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Bioactive amines formation in milk by *Lactococcus* in the presence or not of rennet and NaCl at 20 and 32 °C

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Abstract

The influences of temperature, rennet and NaCl on bioactive amines formation in milk by a commercial starter culture containing *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* were investigated. Sterilized reconstituted dry milk was inoculated with 1% culture in the presence or not of rennet and NaCl and incubated at 20 and 32 °C/24 h. The milk had pH 6.69, acidity 17.58 °D and low levels of agmatine, spermidine, spermine, and cadaverine. There was a decrease of pH, increase in acidity and change in the levels of natural-to-milk amines. Biogenic amines—histamine, serotonin, 2-phenylethylamine and tryptamine—were also formed. Tyramine was only formed at 32 °C. Rennet favoured the formation of natural-to-milk amines and also of putrescine, tyramine, and tryptamine. NaCl decreased agmatine, spermidine, putrescine, histamine and tyramine levels. By decreasing incubation temperature and adding NaCl, it is possible to minimize bioactive amines formation.

Keywords: Amines; *Lactococcus*; Temperature; Histamine; Tyramine; Rennet; NaCl

1. Introduction

Bioactive amines are organic bases of low molecular weight which participate in normal metabolic processes in living tissues (Halász, Baráth, Simon-Sarkadi & Holzapfel, 1994; Lima & Glória, 1999). They can be classified as polyamines and biogenic amines. The polyamines spermine, spermidine and putrescine are naturally occurring amines in all living cells. They are involved in nearly every step of DNA, RNA and protein synthesis, being essential for cell growth and proliferation (Bardocz, Grant, Brown, Ralph, & Pusztai, 1993; Halász et al., 1994; Lima & Glória, 1999). Spermine and spermidine have also been implicated in the renewal and functioning of the digestive tract and in the maturation of intestinal tissue (Bardocz, 1993). The biogenic amines (histamine, putrescine, tyramine, tryptamine, 2-phenylethylamine and cadaverine) can be formed during storage or processing of food products by bacterial enzymatic decarboxylation of free amino acids (Halász et al., 1994; Lima & Glória, 1999).

Cheeses with high levels of biogenic amines have been described in the literature. However, the presence of these amines in cheese is undesirable from a toxicological standpoint. Cheeses with high histamine levels have been associated with outbreaks of histamine poisoning. The types of cheese incriminated include Swiss, Gouda, Gruyère, Parmesan and Provolone among others (Taylor, 1986; Bean, Goulding, Daniels & Angulo, 1997; Vale & Glória, 1998). The symptoms associated with intoxication include hypotension, nausea, vomiting, abdominal pain, diarrhea, facial flushing, burning throat, thirst, lip swelling, edema, localized inflammation, rash, itchiness and palpitation. Putrescine and cadaverine, also found in cheese, can increase histamine toxicity, facilitating its passage through the intestinal barrier (Chang, Ayres, & Sandine, 1985; Edwards & Sandine, 1981).

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Cheeses with high tyramine levels have been associated with outbreaks of cheese reaction, which is characterized by hypertension, fever, palpitation, headache, migraine, perspiration, nausea and vomiting (Diaz-Cinco, Fraijo, & Gradeja, 1992). Tryptamine and 2-phenylethylamine can also cause migraine. Furthermore, tyramine constitutes a potential health risk, especially when coupled with additional factors such as monoamine oxidase inhibitor drugs (antidepressants and antihypertensives), alcohol and gastrointestinal diseases (Stratton, Hutkins, & Taylor, 1991). At levels higher than 6 mg/100 g, tyramine can cause hypertensive crisis in patients under treatment with monoamine oxidase inhibitor drugs (Chang et al., 1985). For this reason, cheese has been restricted in diets of such patients (Fuzikawa, Hara, Glória, & Rocha, 1999).

Polyamines are naturally present in cow's milk. According to Sanguansermsri, Gyorgy, and Zilliken (1974), spermine, spermidine and putrescine were detected in fresh milk at levels of 0.8, 1.4 and 3.2 μg/ml, respectively. Bardocz et al. (1993) found the same amines; however, the levels detected were higher and spermine was the prevailing amine (40.4 μg/ml), followed by spermidine (36.25 μg/ml) and putrescine (13.2 μg/ml). No information was provided on the quality of the milk samples, which could account for the differences observed in amine levels and prevalence.

Cheeses represent an ideal environment for biogenic amine production. Whether or not amines accumulate and persist in cheese depends on a number of factors. Biogenic amine formation in cheese can be affected by the availability of free amino acids and the presence of microorganisms capable of amino acid decarboxylation. Conditions favourable for microbial growth and decarboxylase activity are also important, e.g., pH, salt concentration, temperature, water activity, maturation time, amine catabolism and presence of suitable cofactors (Fernández-García, Tomillo, & Nuñez, 2000; Greif, Drdak, & Greifova, 1995; Roig Sagués, Hernández Herrero, Rodríguez Jerez, Quinto Fernández, & Mora Ventura, 1998; Stratton et al., 1991).

Amino acid formation is mainly brought about by the action of proteolytic enzymes from the starter bacteria and the rennet which contribute to the breakdown of casein (Joosten, 1988). Amino acid decarboxylating enzymes show optimum activity at acid pH as a mechanism of environment neutralization against an excessive pH decrease which is incompatible with bacterial growth. Therefore, low pH values can favour amine production (Edwards & Sandine, 1981; Roig Sagués et al., 1998). The production of histamine is accelerated by high temperatures during production and manufacture of cheese (Joosten, 1988; Stratton et al., 1991). Increase in the concentration of salt can decrease amine formation (Edwards & Sandine, 1981; Joosten, 1988; Roig Sagués et al., 1998; Sumner, Roche, & Taylor, 1990). There can be a decrease on biogenic amine levels due to catabolic activity of some microorganisms. Bacteria capable of metabolizing histamine (*Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus vulgaris*, *Serratia flava*, *Clostridium feseri*) and tyramine (*Sarcina lutea*, *Aspergillus niger*, *Trichosporum* spp) have been reported in the literature (Rice, Eitenmiller, & Koehler, 1976; Roig Sagués et al., 1998). The cofactor pyridoxal phosphate is necessary for decarboxylase activity, mainly with tyrosine, lysine and ornithine (Roig Sagués et al., 1998).

