expected evolution profile taking into account the variation of the pH profile. There was no significant difference between the values detected on days 5 and 7, and subsequently the titratable acidity profile showed fairly uniform ($p < 0,05$) values.

Different authors (OLSON, 1990; ALONSO-CALLEJA et al., 2002; CICHOSCKI et al., 2002; VOLKEN DE SOUSA; DALLA ROSA; ZACHIA AYUB, 2003; ARENAS et al., 2004; PINHO et al., 2004) found erratic evolution profiles for the pH or titratable acidity during the ripening of the artisanal cheeses. The absence of logic relationships between pH and acidity values, in Corrientes Artisanal Cheese show the influence of buffer activity, which can occur during the aging process.

The increase in pH and decrease in acidity values after the seventh day of ripening, another risky factor for the survival of contaminating microorganisms, would be the consequence of the proteolytic effect of wild lactic bacteria with the release of alkaline compounds, ammonium production by the amino acid catabolism of *Lactococcus* spp. strains (ARDÖ, 2006), or activity of microorganisms that use lactic acid such as yeasts. These discrepancies suggest that the design of a starter culture with indigenous bacteria would be useful to standardize partially the biochemical changes that occur during the manufacture and ripening of these cheeses.

The pH and titratable acidity values observed between the 1st and 5th days of ripening in the present study, are in total agreement with the previous results in Corrientes Artisanal cheese obtained at the sales points (VASEK et al., 2008), confirming the commonly short maturation times used for this cheese.

Mean values of protein (TN x 6.38) and fat, expressed as g/100 g of the TS, are in agreement with previous data (VASEK et al., 2008) concerning this cheese at the sales points, which were detected at ranges of 49.57-58.78 protein-%TS and 48.98 to 52.38 fat-%TS, respectively. The protein content found in these cheeses was higher than that of other cheeses (CUESTA et al., 1996; PRIETO et al., 2000; FRANCO et al., 2001; MENÉNDEZ et al., 2001; CICHOSCKI et al., 2002) made from cow's milk with similar values of fat. Although Corrientes Artisanal Cheese parameters have not been standardized yet, the high values observed for the protein:fat ratio, specifically casein to fat ratio, have a significant positive effect on cheese yield and obviously on the cheese-makers' economy.

Lipolysis was measured as acid degree of fat reaching the values of 3.76 ± 0.7 mg KOH/g of fat in 30 day-old cheeses. These values, expressed according to American Public Health Association (CASE; BRADLEY JUNIOR; WILLIAMS, 1985), were < 0.4, corresponding to normal fat inferring a very limited activity of lipolytic enzymes from the microbiota or rennet.

The NaCl content (expressed as g/100 g of TS) increased slightly ($p > 0.05$) between the beginning and the end of ripening. Previous results on artisanal cheeses at the sales points in Corrientes (VASEK et al., 2008) showed two very different groups, the most representative, with ≤ 3.4 g NaCl-%TS, and a minor population that included the most salty cheeses (approximately 4.5 NaCl-%TS), providing evidence of the difference in their manufacture and ripening times. The addition of solid salt instead of brine to the grains of curd and the absence of a true mix, from the technological viewpoint, cause a heterogeneous distribution of the salt in the face of each grain of curd for each future cheese.

On the other hand, salt plays different roles in cheese ripening and affect its properties as well as the control of microbial growth. The concentration of salt dissolved in the cheese, rather than the salt added, determines (BERESFORD et al., 2001) its inhibitory effect. The aforementioned considerations indicate that under these conditions, the salt content and the micro-zones with minimal or no salt concentration create an unequal distribution of it, which is not enough to affect extensively the growth of adventitious microorganisms in the cheese enabling the development and action of wild lactic bacteria.

3.2 Proteolytic changes during ripening

Table 2 shows the evolution of the classical nitrogen fractions in four batches of Corrientes Artisanal Cheese.

