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Performance of Crude Coagulants from Adult Rabbit Stomach and Their Influence on the Brazilian Prato Cheese Type Composition

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Abstract

This study aimed to evaluate the milk clotting capacity (MCA), and caseinolytic activity (CA) of crude coagulant from the stomachs of adult rabbits (RC), and their influence on the composition, proteolysis, and texture of type Prato cheese. We tested two ways for salting the abomasum and three levels of pH and times for enzyme activation. The presence of enzymes and caseinolytic activity in RC was assayed by SDS-PAGE. The effects of three pH levels and the milk's temperature on RC's water-holding capacity (WHC) were evaluated. Brazilian Prato cheese type was developed and its proximate composition, texture, and change during ripening were assessed. The saturated NaCl solutions (37%, m/v) were more efficient for enzyme extraction from adult rabbit stomachs, and conditions of pH of 4.3 for 24 h showed better performance for enzyme activation into RC. An early and more active proteolytic reaction of RC on bovine caseinate was appreciated at the first 5 min of digestion when compared to commercial chymosin (CC). The water holding capacity of gel coagulated by RC was optimum at pH 6.4 and 37˚C and is highly dependent on pH and temperature parameters. Total dry extract and fat were higher for treatment coagulated with RC. Conversely, hardness, gumminess, and chewability were lower in the same treatment. Yield, protein content, and changes that occurred during the ripening stage of cheeses were similar for both treatments, demonstrating that casein proteolysis was developed in a similar way. Coagulants from adult rabbits' stomachs can be used to make semi-cooked curd cheeses.

Keywords

Rennet, Milk Clotting Activity, Prato Cheese, Caseinolytic Activity

1. Introduction

During the coagulation step in cheese making, two principal events occur, pro-

moted by enzymatic coagulants. The first of them is the hydrolysis of κ-casein fraction, a protein located on the outer layer of the casein mycelium; and the second is the aggregation of destabilized proteins, consequently forming a gel. This last event occurs when a minimum of 85% to 90% of κ-casein is destabilized [1] [2]. Effects of residual coagulant could still influence the ripening stage, and consequently, some characteristics of the final product since proteolysis activity manifests itself both in rheology and in sensory aspects [3] [4].

Rennet produced from the abomasum of calves is the coagulant used historically for cheese production and consists of the enzymes chymosin (EC 3.4.23.4) and pepsin (EC 3.4.23.1) [5]. The coagulant used for cheese making should have a high milk clotting capacity, specific action on the breaking peptide bonds, and low proteolytic activity, to avoid loss of both protein and fat components in the serum [6] [7].

Some factors can affect the milk clotting and proteolytic activity of enzymes during cheese making, such as the pH of milk, temperature, Ca^{+} concentration, as well a quantity of the coagulant used. Likewise, the type of coagulant used, or the enzyme source can affect the characteristics of the cheeses, not only during manufacture but also in the ripening stage [8] [9].

The enzymes present in the stomach exist in an inactivated form known as proenzyme; therefore, for the preparation of the coagulant, it is necessary to carry out an activation process, which consists of reducing the pH [10]. Liquid rennet from the kid was prepared at a pH of 2.0 and 30 min for preparation [11]. For the preparation of the coagulant based on buffalo abomasum, the activation of the proenzyme is at pH 4.0 [12].

Abomasum of adult animal species such as ovine, porcine, chicken, or adult bovine stomachs are used for the production of coagulants; in theory, coagulants prepared from these may contain a higher pepsin content than those prepared from animals a few weeks old [7] [13] [14].

Research characterized liquid coagulants from young rabbit stomachs [15]. Alihanoglu [16] tested coagulants from young rabbit stomachs, as well as their action on white cheese composition and texture. Scarce information is available on the use of stomachs of adult rabbits with age and commercial weight and the possibility of their use as coagulants. Therefore, this study aimed to assay strategies for liquid coagulant preparation from adult rabbit stomachs (RC) and the milk clotting and caseinolytic activity and their influence on the composition, texture, and ripening of the Prato cheese type. We tested two ways of salt application for enzyme extraction, and several levels of pH and time of incubation for enzyme activation in liquid coagulant.

2. Materials and Methods

2.1. Materials

Commercial Chymosin (CC), produced from the fermentation of *Aspergillus niger* ssp awamori (HA-LA, Chr. Hansen Ind., Valinhos, SP), was used as a posi-

tive control treatment.

Stomachs from New Zealand white rabbits, with aged between 16 and 20 weeks were provided by the Slaughter Center of Small Species, in the Federal Farroupilha Institute located in the municipality of Júlio de Castilhos/RS, Brazil. After slaughter, the stomachs were separated and immediately frozen without removing the gastric contents.

2.2. Methods

2.2.1. Preparation of Liquid Coagulant

For the preparation of the minimally purified coagulants from adult rabbit stomachs, protocol described by Lambert [17] was adopted. After thawing, the stomachs were opened and the gastric contents immediately removed including the excess perivisceral fat, and the rest of the esophagus, then washed with potable water in both external and internal superficies. For the enzyme extraction, two ways for salt applications were tested: directly on stomach superficies (SD) and immersed in a saturated saline solution (SS) (37%, m/v) for 5 min, and a third treatment without salt application (NS). After, all treatments were dried for 48 hours at 35˚C in an oven with continuous airflow, and dried tissues were cut into pieces of 5 × 20 mm approximately.

For the liquid coagulant preparation, five grams of dry tissues were immersed in a NaCl solution (12%, m/v) (1:10 ratio) and boric acid (1%, v/v), and stirred for 30 min.

2.2.2. Influence of pH and Time for Enzyme Activation on Milk Clotting Activity

A methodology of response superficies (MRS) was used to test the influence of the pH and time of enzyme activation. Two levels of both variables (maximum and minimum) were assayed, and the experimental design shown in **Table 1**,

Table 1. Experimental design of two factors central composite design and experimental values of milk clotting activity.

Experimental runs	pH	t (h)	x_1	x_2	MCA (U/mL)
1	2.5	24	−1	−1	3.28
2	2.5	72	−1	1	14.77
3	4.3	24	1	−1	685.71
4	4.3	72	1	1	296.30
5	3.4	48	0	0	266.67
6	2.12	48	−1.414	0	15.96
7	4.6	48	1.414	0	400.00
8	3.4	14	0	−1.414	352.94
9	3.4	82	0	1.414	203.39
10	3.4	48	0	0	252.63

t (h) time in hours; MCA (U/mL) milk clotting activity.

aimed to find the better condition for enzyme activation. Milk clotting activity was the response variable, following before methods described. Tissues of rabbit abomasum in NaCl solution (12%) were adjusted to pH with HCl (1N) and stay for the time of experimental design incubated at 37˚C in a closed oven. Then, the pH was raised to 5.6 with NaOH solution (1N), filtered on C41 filter paper (UNIFIL), and the liquid collected in amber glass bottles and stored at 5˚C.

