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Study of the effects of β-casein polymorphism (A2 vs A1) on acid coagulation properties of milk

Official Master's Degree in Quality of Foods of Animal Origin: ¨Work presented for the overcoming of the 15 credits of the Master's Final Project Module of the Official Master's Degree in Quality of Foods of Animal Origin

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Master degree

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I declare to be the author of this Master's Thesis that is presented to obtain the Master's degree in Food Quality of Animal Origin at the Universitat Autònoma de Barcelona, Spain. This work has not been submitted before to obtain any degree or exam at any other university.

Barcelona, 09-01-2021.

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INFORM

That the work entitled: "Study of the effects of β-casein polymorphism (A2 vs A1) on acid coagulation properties of milk" has been carried out under our supervision or tutelage by Renata Delgado Teixeira within the Master Final Project module of the Official Master's Degree in Food Quality of Animal Origin at Universitat Autònoma de Barcelona.

Barcelona, 09-01-2021.

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Abstract

This study investigated the effect of β-casein (CN) genetic polymorphisms (A2A2 versus control milk (A2A1/A1A1/A2A2) on acid coagulation and acidification properties of milk. There are studies regarding the consumers opinion of milk containing β-CN A1 and A2 variants, as well about the enzymatic coagulation. However, little is known about the effect of these genetic variants in acid coagulation of milk. For that reason, the objective of the present work was to study the acid coagulation and acidification properties of milk A2A2 in comparison of control milk. Acid coagulation and acidfication characteristics of milks were evaluated by Optigraph® and Cinac® devices, respectively, and the water-holding capacity of milk gels were also analysed. Some variables were highly influenced by the farm factor, showing the importance of the effect of other intrinsic parameters that may influence the results.
Milk containing the β-CN A2A2 genotype presented higher gel density index in comparison to that from the control milk. Clotting time and aggregation rate, despite not showing statistically significant differences, were higher in β-CN A2A2 milk than control one. Latency time also were higher in β-CN A2A2 milk, coinciding with a longer time to reach pH 4.6 and lower acidification rate, although these last two parameters were not statistically significant. Both milks presented the same water-holding capacity. Summarizing, β-CN genotype (A1 vs A2) slightly affected some parameters of acid coagulation of milk, being possible to elaborate dairy products with both type of milks.

Keywords: β-CN poymorphism; A2A2 milk; acid coagulation and fermentation characteristics.

Resumen

Este estudio investigó el efecto del polimorfismo genético de la β-caseína (CN), A2A2 frente a una leche control (A2A1/A1A1/A2A2) sobre las propiedades de coagulación ácida y de acidificación de la leche. Existen estudios sobre la opinión de los consumidores sobre la leche que contiene variantes de A1 y A2 de la β-CN, y su efecto sobre la coagulación enzimática. Sin embargo, se conoce poco el efecto de estas variantes genéticas sobre la coagulación ácida de la leche. Por esa razón, el objetivo del presente trabajo fue estudiar las propiedades de coagulación ácida y acidificación de la leche A2A2 en comparación con la leche control. Las características de coagulación ácida y acidificación se evaluaron mediante los equipos Optigraph® y Cinac®, respectivamente, analizándose también la capacidad de retención de agua de los geles ácidos lácteos. Algunas variables fueron altamente influenciadas por el factor granja, mostrando la importancia del efecto de otros parámetros intrínsecos que pueden influir en los resultados.
La leche con el genotipo de β-CN A2A2 presentó mayor índice de densidad de gel en comparación a aquel procedente de la leche de control. El tiempo de coagulación y la velocidad de agregación, aunque no mostraron diferencias estadísticamente significativas, fueron mayores en la leche con la variante β-CN A2A2 que en la leche control. El tiempo de latencia también fue mayor en la leche con la variante β-CN A2A2, coincidiendo con un mayor tiempo hasta alcanzar pH 4,6 y menor tasa de acidificación, aunque estos dos últimos parámetros no fueron estadísticamente significativos. Ambas leches presentaron geles con similar capacidad de retención de agua. En resumen, el genotipo β-CN (A1 frente a A2) afectó levemente algunos parámetros de la coagulación ácida y acidificación de la leche, siendo posible elaborar productos lácteos con ambos tipos de leche.

Palabras clave: polimorfismo de la β-CN; leche A2A2; características de coagulación ácida y acidificación.

Introduction

Milk provides an important source of nutrition for humans, due to its composition that contains vitamins, minerals, proteins, and carbohydrates (Haug et al., 2007; FAO, 2013). It is known, that exists diferences in milk composition between breeds and animal individuality due to genetic variation and that, detailed protein composition is important for the production of dairy products (Heck et al., 2009; Yang et al., 2013). Cow milk proteins are constituted by caseins (CN) and whey proteins. Caseins, which comprises approximately 80% of protein component (Dalgleish, 2011), are divided in α_{S1}-, α_{S2}-, β-, κ-CN (Formaggioni et al., 1999; Ginger & Gringor 1999), where β-CN comprises approximately 30% of total milk protein (Pal et al., 2015). In bovine, β-CN diversed mutations happened leading 12 different genetic variants: A1, A2, A3, B, C, D, E, F, G, H1, H2 and I (Farrell et al., 2004), being the A1 and A2 the most common ones (Truswell, 2005). The difference between the two types differs in their protein structure owing to a subtitution of the amino acid in the position 67 with histidine in A1 and proline in A2 milk (Caroli et al., 2009).

There are studies that indicates that, the presence of A1 variant can be found with more frequency in *Bos taurus* cattle breeds such as Friesian, Ayrshire, British Shorthorn, and Holstein (except in few taurine breeds like Jersey, Guernsey, Charolais, Limousin and others) (Ng-Kwi-Hang & Grosclaude, 1992; Truswell, 2005; Kaminski et al., 2007; Banerjee, 2018) while in *Bos indicus* breeds the incidence of A2 allele is predominant in Zebu cattle of Indian (Mishra et al., 2009). Moreover, the A1 variant of β-CN is a result of a mutation via natural selection that has been reported in bovine milk specie and not in human, as interpreted by Steinerova et al. (2004) by the results of Dev et al. (1994) and cited by EFSA (2009).