Table 2. Nitrogen fractions of Corrientes Artisanal Cheese during ripening (mean value ± standard deviation corresponds to three determinations in four cheeses).

	Cheese ripening time (days)				
	1	5	7	15	30
TN[A]	3.65 ± 0.2[a]	3.96 ± 0.3[a, b]	4.11 ± 0.2[b, c]	4.47 ± 0.1[c]	5.33 ± 0.3[d]
W-NS[B]	4.50 ± 0.3[a]	4.79 ± 0.5[a]	5.80 ± 0.1[b]	7.76 ± 0.3[c]	8.05 ± 0.1[c]
Casein N[B]	95.50 ± 0.3[a]	95.21 ± 0.5[a]	94.20 ± 0.1[b]	92.24 ± 0.3[c]	91.95 ± 0.1[c]
TCA-NS[B]	3.72 ± 0.2[a]	3.90 ± 0.2[a, b]	4.24 ± 0.8[b]	5.22 ± 0.7[c]	5.72 ± 0.6[d]
Larger peptide N[B]	0.78 ± 0.2[a]	0.89 ± 0.3[a]	1.56 ± 0.0[b]	2.54 ± 0.2[c]	2.33 ± 0.3[c]
PTA-NS[B]	2.49 ± 0.7[a]	2.72 ± 0.5[a]	2.92 ± 0.2[a, b]	3.63 ± 0.4[b]	4.58 ± 0.7[c]
Smaller peptide N[B]	1.23 ± 0.5[a]	1.18 ± 0.2[a]	1.32 ± 0.2[a]	1.59 ± 0.3[a]	1.14 ± 0.2[a]

TN: total nitrogen, W-NS: soluble fraction in water, Casein N: nitrogen from casein, TCA-NS: soluble fraction in (12%) trichloroacetic acid, Larger peptide N: nitrogen from larger peptides, PTA-NS: soluble fraction in (5%) phosphotungstic acid, Smaller peptides N: nitrogen from smaller peptides. [A]Expressed as g 100 g^{-1} of cheese; [B]Expressed as g 100 g^{-1} of TN. [a-d]Means in the same row with different superscripts differ significantly ($p < 0.05$).

The W-SN content increased ($p < 0.05$) during ripening. The average of the final values (8.05 ± 0.1 %TN) for the Corrientes Artisanal Cheese was close to that found in Afuega'l Pitu (CUESTA et al., 1996) and León (PRIETO et al., 2002), but lower than that found in Peñamellera (ESTEPAR et al., 1999) and Prato (GOROSTIZA et al., 2004) starter-free cheeses made from raw cow's milk at the same ripening time.

TCA-NS fraction indicates the intensity degree of proteolysis in cheeses. The mean values at 30 days of ripening (5.72 ± 0.6) were similar to those of some authors (MENÉNDEZ et al., 2001; PRIETO et al., 2002) but lower than others' (ESTEPAR et al., 1999; GOROSTIZA et al., 2004), who investigated cheeses made from raw cow's milk without the addition of starter and at the same ripening time. The variability found is predictable due to the diversity of LAB and rennet enzymes of each particular cheese, which are responsible of the gradual breakdown of high and medium molecular mass peptides and caseins into lower molecular mass peptides and amino acids.

The nitrogen content of the larger peptide underwent a great increase ($p < 0.05$) during ripening showing values three times higher in the cheese after 30 days of ripening than those at the beginning of the manufacturing process. This fraction is the residual result between its production, mainly due to chymosin and its degradation by enzymes of microbial origin. This high content at the end of the ripening time of the cheeses (2.33 ± 0.3), an important factor in the development of cheese texture, makes evident the contribution of the rennet activity to this fraction.

