ORIGINAL RESEARCH

Influence of milk-clotting enzymes on acidification rate of natural whey starter culture

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The aim of this work was to study the influence of milk-clotting enzymes on whey starter culture and hard cheesemaking. Four cheeses were prepared simultaneously per cheesemaking day, and the experiment repeated on eight consecutive days. Adult bovine rennet was used in the control cheeses, and three formulations of fermentation-produced chymosin (FPC) in the experimental cheeses. Whey cultures were obtained from the whey of the preceding cheeses, incubated individually for 24 h at 42°C. pH values of the control and experimental whey did not differ significantly before incubation, but after 24 h incubation the pH of the FPC whey was significantly higher than that of the control. Soluble nitrogen content in trichloroacetic acid 2% and 12% and in phosphotungstic acid 2.5% was significantly lower for all experimental whey samples than for control whey. This probably explains the decrease in the acidification rate of the whey cultures when bovine coagulant is replaced by FPC in hard cheesemaking.

Keywords Hard cheeses, Milk-clotting enzymes, Natural whey starter culture.

INTRODUCTION

The role of lactic acid bacteria starter cultures is the metabolism of lactose to lactic acid, a process that improves milk clotting and whey syneresis and protects the final product against bacterial contamination. Starter cultures also play a major role in ripening, because they contribute to the aroma and flavour of the cheese through proteolysis, carbohydrate metabolism and to a lesser degree lipolysis.[1–3]

In old cheesemaking procedures starter cultures were not used and acidification was carried out by the indigenous bacteria present in raw milk. More recently, pasteurized or raw milk cheese is inoculated with whey, so-called natural starter cultures because they consist of whey containing natural lactic microflora, and incubated overnight.[4] Several cheeses using these kinds of natural starter cultures are protected by 'Denomination of Origin', e.g. Comté, Beaufort, Grana Padano and Parmigiano Reggiano.[5–7] Natural whey starter cultures are still widely used, mainly in Italy, France and Argentina, for the manufacture of traditional hard cheeses.[8–11] To prepare the culture, a predetermined volume of whey is recovered directly from the cheese vat at ~52°C after the cooking stage, and held for 24 h at decreasing temperature (from ~52°C to ~35°C). The microbiological composition of natural starter cultures can be affected readily by ecological or manufacturing parameters.[12] As a consequence of multiple equilibriums and natural selection a complex starter is obtained after 24 h (pH ≈ 3). Thus, the supply of whey starter cultures is readily available from the whey of the previous cheesemaking day.

Reggianito Argentino cheese is a typical Argentinian hard cheese that was introduced to Argentina in the late 19th and early 20th centuries by Italian immigrants. The manufacturing stages were modified and adapted to Argentinian raw materials and environmental characteristics to give a distinct product, indexed in the *Código Alimentario Argentino*.[13]

Natural whey starter cultures are used to prepare Reggianito and the microflora consisting mainly of thermopilic lactobacilli, 66% *Lactobacillus helveticus* and 33% *Lactobacillus delbrueckii* ssp. *lactis* strains, unlike Italian whey starter cultures, where the most common species are *L. helveticus* and *L. delbruekii* ssp. *bulgaricus*.[14,15]

In Argentina, the most popular milk-clotting enzyme used was the extract from adult bovine stomach, that is a mixture containing about 80% bovine pepsin and 20% chymosin. Most natural whey starter cultures were prepared in whey originating from cheesemakings that had used this milk-clotting enzyme. In the 1990s, fermentation-produced chymosin (FPC) obtained from genetically modified organisms began to replace adult bovine coagulant in the Argentinian dairy industry, causing changes not only in coagulation parameters[16,17] but also in the characteristics of natural whey starter cultures. Cheesemakers noticed that the acidification rate of natural whey starter cultures was slower when adult bovine coagulant was replaced by FPC. As a consequence, the coagulation time in most

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dairy plants was altered because of the delay suffered by the cultures.

The objective of the present study was to determine the influence of different milk-clotting enzymes used in cheesemaking on the composition and acidification rate of natural whey starter cultures. The technological aspect of the study was to provide theoretically based information that would be useful for changes in cheesemaking processes or whey starter culture manipulation when adult bovine rennet is replaced by FPC enzymes in Reggianito Argentino cheese production.

