

Histidine decarboxylase-positive lactic acid bacteria strains and the formation of histamine in ripened cheeses

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Abstract

The relationship between histidine decarboxylase-positive (*hdc*+) lactic acid bacteria (LAB) counts and the amounts of biogenic amines, particularly histamine, in cheeses made with and without LAB starter were investigated. Sixty LAB strains were isolated from ripened cheeses made with raw milk inoculated with lactic starter and cheeses without starter. The strains were identified by carbohydrate fermentation test and the histidine decarboxylase gene was determined by PCR. Biogenic amine contents from the cheeses were determined by HPLC. LAB counts, and their diversity were higher in the cheeses without starter. The cheeses contained low concentrations of the studied amines. The lowest concentrations were found for ethylamine and putrescine followed by cadaverine, tyramine, phenylethylamine and histamine, especially in the cheeses with starter. There was a direct relationship between the percentage of *hdc*+ LAB strains and histamine; and between this percentage and the sum of tyramine, phenylethylamine and ethylamine concentrations.

Keywords: lactic acid bacteria, histidine decarboxylase, biogenic amines, histamine, cheese

PRACTICAL APPLICATIONS

It is a well-known fact that the content of biogenic amines in cheeses should be controlled because these compounds can cause toxicity and undesirable flavor. The results of the present study show that LAB starter cultures do not completely prevent the formation of biogenic amines in cheeses manufactured with raw milk. Although the LAB starter helps to reduce the total amount of biogenic amines, histamine concentration is not clearly linked to the use of starter. In order to ensure the safety of cheeses, it is necessary to use high quality milk, follow hygienic practices and take advantage of the selective pressure of the physico-chemical and technological parameters that occur during cheese making.

Introduction

Biogenic amines are compounds of biological origin with low molecular weight that have in common the presence of at least one amino group. Biogenic amines are formed during the metabolism and play an important role in cellular physiology. They are present in living organisms and in different amounts in a variety of foods. Biogenic amines in foods are mainly produced from amino acid precursors by the action of decarboxylase enzymes of microorganisms. The expression and activity of enzymes depend not only on the presence of the decarboxylase gene, but also on the conditions in which the microorganism grows. The ability to form biogenic amines has been described for some microbial groups related to foods, especially fermented foods like cheeses. The main biogenic amine producers in cheese are Gram positive bacteria, such as LAB (e.g. *Lactobacillus* or *Enterococcus*), and most Gram negative bacteria described as usual contaminants of milk, such as *Enterobacteriaceae* (e.g. *Escherichia*, *Salmonella* or *Enterobacter*) and psychrotrophic bacteria (e.g. *Pseudomonas*). The content of biogenic amines such as histamine, tyramine, ethylamine or phenylethylamine in foods should be controlled since, in susceptible consumers, their presence can cause health problems such as headaches, nausea and vomiting, allergies, an increase of arterial blood pressure. Moreover, some biogenic amines like cadaverine and putrescine are responsible for the undesirable flavor in some foods (Brito *et al.* 2014). The degree of toxicity depends on the amine type and concentration and may be increased by the presence of other amines; for example, cadaverine and putrescine facilitate intestinal absorption of histamine due to blockage of monoamine oxidase enzyme activity. There is neither consensus nor enough information as to what levels of amines in foods would be safe for consumption, with the exception of histamine (EFSA 2011). Specific legislation for biogenic amines only covers histamine in fishery products, and although some countries have set a limit for histamine levels in alcoholic drinks such as wines, no other levels of toxic amines are defined in any legislation. All that is available is a few recommendations by some studies (Spano *et al.* 2010; Leuschner *et al.* 2013).

The most common amines found in ripened cheeses are histamine, tyramine, putrescine and cadaverine and they are produced mainly by LAB (Brito *et al.* 2014), but phenylethylamine, ethylamine and tryptamine can be present, too (Fernández *et al.* 2007; Schirone *et al.* 2012; Poveda *et al.* 2015). There are many studies concerning amine presence in cheeses, which depends on the origin and treatment of the milk and

on the manufacturing process, in which the hygienic conditions and the length of the ripening period play a significant role (Fernández *et al.* 2007). During the ripening of cheeses, free amino acids are produced as a result of proteolysis. The presence of these amino acids and of decarboxylase-positive microorganisms and the favorable environmental conditions (technological process, pH, temperature, water availability, etc.) allow the formation of biogenic amines (Linares *et al.* 2012).