The decarboxylase-positive microorganisms can be part of the natural population of the milk used in cheese making, or can be introduced by contamination, before, during and after processing. According to Joosten and Northold (1987), it is necessary to have densities of 10^7 to 10^9 CFU/g in order to produce high concentrations of amines.

The hygienic quality of milk is very important in the formation of biogenic amines in cheese (Roig Sagués et al., 1998). Cheeses made with milk of poor microbial quality contain higher amine levels than cheeses made with high quality milk (Joosten, 1988; Petridis & Steinhart, 1996). *Enterobacteriaceae* species have been observed to produce histamine, tyramine, putrescine, cadaverine and 2-phenylethylamine (Edwards & Sandine, 1981; Joosten & Northold, 1987; Marino, Manfreni, Moret, & Rondinini, 2000; Stratton et al., 1991). *Clostridium* can produce histamine, tryptamine and tyramine (Edwards & Sandine, 1981). Gram-negative microorganisms can increase the concentrations of putrescine and cadaverine in cheese (Joosten, 1988; Marino et al., 2000; Petridis & Steinhart, 1996).

According to Fernández-García et al. (2000) and Greif et al. (1995), lactic starter cultures used in the production of cheese can be an important critical point in the production of biogenic amines. The production of acid is the first manifestation of their growth. The lowering of pH by acids produces a less favourable environment for the growth of undesirable microorganisms. A second major change effected by starter cultures is the alteration of proteins. Starter cultures hydrolyse proteins and increase free amino acid levels which can be used for their growth (Roig Sagués et al., 1998; Speck, 1981). Several starter cultures have been observed to possess amino acid decarboxylase activity (Joosten, 1988; Stratton et al., 1991). According to Straub, Kicherer, Schilcher, and Hammes (1995), several fermentation organisms failed to produce amines in phosphate buffer. However, in milk, various species of *Lactobacillus* produce histamine, for example, *L. bulgaricus*, *L. casei* and *L. acidophilus* (Edwards & Sandine, 1981; Petridis & Steinhart, 1996; Stratton et al., 1991). *Lactococcus lactis* was observed to produce histamine, tyramine and tryptamine (Chander, Batish, Babu, & Singh, 1989). Wild lactococcal and leuconostoc strains

are capable of tyramine formation (González de Llano, Cuesta, & Rodríguez, 1998). According to González de Llano et al. (1998), Halász et al. (1994), Straub et al. (1995), the amine-forming ability should be taken into account when selecting starter cultures.

Although it is very difficult to elaborate cheese free of biogenic amines, it is possible to reduce the levels of the most dangerous ones (Roig Sagués et al., 1998). Despite its importance, very few studies have been undertaken to determine the biogenic amine potential and how to minimize amine formation by lactic cultures. The objective of this study was to investigate the formation of bioactive amines by the most commonly used starter lactic bacteria in cheese making—*Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *Lactis*—as affected by incubation temperature (20 and 32°C, which are the temperatures recommended for the starter culture and rennet, respectively) and by addition of rennet and sodium chloride.

2. Material and methods

2.1. Materials

2.1.1. Samples

Mesophilic homofermentative commercial starter culture type DVS, containing *Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*, non-fat dry milk (four different lots), liquid swine pepsin rennet (Chr. Hansen SA, São Paulo, Brasil), calcium chloride and sodium chloride were used.

The powdered milk was reconstituted with distilled water (10%), sterilized at 110 °C/10 min and analysed for microorganisms, pH, titratable acidity and bioactive amines. This was used in all the experiments. The rennet was analysed for bioactive amines.

The starter culture was aseptically added to 1 l of the sterilized reconstituted milk and incubated at 20 °C/18 h for activation. Samples of the sterilized reconstituted milk were aseptically (i) inoculated with 1% activated culture and stored at 20 and 32 °C/24 h; (ii) inoculated with 1% activated culture, incorporated with 3 ml 85% $CaCl_2$/10 l and 0.25 ml of rennet/10 l and stored at 20 and 32 °C/24 h; (iii) inoculated with 1% activated culture, incorporated with 3 ml 85% $CaCl_2$/10 l, 0.25 ml of rennet/10 l and 50 g NaCl/10 l and stored at 20 and 32 °C/24 h. Samples were taken at 6 h intervals and analysed for pH, titratable acidity and bioactive amine levels. The experiment was repeated four times and the analysis was performed in duplicate.

2.1.2. Chemicals

Bioactive amine standards were purchased from Sigma Chemical Co. (St. Louis, MO, EUA). They included putrescine (PUT) dihydrochloride, spermidine (SPD) trihydrochloride, spermine (SPM) tetrahydrochloride, agmatine (AGM) sulfate, cadaverine (CAD) dihydrochloride, serotonin (SRT) hydrochloride, histamine (HIM) dihydrochloride, tyramine (TYM), tryptamine (TRM) and 2-phenylethylamine (PHM) dihydrochloride.

All reagents were of analytical grade, except HPLC reagents were LC grade. Ultrapure water was obtained from Milli-Q. The mobile phases were filtered through HAWP and HVWP membranes, used, respectively, for aqueous and organic solvents (47 mm diameter and 0.45 μm pore size, Millipore Corp., Milford, MA, EUA).

2.2. Methods of analysis

2.2.1. Microbial count

Samples of the sterilized reconstituted non-fat dry milk were analysed for psychrotrophic, mesophilic and thermoduric microbial counts (APHA, 1992). Plate count agar was used for 1.0 and 0.1 ml of samples, corresponding to dilutions of 10^0 and 10^{-1}, respectively. The microbial counts were determined after incubation at 20±1 °C/24 h for psychrotrophic, and 36±1 °C and 55±1 °C/48 h, respectively, for mesophilic and thermoduric microorganisms.

2.2.2. Determination of titratable acidity and pH

Titratable acidity, in degree Dornic (°D) was determined by titration with *N*/9 NaOH. The pH value was determined using a potentiometer (IAL, 1985).