The milk clotting activity was assayed using caseinate solutions as substrate. Ten mL of caseinate solutions, with sodium azide and $CaCl_2$, were put into glass tube. Then, 200 µL of coagulant were put and shaking on 1 min with stirred. Time of first coagulum appeared over glass tube was registered and MCA was calculated as followed equation:

$$\text{MCA} = \left(2400 * V\right) / \left(t * E\right) \quad (1)$$

Where:

MCA is the milk clotting activity; V is volume of milk (mL), t is the time of coagulation (min); E is the volume of coagulant (mL).

2.3. Caseinolytic Activity

2.3.1. Preparation of Substrate

Calcium caseinate from bovine milk was used as a substrate for proteolytic activity assay following the procedure described by Ahmed [18]. Fresh whole milk was defatted by centrifugation at 2800 g for 10 min and 25˚C. Caseinate was obtained by isoelectric precipitation with milk acidification to pH 4.3, using an HCl solution (6N). The acidity milk was heated to 37˚C for 30 min and centrifuged at 2800 g for 10 min. The supernatant was discarded and the precipitated was washed three times with distilled water. Then the precipitated was rehydrated in distilled water (12% w/v) and the pH was adjusted to 6.5 using NaOH solution (1N), and finally left to stand for three hours at 4˚C to be subsequently frozen and dehydrated by lyophilization.

2.3.2. Polyacrylamides Gel Electrophorese for Casein Hydrolysis

The substrate (1% w/v) was prepared from calcium caseinate in a buffer phosphate solution (pH 6.5) and stabilized at 30˚C of temperature in a water bath. The test started with the addition of RC in a ratio of 0.5 mL for 10 mL of the substrate. Samples were collected after the 5, 30, and 60 min of incubations and immersed in water at 95˚C, for the enzymatic inactivation. This assay included CC as control treatment following the same procedure described in the section before using de volume according to the manufacturer's specifications.

Hydrolysis of casein with RC and CC was monitored using SDS-PAGE following the method described by Sams [19] with minor modifications. A volume of 75 µL of the digested substrate was collected and mixed with 25 µL of sample buffer (4× Laemmli, Biorad), and heated to 95˚C, for 10 minutes. Electrophoresis was developed in stacking and resolving polyacrylamide/bisacrylamide gels (5%, and 14%, respectively) in a Mini PROTEAN cell (Bio-Rad). After the electrophoretic run, the gels were stained with Coomassie Blue, with shaking for 1 h

and decolorized with ethanol (30%), acetic acid (7.5%) and trichloroacetic acid (5%) for 12 hours. To compare molecular weights, a standard containing a mixture of 12 recombinant proteins with molecular weights between 2 kDa and 250 kDa was included in electrophorese gel. Gels' images were analyzed using image density software (ImageJ).

2.4. Water Holding Capacity (WHC)

The effect of pH, temperature, and coagulant volume on the WHC was assayed, on the curd yield of skimmed bovine milk following the method described by Ben Amira [20] with some modifications. Each variable was tested at three levels: pH (5.9, 6.4 and 6.9), temperature (27˚C, 35˚C and 42˚C), RC volume (50, 125 and 200 μL). Bovine milk was skimmed by centrifugation at 2800 g and pasteurized (63˚C for 30 min). The skimmed milk was adjusted to pH and temperature conditions as previously described. Ten (10) mL of skimmed milk into glass tubes were placed in a water bath. Afterwards, RC was added, and coagulation occurred for 45 minutes, and then the tubes were centrifuged at 2800 g for 5 minutes. Supernatant discarded and the curd was weighed. Equation (2) was used to calculate the WHC:

$$\mathrm{WHC}(\%) = (w * 100)/v \quad (2)$$

where:

WHC = is the water holding capacity, w = curd weight, v = coagulant volume.

2.5. Development and Characterization of Prato Cheese Type

2.5.1. Milk Composition

Fresh whole milk was supplied by the Tambo (cow milking area) of the Federal University of Santa Maria/RS, Brazil. Defatted dry matters (DFDM), protein, fat, lactose, mineral residue, and density were determined using the milk analyzer (MASTER MINI, Higmed, Tatuapé, São Paulo, SP). The pH was measured using a pH meter (Digimed DM20 SPLabor, Presidente Prudente, SP, Brazil), and acidity through titration with 0.1N NaOH, with phenolphthalein as an indicator.

2.5.2. Prato Cheese Making

For making of Prato type cheese coagulated by RC and CC, method described by Baptista [21] was followed, with minor modifications. The fresh milk was pasteurized (65˚C/30min) and cooled to 37˚C, then pH was adjusted to 6.5 with a lactic acid solution (1N). The mesophilic culture (Lactococcus lactis ssp. Lactis and L. lactis ssp. Cremoris, Rica Nata, Piracicaba, SP), were added (1%, v/v). Afterward, calcium chloride solution ($CaCl_2$, 50%) (2 ppm w/v) was added and, finally, the RC (3.6 $mL \cdot L^{-1}$) or CC coagulant (0.9 $mL \cdot L^{-1}$), with agitation for a maximum of 2 min, and left to rest for 45 min. Subsequently, the gel was cut with a knife to determine the clotting point. After 5 min of having been cut, the curd was treated, starting with gentle agitation for 5 min, which was repeated for 15 min with rest intervals of 3 min. The temperature was raised to 42˚C and the

agitation and rest sequence was repeated for another 15 min. The whey was separated by draining, and the mass was placed in specific cylindrical molds. The cheeses were pressed applying at 10 times their weight for one hour, followed by turning and applying a pressure equivalent to 15 times their weight for another 12 hours. Pieces of cheese were salted in 20% saline solution for 3 hours and dried in the refrigerator at 8˚C for 3 hours. Finally, the pieces were vacuum-packed and stored in a refrigerator at 8˚C for sixty days.

2.5.3. Proximal Composition of Cheese

To quantify the dry mass in samples of cheese and whey, 5 g of the sample was dried in an oven at 105˚C until constant weight [22] (method 925.23; AOAC, 2007). Protein was estimated by means of the Kjeldahl method (N * 6.38; method 954.01; [22] AOAC, 2007). Fat was determined by treating the sample with sulfuric acid and isoamyl alcohol [23] (IAL, 2008). Ashes were quantified by gravimetry after heating until the complete incineration of 2 g of the moisture-free sample in a muffle furnace at 550˚C [22] (method 935.42; AOAC, 2007). Acidity was determined by titrating the sample with an alkaline sodium hydroxide solution (0.1 N) (Brazil, 2018). The pH was estimated with a digital pH meter calibrated at 25˚C (Digimed DM20 SP Labor; Presidente Prudente, SP, Brazil) mixing 10 g of cheese with 40 mL of distilled water. All analyses were performed in triplicate.

2.5.4. Texture Profile of Cheese

The texture profile was performed according to Alves [24] (2017), with modifications. To cut the samples cylinders molds (20 mm in diameter and 20 mm in high) were used, and then the pieces were packed in plastic film, and then placed in a plastic bag, and kept in a polystyrene box with ice (5˚C ± 1˚C) for 1 h, in order to stabilize the temperature before the test. Texture profile parameters were analyzed with a TA-XT2 (Stable Micro System, Haslemere, United Kingdom) texture equipment using a 36 mm diameter aluminum probe (PB36). Three measurements were made for each treatment replica. The recorded parameters were hardness (N), cohesiveness, adhesiveness (J), elasticity, gumminess (N), and chewability. Data was collected by Texture Expert Software for Windows – 1.2 (Stable Micro Systems).