The peptides of low molecular mass (<600Da) and amino acids present in the cheese are soluble in 5% PTA. They are produced, mainly, by the hydrolytic action of LAB and may be regarded as indicators of aroma and taste. The values obtained for 30 day-old cheeses (4.58 ± 0.7 %TN) were abnormally higher than those of other cheeses (CUESTA et al., 1996; MENÉNDEZ et al., 2001; GOROSTIZA et al., 2004) of similar characteristics, which indicates an intense enzymatic activity of LAB for this production. In fact, the proteolytic metabolism of LAB, necessary for their survival, involves numerous biochemical reactions with a greater biosynthetic potential in wild strains (SMIT; SMIT; ENGELS, 2005) than in industrial strains.

Comparing these values with those reported for this cheese at the sales points (VASEK et al., 2008), it can be said that this cheese is mainly available to consumers in the fresh form.

On the other hand, the use of artisanal coagulant often entails problems due to the lack of standardized activity. Some authors (PRIETO et al., 2004; PEREIRA et al., 2008; JACOB; JAROS; ROHM, 2011) founded lower values of proteolysis index for cheeses made with farmhouse rennet than those of cheese made with commercial rennet. Although the most of clotting enzymes are lost in the whey during the manufacture of cheese with mild manual pressing, the contribution of artisanal rennet is undoubtedly. According to current knowledge on this specific cheese, both the high number of microorganisms and their enzymes activity as the own enzymes of coagulant agent contribute to proteolysis.

3.3 Microbial population dynamics

The microorganisms which indicate deficient hygienic-sanitary conditions, such as coliforms, staphylococci, moulds, and yeasts were present in high numbers (Table 3) in the Corrientes Artisanal Cheeses studied. These high levels are in accordance with the intrinsic parameters (high humidity and pH) poorly restrictive for adventitious microbial growth during the manufacture and ripening of these cheeses.

The mean values of all microorganisms evaluated in the milk and rennet were within the range of data previously mentioned (VASEK et al., 2004) for artisanal cheese marketed in Corrientes. The high counts in both ingredients, which play an important role in the cheese populations, could arise from poor hygienic practices during milking or during the manufacturing of homemade rennet.

The microbial levels observed in the rennet, with the exception of *Staphylococcus aureus*, were slightly higher ($p > 0.05$) than those observed in the milk. Although the pH of rennet is low (3.93 ± 0.2), this raw material is a key factor contributing to the microbial composition of the cheeses.

The number of coliforms present in the curd increased ($p < 0.05$) 2 $\log_{10}$ cycles reaching its highest value at the first day of cheese manufacture. Although the values observed were high, owing to improper quality of raw material and poor handling practices, the average of these microorganisms

Table 3. Microbiological composition of Corrientes Artisanal Cheese (mean value ± standard deviation corresponds to three determinations in four cheeses).

	Milk	Rennet	Curd	Cheese ripening time (days)				
				1	5	7	15	30
Coliforms[A]	3.37 ± 0.7[a]	3.51 ± 0.2[a]	3.34 ± 0.4[a]	5.63 ± 1.1[b]	4.87 ± 0.7[a,b]	4.78 ± 0.8[a,b]	4.42 ± 1.6[a,b]	3.71 ± 2.4[a,b]
E. coli [A]	2.23 ± 1.5[a]	3.51 ± 0.2[a,b]	3.06 ± 0.8[a,b]	4.40 ± 1.4[b]	4.37 ± 0.6[b]	4.13 ± 0.9[b]	3.90 ± 1.2[a, b]	2.21 ± 0.8[a]
Estafilococci[B]	2.78 ± 1.0[a,b]	<2.00[a]	2.65 ± 0.6[a,b]	3.21 ± 1.0[a,b]	3.97 ± 1.3[b]	3.72 ± 1.4[b]	3.04 ± 1.5[a,b]	<2.00[a]
Moulds[B]	2.10 ± 0.0[a]	2.24 ± 0.3[a]	2.65 ± 0.6[a]	2.59 ± 1.2[a]	4.50 ± 1.0[b]	2.65 ± 0.9[a]	<2.00[c]	<2.00[c]
Yeast[B]	2.84 ± 0.7[a]	3.24 ± 1.8[a,b]	4.18 ± 0.8[a,b,c]	5.88 ± 2.1[c,d]	5.25 ± 2.0[b,c,d]	5.65 ± 0.5[c,d]	6.35 ± 1.1[c,d]	7.21 ± 0.2[d]
LAB[B]	6.73 ± 0.6[a]	8.28 ± 0.4[b]	7.01 ± 0.7[a]	8.72 ± 0.5[b,c]	9.04 ± 1.4[c]	9.07 ± 0.3[b,c]	8.96 ± 0.3[b,c]	8.24 ± 0.5[b]