In cheeses made with raw milk and without starter, fermentation depends on the spontaneous development of the indigenous microbiota. Therefore, the quality of the final product relies on the microbial load and the variety of microorganisms present in the milk. Frequently, these types of cheese have more intense flavors than cheeses made with pasteurized milk. Nevertheless, the sensory characteristics vary notably among batches and it is not easy to control them. Some authors explain that the use of commercial ferments in the making of traditional cheeses has caused some loss of typical sensory characteristics of cheeses because of the reduction of microbiological biodiversity (Sheehan *et al.* 2008). A way of solving this problem is to design a starter formed by autochthonous LAB, isolated and selected among the microbiota of cheeses made with raw milk, which would provide the flavor and aroma differentials responsible for the particular identity of each cheese. A possible biosafety criterion could be to select strains that do not produce histamine or other toxic amines (Mesthri *et al.* 2011) and with the capacity to inhibit the growth of undesirable microorganisms biogenic amines producers (Rabie *et al.* 2015; Özogul *et al.* 2016).

Many LAB can be producers of biogenic amines, and therefore biochemical identification of amine presence is not sufficient to determine food safety. A reliable tool for this purpose could be the detection of amino-acid decarboxylase genes (e.g. presence of the histidine decarboxylase gen, *hdc+*). However, it is important to take advantage of the selective pressure of the physico-chemical and technological parameters that occur during cheese making to prevent decarboxylase activity of native strains.

The objectives of this study are: a) to evaluate the relationship between *hdc+* LAB counts in ripened cheeses, isolated using three different agar media, and the amount of histamine in cheeses, and b) to determine the relationship between LAB starter and biogenic amine amounts in cheeses.

Material and methods

Samples

Four cheeses made by different small producers recognized for the quality of their products were analyzed. The cheeses were prepared with raw goat milk and ripened for 5-6 months and weighed 250 g. The main difference between them was that two were made using a commercial LAB starter composed of *Lactococcus lactis lactis*, *Lactococcus lactis cremoris*, *Lactococcus lactis diacetylactis*, and *Streptococcus salivarius thermophilus*. The samples were called S (with starter) and NS (without starter).

LAB counts

The surface of each piece of cheese was removed with a sterile knife. Ten grams was taken aseptically and homogenized mechanically with 90 mL of peptone water in a Stomacher Lab-blender 400 for 2 min. Then, decimal dilutions were made using quarter strength ringer's saline solution and microbiological seeding was performed in duplicate on MRS agar, M17 agar and Mayeux agar. The culture media were purchased from Sharlab (Barcelona, Spain). The agar plates were incubated at 30 °C for 2-5 days in an atmosphere of reduced oxygen at less than 10% using a candle jar. The countable plates (between 30 and 300 colonies) were used to quantify the LAB populations.

Isolation and biochemical identification of LAB

Five colonies were isolated from countable plates of the above media. They were randomly picked and purified by streaking on the same isolation medium. A total of sixty strains (fifteen per cheese) were studied. Bacterial growths were checked by Gram stain and catalase test. After microscopic observation, colonies were transferred onto three fresh plates of MRS, M17 and Mayeux agar, respectively. Identification was accomplished by API 50 CHL test (BioMerieux, France) according to the manufacturer's instructions and gas production (CO₂) from glucose was determined in MRS broth in a test tube sealed with paraffin cap.

Identification of *hdc*+ LAB strains

DNA purification

Detection of *hdc*+ cheese bacteria was performed by a polymerase chain reaction (PCR) assay using purified DNA. First, 10 mL of Elliker broth was inoculated with an isolated

bacterial colony. Cells were grown for 3-4 days at 30 °C until a sufficient population for DNA purification was achieved. Culture cells were harvested by centrifugation at 5000 g for 15-30 min at 4 °C and washed with 10 mL of phosphate buffer saline (PBS). Then, the pellet of bacteria was suspended in 1.5 mL of PBS and recovered in a microtube by centrifugation at 5000 g for 5 min at 4 °C. The pellet was suspended in 100 µL of buffer P1 (0.05 mol/L Tris-Base, pH 8.0, 4 g/L EDTA, and 100 mg/L RNAase, stored at 4 °C) for 5 min at 4 °C. Next, 150 µL of lysis buffer P2 (8 g/L NaOH, and 10 g/L SDS) and 60 µL of 10 mg/mL Lysozyme (prepared in 10mM Tris, pH 8) were added and the mix was maintained for 1 h at 37 °C. The reaction was then neutralized with 200 µL of buffer P3 (294.5 g/L potassium acetate, pH 5.5) and the suspension was centrifuged at 10000 g for 5 min. The supernatant was obtained to precipitate the DNA using ethanol absolute and DNA quality was determined by the ratio A_{260}/A_{280} .