Critical changes are verified in the secondary conformation of the expressed A1 β-CN by releasing a bioactive peptide called beta-casomorphin-7 (BCM-7) in the process of gastrointestinal human proteolysis of A1 β-CN. In the meanwhile, the presence of proline in A2 protein in position 67 prevents the polypeptide sequence from breaking at this critical site (Elliot et al., 1999). A1 β-CN digestion by digestive enzymes develops BCM-7 and on the other hand A2 β-CN digestion results in slight development and less release of BCM-7 (Banerjee, 2018).

In some research studies that can be found in literature, it was identified a relationship between consumption and a health risk factor linked to the realease of BCM7 mentioned before found specially in A1 variant, that can potentially affect opioid receptors in the nervous, endocrine and imune system (Kaminski et al., 2007; Shodi et al., 2012). Moreover,

studies suggest that the specific effects of BCM-7 on human cognition are still unknown. Nevertheless, studies performed by Kost et al. (2009) revealed that, infants presenting delays in psychomotor development after consuming formula containing cow's milk presented elevated basal BCM-7. Futhermore, Sheng et al. (2019) investigated some effects of milk consumption containing A1 and A2 β-CN (conventional milk) or only A2 β-CN in children and some of their findings were that A2 β-CN only has a positive effect on gastrointestinal system, but also on cognition in preschoolers.

He et al. (2017) was prompted the hypothesis that the acute symptoms of milk intolerance (including selfreported lactose intolerance) in some people might be related to the presence of A1 β-CN in cow's milk and that, eliminating A1 β-CN could avoid these symptoms. Theirs findings suggest the consequence of adverse gastrointestinal symptoms after milk intake containing lactose intolerance, might be related to the presence of A1 β-CN in milk rather than lactose itself and that, A2 β-CN, the symptoms were consistently reduced in both lactose absorbers and lactose malabsorbers, specially related to abdominal pain.

Some patients (8-20%) suffer from lactose intolerance, cow milk allergy (people who have allergies againts bovine milk proteins), other gastrointestinal problems and skin allergies derivated from milk consumption. The BCM-7 found in milk A1 causes the development of a gut-associated immune tolerance allergic effect by inhibiting human intestinal lymphocyte proliferation, whereas lactose intolerance is because of the inability of lactose digestion due to the absence of the digestive enzyme lactase (Caroli et al., 2009; Park et al., 2021).

A regularly reported gastrointestinal disorder in dary products is the intolerance and is routinely assigned to lactose intolerance (Jianqin et al., 2016). Although, some investigators realized that, based on the gastrointestinal effects of BCM-7 (milk containing A1 β-CN), it is probable that, the intolerance to dairy products reported, in some cases, may be related to the presence of A1 β-casein in milk instead of lactose itself (Jianqin et al., 2016; He et al., 2017).

Apart from people who suffer to some extent from milk consumption, it is known that worldwide, more than 6 billion people consume milk and dairy products. In addition to milk, a variety of dairy products are produced from fluid milk and consumed such as cheese, butter, cream, milk powder, fermented milk and others. The fermented milk is obtained by the action of suitable micro-organisms and resulting in reduction of pH with or without coagulation (FAO and WHO, 2010). In the process of acid coagulation of milk, casein micelle properties are modifyed by the reduction of milk pH. Milk and casein micelles are in a neutral pH and in this stage neutral casein micelles are dispersed. When acidification of milk occurs, colloidal calcium

phosphate present in casein micelles dissociate and the negative charges in casein micelles are neutralised, with aggregation occuring as the isoelectric point of the casein micelle approaches (pH 4.6), making the protein less soluble and stable, resulting in casein micelle destabilisation and precipitation (Lucey & Singh, 1998; Sinaga et al., 2017). The aggregation of micelles (clusters) becomes denser and to form a comparter structure until starts eventually to develop into a gel (Sinaga et al., 2017). The acid coagulation of milk is in the development for a widevariety of cultured dairy products.

The texture and physical properties of acid-induced gels are dependent on some specific conditions that includes the rate of acidification, protein content and whey protein denaturation (Lucey, 2016). For the acidification process, milk can be acidified by bacterial cultures, which ferment lactose to lactic acid, or other methods that promotes the decrease of pH. Whey separation refers to the appearance of liquid (whey) on the surface of a milk gel and is a common defect in fermented milk products this is related to instability of the gel network (Lucey, 2016).

The impact of milk protein genetic polymorphisms on milk composition and its coagulation properties, both enzymatic and acidic, is wide documented; however, there is a lack of knowledge about the influence of the β-CN and a comparison of theirs variant A1 and A2 on acid coagulation properties. There is an increase interest to get to know more about A1 and A2 milk, which is confirmed to the existence of companys in dairy industry who has the aim of to produce milk containing pure A2 but not A1 β-CN.

Regarding the milk protein genotypes on the milk coagulation properties, there are studies that has been demonstrated that, genetic polymorphism in β-CN genotypes did not influence the acid coagulation properties of the milk from the investigated Norwegian Red cattle (Ketto et al., 2017a) or Swedish Red and Swedish Holstein breeds (Hallén et al., 2009). However, Nguyen et al. (2018) evaluated the effects of milk A1 and A2 in the properties of acid gelation in yogur, and obtained that both milk variants required a similar fermentation time but a longer gelation time in milk A2 with a softer gel, which might enhance digestion. Futhermore, in a study performed by Sebastiane et al. (2020), found out that, the β-casein variant A2 increases the digestibility of milk, which is a desirable functional propertie in milk. Heck et al. (2009) also described an association with increased protein yield and β-CN A2 allele, which could be used in the production of dairy products.

1.1.Objectives

β-CN A1 and A2 variants recently been gaining increasing interest from both researchers and consumers, stimulating a selection to A2 in farm. However, there are few

information about the effect of this selection in milk technofunctional characteristics. For this reason, the objective of the present work was to study the acid coagulation properties of milk A2A2 in comparison of control milk (blend of A1A2, A1A1 and A2A2). To achieve the main objective, the following specific objectives were raised:

- To evaluate de coagulation properties (onset of coagulation, CT; aggregation rate, AR; gel density index, GD) by using Optigraph device of A2A2 and control milk.
- To evaluate de acidification properties (coagulation time, *Te*; mean acidification rate, *Vmar*; maximum acidification rate, *Vm*, time at the maximum acidification rate, *Tm*, latency time, *Ta*) by using Cinac device of A2A2 and control milk.
- To determine the water holding capacity of acid gels from A2A2 and control milk.