2.2.3. Determination of bioactive amines

Amines were extracted from samples (18 ml) by adding 2 ml 5 N HCl. After agitation for 5 min in a Vortex mixer, the slurry was centrifuged at 10,000×*g* at 4 °C, the supernatant was collected and filtered through Whatman No. 1 filter paper. The amines were separated by ion-pair reverse phase HPLC and quantified fluorimetrically after post-column derivatization with *o*-phthalaldehyde (Vale & Glória, 1997).

Liquid chromatography was performed on a LC-10AD system connected to a RF-551 spectrofluorimetric detector at 340 and 445 nm of excitation and emission, respectively, and to a CBM-10AD controller (Shimadzu, Kyoto, Japan). A reversed-phase μBondapak C18 column, 300×3.9 mm i.d., 10 μm, was used with a μBondapak C18 guard-pak insert (Waters, Milford, MA). The mobile phases were: A, solution of 0.2 M sodium acetate and 10 mM 1-octanesulfonic acid sodium salt adjusted to pH 5.0 with acetic acid, and B, acetonitrile. The flow rate was set at 0.6 ml/min and the gradient was: 20 min at 12% B, 22 min at 13%, 35 min at 13%, 43 min at 26%, 66 min at 26%, 71 min at 12% and 80 min at 12%. The post-column derivatization reagent was delivered at 0.4 ml/min. It consisted of 1.5 ml Brij-35, 1.5 ml mercaptoethanol and 0.2 g

o-phthalaldehyde dissolved in a 500 ml solution of 25 g boric acid and 22 g KOH (pH adjusted to 10.5 with 3% KOH). The column and the post-column reaction apparata were at room temperature (22±1 °C). The identification of amines was performed by comparison of retention times of amines in samples to standard solutions and also by addition of the suspected amine to the sample. Amine levels were calculated by direct interpolation in the standard curve.

2.3. Statistical analysis

The experiment was designed in random blocks with subdivided parcels. The results were submitted to analysis of variance and the means compared by the Fisher *t* test at 5% probability. Spearman correlation, at 5% of probability, was used to determine relationships among investigated parameters (Sampaio, 1998).

3. Results and discussion

3.1. Characteristics of the reconstituted and sterilized non-fat dry milk and rennet

The microbial counts of the reconstituted and sterilized non-fat dry milk are shown in Table 1. Very low counts were detected; therefore they would impart very little influence during fermentation, and amine formation by these microorganisms would be irrelevant (Fernández-García et al., 2000). The milk samples exhibited pH values ranging from 6.63 to 6.75 (average 6.69) and titratable acidities from 16.6 to 18.5 °D (average 17.6 °D).

Among the 10 bioactive amines investigated in the milk samples, only agmatine, spermine, spermidine, and cadaverine were detected (Fig. 1). The prevailing amine was spermidine (74.5 μg/100 ml), followed by agmatine (22.4 μg/100 ml), spermine (19.5 μg/100 ml), and cadaverine (3.67 μg/100 ml). The presence of spermidine, spermine and putrescine in cow's milk has been reported in the literature (Bardocz, 1993; Sanguansermsri et al., 1974). Putrescine was not detected in this study. It may have been lost due to volatilization during dehydration (Glória, Daeschel, Craven, & Hilderbrand, 1999). The presence of cadaverine in milk has also been reported in the literature (Sanguansermsri et al., 1974). However, according to Marino et al. (2000), the presence and levels of cadaverine in milk and dairy products is correlated with *Enterobacteriaceae* counts, an indication of contamination during processing. The presence of agmatine in milk has been reported. Its presence in milk is expected since it is a precursor of putrescine, spermidine and spermine (Bardocz, 1993; Lima & Glória, 1999). However, it was not determined because the analytical methods used did not detect agmatine. The prevalence of spermidine over spermine is in accordance with Sanguansermsri et al. (1974) but contradictory to Bardocz (1993). The levels of amines obtained in this study are higher than those reported in the literature.

High levels of amines were present in the rennet: 205 μg/100 ml of spermidine, 693 μg/100 ml of tyramine, 85 μg/100 ml of histamine, 42.5 μg/100 ml of agmatine and 77.5 μg/100 ml of tryptamine. Since the rennet is diluted by a factor of 40,000 when incorporated into the milk, the levels of amines in the final product would be irrelevant. This is in agreement with Roig Sagués et al. (1998), who concluded that rennet itself could not be considered a source of high levels of amines in cheese.

3.2. Bioactive amines formation by Lactococcus at 20 and 32 °C

The titratable acidity and pH values of the milk during fermentation at 20 and 32 °C are shown in Fig. 2. At both temperatures, there was significant ($P < 0.05$) increase in acidity and decrease in pH with time. Such behaviour was expected, since production of acid by starter cultures is one of the first manifestations of growth. According to Speck (1981), the increase in acidity is a useful parameter for following starter culture growth.

The changes in pH and acidity were faster at 32 °C than 20 °C. These results are supported by the litera-

Table 1
Colony-forming units of psychrotrophic, mesophilic and thermoduric microorganisms in the sterilized reconstituted non-fat dry milk samples

Milk sample	Colony-forming units (CFU) / ml					
	Psychrotrophic		Mesophilic		Thermoduric	
	10^0	10^{-1}	10^0	10^{-1}	10^0	10^{-1}
A	0	0	0	0	1	0
B	0	0	0.5	0	0	0
C	0	0	0	0	0.5	2.5
D	7	0	0	0	0.5	0

Mean values of duplicate analyses.

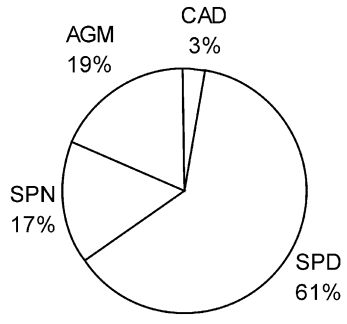

Fig. 1. Profile of bioactive amines in the sterilized reconstituted non-fat dry milk samples (SPD = spermidine; SPN = spermine; AGM = agmatine; CAD = cadaverine).