2.5.5. Changes during Ripening of Cheese

For 60 days, pH and acidity (mg lactic acid 100 g^{-1}) were registered following method described in the proximate composition of the cheeses (2.5.3).

The Proteolysis was described through the ripening extension index (REI) and ripening depth index (RDI) parameters that were determined by the Kjeldahl method, which allowed obtaining the amount of water-soluble nitrogen at a pH of 4.6 (SN-pH 4.6), and the soluble nitrogen in trichloroacetic acid (SN-TCA), and total nitrogen (TN), starting from a standard solution [25].

The ripening extension index (REI) is obtained by Equation (3), below:

$$\mathrm{REI}(\%) = \frac{\text{SN-pH 4.6} \times 100}{\mathrm{TN}} \quad (3)$$

where:

SN-pH 4.6 = water-soluble nitrogen content at pH 4.6;

TN = the total nitrogen.

The ripening depth index (RDI) was determined by Equation (4), below:

$$\mathrm{RDI}(\%) = \frac{\text{SN-TCA} \times 100}{\mathrm{TN}} \quad (4)$$

where:

SN-TCA = soluble nitrogen content in trichloroacetic acid (SN-TCA);

TN = is the total nitrogen.

2.6. Experimental Design and Statistical Analysis

Response surface methodology (RSM) was used to study the better condition of enzyme activation. Different pH and time of activation (t) were assayed, and the highest milk-clotting activities (MCAs) of the obtained extracts were tested. In this work, a central composite design belonging to response surface methodology (RSM) was applied to determine the optimum levels of two factors. This experimental design consists of three parts: Four (4) experiments corresponding to a two-factor factorial design 22, four (4) axial experiments symmetrically spaced at $\pm a$ along the variable axes, and two (2) experiment at the center of the experimental domain.

To determine the WHC, a 3^3 factorial experiment was carried out. Then, the three-way ANOVA was performed, and the significance of the interaction was analyzed as a priority. When significant, the interaction was unfolded, and the mean difference analysis was tested using the HSD-Tukey posthoc test ($p \leq 0.05$).

Cheeses coagulated with RC and CC were developed in three batches, with three repetitions of each, totaling 18 pieces. A completely randomized design was used, considering the type of coagulant (RC, CC) and the ripening time (2, 30, and 60 days) as the source of variation. The response variables were pH, total nitrogen (TN), ripening extension index (REI), and ripening depth index (RDI). The differences were analyzed using two-way ANOVA, with repetition, using the data analysis tool of Statistic 8.0 (StatSoft Inc., USA) software (2008).

3. Results and Discussion

3.1. Influence of Salting on Enzyme Extraction

Salt is a common ingredient in the preparation of both paste and liquid coagulants from abomasa stomachs with two purposes, to facilitate the extraction of enzymes from the tissue and, preserve the shelf-life of the coagulant [10]. In this study, were tested two ways to incorporate salt in the fresh stomachs before liquid coagulant preparation; direct application on the surface and submersion in saline saturated solution for 5 minutes. **Figure 1** show in the gel electrophorese

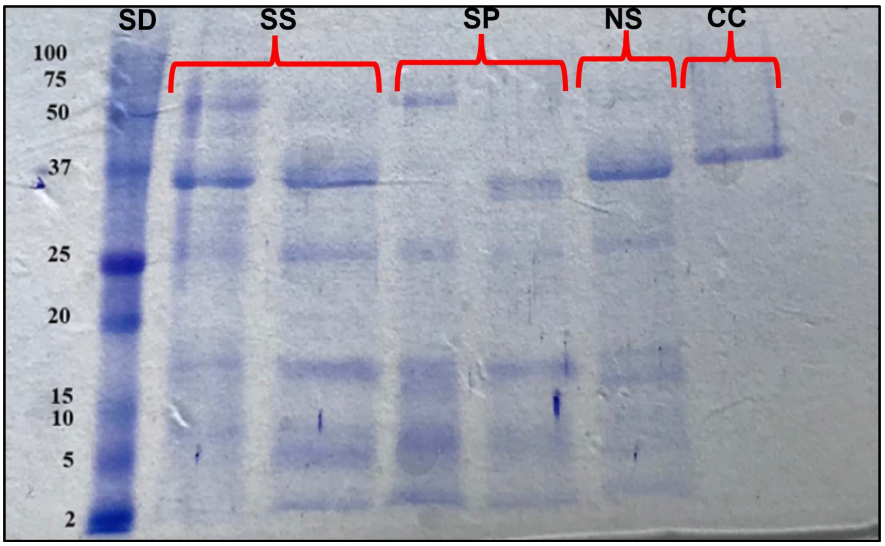

Figure 1. Electrophoretic profile of liquid coagulant from adult rabbit using two ways of salting for enzyme extraction. SD standards of proteins; SS extraction with saturated saline solutions (37%) SP extraction with salt directly into superficies; NS no salt addition, CC commercial chymosin.

(SDS-PAGE), proteins registered. Consistent bands were identified for the treatment where the saturated saline solution was used (SS), leaving evidence of its effectiveness in comparison with the extract made with salt directly placed on the stomach surface (SP). It is possible to deduce that the fact that the salt in saturated solution allowed a greater penetration and interaction with the existing enzymes in the gastric tissue.

The treatment without the addition of salt showed a more marked band, however, this was not considered for subsequent tests due to its susceptibility to deterioration by decomposition.

The approximate molecular weight of the principal's band in SS, SP, and NS was 35 KDa, lower than CC wich showed 37 KDa. These results are like those found by Sams *et al.* (2018) who obtained bands of 35 KDa in the commercial rabbit gastric extract.

3.2. Influence of pH and Time of Activation Enzyme on Milk Clotting Activity of Coagulant

The enzymes within the stomach remain in either active or inactivated form, the last known as a proenzyme. A necessary step in protocols for the preparation of coagulants is a reduction in pH for a period not clearly defined. Therefore, it is necessary to evaluate the conditions to guarantee the maximum efficiency of the coagulant available for use [26] (Hill, 2003). In this study we investigate some pH conditions for enzyme activation using MRS as tool to assess the optimum condition for highest MCA as response variable. **Figure 2** show the graphic of response surface, where is possible observe that the pH was the most influent variable on MCA than the time of activation. The pH with de maximum MCA (685 U/mL) was 4.3, independent to the time of activation. This demonstrates