2. Materials and methods

2.1. Preparation of milk

This experiment used milk A2 (β-CN A2A2) and milk control (β-CN A2A1, A1A1 and A2A2) obtained from local farms named "La Cavalleria" located in Manlleu, "Can Barrina" which is located in Santa Cecília de Voltregà, and the farm called "Compte Isern'' which is located in Vic. Milk was obtainded from individual Frisona cows collected from April to July. The cows were selected based on β-CN genotype. Information of animals (days and number of lactation and distribution of casein genotypes) can be seen in Table 1 and 2.

Inmediately after milking, milk samples were transported to the laboratory. Once at the laboratory, the milk was tempered at 50 ºC to be skimmed and immediatelly cooled in cold water and stored in a cold chamber (4 ºC) before being subjected to analysis.

Before fermentation, milk was heated at 80 °C for 20 min in order to eliminate possible pathogens present in this milk and improve the visco-elastic properties of the product (commonly used in the industry to make dairy products). The heat treatment causes a denaturation of whey proteins, especially β-lactoglobulin (β-LG) that associates with κ-CN on the surface of casein micelles (Haque & Kinsella, 1988).

2.2.Composition of milk

Milk samples were analysed for total solids (ISO-IDF, 2010) and pH with a pHmeter (Crison Micro-pH 2001). The total nitrogen was analysed using the Kjeldahl method (FIL-IDF,

1993) and protein content was calculated by multiplying the total nitrogen content by 6.38. All the analysis were made in duplicate.

2.3.Preparation of starter culture

This experiment used the commercial lyophilized culture of *Lactobacillus delbruekii* subsp *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* (DANISCO FRANCE SAS, YO-MIX 300 LYO 10 DCU), in the proportion of 1:1. Skim milk was heated to a temperature around 44 °C and 0.2 g of the starter culture was added, mixed, and incubated at 43 °C until it reached a pH around 4.8.

2.4. Evaluation of coagulation parameters

2.4.1 Sample preparation for Optigraph and process

In brief, skim milk samples were pre-heated in a water bath until hits the temperature of 43 °C, and subsequently were acidified with 2% of the starter culture. The cuvettes were each filled with 10 mL of inoculated milk and monitored for 4 h at 43 °C ± 2 °C. Both of milk samples were analyzed in quadruplicate (4 cuvettes).

Acid coagulation was monitored by Optigraph® System (Ysebaert, Frépillon, France) that is based on a near-infrared optical device. The Optigraph calculates the coagulation parameters and from the coagulation curves three parameters were obtained: time for detecting the onset of coagulation (CT), which is indicated by the maximum of the firt derivate curve, aggregation rate (AR), calculated from the slope of the linear region of the curve and the gel density index (GD), calculated as the diferences between D1 and D0 (Fig. 1).

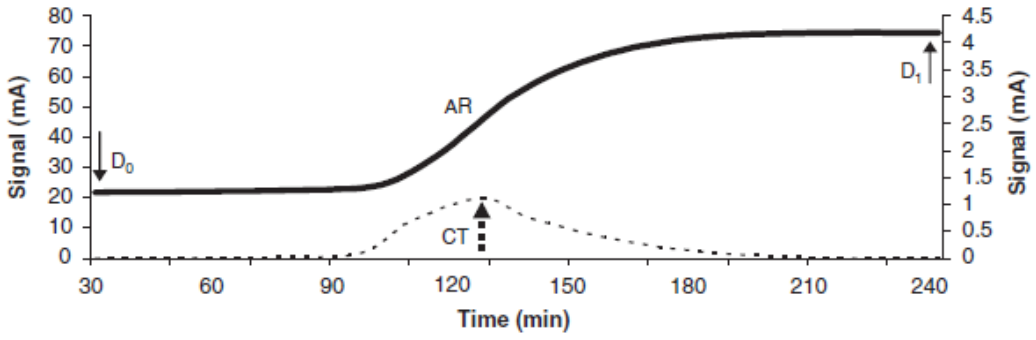

Figure 1. Coagulation curves from the Optigraph (black line) expressed as signal attenuation as a function of time (min). CT is the time at the maximum of first derivative value (dotted line). AR is the aggregation rate, which is the slope of the plot over the coagulation period. Gel density (GD) was calculated as D1–D0 (Serra et al., 2007).

2.4.2. Sample preparation for Cinac and process

Skim milk samples were fermented with 2% of latic culture (prepared the day before) in three replicates bottle flasks with 50 mL of volume and monitored until reaching the pH of 4.6. The acidification kinetics were monitored using a CINAC® System (Ysebaert, Frépillon, France) wich allows during milk fermentation to observe the acidifying activity of starter cultures lowering it the pH value. The duration of the analysis was determined by the time needed to reach pH 4.6. The parameters assessed from the acidification curves process were the time of coagulation (T_e) which is the time to reach a pH 4.6, the mean of acidification rate (V_{mar}), defined as the slope of the straight section of the curve, the maximum acidification rate (V_m), which corresponds to the inflection point of the pH curve versus time, the time required to reach V_m , being the time at which the maximum acidification rate was observed (T_m), and the latency time (T_a) which is the time necessary for a pH decrease of 0.08 units to be produced (Fig. 2).