[A]The results are expressed as $\log_{10}$ NMP mL^{-1} for milk and rennet, or g^{-1} for curd and cheese. [B]The results are expressed as $\log_{10}$ cfu mL^{-1} for milk and rennet, or g^{-1} for curd and cheese. [a-d]Means in the same row with different superscripts differ significantly ($p < 0.05$).

decreased slightly ($p > 0.05$) reaching, in 30-day old cheeses, similar levels to those found in milk; these values were much lower than those reported previously (PEREIRA; VASEK; FUSCO, 1995; VASEK et al., 2004, 2008) for artisanal cheeses at the sales points in Corrientes. Numerous authors reported higher levels (ZÁRATE et al., 1997; ESTEPAR et al., 1999; GARCÍA FONTÁN et al., 2001; MENÉNDEZ et al., 2001; PSONI; TZANETAKIS; LITOPOULOU-TZANETAKI, 2003; VOLKEN DE SOUZA; DALLA ROSA; ZACHIA AYUB, 2003) for starter-free cheeses made from raw milk at the same ripening time. *E. coli* number (approximately 1 $\log_{10}$ cycle lower than total coliforms) remained at levels of 10^4 MPN/g during the first week of cheese manufacture. Although they had a significant decline ($p < 0.05$) remained present until the end of ripening.

Even though high numbers for coliforms and *E. coli* were observed during the process, it was noticed that in 30 day-old cheeses both populations decreased to similar values to those founded in the milk. Taking these results into account, it would be interesting to try to improve the quality of the milk and the milking practices employed.

Staphylococcus aureus, scarcely present in the milk and curd, increased ($p > 0.05$) their population reaching up to 9.33×10^3 cfu/g in 5 day-old cheeses; it then gradually decreased and was not detected at 30 days. Although enterotoxins production was not tested, the counts of *Staphylococcus* observed during cheese manufacture and ripening were not close to the level in which (BRYAN; GUZEWICH, J. J.; TODD, 1997) the enterotoxin concentrations lead to clinical symptomatology.

After reaching the highest count on the fifth day, the mould number (Table 3), decreased ($p < 0.05$) quickly (speed = 1.55×10^4 cfu/g/d) until no mould presence was detected, probably due to the absence of the oxygen necessary for growth. Anaerobic conditions, developed inside the cheese with the ripening progress, enabled the proliferation of yeasts, which remained unaffected for the metabolites from LAB.

Yeasts are widely distributed in the dairy environments and appear as natural contaminants in raw milk, air, and dairy utensils. Some important features able to explain their occurrence in cheese are attributable to their tolerance towards low pH, high salt concentrations, and their ability to grow on organic acids.

The increase ($p < 0.05$) in the number of yeasts during Corrientes Artisanal Cheese ripening agrees with that of other studies (CUESTA et al., 1996; ZÁRATE et al., 1997; PEREIRA-DIAS et al., 2000) inside the starter-free cheeses made from raw milk up to 30 days of storage.

Their presence would contribute to the alkalinization of this cheese, explaining the high values of pH observed (5.50 ± 0.0) at 30 days of ripening. Although their role in this ecosystem is not completely understood (WOUTERS et al., 2002), some positive contributions of yeasts to the final product characteristics, strongly dependent on the type of cheese, were attributed (FERREIRA; VILJOEN, 2003) to interactions with the LAB and the secondary flora.