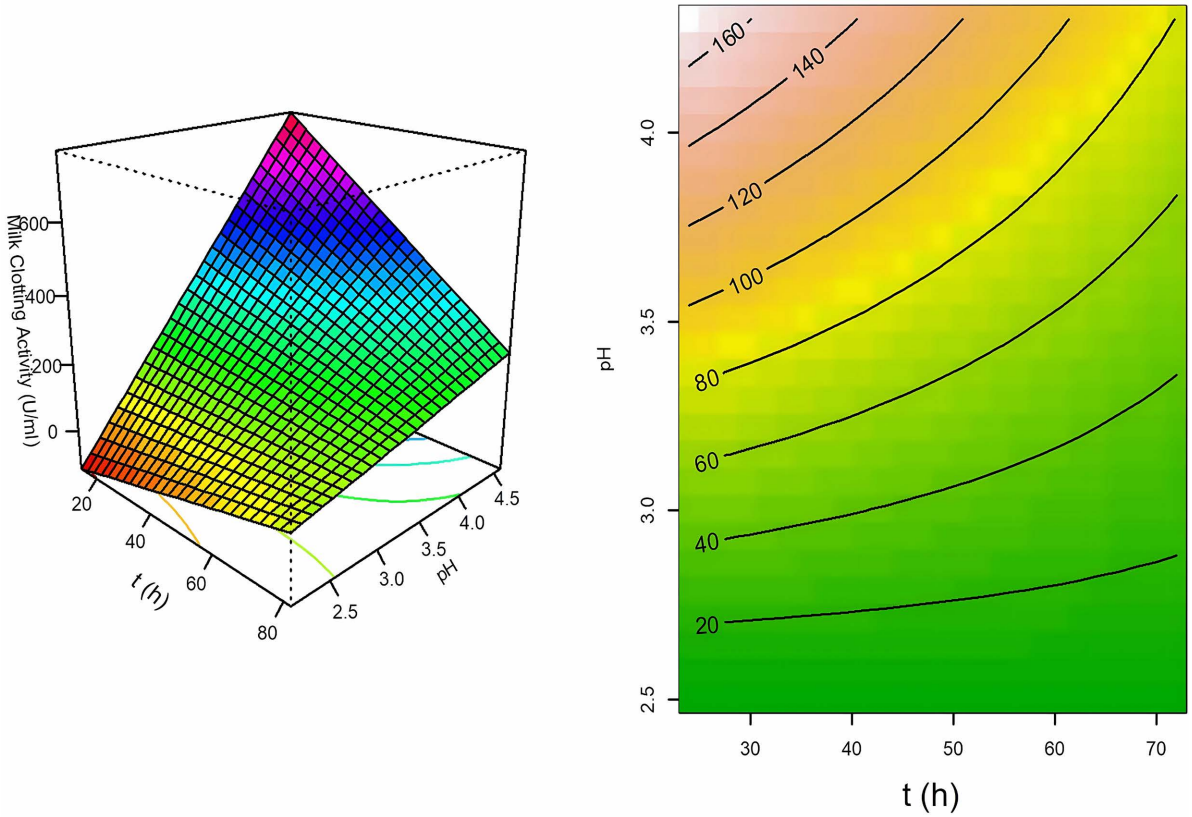

Figure 2. Influence of pH and time of incubation on milk clotting activity (MCA) of adult rabbit coagulant.

the sensitivity of the enzymes of the gastric extract to changes in pH to extreme levels. In the isolation of chymosin and pepsin from goat abomasum Moschopoulou *et al.* (2006) acidified the extract was at pH 2 [11]. For [26] Hill (2003), the pH of enzyme activation ranges from 2 to higher pH.

3.3. Caseinolytic Activity of Coagulant on Bovine Casein

The electrophoresis profile of hydrolysis of casein is showed in **Figure 3**. The hydrolysis of bovine milk caseinate in solution (1%) with RC and commercial chymosin (CC) showed that the digestion of protein occurred in a similar way for both treatments. After 5 min the formation of a peptide product of hydrolysis with approximate molecular weight of 11 kDa was observed, occurring for RC and CC. However, at the same time, a more intensive degradation was observed in RC (column 1). This performance was observed at 30 minutes, with the formation of a peptide product from the digestion of caseinate with an approximate weight of 22 kDa, specifically for treatment with RC (column 4), not being observed for the control treatment. This behavior was maintained until the 60 minutes point of the test. Sams *et al.* [19] identified the presence of pepsin in RC and analyzed the proteolytic action on β-casein, obtaining hydrolysis patterns similar to that observed with porcine pepsin, supporting the results that can be found in our work. Futures studies are necessaries for isolating and identifying the present of enzymes in the RC.

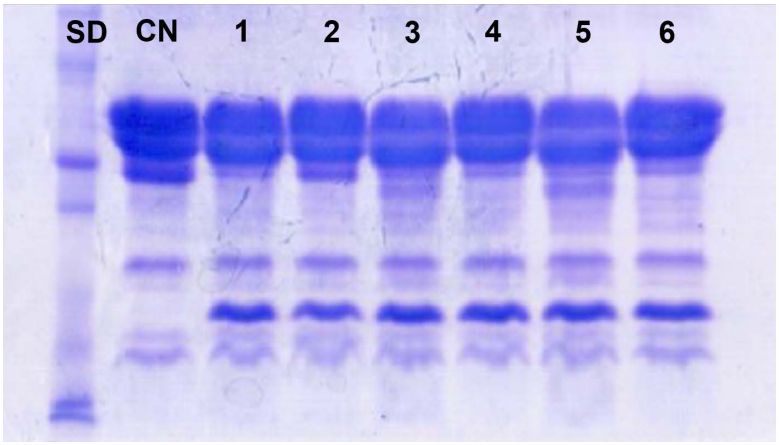

Figure 3. Caseinolytic activity of Rabbit Gastric Extract vs Commercial Chymosin. SP Standard of proteins, CN casein, 1-3-5 hydrolysis of casein using RG collected at 5, 30, 60 min; 2-4-6 hydrolysis of casein collected at 5, 30 and 60 min.

3.4. Effect of pH, Temperature, and Volume of RC on Water Holding Capacity (WHC)

Results of the WHC in 60 minutes after milk coagulation in three levels of the factors pH, temperature, and volume of the RC are showed in **Figure 4**. Those parameters analyzed in this study are theoretically considered as possible influencers in milk coagulation, pH and volume of RC were the ones that most explained the variance ($p < 0.01$). According to Law & Tamime [14], the pH of the milk is decisive during the first phase of hydrolysis, and changes can be seen in the rate of aggregation and curd yield.

The maximum WHC was reached at pH 6.4, combined with a volume of extract of 50 µl and a temperature of 27˚C. The WHC of RC was affected ($p < 0.001$) at pH 6.9, causing a drop in the WHC, regardless of the temperature and volume of the extract. This response is similar to those obtained by Green [27], which observed that the coagulant capacity of chicken gastric extracts decreased with increasing pH; however, at pH 6.8 it still showed activity. For Dalgleish [28], chymosin activity is still maintained with milk at pH 6.8. However, the author states that other proteolytic enzymes lose their activity above this pH. The temperature range in this test (27˚C - 42˚C) was not significant ($p > 0.05$). Law & Tamime [14] emphasize that coagulation can occur in wide intervals between 20˚C and 60˚C, however, below 15˚C, the coagulant activity can be reduced or null.