PCR analysis

Purified DNA was used as template in the PCR experiments using 3 pairs of primers (Figure 1). These primers were chosen by studying amino acid and nucleotide sequences of the histidine decarboxylase genes of *Lactobacillus* 30a and *Clostridium perfringens* in the conserved regions (Le Jeune *et al.* 1995). PCR conditions were as follows: 40 cycles, DNA denaturation 1 min at 94 °C, annealing 30 seconds at 48 °C, and DNA polymerization 2 min at 72 °C. PCR was finished with an extension of 5 min at 70 °C. Amplification products were analyzed on 1.5% agarose gels. PCR was controlled using the ribosomal gene 16S and primer assays were 8F (5'-AgAgTTTgATCCTggCTCAg-3') and 1510R (5'-ggTTACCTTgTTACgACTT-3'). Positive control was performed using DNA purified from *Lactobacillus buchneri* as *hdc+* (INCAVI, Spain). To determine false products during PCR reaction, PCR products were sequenced and nested PCR was carried out.

Formation of histamine in MRS broth medium

Amine production in a synthetic medium was determined by studying *hdc+* bacteria. A loop full from pure culture was inoculated into 8 mL tubes of MRS broth with 50 mg/L of histidine and pH adjusted to 6. The tubes were incubated at 30 °C for 48-72h. Histamine formation was determined using a Veratox histamine kit (Neogen Corporation, USA). A microwell reader (Synergy HTX MultiMode Reader, Biotek

Instruments, Inc., Winooski, VT, USA) was used to yield optical densities at 650 nm. Control optical densities were used to form a standard curve, and optical densities of samples were plotted against the curve to calculate the concentration of histamine.

Determination of biogenic amines in cheeses by HPLC

Ten grams of each cheese sample was blended with 20 mL of 0.1 M HCl. After cooling to 3 °C to allow crystallization of most of the fat, the sample was centrifuged at 4500 rpm at 3-5 °C for 30 min. The creamed layer was removed and the supernatant filtered through qualitative filter paper. Five mL of the filtrate was poured into 1 mL of NaOH 0.5 M. Then, Na₂CO₃ was added in excess (about 1 g) and homogenized in a shaker, and 6 mL of n-butyl alcohol was added and shaken again. The aqueous phase was again extracted twice with 6 mL of n-butyl alcohol for each time. The organic extracts were then combined and 5 mL of 0.1 M HCl was added and homogenized by stirring. Finally, the phases of this mix were allowed to separate, and the acid phase was filtered with a 0.45 µm filter and stored in tubes at -20° C until being analyzed by HPLC. The amines were separated by reverse phase HPLC and the method involved pre-column derivatization of the amines by treatment with o-phthaldehyde (OPA). The volume injected was 14 µl (7 µl of OPA mixed with 7 µl of sample). The OPA was prepared from 50 mg of OPA and 1 mL of methanol in 10 mL of borate buffer (0.4 M, pH 10), with the final addition of 40 µL of mercaptoethanol. HPLC was equipped with two pumps Beckman 110B System Gold (San Ramon, CA, USA), an injector Hewlett Packard series 1100 and fluorescence detector Hewlett Packard 1046A (Agilent Technologies, Waldbronn, Germany).

HPLC separations were performed at 45 °C using a 25 cm x 0.46 cm Spherisorb ODS 2 C18 column with particles of 0.5 µm of diameter and gradient mobile phase composition with a flow rate of 0.85 mL per minute. As a mobile phase for chromatographic separation, methanol:acetate buffer solution (0.05 M, pH 6.6) was used. Biogenic amine detection was performed using a fluorescence detector with an excitation wavelength of 230 nm and an emission wavelength of 440 nm.

Amines were identified by comparison of retention times of amines in samples to standard solutions and also by addition of the suspected amine to samples. Amine levels were calculated by direct interpolation in the standard curve for histamine, phenylethylamine, ethylamine, tyramine, putrescine and cadaverine.

Statistical analysis

PCR results were tabulated as presence or absence of the histidine decarboxylase gene. Statistical analysis of data was conducted by graphical and numerical summaries. Variance tests (ANOVA) were used to evaluate the influence of the two main factors, starter and medium (use or no use of starter and the three different agar media used), on the LAB counts and their interactions. Separation of means was evaluated by variance tests according to the Tukey-Kramer procedure. Data were analyzed using Minitab® Statistical Software (Minitab Inc 2012). The probability significance level was set at 0.05.