LAB were the most abundant microbial group (Table 3) during the experiments (3-4 $\log_{10}$ cycles over all residual groups) and reached their highest value in the order of 10^9 cfu/g at 7 days of cheese ripening.

The high number ($p < 0.05$) of LAB which provides the coagulant is remarkable, which could be acting as a natural whey starter, unknown in this specific cheese.

Three hundred isolates from four batches of Corrientes Artisanal Cheese manufacture were subjected to primary recognition. All strains were Gram-positive, catalase negative, and non-spore-forming; two hundred fourteen and eighty six showed microscopic cellular morphology of cocci and rods, respectively.

For the purpose of this paper, the key characteristics used to assign a genus to the isolates made it possible to classify (Table 4) 196 (91.6%) and only 18 (8.4%) spherical strains as *Lactococcus* and *Leuconostoc*, respectively. The absence of growth when the strains were cultured in 6.5% NaCl, pH 9.6 and 45 °C exclude the presence of enterococci.

The rods family was divided into four groups: heterofermentative thermophilic (2.32%) and mesophilic (5.81%) strains and homofermentative thermophilic (4.65%) and mesophilic (87.21%) strains, which were classified as *Lactobacillus* according to their additional inability to grow at pH 9.0 and their ability to grow on acetate media.

The most abundant lactobacilli types (79 isolates) present in Corrientes Artisanal Cheese were mesophilics. They were considered (BERESFORD et al., 2001; WOUTERS et al., 2002) as secondary flora, but in general they play a significant role during cheese ripening due to their proteolytic activity, which affects the attributes of flavor.

In the last decade, they have been detected (COGAN et al., 1997; MAS et al., 2002; PSONI; TZANETAKIS; LITOPOULOU-

TZANETAKI, 2003; BALLESTEROS et al., 2006; PISANO et al., 2006; VELJOVIC et al., 2007) in different starter-free cheeses made from raw milk, and currently, their use in dairy applications as adjunct starter/cultures have increased significantly. These considerations will be important for a future design of a starter culture from wild strains for Corrientes Artisanal Cheese.

The viable counts of the lactic bacteria genus during manufacture and ripening of Corrientes Artisanal Cheeses are shown in Table 5. Lactococci was the dominant member of lactic bacteria, representing 67.67% of the LAB population during the first 5 days, showing a small subsequent decrease ($p > 0.05$). Some authors (BERESFORD et al., 2001; WOUTERS et al., 2002; PELÁEZ; REQUENA, 2005) reported the predominance of *Lactococcus* strains in a great variety of cheeses. Other authors (COGAN et al., 1997; ZÁRATE et al., 1997; ESTEPAR et al., 1999; MAS et al., 2002; PESIC-MIKULEC; JOVANOVIC, 2005) reported their preponderance in cheeses made from raw milk and spontaneous fermentation up to 1 month of ripening.

When the lactococci grow in milk, they produce acid by the sugar metabolism and, as a consequence, the growth of other indigenous bacteria is largely inhibited. In most cases, the wild lactococci do not produce sufficient amount of acid (COGAN et al., 1997; PELÁEZ; REQUENA, 2005) when they are tested**.** In fact, the lactic acid formed from the wild strains of Corrientes Cheese was not enough to inhibit the coliforms and *E. coli* population even in cheeses with 1 month of storage. To improve the safety of these products, it would be interesting to select some wild lactococci strains showing high acidifying activity with the aim of formulating a starter culture that, simultaneously, standardize the process.