3.5. Prato Cheese Manufacture

3.5.1. Whole Bovine Milk Composition

Whole bovine milk presented contents in the percentage of defatted solids of 8.79 ± 0.13, protein 3.24 ± 0.05, lactose 4.82 ± 0.07, and mineral residues 0.72 ± 0.01. Density (g/mL) was 1.032 ± 0.001, cryoscopy point (˚H) −0.550 ± 0.01 and pH was 6.94 ± 0.01. In general, the results obtained were within the values required by Brazilian legislation [29], except for the fat content, which was 2.56 ±

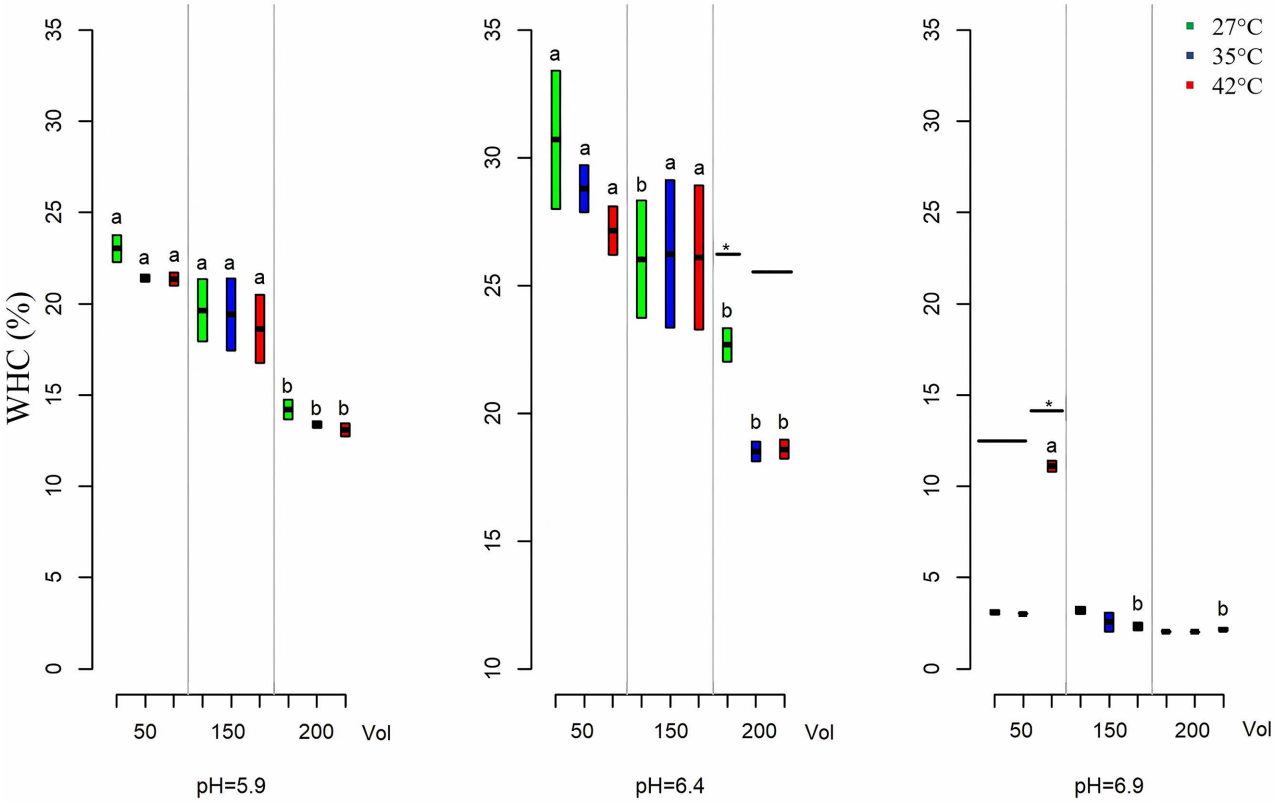

Figure 4. Influence of pH, temperature and coagulant volume on water holding capacity (WHC) of curd coagulated by rabbit gastric extract.

0.08, below the recommended. This fat value could be associated with the factor known as depression in fat in milk, which means that for the period of milk collection, factors such as diet or seasonality influenced the decrease in the production of this compound in milk [30].

3.5.2. Physical and Chemical Characteristics of Cheeses

Initially, a rapid coagulation test was necessary for determinate de MCA due to high pH (6.94) of raw milk and was confirmed the needing of adjust the pH of milk to 6.5, like this was demonstrated in the Section 3.2. For attempt the same coagulation time, the volume of RC was 4 times the volume than the CC used to coagulate the milk within 45 minutes. During coagulation, the principal solid constituents of milk remain occluded in the proteinic matrix formed, a product of casein digestion, except for soluble proteins. Factors such as pH and temperature of milk influence the aggregation stage of protein fractions since κ-casein was hydrolyzed by enzymatic action [14]. Data from physical chemical analyzes of cheeses are shown in **Table 2**. Cheese composition generally did not show differences between treatments coagulated with RC and CC. The parameters considered as protein, fat, ash, and total solids obtained similar values for both treatments.

These results demonstrate that despite the possible presence of pepsin as the main enzyme present in the coagulant of the stomach of adult rabbits, RC can be

Table 2. Effect of the type of coagulant on chemical composition and physical-chemical parameters of cheese coagulated with RGE and CC after two days of manufacture (means ± SD; n = 9).

Item	RC	CC	CV (%)
TS (%)	48.93 ± 3.12[a]	48.17 ± 0.87[a]	2.75
Total protein (%)	25.31 ± 1.66[a]	24.35 ± 1.23[a]	3.95
Fat (%)	18.77 ± 2.01[a]	17.90 ± 1.24[a]	8.70
Salt (%)	1.270 ± 0.19[b]	1.65 ± 0.21[a]	13.83
Ash (%)	3.93 ± 0.54[a]	4.33 ± 0.44[a]	11.88
Yield (%)	8.56 ± 0.22[a]	8.51 ± 0.39[a]	3.72
Acid	0.188 ± 0.027[a]	0.174 ± 0.057[a]	24.76
pH	6.16 ± 0.34[a]	6.44 ± 0.43[a]	5.41
Aw	0.978 ± 0.01[a]	0.979 ± 0.002[a]	0.35
WTS (%)	7.33 ± 0.19[a]	7.38 ± 0.16[a]	1.79

used as a coagulant for cheese formulation without loss of components due to probability excessive hydrolysis.

Another indicator of excessive hydrolysis can be reflected in the total solids on the whey [31]. In this study, the values found for total solids in the whey did not show differences between treatments.

These results express the conformation of a matrix with a greater capacity for retaining solids reflected in the coagulated treatment with RC. In contrast, when the bovine rennet made up mainly of pepsin used for cheese production, it can present a high proteolytic activity that can be reflected in a low yield of the product [31]. Alihanoğlu [16] found higher fat content in fresh cheeses (White Cheese) coagulated with gastric extract of young rabbits with a few weeks of age in comparison with coagulated treatments with renin bovine and camel renin.