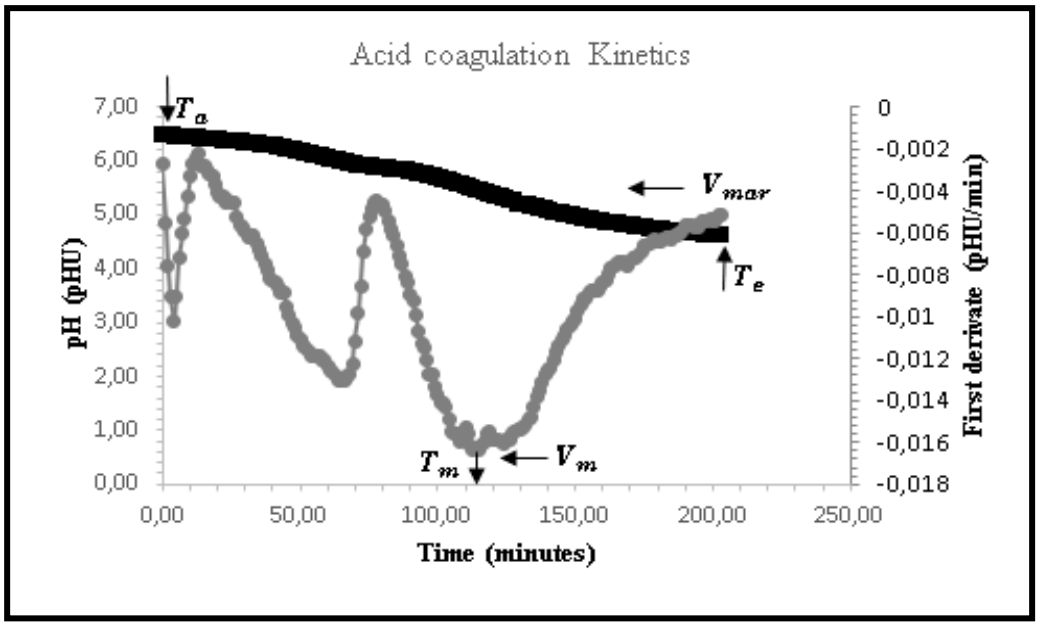

Figure 2. Acidification curves from Cinac (black line) expressed as a function of time. T_e: the time needed to reach a pH 4.6; V_{mar} is the mean of acidification rate which is the slope of the plot over the acidification period; V_m:maximum acidification rate: T_m: the time at which the maximum acidification rate was observed and T_a: the latency time which is the time necessary for a pH decrease of 0.08 units to be produced.

2.5. Water-holding capacity (WHC) of gels

Samples of 200 mL of each type of skim milk was heated to 45 °C and next inoculated with the starter culture at 2 %. Inoculated milk (40 g) was distributed in three centrifuge tubes for each sample and placed to incubate at 43 °C for 4 h. Subsequently, the tubes were placed in cold chamber at 4 °C for 24 hours. Before centrifugation, coagulated milks were warmed at room temperature an afterwards centrifuged at 5000 *g* for 20 min at 20 °C. Succeeding centrifugation, whey was weighed, and results were expressed as grams of expelled whey per gram of milk.

2.6. Statistical analysis

Data were processed by multifactor analysis of variance (ANOVA) using the randomized block desing of R Commander (*Rcmdr*, Fox & Bouchet-Valat, 2020) and agricolae (Mendiburu, 2015), considering both genotypes of β-CN (A2 and control), farm and their interaction. Tukey's range test was used for comparison the medias of genotypes, and evalutations were based on a significance level of $P \leq 0.05$. Data of milk composition were compared by Student's T-test for independent samples. The complete experiment was repeated on two independent occasions.

3. Results and discussion

3.1. Information of animals used to collect milk for the study

Table 1 shows different information of animals used in this study.

Table 1. Animals' information for each farm.

Farm[1]	Production	Milk[2]	Nºanimals	Days of lactation	Nº of lactation	Liters of milk
A	1	A2	16	116.38	1.69	8460
		C	16	119.19	1.69	8050
	2	A2	29	272.50	2.10	7250
		C	29	264.50	2.60	7250
B	1	A2	70	179.44	1.40	7000
		C	57	173	1.80	5700
	2	A2	66	145.45	1.83	8580
		C	62	176.19	1.67	8060
C	1	A2	28	140.75	1.00	7000
		C	31	159.90	1.07	7750
	2	A2	34	149.18	1.12	8500
		C	33	159.88	1.06	8250

[1]A: La Cavalleria, B: Can Barrina, C: Compte Isern; [2]A2: milk with A2A2 β-CN, C: control milk.

It can be observed that, the days of lactation, number of lactation and liters of milk collected for the two types of milk are similar, in order to not present differences that could affect milk´s composition and coagulation parameters for the study (Table 1).

Table 2 lists the genetic cow's information that was facilitated by FEFRIC (Federació Frisona de Catalunya).

Table 2. Genetic information of animals of each farm.

Farm[1]	P[3]	Milk[2]	β -CN			κ-CN						β-LG		
			A2A2	A1A2	A1A1	AA	AB	BB	BE	AE	EE	AA	AB	BB
A	1	A2	16			8	7	1				3	10	3
		C	3	11	2	2	5		7	1	1	4	9	3
	2	A2	29			9	13	6				5	16	8
		C		24	5	3	4	2	10	8	2	4	13	12
B	1	A2	70			20	33	17				36	25	9
		C		45	12	14	14	5	10	11	1	25	28	5
	2	A2	66			18	33	15				31	25	10
		C	6	47	9	16	17	7	7	12	1	27	30	6
C		A2	28			10	13	5				12	13	3
	1	C		30	1	4	11	7	7	2		10	16	5
	2	A2	34			10	18	6				11	19	4
		C		33	0	4	12	7	8	2		12	16	5

[1]A: La Cavalleria, B: Can Barrina, C: Compte Isern. [3]P = Production. [2]A2: milk with A2A2 β-CN, C: control milk.

Control milk was a mixture of milks from animals genotyped for β-CN A1A1, A2A2, and mostly A1A2 (Table 2). Expression of the three β-LG variants in milk not presented bigger differences between A2 and control milk. Moreover, the tendency for genotype found in β-LG is in order AB> AA > BB. As regards κ-CN, the allelle AA, AB and BB are expressed in a higher level then BE, AE and EE, being the majority the AB. In addition, it can be observed that for β-CN A2A2 the allele for κ-CN BE, AE and EE it can´t be found, being only found in control milk, agreeing with the investigation of Hallén et al. (2009). Overall, the most common genotypes of κ-CN and β-LG found in milks were AB.

3.2. Composition of milk

Milk samples had pH and protein values around 6.71 ± 0.05 and 3.13 ± 0.13, respectively, being the expected values for bovine milk (Gellrich et al., 2014; Alba, 2017). The results of total solids-non-fat were consistent to the findings of Alba (2017), with values around 9.12 ± 0.18 (Table 3). Cows from farm A produced milk with a slightly higher amount of protein than farm C. No statistical differences were observed for pH, total solids, or protein between A2 and C milks in any of the farms. These results are in agreement with those of Nguyen et al. (2018) who didn't find significant differences in the concentration of fat, protein and total solids between milk with two different β-CN phenotypes (A2A2 and A1A1).

Table 3. pH, total solids-not-fat, and protein of milks.