The subgroup of lactobacilli detected in the curd increased significantly ($p < 0.05$) and constituted the second most numerous microbiota. The lactobacilli number (Table 5) in 30-day old Corrientes Artisanal Cheese was similar (CUESTA et al., 1996; ESTEPAR et al., 1999) or lower (GARCÍA FONTÁN et al., 2001; MENÉNDEZ et al., 2001; MARINO; MAIFRENI; RONDININI, 2003; VOLKEN DE SOUZA; DALLA ROSA; ZACHIA AYUB, 2003; ARENAS et al., 2004) than those found in other starter-free cheeses made from raw cow's milk.

Leuconostocs showed lower incidence. Since leuconostocs grow poorly in milk, this fraction of LAB was multiplied, reaching 1.41×10^8 cfu/g at 7 days of cheese ripening and decreasing slightly ($p > 0.05$) later to 1.99×10^7 cfu/g at 30 days. The counts were higher than those determined by Estepar et al. (1999), Marino, Maifreni and Rondinini (2003) and Arenas et al. (2004) and lower than those found by García Fontán et al. (2001) and Alonso-Calleja et al. (2002) in starter-free cheeses made from raw milk at the same ripening times.

Although *Leuconostoc* is not often used as a starter in cheese production, they have currently been considered for this purpose (SÁNCHEZ; MARTÍNEZ; RODRÍGUEZ, 2005) acting as flavor producers in combination with other LAB through their lactose and citrate co-metabolism.

The order of importance in the LAB population fractions isolated in Corrientes Artisanal Cheeses, lactococci > lactobacilli > leuconostocs, was maintained during all the entire

Table 4. Differential characteristics of lactic acid bacteria isolated from Corrientes Artisanal Cheese.

Test	Cocci (71.33)		Rods (28.66)			
n	18 (6.00)	196 (65.33)	2 (0.66)	5 (1.66)	4 (1.33)	75 (25.00)
Tetrad formation	-	-	-	-	-	-
CO_2 from glucose	+	-	+	+	-	-
Growth at 10 °C	+	+	-	+	ND	ND
Growth at 15 °C	ND	ND	-	+	-	+
Growth at 45 °C	-	-	+	-	+	-
Growth at 6.5% NaCl	-	-	ND	ND	ND	ND
Growth at pH 9.0	ND	ND	-	-	-	-
Growth at pH 9.6	-	-	-	-	-	-
Growth in acetate agar	ND	ND	+	+	+	+

The numbers in parentheses correspond to the isolates percentage of total lactic acid bacteria; ND: Not determined.

Table 5. Microbial populations and incidence of lactic acid bacteria during Corrientes Artisanal Cheese manufacture and ripening (mean value ± standard deviations of two replicates of four cheese expressed as $\log_{10}$ cfu mL^{-1} for milk or rennet and $\log_{10}$ cfu g^{-1} for curd and cheeses).

	Milk	Rennet	Curd	Cheese ripening time (days)				
				1	5	7	15	30
Lactococcus genus[A]	6.49 ± 0.4^{a} (57.00)	$8.07 \pm 0.3^{b,c}$ (62.00)	6.81 ± 0.1^{a} (63.00)	$8.55 \pm 0.2^{b,c,d}$ (67.67)	$8.87 \pm 0.9^{c,d}$ (67.67)	8.89 ± 0.3^{d} (65.76)	8.78 ± 0.2^{d} (65.40)	8.05 ± 0.3^{b} (63.78)
Lactobacillus genus[A]	6.13 ± 0.2^{a} (25.00)	7.58 ± 0.3^{b} (20.00)	6.33 ± 0.1^{a} (21.00)	$8.00 \pm 0.4^{b,c}$ (20.02)	$8.34 \pm 1.0^{b,c}$ (20.00)	8.42 ± 0.2^{c} (22.29)	$8.32 \pm 0.3^{b,c}$ (22.67)	7.64 ± 0.2^{b} (24.89)
Leuconostoc genus[A]	5.99 ± 0.7^{a} (18.00)	$7.54 \pm 0.4^{b,c}$ (18.00)	$6.21 \pm 0.6^{a,b}$ (16.00)	7.81 ± 0.7^{c} (12.31)	8.13 ± 1.5^{c} (12.33)	8.15 ± 0.9^{c} (11.95)	8.04 ± 0.3^{c} (11.93)	$7.30 \pm 0.5^{a,b,c}$ (11.33)