During gel formation, fat, as well as other milk compounds, remain immersed in the protein structure derived from enzymatic hydrolysis on the casein mycelium, therefore, the retention of fat, minerals, water, and other elements may vary depending on the type of clot formed and is reflected in product characteristics and manufacturing performance [6]. Garg & Johri [32] consider that pepsin has some technological limitations to be used as a coagulant in cheese making, highlighting an excessive loss of fat during processing. The salt content was shown to be higher in the CC ($p < 0.05$), a response that could be directly associated with the moisture content, which also showed higher levels for the same treatment.

Cheese structure is highly dependent to casein and the way that it is affected by enzymatic action [6], and the effects of hydrolysis can be observed through several aspects such as proximate composition. The protein content was similar in both treatments ($p > 0.05$), varying between 25.31% and 24.35% for cheese coagulated with RC and CC, respectively. Moatsou [33] tested artisanal rennet

from goats and lambs abomasum, finding no differences compared between commercial rennet on the principal compounds as total solids, ash, sodium chloride (NaCl), fat, and total protein. [24] Alves *et al.* (2013) compared the coagulant capacity of enzymes produced from the *Termomucor indicae* fungus with a commercial coagulant, the latter is similar to that used as a control in the present study. These authors determined that the hydrolytic action of the enzymes produced by the fungus was alike to that of commercial chymosin.

The yield of cheese expressed as the final weight of the product in relation to the volume of milk used for their preparation can be considered an indicator to evaluate the effectiveness of the coagulant in the hydrolysis of the protein [34]. Excessive proteolytic activity of the enzyme on the casein matrix can result in low yield, which can be observed in the increase of the solids content during whey draining [1]. In our finding was clear that the action of RC was like the commercial coagulant CC, with no differences in total yield for both treatments ($p > 0.05$). For both treatments no differences were detected in total solids of whey and water activity (Aw), reinforcing the idea of similar action of RC and CC.

3.5.3. Texture of Cheeses

An effect of enzymatic activity on the casein can be seen in the physical structure of the cheeses, which can be assessed through the texture profile. **Table 3** shows the results of the evaluation of the texture profile in the RC and CC cheeses. When analyzing hardness, differences between the samples were evidenced, being higher ($p < 0.05$) in the CC with values of 34.37 ± 4.37 N, a result that shows the composition of a more rigid structure compared to RC. This result, probably, could be associated with a higher intensity of proteolysis on the casein matrix and fat retention in the treatment with RC.

The gummy and chewable parameters were also lower ($p < 0.05$) on the RC cheese. These parameters reflect that this treatment has a less compact and less firm structure, and less effort is required to generate the deformation that is necessary for chewing. The result also confirms that the impact of the hydrolysis product of coagulation with RC is relatively greater on casein when compared to CC, giving a more fragile structure in cheese.

Alihanoğlu *et al.* [16] compared cheeses coagulated with bovine chymosin, camel's chymosin, and gastric extract of young rabbits showing a texture profile where parameters such as hardness, gumminess, and chewability were superior in treatments when coagulated with RC and camel chymosin and lower in cheeses which were coagulated with bovine chymosin. Although these authors did not specify the age of the rabbits, it is likely that there is still residual enzyme chymosin, acting in a similar way to bovine chymosin.

3.5.4. Evolution of pH during Cheese Ripening

The pH of the cheeses was monitoring during for 60 days of storage, showed changes due to the ripening process (**Figure 5**). It is important to highlight the need to adjust the pH of the milk to 6.5 in the initial preparation stage, since the

Table 3. Texture profile results of cheese coagulated with rabbit gastric extract and commercial chymosin.

Item	RC	CC	CV
Hardness (N)	34.37 ± 4.37[b]	43.35 ± 6.47[a]	14.22
Cohesivity (−)	0.69 ± 0.03[a]	0.69 ± 0.04[a]	3.27
Gumminess (N)	23.88 ± 3.58[b]	30.33 ± 4.82[a]	16.06
Chewiness (J)	25.33 ± 3.79[b]	32.19 ± 5.61[a]	15.14

RC cheese coagulated with rabbits gastric extract; TC Cheese coagulated with commercial chymosin; CV coefficient of variation. Different lower case letters indicate significant differences by the Tukey test (5%).

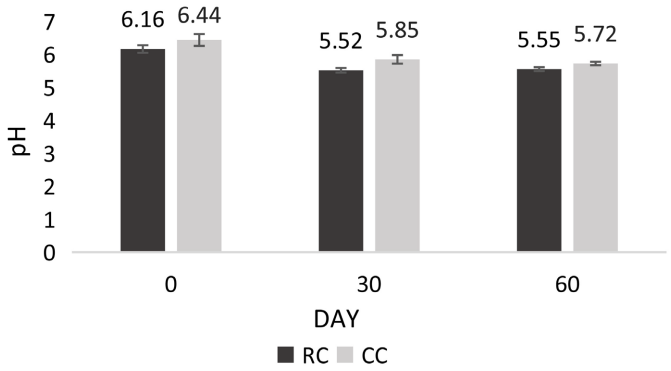

Figure 5. Evolution of pH during the ripening time of cheeses coagulated by rabbit gastric stomach and commercial chymosin.

milk had a pH of 6.9, and almost zero coagulant activity of the RC at pH above 6.9 was demonstrated. After two days of storage, the pH decreased for both RC and CC varied between 6.1 and 6.4, respectively, showing slight differences ($p < 0.05$). This comportment is probably associated with the residual of coagulant or the action of added starter cultures, which act on milk sugar, transforming it into lactic acid [14].

On day 30, there was a decrease in pH compared to the first period, thus showing differences between treatments as well. At the end of day 60, the pH remained the same in both treatments, RC and CC, maintaining differences between both treatments. The stability of the pH value can be explained by the decrease in the transformation of lactose into lactic acid.

In a study carried out by Alihanoglu [16] with the application of a gastric extract of young rabbits in the formulation of fresh cheese (White cheese), there was a more intense pH decrease, obtaining a cheese with a pH of 4.57 at 60 days of ripening in the treatment elaborated from RC. Alves [24] obtained pH values of 5.3 in the first 5 days after the manufacture of Prato cheese when they evaluated the coagulant capacity of the extract produced by fermentation of the fungus *Thermomucor indicae*. The pH values on the 60th day of ripening were within the values found by Batista *et al.* [35] in the evaluation of commercial-branded Prato cheeses.

During the ripening of cheese occur degradation process on protein, lipids, and carbohydrates, the main components of curd, because of starter cultures added, as well as residues of the coagulant used. The product results in the generation of other substances such as medium-chain peptides and free amino acids, as well as such as short-chain fatty acids and phospholipids, and lactic acid, derived from the biochemical processes known as proteolysis, lipolysis, and glycolysis, respectively [36].