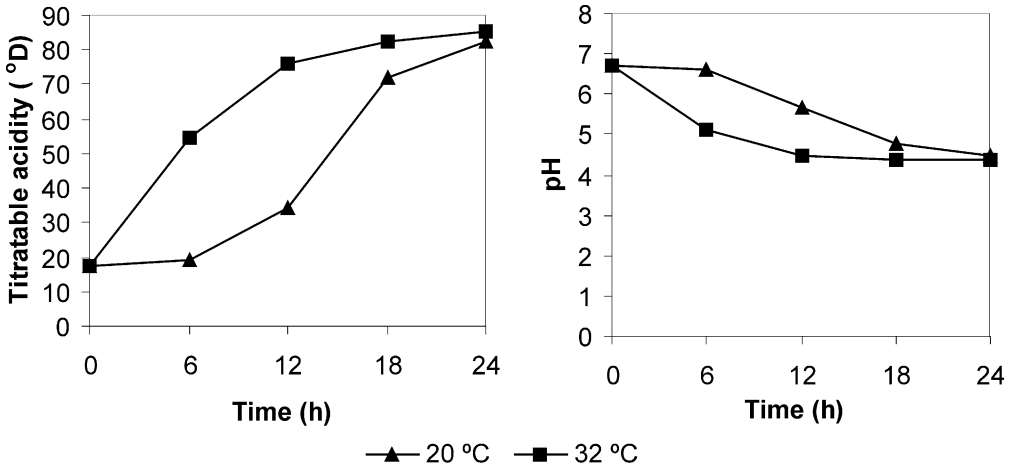

Fig. 2. Titratable acidity and pH of reconstituted and sterilized non-fat dry milk inoculated with *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* at 20 and 32 °C.

ture. At 32 °C, highest acidity (~83°D) and lowest pH (~4.5) values were achieved in 18 and 12 h, respectively. After that time, the values were not statistically different ($P > 0.05$). At 20 °C, similar values for highest acidity and lowest pH were obtained in 24 h of incubation. According to Speck (1981), the lowest pH range for *L. lactis* growth is 4.1–4.8. Therefore, the environment could have become unfavourable for growth in 12 h at 32 °C and in 24 h at 20 °C.

During fermentation by the starter culture, there was a significant ($P < 0.05$) change in the levels of the amines that were initially present in the milk (Fig. 3). Spermidine levels increased slightly with time, while spermine levels increased at faster rates up to 18 h and then decreased. According to Sanguansermsri et al. (1974) and Hamana, Akiba, Uchino, and Matsuzaki (1989), spermine and spermidine are essential growth factors for several microorganisms. Therefore, the increase of these polyamine levels is expected since the starter culture is growing. They can be synthesized *de novo* via ornithine or agmatine (Löser, 2000). However, a decrease in spermine levels could indicate a decrease in growth, or that the culture reached the stationary phase of growth (Bardocz, 1993; Halász et al., 1994).

Agmatine decreased, reaching lowest levels at 6–12 h of incubation, increasing thereafter. The decrease in agmatine levels could be related to its role as a precursor of spermidine and spermine. It is known that putrescine is an intermediate in the production of spermidine and spermine (Bardocz, 1993; Lima & Glória, 1999). The fact that it was not detected in this study, suggests that it is a very unstable intermediate, favouring the build-up of spermidine and spermine or of the precursor agmatine.

There was a significant increase in cadaverine levels with time at both temperatures, followed by a decrease ($P < 0.05$). The decrease in cadaverine levels could be associated with the presence of cadaverine catabolic enzymes (Roig Sagués et al., 1998). Temperature significantly affected the levels of amines at specific fermentation times. Amine levels at 20 °C were lower than at 32 °C for cadaverine at 6, 18 and 24 h, agmatine at 12, 18 and 24 h, spermidine at 12 and 24 h and spermine at 6, 12 and 18 h.

Several biogenic amines, which were not detected in the milk, appeared during fermentation, among them histamine, tyramine, serotonine, tryptamine and 2-phenylethylamine. According to Straub et al. (1995), these compounds are formed by the metabolic activity of microorganisms. As indicated in Fig. 4, histamine was detected at 12 h during fermentation at 20 °C compared to 18 h at 32 °C. Even though it took longer for histamine to be formed at 32 °C, it accumulated higher levels in 24 h ($P < 0.05$). Tyramine was detected only at 32 °C, reaching levels of 8.04 μg/100 ml. At both temperatures, serotonin was detected at 12 h. The levels increased significantly ($P < 0.05$) with time, reaching higher levels (~165 μg/100 ml) than the other amines investigated.

Tryptamine and 2-phenylethylamine were only detected at 24 h at both temperatures investigated. Tryptamine levels produced at 20 °C were 20.16 μg/100 ml and at 32 °C, 28.1 μg/100 ml. 2-Phenylethylamine levels detected were 9.55 μg/100 ml at 20 °C and 17.6 μg/100 ml at 32 °C. According to Joosten (1988), bacteria with phenylalanine decarboxylase have not been described in the literature. However, 2-phenylethylamine can be formed as a result of the slight activity of tyrosine-decarboxylating bacteria towards this structurally-related amino acid. This might explain its late formation.

Based on these results, biogenic amine accumulation during fermentation occurs after 6–12 h (histamine, tyramine and serotonin) or after 24 h (tryptamine and 2-phenylethylamine). These results are in agreement with the observation that the formation of biogenic amines happens at advanced stages of fermentation. According to Halász et al. (1994), in the stationary phase of microbial development, there is an increase in decarboxylase