Farm[1]	Milk[2]	pH	Total solids (%)	Protein (%)
A	A2	6.71 ± 0.05	9.40 ± 0.02	3.33 ± 0.03 [a]
	C	6.73 ± 0.05	9.37 ± 0.07	3.29 ± 0.12 [a]
B	A2	6.74 ± 0.05	8.74 ± 0.03	3.15 ± 0.02 [ab]
	C	6.78 ± 0.05	9.02 ± 0.03	2.98 ± 0.16 [b]
C	A2	6.61 ± 0.16	9.13 ± 0.11	3.03 ± 0.08 [b]
	C	6.72 ± 0.04	9.08 ± 0.02	2.99 ± 0.20 [b]

[1]A: La Cavalleria, B: Can Barrina, C: Compte Isern; [2]A2: milk with A2A2 β-CN, C: control milk. [a]Mean for the same parameter followed by different letters are significantly ($P \leq 0.05$) different.

3.3. Acid coagulation properties of milks

Significance of farm, genotype and their interaction in coagulation milk variables was showed in Table 4. Not statistical diferences between the two types of milk were found in global results for CT parameter, although A2 milk presented higher values than control (Table 4, Fig. 3). These results agree with those found by Ketto et al. (2017a) who found that β-CN polymorphism did not affect the coagulation time of milks.

Table 4. Coagulation properties from Optigraph of A2 and control milks.

Milk[1]	T (°C)	CT (min)	AR (mA/min)	GD (mA)
A2	43 ± 0.02	108.83 ± 29.38	0.49 ± 0.04	26.66 ± 2.48[a]
C	43 ± 0.02	99.66 ± 22.88	0.48 ± 0.03	24.75 ± 3.37[b]
***SE*[2]**				
Farm			*	**
Genotype				*
Farm*Genotype			*	*

[1]A2: milk with A2A2 β-CN, C: control milk; [2]SE: statistical significance ***$P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$. [a]Mean value ± standard error (for T, mean value ± s.e.). [ab]Mean for the same parameter followed by different letters are significantly ($P \leq 0.05$) different. T = coagulation temperature, CT = clotting time, AR = agregation rate and GD = gel density.

In Figure 3 we can observe the average of the profiles of the light scattering ratio and its first derivative for the A2 and control milk. The coagulation time vas shorter in control than A2 milk, however, there were not differences in the agregation rate.

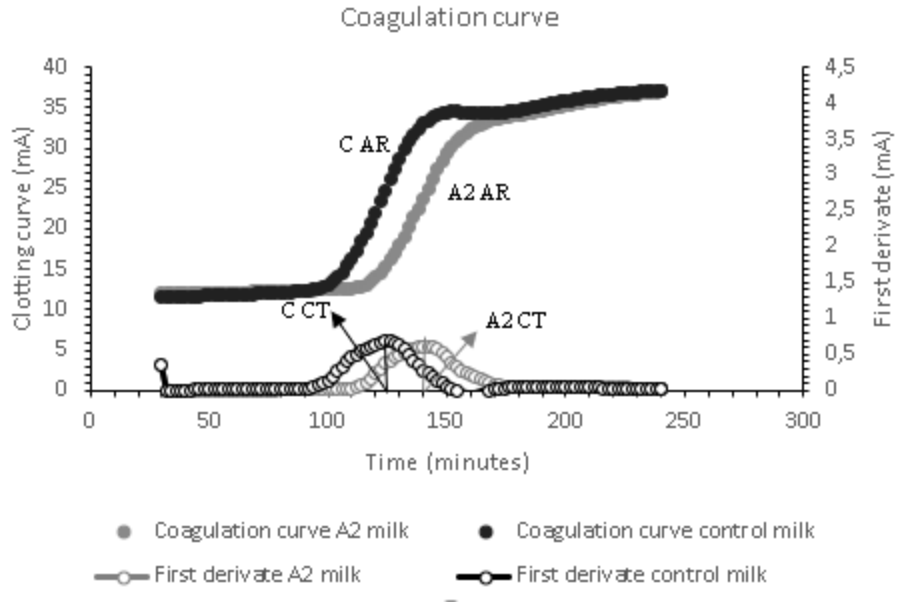

Figure 3. Coagulation parameters obtained from Optigraph of A2 (grey) and control milk (black). Coagulation curve (full dots) and first derivative (empty dots). A2: milk with A2A2 β-cn, C: control milk. AR: aggregation rate, CT: coagulation time.

When comparing the two types of milk for each independent farm, there was a statistical difference in farm C ($P \leq 0.05$), showing higher CT in A2 than control milk (Fig. 4). Although there were no statistically significant differences in farm A and B, it was also observed a tendency for A2 milk to show higher CT than control milk.

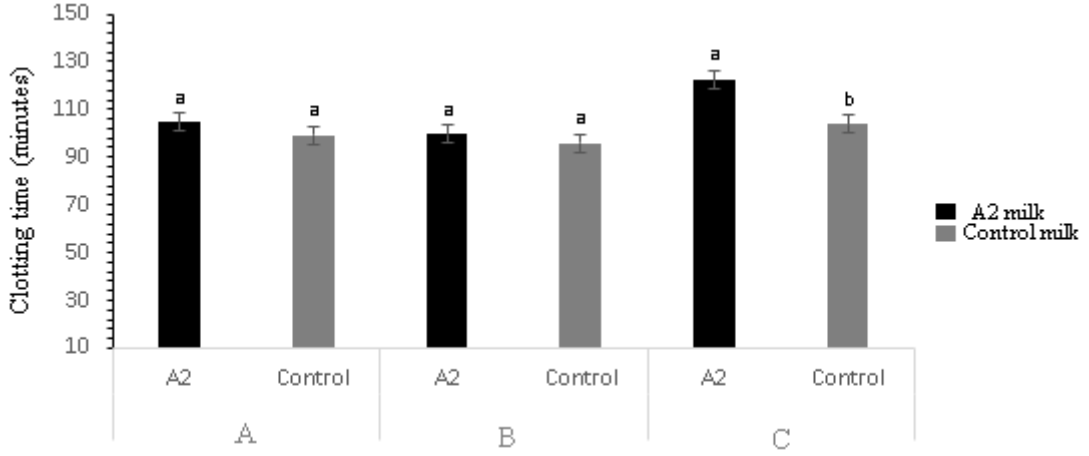

Figure 4. Mean of Clotting time (CT) of different farms obtained from Optigraph of A2 and control milk. [1]A: La Cavalleria, B: Can Barrina, C: Compte Isern. Values of the same farm with different superscripts letters were significantly different ($P \leq 0.05$).