MATERIALS AND METHODS

Cheesemaking

Cheesemaking was carried out in an ensemble of four laboratory-scale 5-L vats, equipped with a system for heating and cooling the vats simultaneously. In the first vat, adult bovine liquid coagulant was used (control); in the second to fourth vats, FPC formulations were added (experimental vats). Cheesemaking was carried out on eight subsequent days, and followed Reggianito Argentino cheesemaking technology.[9,10]

Raw milk was supplied by Milkaut Coop. Ltda (Colonia Nueva, Santa Fe, Argentina), skimmed by centrifugation (Alfa Laval Separator Co., Tumba, Sweden), standardized to 2.50% of fat and batch pasteurized at 65°C for 20 min. After cooling to 33°C, $CaCl_2$ was added to a final concentration of 0.02% w/v. To increase the initial milk acidity from 0.04% to 0.06%, 25–30 ml/L of natural whey starter culture was added. After stirring, each milk-clotting enzyme was added at a different dose so that a similar curd strength was reached simultaneously in the four vats. After 18–20 min, the curd was cut to the adequate grain size (approximately half a rice grain), and the mixture of curd particles and whey stirred gently and heated to 44°C to reduce the moisture content in the curd. The mixture was then heated rapidly to 52°C, and stirring was ceased. Whey samples were taken at this point, after which the curd was separated and moulded and the remaining whey discarded. The curd was pressed into moulds, salted in saturated brine and stored at 12°C and 80% relative humidity.

Starter cultures

A local dairy plant (Milkaut Coop. Ltda) supplied the natural whey starter culture for the first cheesemaking day in our laboratory. This culture originated from cheese made with adult bovine coagulant. Starters for the subsequent days were obtained by incubation of whey from laboratory cheesemakings, and each vat provided the whey for preparing the next batch. To prepare cultures, a 500-mL whey sample was taken from each vat after curd cooking and incubated at 42°C for 24 h.

Milk-clotting enzymes

Adult bovine coagulant (Naturen®, Chr. Hansen Argentina S.A.I.C., Quilmes, Argentina) was used as the milk-clotting enzyme for the control cheese. Experimental cheeses were prepared with FPC coagulants obtained from the local market, for example ChyMax® (Chr. Hansen Argentina S.A.I.C., Quilmes, Argentina), Chymogen® (Chr. Hansen, Horshølm, Denmark) and Maxiren® (Gist Brocades, Seclin, France). The enzymes Chymax® and Chymogen® were in liquid form while Maxiren® was dried.

In order to add equivalent amounts of each enzyme to the milk, it was necessary to know their activity. The coagulants were compared by means of a Formagraph (Foss Electric, Höganäs, Denmark), as coagulation time and curd firmness depend on the milk-clotting activity of the enzyme. The concentration of each coagulant (mg or mL/L) used in cheesemaking was then established to obtain similar gel firmness at the same time in all the cheese vats.[16,18]

In the Formagraph apparatus, samples of milk to be coagulated are placed in cells in an electrically heated metal block. The milk-clotting enzyme is added and a loop-shaped pendulum is immersed in the milk. The metal block is then moved back and forth, creating a 'drag' on the pendulum in the milk. The arm to which the pendulum is attached contains a mirror from which a flashing light is reflected onto a photosensitive paper, creating a mark. While the milk is fluid, the viscosity is low and the drag on the pendulum is slight and it scarcely moves from its normal position, hence a straight line appears on the paper. As the milk coagulates, the viscosity increases and the pendulum is dragged out of position, resulting in bifurcation of the trace. A Formagraph diagram and a typical trace are shown in Figure 1. The coagulation time is represented by r, and k_{10} is the time required from coagulation for the arms of the diagram to bifurcate by 10 mm.[19] The value $r + k_{10}$ is usually used as an indicator of the time to reach a given firmness in the curd. Low-heat skim milk powder reconstituted in $CaCl_2$ 0.01 M, pH adjusted to 6.5, was used as the substrate for the Formagraph analysis.

Whey analysis

Whey analysis was performed on each sample obtained from the fourth to the eighth cheesemaking day. From the first to the third cheesemaking day the whey was not analysed to avoid any carry-over effect caused by the use of the natural whey starter culture from the dairy industry.