[A]The number in parenthesis corresponds to genus incidence for each analysis time. [a-d]Means in the same row with different superscripts differ significantly ($p < 0.05$).

manufacture and ripening process. With regard to starter-free cheeses made from raw milk, there are few studies available in the literature were found (ZÁRATE et al., 1997; ESTEPAR et al., 1999; VOLKEN DE; DALLA ROSA; ZACHIA AYUB, 2003) which report stability in the relationship of LAB types during and up to 30 days of cheese ripening. In the most of the cases, a predictable change of genus supremacy over time was found, mainly between 15 and 30 ripening days. Given the recognized sturdiness of wild strains, it would be helpful to take this biodiversity and design a starter culture with mixtures of these strains to contribute naturally to the safety of these products preserving their typical characteristics.

In order to reduce space dimensions to evaluate the variability and the relationship among the different variables during the ripening of the cheeses, the variables presented in Tables 1, 2, 3, and 5 were standardized and used in the Principal Component (PC) Analysis. Variability between the composition at 1, 5, 7, 15, and 30 days of ripening, and the relationships between the variables in the two first PC are shown in a biplot (Figure 1).

The two first components explained the 94% of the variance, with a cofenetic correlation coefficient of 0.998.

The first component accounted for 74.7% of the original variation and divided the microbiological variables, with exception of yeasts, associated to the first 7 days of ripening and the majority of physicochemical variables associated with the last 15 days of ripening. Therefore, the highest variability during ripening is explained with these groups of variables. Variations of TS (correlation (r) = 0.97), Fat and ADV (r = 0.88 and 0.97, respectively), pH (r = 0.87), Protein, W-SN, TCA-SN, and PTA-SN (all r > 0.87) can be explained by the first component, as well as the variation in the number of yeasts (r = 0.98). The negative associations with the microbiological components studied were coliforms and *E. coli* (r = –0.85 and –0.97, respectively), *Staphylococus aureus* (r = –0.97), LAB (r = –0.87), lactococci (r = –0.86), lactobacilli (r = –0.78), leuconostocs (r = –0.86), and moulds (r = –0.86).

As expected, the number of different LAB genus showed a strong correlation between them, while the number of yeasts was inversely correlated with the LAB, although both groups were grown at high levels during ripening. This PC separated the mould and yeast charge with an inverse correlation, as previously mentioned.

The second PC, conserved 19.5% of the original variability and showed a positive correlation with NaCl (r = 0.86) and acidity (r = 0.91) variations.

In conclusion, the variation of coliforms and mould counts was associated with the beginning of ripening; the *Staphylococcus aureus* and LAB content variations were associated with 5-7 days of ripening; TS, acidity, fat, protein, and nitrogen fraction variations were mostly associated with 15 days of ripening. The variation of yeasts and the pH increase were associated with the end (30 days) of ripening.