A significant effect between farm and interaction between farm and genotype was observed for AR ($P \leq 0.05$) (Table 4), but not statistical differences were observed between A2 and control milk in global results (Table 4, Fig. 3).

However, comparing different milks separately by farms, it was observed a statistical difference between milks in farm B ($P \leq 0.05$), being higher in A2 compared to control milk. The same pattern was observed in farm A, but it was the opposite in farm C.

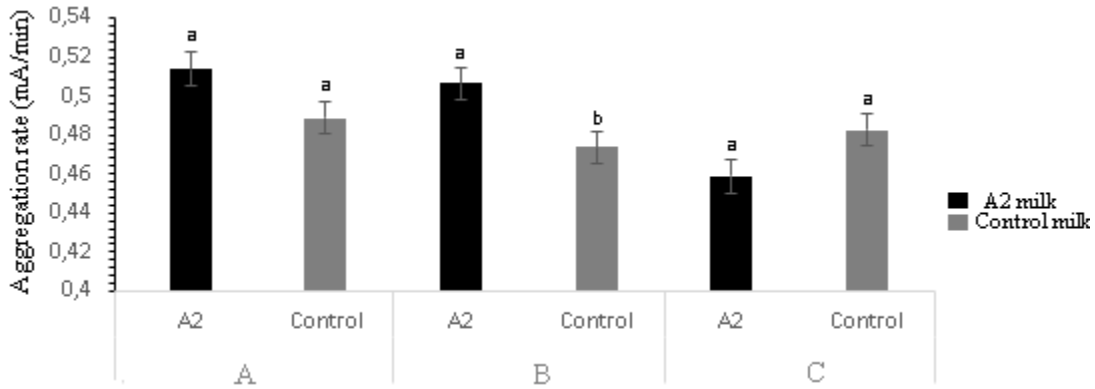

Figure 5. Aggregation rate (AR) of different farms obtained from Optigraph of A2 and control milk. [1]A: La Cavalleria, B: Can Barrina, C: Compte Isern. Values of the same column with different superscripts letters were significantly different ($P \leq 0.05$).

In the analysis of GD, farm, genotype, and their interaction were significant (Table 4). A2 milks presented higher GD than control milk. In the comparison of two milks by farms, this tendency was observed in farm A and B, being only significant in the last one (Fig. 6).

Our results of GD index, which is related to gel firmess, disagree with the study of Nguyen et al. (2018) who found that yoghurt elaborated with A2A2 milk presented a gel firmness lower than that elaborated by A1A1 milk, indicating that A2A2 milk induced and acid gel less dense than gel from control milk.

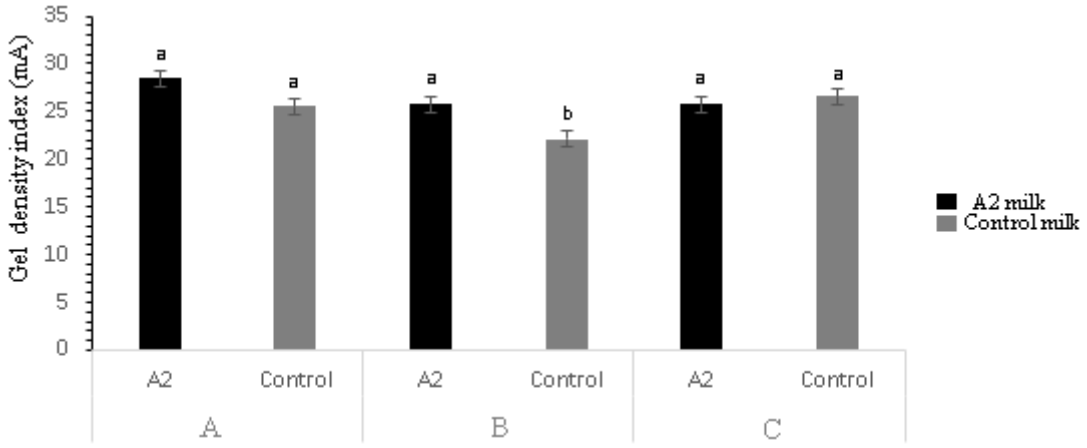

Figure 6. Gel density index (GD) of different farms obtained from Optigraph of A2 and control milk. [1]A: La Cavalleria, B: Can Barrina, C: Compte Isern. Values of the same farm with different superscripts letters were significantly different ($P \leq 0.05$).

The influence of casein genotypes on enzymatic coagulation of milk has been extensively studied. The A2 variant of β-CN has been reported to be associated with milk that has poor rennet coagulation properties (Jensen et al., 2012; Jensen, Holland, Poulsen, & Larsen, 2012; Poulsen et al., 2013). However, limited research has been reported on the effects of the A1 and A2 variants of β-CN on acid coagulation. In this sense, it is known that β-LG genotype is an influent parameter on acid coagulation process of milk (Allmere et al., 1997, 1998a, Hallén et al., 2008), being AA genotype associated with shorter coagulation time compared with AB and BB (Hallén et al., 2009).

On the other hand, β-LG BB were associated with higher curd firmness compared with AA (Bikker et al., 2000, Hallén et al., 2009), and the β-LG variant B presented higher aggregation rates towards κ-CN during heating of milk than variant A (Allmere et al., 1997, 1998b). Respect to κ-CN, not differences have been observed between κ-CN A and B variants to acid coagulation of milk (Allmere et al., 1998), and no significant effect of β-/κ-CN genotype on acid coagulation was observed (Hallén et al., 2009).

To our knowledge, only Nguyen et al. (2018) assessed the differences in acid milk gel containing A1A1 or A2A2 β-CN and described longer coagulation time in milk β-CN A2A2 than A1A1, with more porous microstructure and thinner protein strands in gel by β-CN A2A2, contrary to our results.