The acidification rates of the control and the experimental whey starter cultures were determined during incubation by titration with a 0.1111 N NaOH solution using phenolphthalein as indicator.

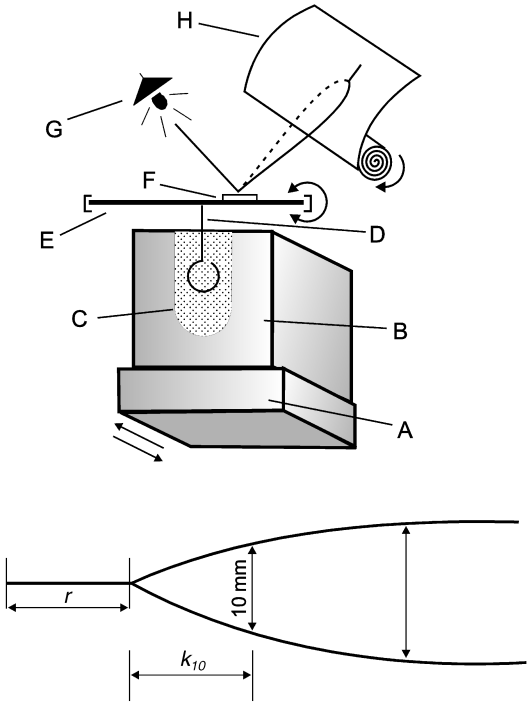

Figure 1 Formagraph illustration and a typical trace. A, moving plate; B, heated metal block; C, milk sample; D, pendulum; E, axe attached to the pendulum; F, mirror; G, lamp; H, photosensitive paper. *r*, coagulation time; k_{10}, time to reach a curd firmness represented by 10 mm width in the trace.

The pH was measured with a Horiba pH meter (Kyoto, Japan). Whey samples were taken aseptically every hour during the incubation period, with an interruption between the 10th and 20th hours.

The absence of milk-clotting activity in the whey after curd cooking was checked by inoculating 10% (v/v) whey into reconstituted low-heat skim milk, incubating at 30°C for 2 h, and periodically testing by gentle bending to determine whether casein floccules appeared on the test-tube walls.

Whey from all the cheese vats was sampled at the end of each cheesemaking process from the fourth to the eighth day, and analysed to obtain the nitrogen soluble fractions. The soluble fractions were trichloroacetic acid (TCA) 2% and 12% and phosphotungstic acid (PTA) 2.5%.[20] The fractions were analysed by the Kjeldahl method (IDF, 1993).[21] The average nitrogen content of four cheesemakings was calculated for each fraction and expressed as mg/L.

Whey from the dairy plant (Milkaut Coop. Ltda) was sampled on three different days and analysed for nitrogen fractions as described earlier. Its composition was compared to that of whey obtained from control cheeses in our laboratory.

Data analysis

Means and standard deviations were calculated for the acidity, pH and nitrogen content in the whey fractions. Means were compared by the one-way ANOVA test. The Duncan test of multiple range was used to detect treatments that caused significant differences.[21,22]

RESULTS AND DISCUSSION

Milk-clotting enzyme concentration

Reggianito Argentino cheese is usually made with adult bovine rennet at a rate of 0.5 mL/L of milk (milk-clotting power ~230 IMCU/mL). This enzyme concentration resulted in a coagulation time plus curd firmness ($r + k_{10}$) of 19.8 min measured by using the Formagraph. Several dilutions of each FPC enzyme were prepared to assess the concentration that clots the milk with the same $r + k_{10}$ as adult bovine rennet, i.e. ~19.8 min. Figure 2 shows a Formagraph trace from which the appropriated concentration of Chymax® was obtained, and Formagraph diagrams of other enzymes were similar (data not shown). Hence, the amount (mL or g/L) required for each FPC was as follows: 0.500 Chymax®, 0.450 Chymogen® and 0.012 Maxiren®.