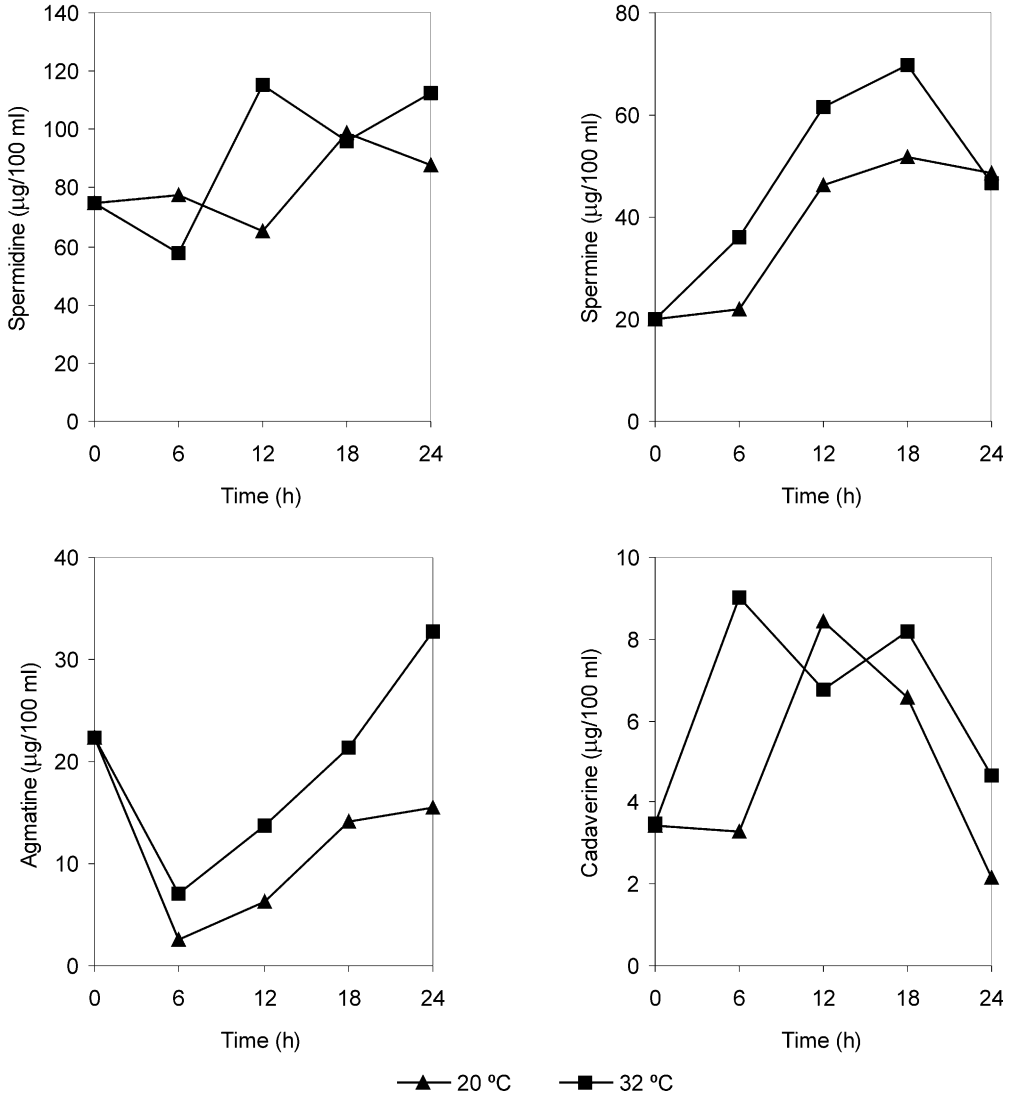

Fig. 3. Levels of spermidine, spermine, agmatine and cadaverine in reconstituted and sterilized non-fat dry milk fermented by *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* at 20 and 32 °C.

activity. This could be due to the absence of fermentable carbohydrates, which causes lactic acid bacteria to decarboxylate free amino acids as an alternative energy source (Fernández-García et al., 2000). Babu, Chander, Batish, and Bhatia (1986) and Chander et al. (1989) observed that *L. cremoris* and *L. bulgaricus-52* produced histamine, tyramine and tryptamine after a 24 h incubation at 30 and 37 °C, which corresponded to the apices of the log growth phase.

With respect to the effect of temperature, tyramine was only formed when fermentation was carried out at 32 °C. Histamine levels accumulated at 18 and 24 h were higher at 32 than at 20 °C. Several investigators have reported that, at higher temperatures, there is an increase in amine production (Halász et al., 1994; Joosten, 1988). According to these results, in order to prevent the formation or build-up of amines during fermentation, lower temperatures should be used.

The Spearman correlation matrix of the data obtained in this study is shown in Table 2. There was a significant negative correlation between pH and acidity. As acidity increased, there was an increase in the levels of serotonin, histamine, spermidine and spermine. According to Babu et al. (1986) and Chander et al. (1989), pH values around 5.0 can enhance histamine and other amine formations. Furthermore, according to Chen, Wei, Koburger, and Marshall (1989) and Halász et al. (1994), the growth of bacteria in an acidic medium stimulates the formation of decarboxylases and, therefore, the formation of amines from amino acids, in order to protect the bacteria against the acidic medium. The significant positive correlations between serotonin × histamine, serotonin × spermine, and serotonin × spermidine suggest that these amines are formed by the same mechanism or are affected by the same factors.