3.4. Acidification characteristis of milks

The analysis for acidic milk coagulation was performed the day after receiving the milk. Significance of farm, genotype and their interaction in coagulation parameters accessed from Cinac device are showed in Table 5. Not statistical diferences were found in global results for fermentation time (T_e), time to reach acidification rate (T_m), maximum of acidification speed (V_m) and mean of acidification rate (V_{mar}) between A2 and control milks ($P > 0.05$). However, A2 milk presented a higher latency time (T_a) than control milk. This parameter was very influencied by the genotype of milk (Table 5) and there were neither difference in the agregation rate.

Table 5. Acidification properties of A2 and control milks.

Milk[1]	V_m (pHu/min)	V_{mar} (pHU/min)	T_e (min)	T_m (min)	T_a (min)
A2	0.0192±0.0024 [a]	0.0186±0.0016[a]	191.11±15.80[a]	112.67± 8.55 [a]	23.78±5.61[a]
C	0.0200±0.0027 [a]	0.0188±0.0028[a]	184.88±21.89[a]	105.63±12.51[a]	12.35±5.83[b]
***SE*[2]**					
Farm			**		
Genotype					***
Farm*Genotype				*	

[1]A2: milk with A2A2 β-cn, C: control milk; [2]SE: statistical significance ***$P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$. [a]Mean for the same parameter followed by different letters are significantly ($P \leq 0.05$) different. V_m =maximum acidification speed; V_{mar}= mean of acidification rate; T_e= coagulation time at pH 4.6; T_m = time to reach V_m; T_a = latency time.

In Figure 7 can be observed that, comparing the A2 and control milks, there were not significant differences in the coagulation curve.

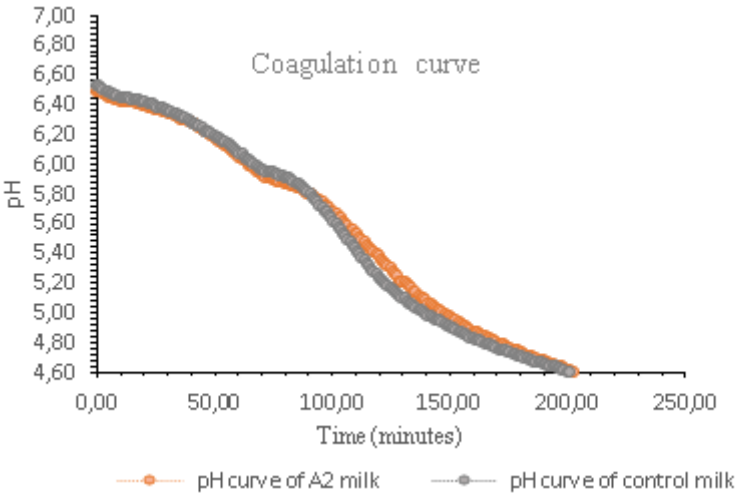

Figure 7. Coagulation parameters obtained from Cinac device of A2 (orange) and control milk (grey) until reach a 4.6 pH.

As we have mentioned previously, not statistical differences were found in V_{mar} , V_m and T_m , showing a medium value of 0.0196 pHU/min, 0.0187 pHU/min and 109.15 minutes, respectively. The results for V_m was consistet with Nguyen et al. (2018), which discovered no significant difference in the maximum of acidification rate (V_m) between the two types of milk ($P > 0.05$).

Fermentation time (T_e), defined as the time for the milk to reach a pH 4.6, was aproximately 188 min, without statistical diferences between A2 and control milks (Table 5). These values were similar that those obtained by Serra et al. (2007), but shorter that those of Nyugen et al. (2018) with a fermentation time ~300 minutes. Moreover, the latter descrived a longer gelation time in A2A2 in comparison to A1A1 milk.

Because farm was significant for this variable, values were also analyzed individually by farm (Fig. 8).

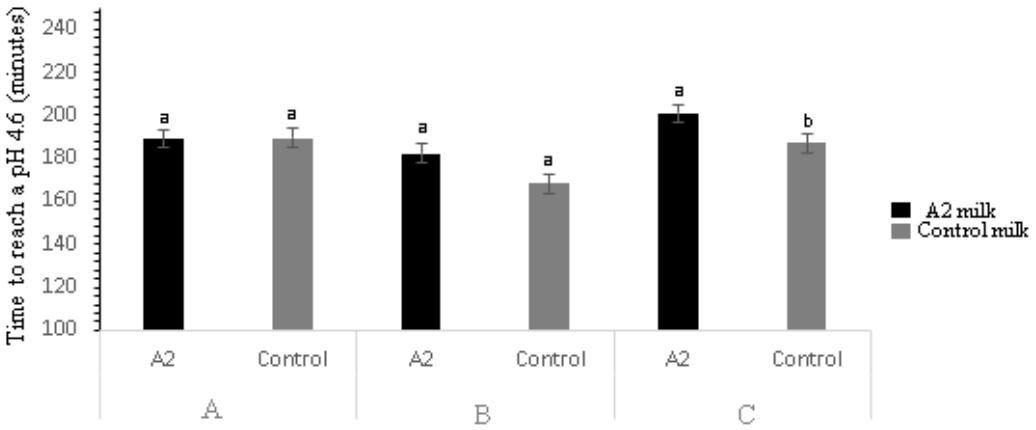

Figure 8. Mean of fermentation time (T_e) of different farms obtained from Cinac between A2 and control milk. [1]A: La Cavalleria, B: Can Barrina, C: Compte Isern. Values of the same column with different superscripts letters were significantly different ($P \leq 0.05$).

Fermentation time was statisticaly different in farm C, being higher in A2 than control milk (Fig. 8). This tendency also can we observed in farm B. Therefore, the acidification time follows the same pattern both in Cinac (Fig. 8) and Optigraph results (Fig. 4).

As shown in Table 5 and Figure 9, there was statistical difference for T_a between the two types of milk ($P \leq 0.05$), being clearly longer in A2 milk compared to control milk. This faster acidification rate at the beginning of fermentation in control milk, agrees with the higher V_m, V_{mar} and shorter T_e and T_m of control milk, although differences were not statisticaly significant (Table 5).