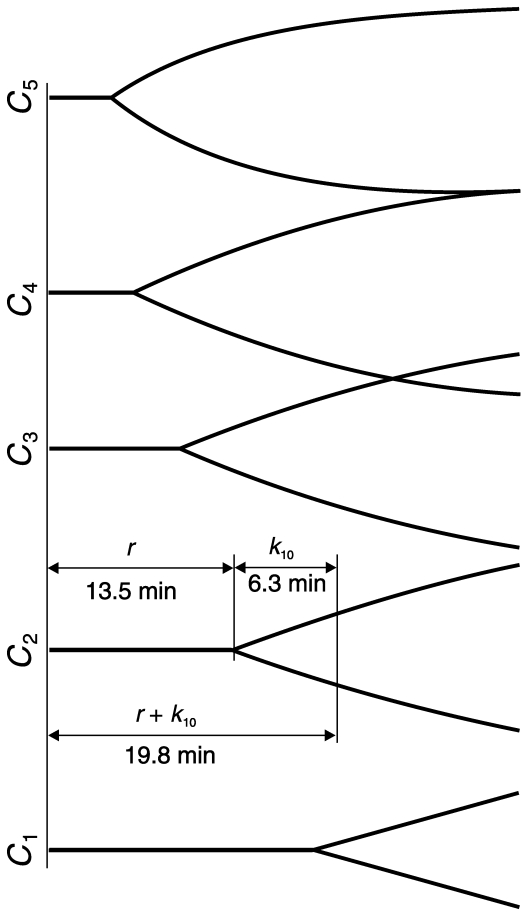

Figure 2 Formagraph traces obtained with Chymax® at different concentrations: $C_1 = 0.350$, $C_2 = 0.500$, $C_3 = 0.650$, $C_4 = 0.950$ and $C_5 = 1.150$ mL/L of milk, respectively. C_2 is the dilution chosen in the cheesemaking experiment because it gives the same $r + k_{10}$ value (19.8 min) as adult bovine coagulant.

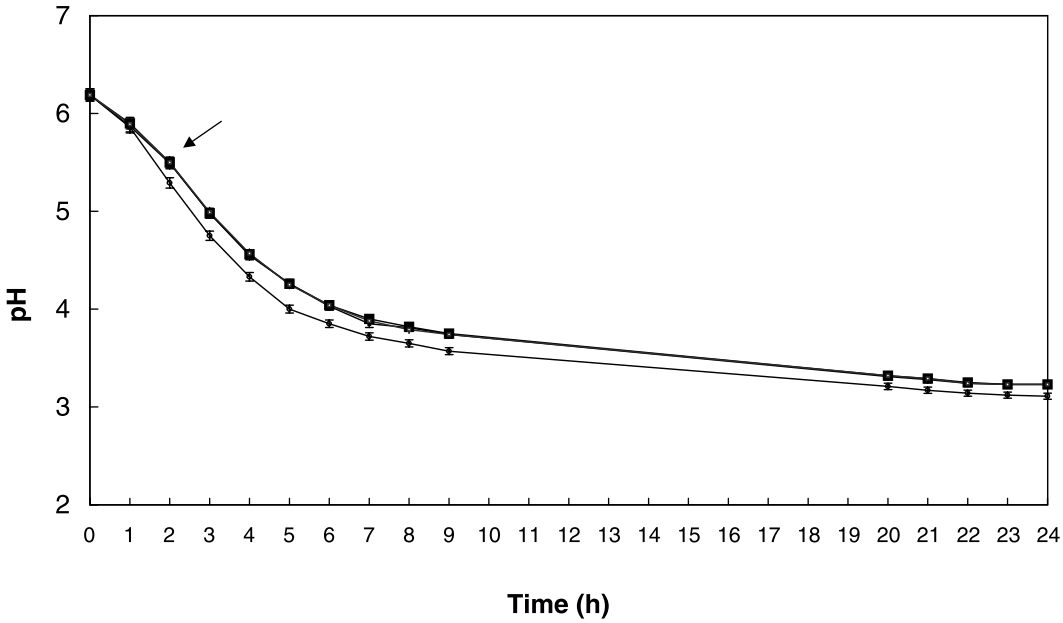

Figure 3 pH evolution during incubation of whey from different cheese vats on the fourth cheesemaking day. (○) Adult bovine coagulant enzyme, (●) Chymax®, (■) Chymogen®, (◆) Maxiren®. The pH of fermentation-produced chymosin (FPC) whey decreased slower than that of control whey after 2 h (*arrow*) of incubation ($P > 0.05$).