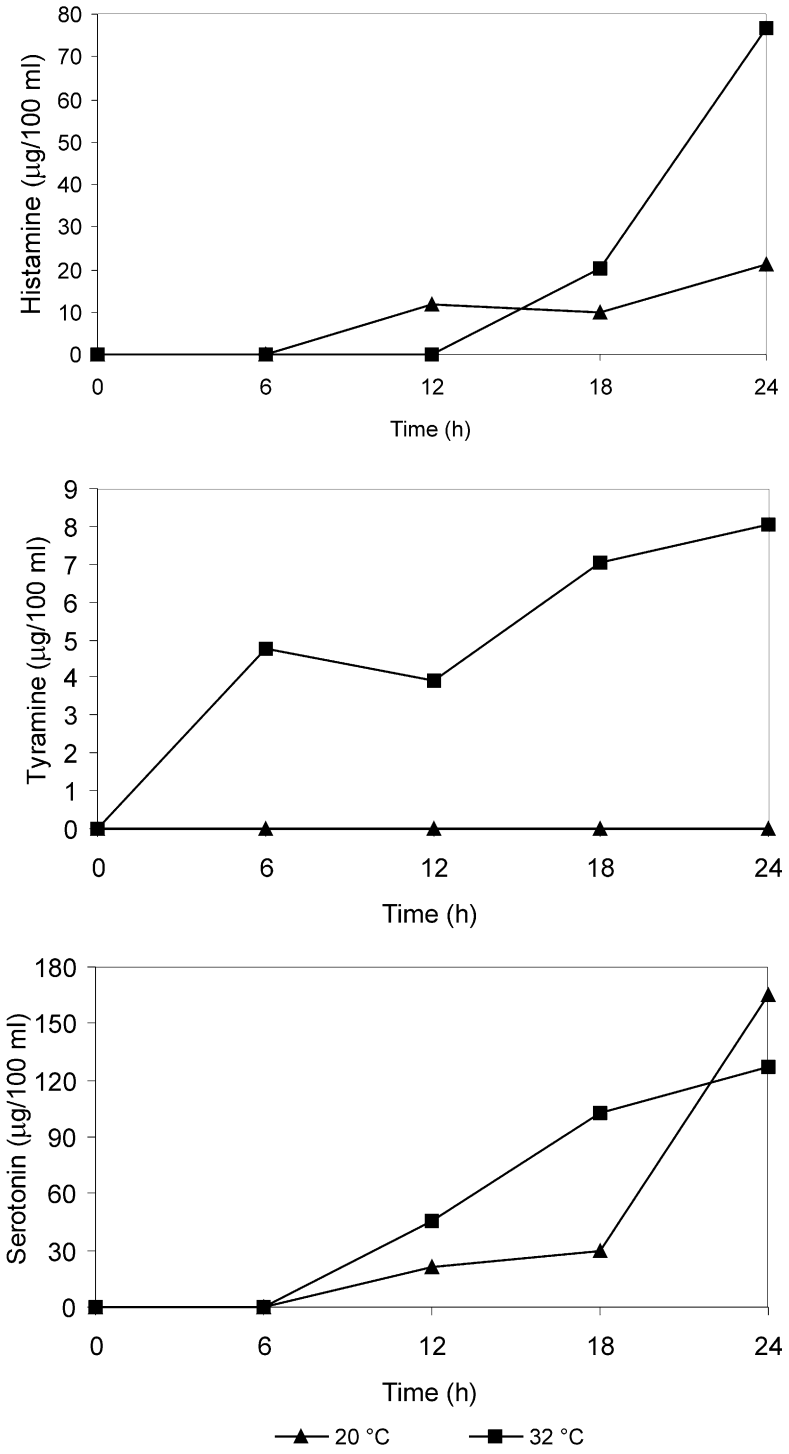

Fig. 4. Levels of histamine, tyramine and serotonin in reconstituted and sterilized non-fat dry milk fermented by *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* at 20 and 32 °C.

3.3. *Bioactive amine formation by* Lactococcus *in the presence of rennet and/or NaCl at 20 and 32 °C*

The titratable acidity and pH values of the milk with rennet and in the presence and absence of NaCl are shown in Fig. 5. The addition of rennet accelerated the decrease in pH and the increase in acidity at 20 °C, but highest and lowest values obtained for acidity and pH, respectively, were not significantly different from samples without rennet. At 32 °C, however, no significant difference was observed between samples with and without rennet. The addition of salt to samples containing rennet did not affect pH and acidity throughout time.

Table 2
Spearman correlation among amine levels, acidity and pH of reconstituted and sterilized non-fat dry milk fermented by *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* at 20 and 32 °C

	CAD	HIM	SRT	AGM	SPD	SPN	Acidity
HIM	0.010						
SRT	0.028	**0.859**					
AGM	−0.118	−0.080	−0.079				
SPD	−0.068	0.341	**0.589**	−0.044			
SPN	**0.329**	0.499	**0.541**	−0.189	0.115		
Acidity	0.132	**0.740**	**0.873**	−0.180	**0.588**	**0.441**	
PH	−0.204	**−0.741**	**−0.880**	0.136	**−0.627**	**−0.479**	**−0.960**

CAD = cadaverine, HIM = histamine, SRT = serotonin, AGM = agmatine, SPD = spermidine, SPN = spermine. Values in bold are significant at 5% probability.

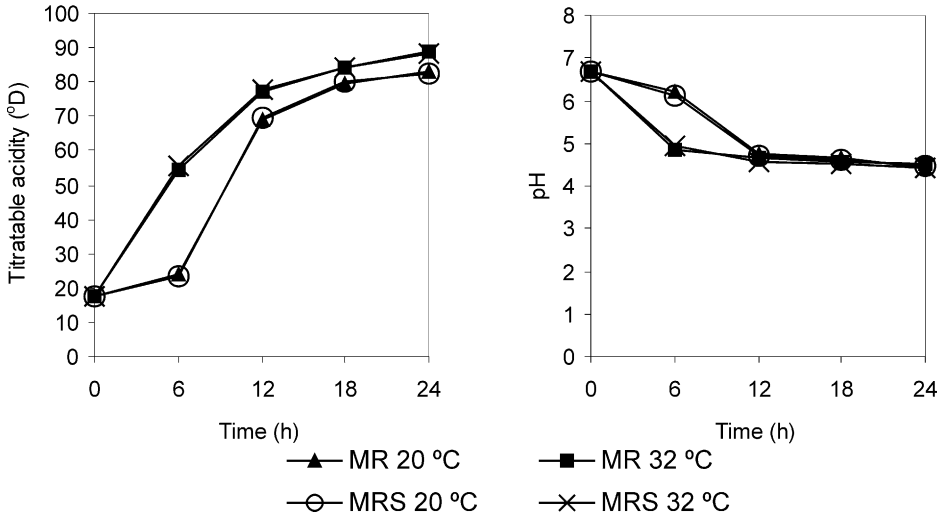

Fig. 5. Titratable acidity and pH of reconstituted and sterilized non-fat dry milk inoculated with *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* at 20 and 32 °C (MR = milk + rennet; MRS = milk + rennet + NaCl).

The addition of rennet and NaCl significantly affected ($P < 0.05$) the levels of the amines initially present in the milk samples (Fig. 6). The levels of spermidine increased constantly with time in samples with rennet, accumulating levels statistically higher than those from samples without rennet and with rennet and NaCl at 24 h. There was also an increase in spermine levels at 12 and 18 h of fermentation. However, no significant difference was observed in the levels of spermine during fermentation of samples with rennet and NaCl. This result suggests that addition of NaCl prevented the increase in spermine levels. The temperature of incubation did not affect spermine formation. The changes in agmatine were only statistically different at 24 h, for samples containing rennet and incubated at 32 °C.