Results and discussion

Counts and isolation media

The LAB counts for the four cheeses on the three different agar media are shown in Figure 2. As can be observed, they ranged between 8.52 and 6.40 Log CFU/g. The averages (and standard deviation of the mean) of the cheeses without starter (NS1 and NS2) and the cheeses with starter (S1 and S2) was 7.81 (0.50) Log CFU/g and 7.57 (0.46) Log CFU/g, respectively. LAB counts for the cheeses without starter on MRS agar were slightly higher than those for the cheeses with starter. According to the medium used, the highest counts were obtained mostly on MRS agar, with an average (and standard deviation of the mean) equal to 8.28 (0.09) Log CFU/g, followed by LAB counts on M17 agar, with 8.04 (0.22) Log CFU/g. The lowest counts were obtained on Mayeux agar, with 6.75 (0.34) Log CFU/g. ANOVA applied to LAB count showed that the interaction of the two factors (starter and agar medium) was not significant (p -value = 0.968), and neither was use or no use of starter. On the other hand, differences were significant depending on the type of agar medium (p -value = 0.017). Comparison of the three means by the Tukey method revealed that the count on Mayeux agar was significantly different from the counts on MRS and M17 agar. However, the difference between these two last media was not large. The difference in growth depending on the medium is a qualitative attribute since lactic cocci were favored in Mayeux and M17 agar and lactobacilli were favored in MRS agar although they are not selective media. The different LAB counts on different culture media are in accordance with results of other studies (Aponte *et al.* 2008; Van Hoorde *et al.* 2008), and the use of the three culture media seems a good strategy to achieve more diversity of grown strains.

LAB identification

Biochemical API 50CHL test allowed the identification of 48 of 60 isolated lactic strains, with 29 of them being named at species level (Table 1). The test kit was specific for lactobacilli but was used for identification of all isolated LAB as in other studies carried out (Trias *et al.* 2008; Hong *et al.* 2015). In order to allow growth and partial or total identification of cocci, incubation time was extended up to 5 days. The carbohydrate fermentation profiles of some cocci strains did not allow differentiation between *Streptococcus* and *Lactococcus*. In fact, for data processing purposes, all cocci were included in the *Lactococcus* group because the galleries were incubated at 30 °C. All LAB strains isolated from MRS agar corresponded to *Lactobacillus*; strains isolated from M17 agar were identified as *Lactobacillus*, *Lactococcus* and *Leuconostoc*; and strains isolated from Mayeux agar were *Lactobacillus* and *Lactococcus*. In fact, the test media displayed poor selectivity, favoring the growth of specific LAB genera but also of other genera.

The most important finding in bacteria identification was that the diversity of LAB in the cheeses manufactured without starter was higher than in those with starter (see NS and S samples, respectively; Table 1). In the cheeses made without starter, 25 of the 30 strains were identified at genus level; the highest proportion belonged to *Lactobacillus* (60%), followed by *Lactococcus* (32%) and *Leuconostoc* (8%). Also, four *Lactobacillus* species (*plantarum*, *brevis*, *casei* and *buchneri*) and one *Lactococcus* species (*lactis*) were identified. As for the cheeses made with starter, 23 of the 30 strains were identified at genus level, all of which belonged to *Lactobacillus*. In these cheeses, four *Lactobacillus* species (*plantarum*, *brevis*, *casei* and *curvatus*) were identified too. Three of the four species of *Lactobacillus* identified in the cheeses with and without starter were the same. Generally, species variety is greater at the beginning of ripening and decreases as the process advances. The greater presence of lactobacilli in ripened cheeses made with raw milk is shown in several works (Gala *et al.* 2008; Van Hoorde *et al.* 2008; Aydemir *et al.* 2015; Gobbetti *et al.* 2015). Total or partial loss of starter during ripening has been reported in some studies (Novella-Rodríguez *et al.* 2002; Pogačić *et al.* 2013; Terzić-Vidojević *et al.* 2015). Also, it is known that, in ripened cheeses, the prevailing bacteria are not LAB starters but mainly mesophilic lactobacilli (Gobbetti *et al.* 2015). This is why the *Lactococcus* and *Streptococcus* starters were not

isolated in this study. If they had been isolated, they would have been incubated at 42 or 45 °C (Aponte *et al.* 2008).

The starter enabled rapid decline in pH, and probably the LAB diversity succession was lower. The cheeses without starter showed more bacteria diversity probably because the succession of microorganisms was slower and the wild strains had more ability to adapt to changes, as also reported in Gobbetti *et al.* (2015).

Potential LAB histamine producers

PCR test for *Lactobacillus buchneri* as positive control of *hdc*⁺ LAB was standardized (Fig. 1). Using this test to detect the *hdcA* gene, the *hdc*⁺ LAB strains isolated from the cheeses were identified and quantified. 46.7% of total isolated LAB had the *hdcA* gene, and therefore they were potential histamine producers although the production of histamine requires the presence of *hdc*⁺ strains, as well as favorable conditions for the expression of the histidine decarboxylase gene. The highest percent of *hdc*⁺ strains corresponded to *Lactococcus* (62.5%), followed by *Lactobacillus* (55.3%) and *Leuconostoc*, with only one of the two species identified.