As the Formagraph analysis was carried out on reconstituted low-heat skim milk but the cheeses were made with whole pasteurized bulk milk, the coagulation time and curd firmness were checked to be similar for all vats during cheesemaking. To do that, the curd was cut with a spatula and the cleanness of the cut and strength of the gel were observed empirically. The gross composition of the cheeses (moisture content, fat and protein) did not differ significantly ($P < 0.05$) among the experimental and the control cheeses, confirming that the cheesemaking processes were equivalent. The means and standard deviations for the moisture content, fat matter and protein content were 37.13 ± 0.76, 21.90 ± 1.2 and 31.45 ± 0.40, respectively, for the control cheese, and 36.84 ± 0.89, 23.20 ± 0.73 and 31.68 ± 0.35, respectively, for the FPC cheese.

Acidification rate

The acidification rates (pH and titratable acidity) of the control and experimental whey during incubation are shown in Figures 3 and 4. The means and standard deviations from the fourth to the eighth cheesemakings are shown.

The initial pH and acidity values were not significantly different: 6.12 ± 0.07 and 0.150 ± 0.003%, respectively, for the control whey, and 6.12 ± 0.05 and 0.149 ± 0.006%, respectively, for the experimental whey. This is not surprising taking into account that the cheesemaking process was identical in all the vats. Nevertheless, acidification differences occurred after 2 h of incubation, where the pH of the control whey decreased faster. Final pH and acidity values showed significant differences: 3.12 ± 0.04 and 1.699 ± 0.006%, respectively, for adult bovine coagulant, and 3.23 ± 0.01 and 1.435 ± 0.004, respectively, for all the FPC in the experimental whey samples. A Duncan test of multiple range showed that the treatment that caused significant differences in final pH and acidity was the use of adult bovine coagulant.

Nitrogen fractions

The nitrogen (N) content in TCA 2% and 12% and PTA 2.5% (means and standard deviations) of whey originating from control and experimental cheesemaking, before the incubation period, is shown in Table 1. Whey samples of the experimental cheeses had significantly lower concentrations of nitrogen compounds than whey of the control cheese for all the tested fractions ($P < 0.05$). Kuchroo and Fox[24] reported that the soluble nitrogen fraction in TCA 2% was similar to the water soluble extract, and was composed of proteins (excluding most caseins), all peptides, amino acids and smaller N compounds. Almost all N compounds in the whey are soluble in 2% TCA. TCA 12% represented medium-sized to small peptides, amino acids and smaller N compounds, while the PTA results applied to very small peptides, amino acids and smaller N compounds, except for dibasic amino acids and ammonia.[25] This indicates that the whey from FPC cheesemakings had less nitrogen material in all the range of molecular weights than whey from adult bovine cheesemaking. The FPC's higher ratio of milk-clotting activity to proteolytic