There was a significant random change in cadaverine levels with fermentation time, especially for samples without NaCl; however no significant difference in levels was observed at the end of the experiment at either temperature.

The formation of biogenic amines during fermentation of samples with rennet in the presence or not of NaCl is shown in Fig. 7. Besides the five amines formed by the starter culture, another amine—putrescine—was detected when rennet was added. It was detected at 6 h of incubation. The levels of putrescine increased significantly ($P < 0.05$) with time in samples with or without NaCl. However, higher rates of putrescine formation were observed in samples without NaCl. The temperature only significantly affected putrescine formation in the first 6 h in the sample without NaCl. No significant difference was observed in putrescine levels from 12 h until the end of the experiment. These results suggest that the formation of putrescine could be associated with the addition of rennet and also that it was prevented by NaCl.

Histamine and tyramine were detected after 12 h of fermentation in samples containing rennet at both temperatures and in the presence or absence of NaCl. However, the presence of NaCl lowered the speed of formation and the build up of histamine and tyramine. Serotonin was detected after 12 h in most of the samples and the levels increased significantly ($P < 0.05$) with time. Tryptamine was detected at low levels at 12 h of fermentation at 32 °C, either in the presence or in the absence of NaCl. 2-Phenylethylamine was not detected in any of the treatments. According to these results, the

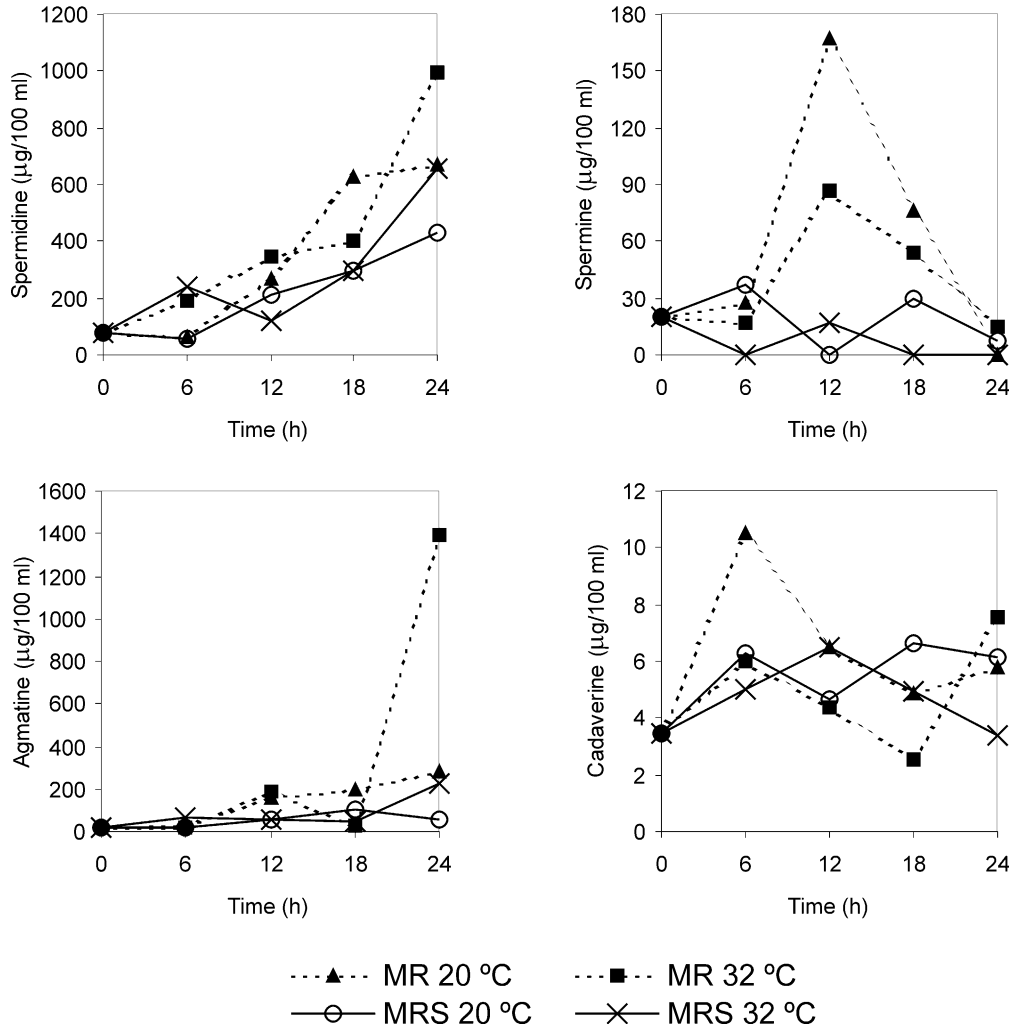

Fig. 6. Levels of spermidine, spermine, agmatine and cadaverine in reconstituted and sterilized non-fat dry milk fermented by *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* at 20 and 32 °C (MR = milk + rennet; MRS = milk + rennet + NaCl).

addition of rennet promoted putrescine formation and accelerated tryptamine formation. This is in accordance with reports by Fernández-García et al. (2000) and Stratton et al. (1991), that addition of rennet promotes proteolysis, which can enhance biogenic amine formation. The formation of tryptamine was only observed at 32 °C. The addition of NaCl decreased the rates of putrescine, histamine and tyramine formation.

The Spearman correlation matrix of the data obtained in this study is shown in Table 3. There was significant

Table 3
Spearman correlation among amine levels, acidity and pH of reconstituted and sterilized non-fat dry milk fermented by *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* with rennet in the presence, or not, of NaCl at 20 and 32 °C

	PUT	CAD	HIM	SRT	AGM	SPD	SPN	Acidity
CAD	0.121							
HIM	0.620	0.075						
SRT	0.557	0.176	**0.747**					
AGM	**0.515**	0.051	0.695	0.654				
SPD	**0.591**	0.060	0.715	0.645	0.573			
SPN	−0.208	0.054	−0.074	−0.205	−0.186	−0.231		
Acidity	**0.590**	0.166	**0.753**	**0.789**	**0.656**	**0.684**	**−0.250**	
pH	**−0.632**	**−0.236**	**−0.761**	**−0.782**	**−0.677**	**−0.677**	**0.273**	**−0.919**

PUT = putrescine, CAD = cadaverine, HIM = histamine, SRT = serotonin, AGM = agmatine, SPD = spermidine, SPN = spermine. Values in bold are significant at 5% probability.