Considering the strains grown in each agar medium, the bacteria isolated on Mayeux agar had a high percentage of *hdc*⁺ strains (70%), while the LAB isolated on MRS and M17 with high bacterial counts had a low proportion of *hdc*⁺ strains (40% and 30%, respectively). The results confirm that the ability to produce histamine was strain-dependent and not only related to specific species. It was also found that isolation of the maximum number of LAB strains absolutely requires the use of different media.

The results also showed that the cheeses with starter had a lower proportion of *hdc*⁺ strains (43.3%) than the cheeses without starter (50%). This suggests that the LAB starter could act as an inhibitor of some, but insufficient, autochthonous *hdc*⁺ strains. All isolated *hdc*⁺ LAB produced more than 50 mg/kg of histamine in MRS broth with 50 mg/L of histidine. Therefore, the quality and safety of the cheeses studied could be affected by the growth of isolated strains. Thus, in cheeses manufactured with raw milk, it seems necessary to control the factors that inhibit the ability of LAB to decarboxylate amino acids precursors of biogenic amines; being important factors the pH, the NaCl concentration, the time and the temperature of ripening between others (Komprda *et al.* 2007; Komprda *et al.* 2012; Linares *et al.* 2012). The controlling factors are even more crucial given that some LAB used as a dairy starter could have the ability to form amines (Guarcello *et al.* 2015).

Biogenic amines in cheeses

The cheeses tested contained most of the studied biogenic amines (i.e. histamine, tyramine, ethylamine, phenylethylamine, putrescine and cadaverine), as Figure 3 shows. The lowest concentrations were found for ethylamine in the four cheeses (< 8 mg/Kg), followed by putrescine (≤ 15 mg/Kg). The amounts of cadaverine, tyramine and phenylethylamine were smaller in cheeses with starter (≤ 4 mg/Kg) than in cheeses without LAB starter (between 17 and 50 mg/Kg). These results could be explained by the higher LAB counts obtained in cheeses without LAB-starter. Also Combarros-Fuertes *et al.* (2016) reported that in Zamorano cheeses, biogenic amines contents could be related to higher non-starter LAB counts. The histamine concentration (between 3 and 30 mg/Kg) did not allow differentiation between the cheeses with and without starter. The highest histamine concentration (30 mg/Kg) was found in one of the cheeses with starter. However, the second highest was found in one of the cheeses without starter (21 mg/Kg). Despite the results obtained for histamine, it seems that the use of LAB starter could reduce the presence of some indigenous strains with potential ability to produce toxic amines, such as tyramine and phenylethylamine, or amines responsible for undesirable flavors, such as putrescine and cadaverine. The values found for the four cheeses were similar to or below those reported in other works (Pintado *et al.* 2008; Poveda *et al.* 2015), and the histamine concentrations obtained were considered safe by EFSA (2011).

Figure 4 shows that the cheeses without starter had the highest total amounts of biogenic amines, more than double the total quantity of amines found in the cheeses with starter. The results showed that there was no relationship between histamine and total biogenic amine concentrations, while similar percentages of *hdc*⁺ LAB strains were detected (p-value = 0.392). Therefore, in addition to *hdc*⁺ LAB strains, there could be a greater presence of other undesirable biogenic amine producers that can grow better without the competition of a LAB starter. During the ripening period, there is greater availability of amino acid precursors of biogenic amines, and the action of microbiota decarboxylases tends to take place in this stage (Pinho *et al.* 2004; Fernández *et al.* 2007; Poveda *et al.* 2015).

Figure 5 shows a direct relationship between the number (%) of *hdc*⁺ LAB strains and histamine concentration in both groups of cheeses, with and without starter (S1-S2 and NS1-NS2), although a larger sample size would be required to confirm our findings.

An inverse (or indirect) linear relationship between histamine concentration and sum of amines putrescine and cadaverine is observed, with a Pearson coefficient equal to -0.849. On the other hand, there is a direct linear relationship between the percentage of *hdc*+ LAB strains and the sum of the amounts of tyramine, phenylethylamine and ethylamine, with a Pearson coefficient equal to 0.791. But no relationship between the number (%) of *hdc* + LAB strains and the sum of putrescine and cadaverine concentration can be established. Neither relationship between the histamine and the sum of tyramine, phenylethylamine and ethylamine concentrations, nor between these three toxic amines and the sum of putrescine and cadaverine concentrations can be established. In addition to *hdc*+ LAB strains, other microorganisms as some species of *Enterobacteriaceae* (*E.coli*, *Pantoea conspicua*, *Providencia rettgeri*) or *Enterococci* (*Enterococcus faecalis*, *Enterococcus faecium*) might be involved in the production of histamine or tyramine, phenylethylamine and ethylamine (Torracca *et al.*, 2018). The results also suggest that *hdc*+ LAB strains could have the ability to decarboxylate other amino acids, in addition to histidine. However, strains producing cadaverine and putrescine could be different from strains producing histamine.