3.5.5. Proteolysis Ripening Extension Index (REI) and Ripening Depth Index (RDI)

The ripening extension index (REI) is represented by SN-pH 4.6 fraction and indicates the percentage of soluble nitrogen when the sample is precipitated at pH 4.6. During the early stages of ripening, much of the released nitrogen comes from digestion by the action of the residual coagulant [36]. In this study, the REI was similar between treatments and times of collect the samples, the percentage of soluble nitrogen at pH 4.6 for the first 30 days was between 17.8% and 18.4% for cheese coagulated with RC and CC, respectively (**Figure 6(a)**). The RDI represents the percentage of nitrogen soluble in trichloroacetic acid (TCA), also related to total nitrogen, and it refers to the nitrogen released by the action of lactic acid bacteria. Likewise, no differences ($p > 0.05$) were found in both samples for the REI and RDI (**Figure 6(b)**).

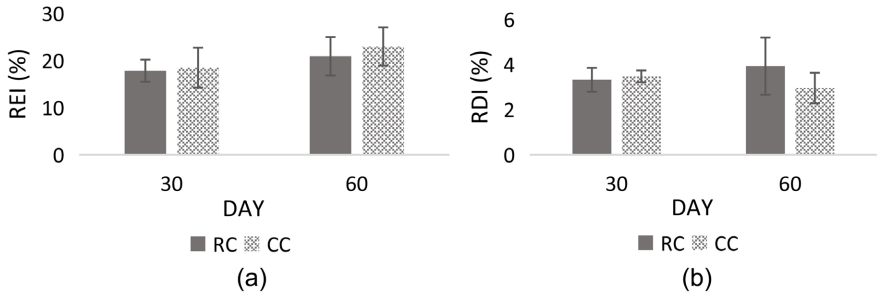

Figure 6. Proteolysis index of Type Prato cheese with ripening during 60 days. REI ripening extension index; RDI ripening depth index; RC rabbit gastric extract; CC commercial chymosin.

4. Conclusions

Salting in saturated solution is an efficient way for the enzyme extraction in the liquid coagulant preparation from adult rabbit abomasum. In this process, the pH can affect the enzyme activation and the more adequate is at 4.3. In relation to the use of RC as coagulant for the manufacture of cheese, the pH of milk together with the temperature affects the performance of the RC seen in the water retention capacity of the gel, showing a better performance between pH 5.9 and 6.5 and 27˚C and 36˚C, respectively. Testing the hydrolysis capacity, RC shows early reaction and intense degradation on the casein compared with CC, and this is not reflected in the composition, and ripening of Prato cheese type, affecting only texture characteristics like hardness, which is less in cheese made with RC.

The yield of cheeses coagulated with RC can be compared with the coagulant based on chymosin produced by fermentation of the fungus *Aspergillus niger* ssp. Awamori.

The early and more intense activity showed by RC in SDS PAGE analysis can be reflected in the texture of the cheeses, especially in parameters such as hardness, gumminess, and chewability. Adult rabbit stomachs are a promising alternative for rennet preparation for semi-ripening cheese preparations, guaranteeing comparable yield with commercial coagulants.

Conflicts of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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References

[1] Ben Amira, A., Makhlouf, I., Flaviu Petrut, R., Francis, F., Bauwens, J., Attia, H., Besbes, S. and Blecker, C. (2017) Effect of Extraction pH on Techno-Functional Properties of Crude Extracts from Wild Cardoon (*Cynara cardunculus* L.) Flowers. *Food Chemistry*, **225**, 258-266. https://doi.org/10.1016/j.foodchem.2017.01.040

[2] Kumar, A., Grover, S., Sharma, J. and Batish, V.K. (2010) Chymosin and Other Milk Coagulants: Sources and Biotechnological Interventions. *Critical Reviews in Biotechnology*, **30**, 243-258. https://doi.org/10.3109/07388551.2010.483459

[3] Ferrandini, E., Castillo, M., de Renobales, M., Virto, M.D., Garrido, M.D., Rovira, S. and López, M.B. (2012) Influence of Lamb Rennet Paste on the Lipolytic and Sensory Profile of Murcia al Vino Cheese. *Journal of Dairy Science*, **95**, 2788-2796. https://doi.org/10.3168/jds.2011-4733

[4] Soodam, K., Ong, L., Powell, I.B., Kentish, S.E. and Gras, S.L. (2015) Effect of Rennet on the Composition, Proteolysis and Microstructure of Reduced-Fat Cheddar Cheese during Ripening. *Dairy Science and Technology*, **95**, 665-686. https://doi.org/10.1007/s13594-015-0250-5

[5] McSweeney, P.L.H. (2011) Cheese: Biochemistry of Cheese Ripening. In: Wouters, J.T.M., Eds., *Encyclopedia of Dairy Sciences*: *Second Edition*, Academic Press, Cambridge, 667-674. https://doi.org/10.1016/B978-0-12-374407-4.00080-7

[6] Banks, J.M. and Horne, D.S. (2004) CHEESES: Chemistry of Gel Formation. In: Caballero, B., Finglas, P. and Toldra, F., Eds., *Encyclopedia of Food Sciences and Nutrition* (*Second Edition*), Academic Press, Cambridge, 1056-1062. https://doi.org/10.1016/B0-12-227055-X/00195-4

[7] Slamani, R., Redouane Labadi, R., Errahmani, M.B. and Bellal, M.M. (2018) Purification and Characterisation of Milk-Clotting and Caseinolytic Activities of Pepsin Isolated from Adult Sheep Abomasa. *International Journal of Dairy Technology*, **71**, 764-770. https://doi.org/10.1111/1471-0307.12492

[8] García-Gómez, B., Vázquez-Odériz, L., Muñoz-Ferreiro, N., Romero-Rodríguez, Á. and Vázquez, M. (2020) Rennet Type and Microbial Transglutaminase in Cheese: Effect on Sensory Properties. *European Food Research and Technology*, **246**, 513-526. https://doi.org/10.1007/s00217-019-03418-6

[9] Lombardi, J., Ciocia, F., Uniacke-Lowe, T., Boeris, V., Risso, P. and McSweeney, P.L.H. (2019) Application of an Enzymatic Extract from *Aspergillus niger* as Coagulant for Cheddar Cheese Manufacture. *Brazilian Archives of Biology and Technology*, **62**, e19180128. https://doi.org/10.1590/1678-4324-2019180128

[10] Moschopoulou, E. (2011) Characteristics of Rennet and Other Enzymes from Small Ruminants Used in Cheese Production. *Small Ruminant Research*, **101**, 188-195. https://doi.org/10.1016/j.smallrumres.2011.09.039

[11] Moschopoulou, E.E., Kandarakis, I.G., Alichanidis, E. and Anifantakis, E.M. (2006) Purification and Characterization of Chymosin and Pepsin from Kid. *Journal of Dairy Research*, **73**, 49-57. https://doi.org/10.1017/S0022029905001470

[12] Mohanty, A.K., Mukhopadhyay, U.K., Kaushik, J.K., Grover, S. and Batish, V.K. (2003) Isolation, Purification and Characterization of Chymosin from Riverine Buffalo (*Bubalos bubalis*). *Journal of Dairy Research*, **70**, 37-43. https://doi.org/10.1017/S0022029902005927

[13] Addis, M., Piredda, G. and Pirisi, A. (2008) The Use of Lamb Rennet Paste in Traditional Sheep Milk Cheese Production. *Small Ruminant Research*, **79**, 2-10. https://doi.org/10.1016/j.smallrumres.2008.07.002

[14] Law, B.A. and Tamine, A.Y. (2010) Technology of Cheesemaking. 2nd, Wiley-Blackwell, Hoboken. https://doi.org/10.1002/9781444323740

[15] Dobler-López, J., Espinosa-Ayala, E., Hernández-García, P.A., López-Martínez, L.X. and Márquez-Molina, O. (2016) Extracto Coagulante De Leche Proveniente Del Estómago De Conejo (*Oryctolagus cuniculus* sp.). *Agrociencia*, **50**, 583-593.