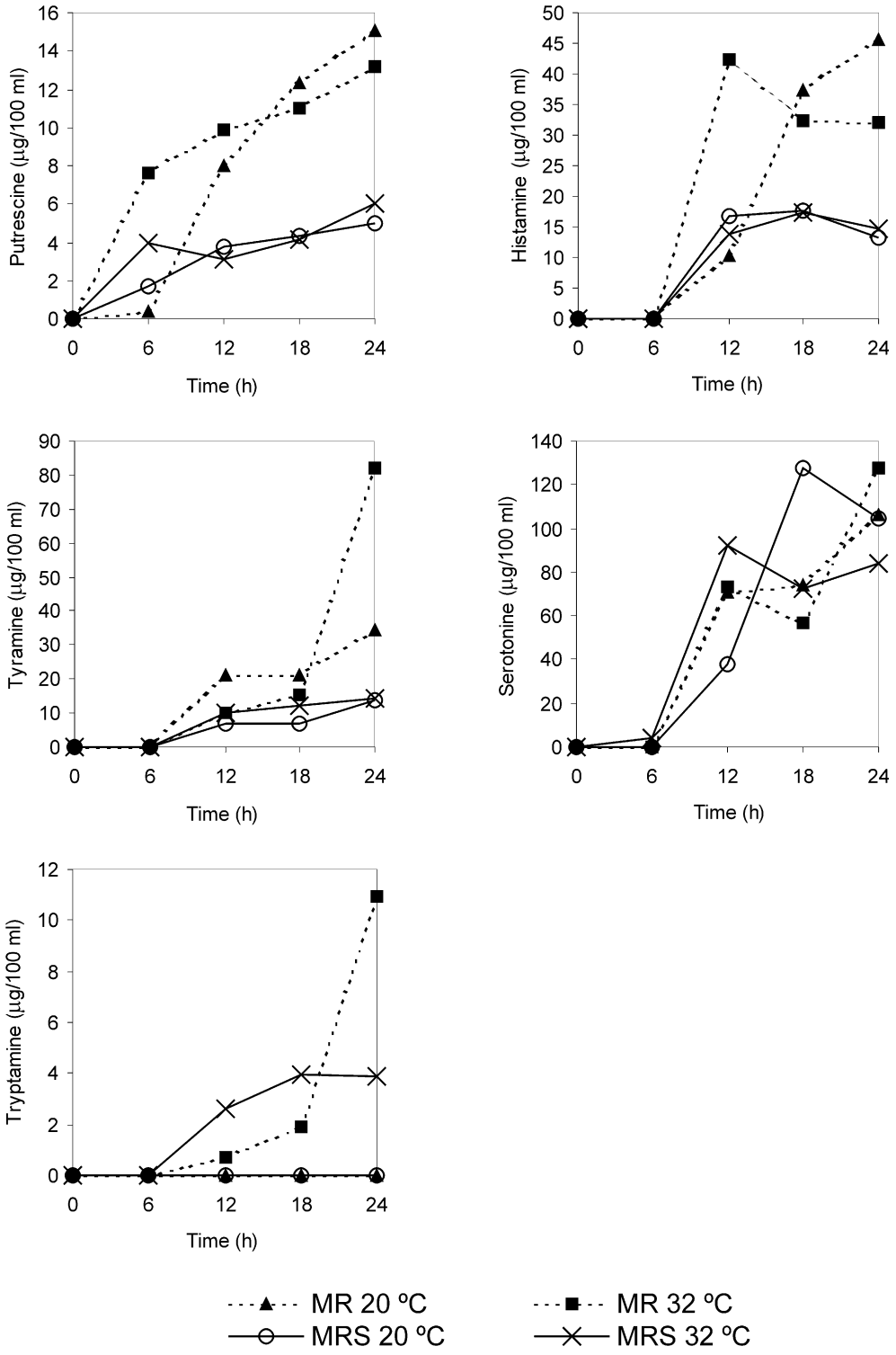

Fig. 7. Levels of putrescine, histamine, tyramine, serotonin and tryptamine in reconstituted and sterilized non-fat dry milk fermented by *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* at 20 and 32 °C (MR = milk + rennet; MRS = milk + rennet + NaCl).

negative correlation between pH and acidity. As the acidity increased, there were increases in the levels of serotonin, histamine, spermidine, agmatine and putrescine and there was a decrease in spermine. There were also positive significant correlation between several different amines, except for cadaverine and spermine.

4. Conclusions

Non-fat dry milk contained the polyamines agmatine, spermidine, spermine, and cadaverine. The prevalent amine was spermidine, followed by spermine, agmatine and cadaverine. During fermentation of *Lactococcus*

lactis ssp. *lactis* and *L. lactis* ssp. *cremoris*, there was an increase in acidity and a decrease in pH. The changes were faster at 32 °C compared to 20 °C, however values obtained at the end of 24 h were not statistically different.

The starter culture was capable of affecting the profile and levels of amines in milk. There were increases in spermidine and spermine levels and a decrease in agmatine, followed by an increase. Cadaverine levels increased and then decreased. Biogenic amines were formed during fermentation. Histamine and serotonin were found within 12 h, whereas 2-phenylethylamine and tryptamine where formed within 24 h. Histamine and serotonin formation correlated positively with titratable acidity. Temperature statistically affected amine formation. Tyramine was only formed at 32 °C. Higher levels of most of the amines were found in samples incubated at 32 °C than at 20 °C. The addition of rennet promoted spermidine build up, favoured the formation of putrescine and tyramine and accelerated the formation of tryptamine. It also prevented 2-phenylethylamine formation. The addition of NaCl slowed down the formation of agmatine, spermidine, spermine, putrescine, histamine, tyramine and tryptamine. Based on these results, in order to minimize biogenic amine formation during fermentation of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*, the temperature should be below 20 °C. The addition of NaCl to the milk can also be used to prevent amine formation.

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