As found in other works (Fernández *et al.* 2007; Schirone *et al.* 2012), the total amount of amines in the four cheeses was considered low (< 120 mg/Kg), which is a clear indicator of good hygienic practices during manufacturing and ripening. Total amine concentrations are not a risk in any of the cheeses analyzed and, although *hdc*+ LAB strains were isolated, these bacteria did not form large quantities of histamine or other amines in the cheese matrix. It has been stated that the acidic medium stimulates bacteria to form amines from amino acids to protect themselves against this medium (Pircher *et al.* 2007; Linares *et al.* 2012). In the present study, it was found that, in MRS broth enriched with histidine, *hdc*+ LAB strains synthesized the maximum histamine concentration according to initial histidine concentration. Not always happen that bacteria with decarboxylase capacity synthesize biogenic amines, even in synthetic medium. Ladero *et al* (2015) showed that some isolated strains possessed the histidine decarboxylase gene, but no histamine was found in the culture supernatant after 24 - 48 h of culture. This means that, during cheese ripening, the pH but mostly a mixture of physico-chemical factors with an inhibitory effect on the amines production acted jointly (Linares *et al.* 2012).

Conclusion

The elaboration of cheeses from raw milk implies a high probability that there are *hdc+* LAB strains with ability to form histamine and other biogenic amines (tyramine, ethylamine, phenylethylamine, putrescine and cadaverine). The use of LAB starter does not guarantee the absence of biogenic amines since other *hdc+* bacteria may be present.

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Primer	Sequence	Size (pb)
CL1	5'-CCWggWAAWATWggAAATggWTA-3'	150
CL2	5'-gAWgCWgTWgTCATATTWATTTgWCC-3'	
JV16	5'-AgATggTATTgTTTCTTATg-3'	370
JV17	5'-AgACCATACACCATAACCTTg-3'	
CL1	5'-CCWggWAAWATWggAAATggWTA-3'	500
JV17	5'-AgACCATACACCATAACCTTg-3'	

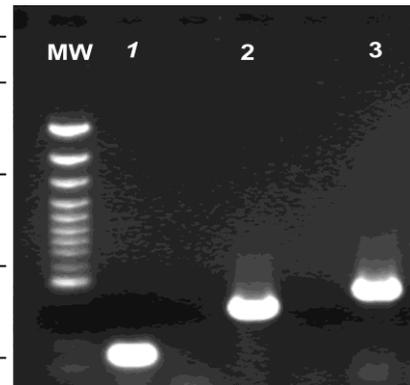


Figure 1. Primers and PCR products that show the detection of the histidine decarboxylase gene (*hdcA*) from *Lactobacillus buchneri* used as positive control of the PCR assay. The DNA template was amplified with the primers: CL1–CL2 (lane 1), JV16–JV17 (lane 2), and CL1–JV17 (lane 3).

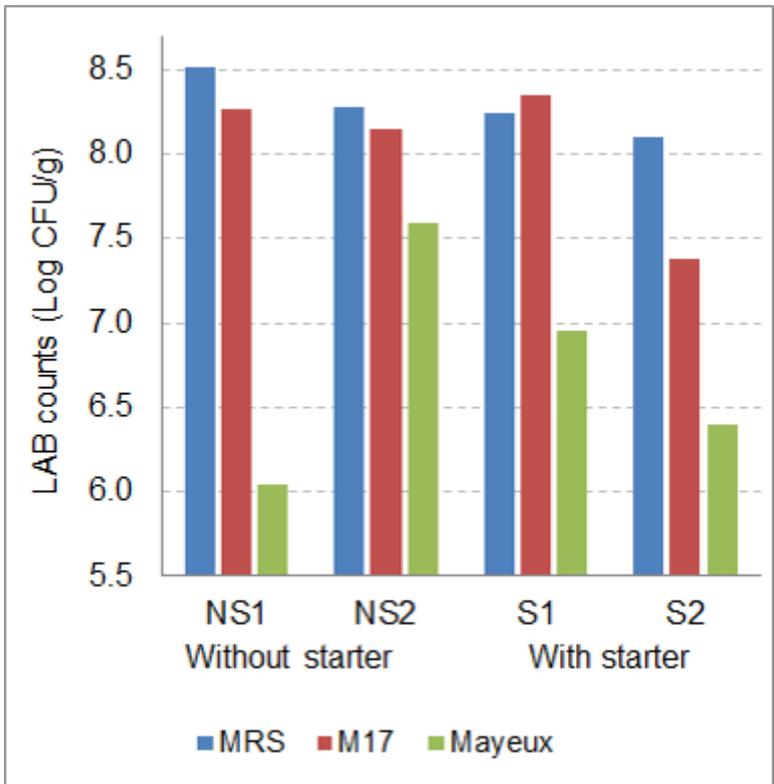


Figure 2. The LAB counts of the cheeses with starter (S1 and S2) and without starter (NS1 and NS2) for the three different agar media.