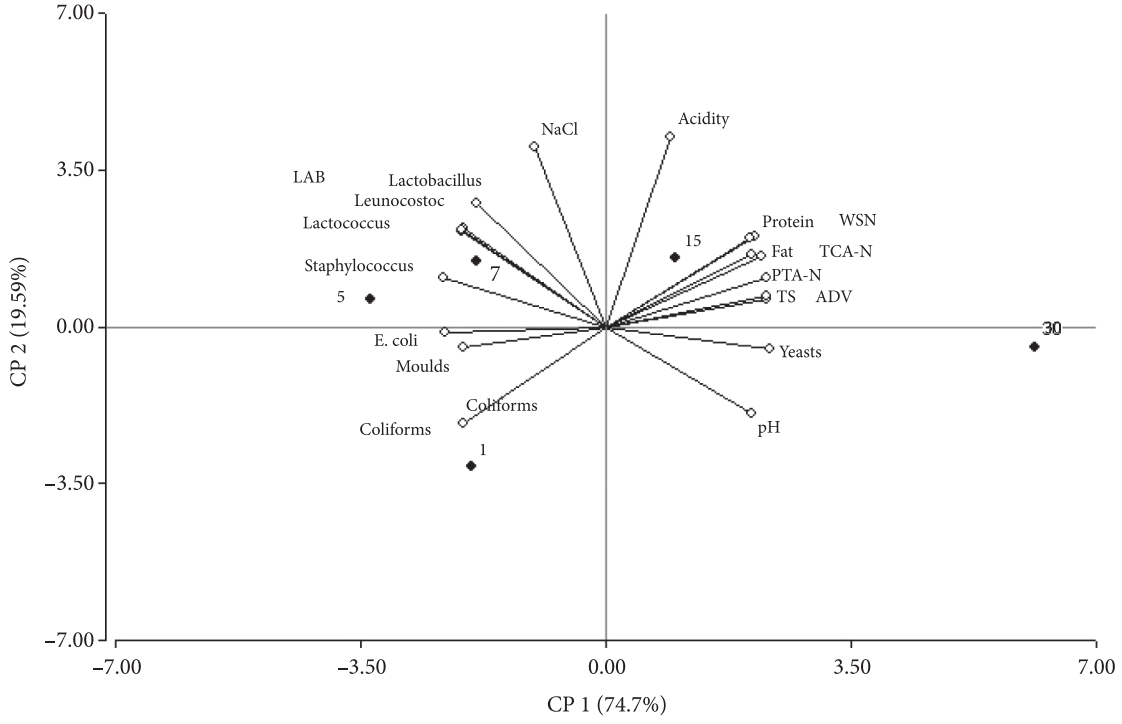

Figure 1. Biplot representing the first two components of the Principal Components Analysis performed on the standardized values of physicochemical (Table 1), nitrogen fractions (Table 2), and microbiological (Tables 3 and 5) composition for four batches of Corrientes Artisanal Cheese during manufacture and ripening. Variable Abbreviations: LAB = lactic acid bacteria; *E. coli* = *Escherichia coli*; WSN = water soluble nitrogen; TCA-N = trichloroacetic acid-soluble nitrogen; PTA-N = phosphotungstic acid-soluble nitrogen; ADV = acid degree value of fat; 1, 5, 7, 15 and 30 = days of cheese ripening.

4 Conclusions

The changes in the physicochemical parameters during ripening of Corrientes Artisanal Cheese do not create strongly antagonistic conditions for the growth of undesirable microbial groups evaluated.

The etiologic agents of the foodborne diseases studied (*Staphylococcus aureus* and *E. coli*) and their detection even at 15 and 30 days of ripening cheeses, respectively, represent a sanitary risk to public health. Bearing in mind the results obtained, and the maximum time recorded for the consumption of these cheeses, nowadays, the hygienic-sanitary quality cannot be guaranteed. The pasteurization of the milk, an easy solution, could provide a reduction of the undesirable microflora, but also of the rich microbial biodiversity responsible of the typical flavor. Unquestionably, 30 ripening days did improve the hygienic quality of the cheeses due to the decrease of undesirable microorganisms, but it is still not long enough.

The lactic acid bacteria genus implicated in this fermentation are lactococci, lactobacilli and leuconostocs in decreasing order of importance. This relationship of prevalence was maintained throughout the fermentation**.** According to the long tradition of these cheeses and their high acceptability, the next aim will be the taxonomic identification and technological characterization of the strains isolated from Corrientes Artisanal Cheeses in order to design an autochthonous specific starter culture that preserves the typical characteristics of the original product and that would be safer from a health point of view.

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