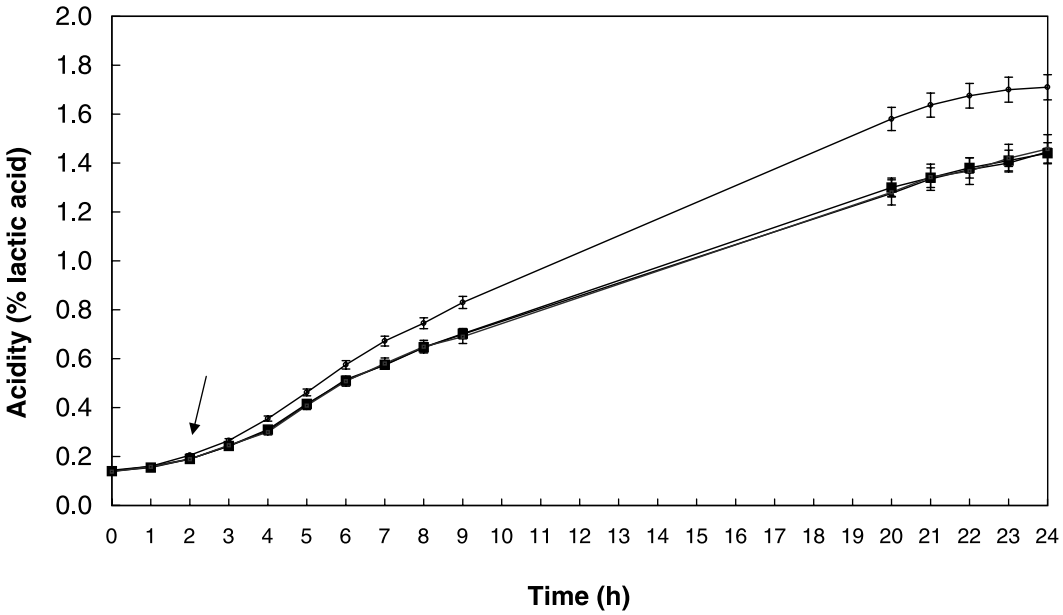

Figure 4 Acidity evolution during incubation of whey from different cheese vats on the fourth cheesemaking day. (○) Adult bovine coagulant enzyme, (●) Chymax®, (■) Chymogen®, (◆) Maxiren®. Fermentation-produced chymosin (FPC) whey acidified slower than control whey after 2 h (*arrow*) of incubation ($P > 0.05$).

Table 1 Means and standard deviations of N content in soluble fractions of whey obtained from cheesemakings using different milk-clotting enzymes, after 24 h of incubation at 42°C

	N content in soluble fractions (mg/100 mL of whey)		
Milk-clotting enzyme	*TCA 2%*	*TCA 12%*	*PTA 2.5%*
Adult bovine coagulant[1]	$105.2^{a} \pm 0.3$	$54.9^{a} \pm 1.4$	$51.9^{a} \pm 0.6$
Chymax[1]	$91.7^{b} \pm 1.3$	$47.9^{b} \pm 0.1$	$44.8^{b} \pm 2.3$
Chymogen[1]	$92.3^{b} \pm 2.4$	$48.4^{b} \pm 0.1$	$43.8^{b} \pm 1.7$
Maxiren[1]	$93.3^{b} \pm 1.1$	$48.6^{b} \pm 0.8$	$44.7^{b} \pm 2.4$
Adult bovine coagulant[2]	$103.7^{a} \pm 0.2$	$53.7^{a} \pm 0.3$	$50.3^{a} \pm 0.3$

TCA, trichloroacetic acid; PTA, phosphotungstic acid

[1]Means were calculated from data from the fourth to eighth cheesemaking days

[2]Mean was calculated from data from three industrial whey samples

[a,b]Means in the same column with different superscripts differ sigificantly ($P < 0.05$)

activity, which prevented nonspecific proteolysis during manufacture,[26] is most probably the cause of the lower soluble nitrogen content in the whey. These results indicate that FPC whey had less nitrogen available for lactic acid microflora growth.

The nitrogen content of the soluble fractions of control whey obtained in the laboratory (105.2 ± 0.3, 54.9 ± 1.4 and 51.9 ± 0.6 mg/100 mL for TCA 2%, TCA 12% and PTA N, respectively) and the whey from the industrial dairy plant (103.7 ± 0.2, 53.7 ± 0.3 and 50.3 ± 0.3 mg/100 mL for TCA 2%, TCA 12% and PTA N, respectively) did not differ significantly ($P < 0.05$).

All the milk-clotting enzymes tested were inactivated by cooking during cheesemaking, which indicates that they were heat labile and did not hydrolyse polypeptides and proteins during the incubation period.

CONCLUSIONS

The acidification rate of whey from cheeses made with FPC has proven to be slower than that from cheeses made with adult bovine coagulant, even though the quantities of milk-clotting enzyme were equivalent. The slow-down in the acidification curves began after 2 h of incubation, and resulted in higher pH and lower titratable acidity for FPC whey at the end of incubation compared with whey from adult bovine coagulant. The composition of all the experimental whey contained a lower concentration of nitrogen compounds in all the analysed fractions, which probably caused the delay in acidification as nitrogen compounds of low molecular weight are substrates for lactic microflora growth.

If the cheesemaker's goal is to obtain similar final products with adult bovine coagulant or fermentation-produced chymosin, a technological solution could be the addition of protein hydrolysate or other nitrogen sources into the whey before its incubation.

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