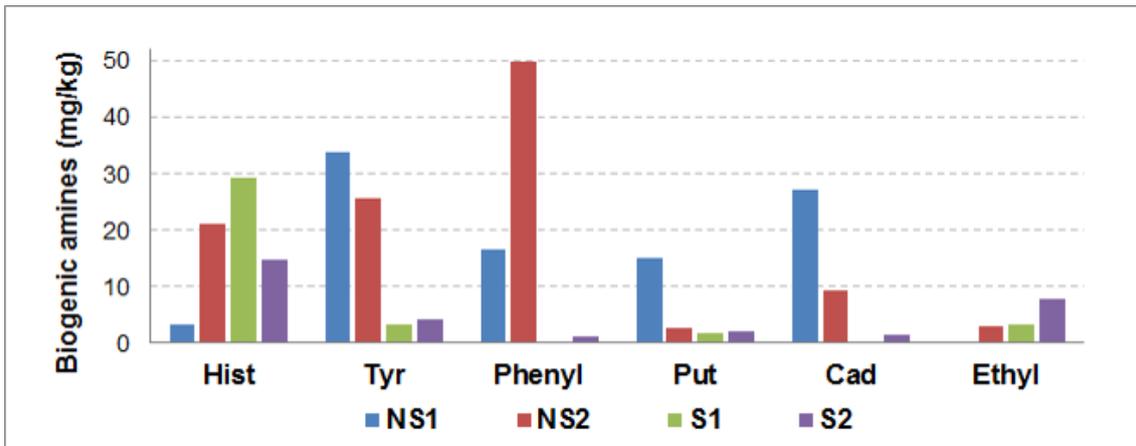


Figure 3. Concentration of biogenic amines, histamine (Hist), tyramine (Tyr), phenylethylamine (Phenyl), putrescine (Put), cadaverine (Cad), and ethylamine (Ethyl), in the four cheeses; with starter (S1 and S2) and without starter (NS1 and NS2).