[16] Alihanoğlu, S., Ektiren, D., Akbulut, Ç., Hasan, Ç., Karaaslan, A. and Karaaslan, M. (2018) Effect of *Oryctolagus cuniculus* (Rabbit) Rennet on the Texture, Rheology, and Sensory Properties of White Cheese. *Food Science & Nutrition*, **6**, 1100-1108. https://doi.org/10.1002/fsn3.649

[17] Lambert, J.C. (1988) Village Milk Processing. FAO Animal Production and Health, 69. https://www.fao.org/3/T0045E/T0045E00.htm

[18] Ahmed, A.B.F., Babiker, E.E., Mori, N. and Ahmed, I.A.M. (2011) Hydrolysis of Ovine and Caprine Caseins by Enzymatic Extract from *Solanum dubium* Seeds. *Australian Journal of Basic and Applied Sciences*, **5**, 331-336.

[19] Sams, L., Amara, S., Mansuelle, P., Puppo, R., Lebrun, R., Paume, J., Giallo, J. and Carrière, F. (2018) Characterization of Pepsin from Rabbit Gastric Extract, Its Action on β-Casein and the Effects of Lipids on Proteolysis. *Food and Function*, **9**, 5975-5988. https://doi.org/10.1039/C8FO01450G

[20] Ben Amira, A., Mokni, A., Yaich, H., Chaabouni, M., Besbes, S., Blecker, C. and Attia, H. (2017) Technological Properties of Milk Gels Produced by Chymosin, and Wild Cardoon Rennet Optimized by Response Surface Methodology. *Food Chemistry*, **237**, 150-158. https://doi.org/10.1016/j.foodchem.2017.05.105

[21] Baptista, P.D., Silva Araújo, F.D., Nogueira, M. and Gigante, M.L. (2017) Reduction of 25% Salt in Prato Cheese Does Not Affect Proteolysis and Sensory Acceptance. *International Dairy Journal*, **75**, 101-110. https://doi.org/10.1016/j.idairyj.2017.08.001

[22] AOAC (2007) Official Methods of Analysis of AOAC International. 18th Editon, Association of Official Analytical Chemists, Washington DC.

[23] Instituto Adolfo Lutz (2008) Métodos físico-químicos para análise de alimentos. São Paulo.

[24] Alves, L.S., Merheb-Dini, C., Gomes, E., da Silva, R. and Gigante, M.L. (2013) Yield, Changes in Proteolysis, and Sensory Quality of Prato Cheese Produced with Different Coagulants. *Journal of Dairy Science*, **96**, 7490-7499. https://doi.org/10.3168/jds.2013-7119

[25] Gorostiza, A., Cichoscki, A.J., Valduga, A.T., Valduga, E., Bernardo, A. and Fresno, J.M. (2004) Changes in Soluble Nitrogenous Compounds, Caseins and Free Amino Acids during Ripening of Artisanal Prato Cheese; a Brazilian Semi-Hard Cows' Variety. *Food Chemistry*, **85**, 407-414. https://doi.org/10.1016/j.foodchem.2003.07.018

[26] Hill, A.R. and Kethireddipalli, P. (2013) Dairy Products—Cheese and Yogurt. In: Michael Eskin, N.A. and Shahidi, F., Eds., *Biochemistry of Foods*, Academic Press, Cambridge, 319-362. https://doi.org/10.1016/B978-0-08-091809-9.00008-X

[27] Green, M. (1972) Assessment of Swine, Bovine and Chicken Pepsins as Rennet Substitutes for Cheddar Cheese-Making. *Journal of Dairy Research*, **39**, 261-273. https://doi.org/10.1017/S0022029900014096

[28] Dalgleish, D.G. (1993) The Enzymatic Coagulation of Milk. In: Fox, P.F., Eds., *Cheese*: *Chemistry*, *Physics and Microbiology*, Springer, Boston. https://doi.org/10.1007/978-1-4615-2650-6_3

[29] Brasil, Ministerio de Agricultura, Pecuaria e Abastecimento (2018) Instrução Normativa MAPA-76. https://www.defesa.agricultura.sp.gov.br/legislacoes/instrucao-normativa-mapa-76-de-26-11-2018,1213.html

[30] Bauman, D.E. and Griinari, J.M. (2003) Nutritional Regulation of Milk Fat Synthesis. *Annual Review of Nutrition*, **23**, 203-227. https://doi.org/10.1146/annurev.nutr.23.011702.073408

[31] Andrén, A. (2011) Cheese: Rennets and Coagulants. In: Fuquay, J.W., McSweeney, P.L.H. and Fox, P.F., Eds., *Encyclopedia of Dairy Sciences*: *Second Edition*, Academic Press, Cambridge, 574-578. https://doi.org/10.1016/B978-0-12-374407-4.00069-8

[32] Garg, S. and Johri, B. (1994) Rennet: Current Trends and Future Research. *Food Reviews International*, **10**, 313-355. https://doi.org/10.1080/87559129409541005

[33] Moatsou, G., Moschopoulou, E., Georgala, A., Zoidou, E., Kandarakis, I., Kaminarides, S. and Anifantakis, E. (2004) Effect of Artisanal Liquid Rennet from Kids and Lambs' Abomasa on the Characteristics of Feta Cheese. *Food Chemistry*, **88**, 517-525. https://doi.org/10.1016/j.foodchem.2004.01.066

[34] Nassar, K.S., Lu, J., Pang, X., Ragab, E.S., Yue, Y., Zhang, S. and Lv, J. (2020) Rheological and Microstructural Properties of Rennet Gel Made from Caprine Milk Treated by HP. *Journal of Food Engineering*, **267**, Article ID: 109710. https://doi.org/10.1016/j.jfoodeng.2019.109710

[35] Baptista, D.P., da Silva Araújo, F.D., Eberlin, M.N. and Gigante, M.L. (2017) A Survey of the Peptide Profile in Prato Cheese as Measured by MALDI-MS and Capillary Electrophoresis. *Journal of Food Science*, **8**, 386-393. https://doi.org/10.1111/1750-3841.13618

[36] Mcsweeney, P.L.H. and Fox, P.F. (1997) Chemical Methods for the Characterization of Proteolysis in Cheese during Ripening. *Le Lait*, **77**, 41-76. https://doi.org/10.1051/lait:199713