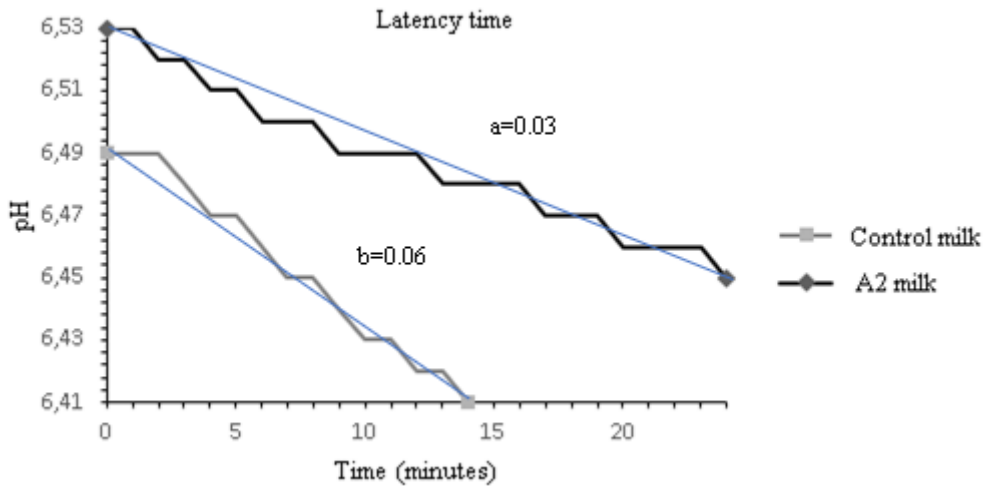

Figure 9. Mean of latency time (T_a) obtained from Cinac of A2 (black) and control milk (grey). a: slope of acidification rate of A2 milk; b: slope of acidification rate of control milk.

In Figure 9 can be observed that, comparing the A2 and control milk, the time needed to achieve the reduction of 0.08 pH units (T_a) was faster in control milk than A2 milk. The acidification speed was double in control compared to the A2 milk, being a slope of 0.06 and 0.03, respectively. As can be observed, initial pH was quite diferent of initial pH of milks (Table 3) due to the addition of starter culture. Moreover, the pH value of the two milks presented differences statisticaly significants ($P \leq 0.05$) being higher in A2 than control milk. This could affect the inicial acidification rate in milks.

3.5. Water-holding capacity of acid gels

The water-holding capacity (WHC) represented, as the quantity of whey expulsion after a forced centrifugation process, is an estimation of the water-retention capacity of the gel. No statiscal diferences were found in global results of WHC between milks (Table 6). Abeykoon et al. (2016) did not observe significant correlation with the values of gel syneresis and β-CN genotype (A1 and A2), which would be related to WHC, coinciding with our results.

Table 6. Results of water-holding capacity (WHC) of A2 and control milk.

Milk[1]	WHC (g of whey/100 g of milk)
A2	81.77 ± 1.15
C	82.46 ± 1.89

[1]A2: milk with A2A2 β-CN, C: control milk

In our study, WHC was clearly influenced by farm ($P \leq 0.001$), and for that reason, a comparison with A2 and control milk were made individualy by farms. There was a significant difference in farm C, observing higher WHC in control milk. This trend was also observed in farm B but it was the opposite in farm A (Fig. 10).

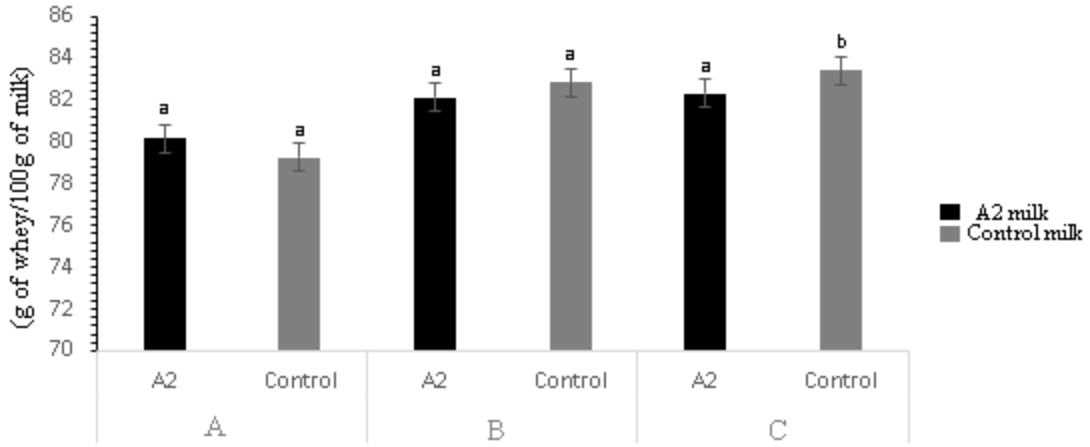

Figure 10. Water-holding capacity of acid-induced coagulation properties of A2 and control milk. [1]A: La Cavalleria, B: Can Barrina, C: Compte Isern. Values of the same farm with different superscripts letters were significantly different ($P \leq 0.05$).

Water-holding properties have been well recognized by food technologists among the diversity of functional properties attributed to milk protein products (Kneifel & Seiler, 1993). For acid coagulation products such as yoghurt, whey separation or syneresis is one of the major problems found because of the undesired texture and instability of processing and storage (Athar et al., 2000). The formation of a more stable gel network is related directly with the strongly ability to retains water (less syneresis) while a weaker gel (less firm) promotes a more whey separation and are more sensitive to syneresis (Lee & Lucey, 2003). In our study, the

higher gel density index found in A2 milk (Table 4) did not correspond with higher WHC (Table 6).

Knowledge related to the topic of WHC respect to β-CN polymorphism in milk is limited to date. Ketto et al. (2017b) studied the influence of milk casein genotypes on the degree of syneresis in order to use protein genomics for improving the WHC of cultured milk. However, only the genotypes of α_{s1}-CN, κ-CN and β-LG were studied. These authors found that the β-LG and κ-CN/β-LG composite genotypes significantly influenced the degree of syneresis, being lower in cultured milk with the AB genotype of β-LG compared to BB.

4. Conclusions

A great variability of results between farms was noticed, being this factor significant for the parameters AR, GD, T_e and WHC. This shows the importance of intrinsic factors (independent of the casein genotype) that can influence the results.

β-CN genotype slightly affected some parameters of acid coagulation of milk. In general, it seems to be that A2A2 milk requires more time to coagulate but produces a higher density gel. However, the variations found between both milks are very small, indicating the feasibility of β-CN A2A2 to elaborate acid coagulation dairy products.

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