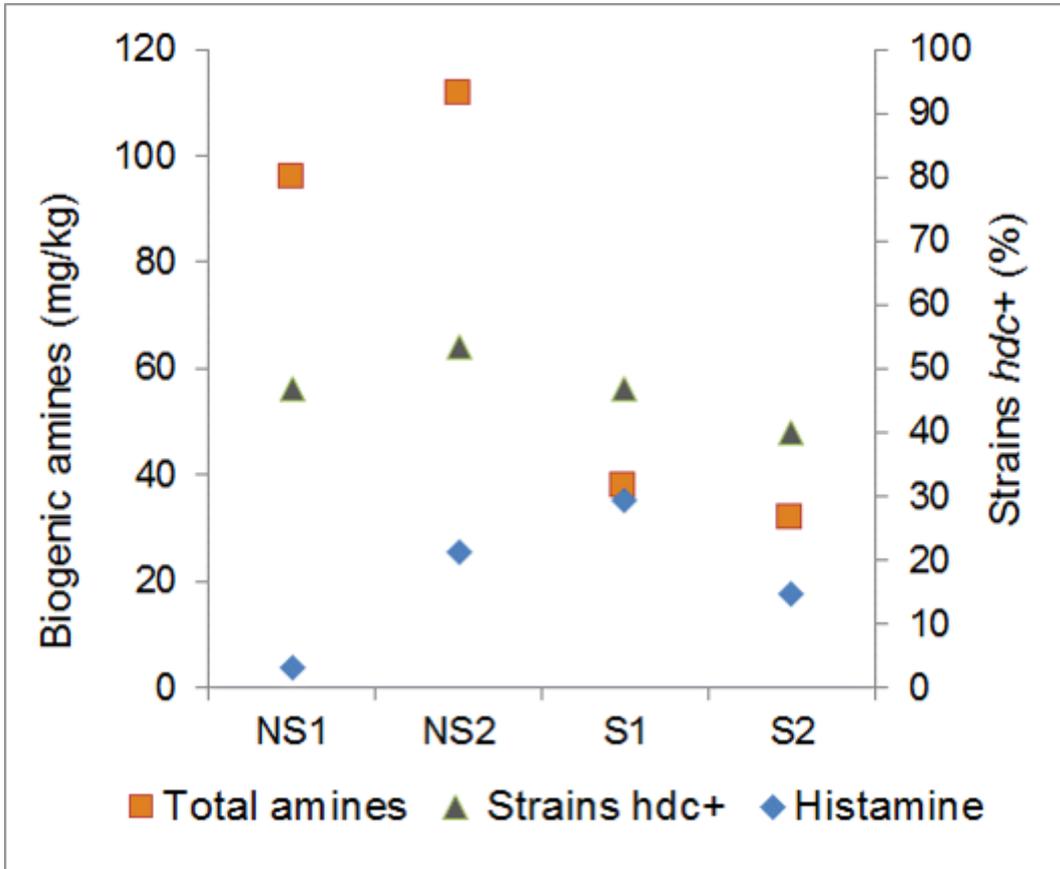


Figure 4. Total amines, histamine and percentage of strains with *hdc+* of the cheeses analyzed; with starter (S1 and S2) and without starter (NS1 and NS2)

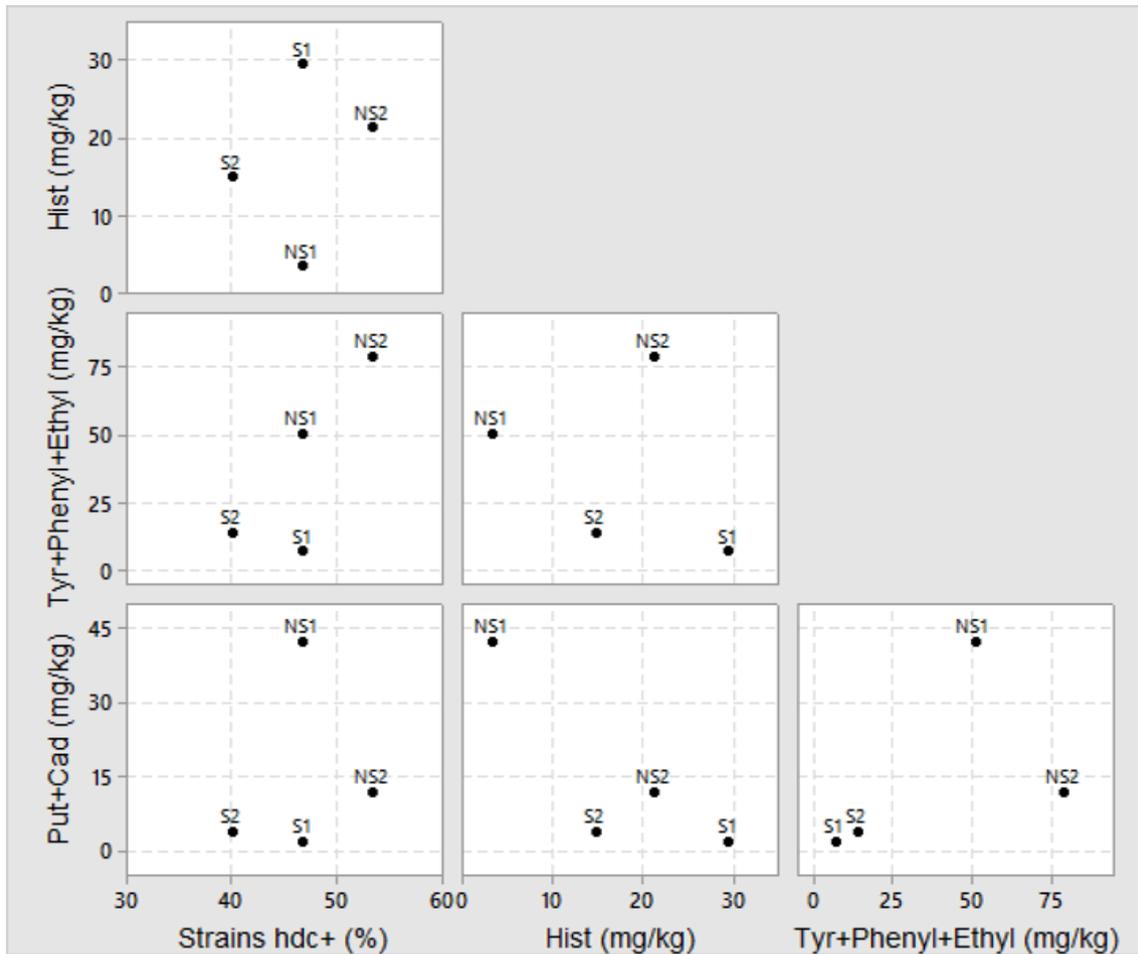


Figure 5. Scatterplots for the relationships of the different types of amines, histamine (Hist), tyramine (Tyr), phenylethylamine (Phenyl), ethylamine (Ethyl), putrescine (Put), cadaverine (Cad), and the percentage of strains with *hdc+*. Cheeses with starter (S1 and S2) and without starter (NS1 and NS2).