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Lactococcus lactis subsp. *lactis* bv. diacetylactis Q5C6 strain as debittering adjunct culture for vegetable clotted cheese

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ABSTRACT

One of the main challenges associated to the use of vegetable coagulants in cheesemaking is represented by the bitter taste conferred to the final product. In this context, the use of aminopeptidases positive strains could lead the hydrolysis of bitter peptides. The present study aimed to setup an experimental cheese clotted by using a kiwifruit enzymatic extract and inoculated with the *Lactococcus lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain as debittering adjunct culture. The optimal amount of kiwifruit enzymatic extract, to be used in cheesemaking, was determined by performing laboratory-scale coagulation tests. Two experimental cheesemaking trials (A and B) were performed and the obtained cheeses were subjected to physico-chemical, microbiological, and sensory analysis. Results showed that, compared to cheeses clotted by animal rennet, the use of the kiwifruit enzymatic extract determined changes in fat, ash, and protein content. The L. *lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain, inoculated at 1%, was able to reduce the bitter taste obtaining a final product with a sensory profile comparable to cheese clotted by animal rennet.

1. Introduction

Milk-clotting enzymes have a key role in cheesemaking, allowing the destabilization, and in turn the consequent aggregation of casein micelles forming the curd. Since ancient times, among milk-clotting enzymes, the animal rennet, obtained from the fourth stomach of suckling ruminants (such as calf), was widely used (Harboe et al., 2010). Nowadays, the use of conventional animal rennet in cheesemaking is limited due to many factors, such as religious restrictions (Islam and Judaism), dietary habits (such as vegetarianism), or economic factors (Jacob et al., 2011; Roseiro et al., 2003). For these reasons, the attention of dairy companies was turned towards other milk clotting enzymes that are not of animal origin, such as microbial, recombinant, or vegetable extract (Liu et al., 2021; Nicosia, Puglisi, Pino, Caggia, & Randazzo, 2022; Vaccalluzzo et al., 2020). Proteases of vegetable origin are traditionally used in cheesemaking in the Mediterranean, West African, and Southern European regions (Roseiro et al., 2003). Compared to chymosin, able to specifically hydrolyze the Phe_{105}-Met_{106} k-casein bond, plant-derived proteases, with a low hydrolysis specificity for caseins, lead to the formation of some defects during cheese ripening (Mazorra-Manzano et al., 2013). In fact, the significant proteolytic activity of vegetable proteases, cause the formation of short peptides mainly conferring acidic and bitter taste (Öner and Akar, 1993). The latter is caused by the presence, in the side chains of peptides, of amino acids, such as lysine, leucine, and proline, which have high hydrophobicity values. According to Ney (1971), a peptide with a Q value higher or equal to 1400 certainly confers bitter taste. Differently, if hydrophobic amino acids are located in a terminal position or are totally free, the Q value is below 1400 resulting in less perceived bitter taste. Among vegetable coagulant the Actinidin (EC. 3.4.22.14), a cysteine protease of kiwifruit, with a molecular mass of 23.5 kDa and an optimal MCA/PA ratio, is considered a promising plant derived clotting enzyme (Lo Piero

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et al., 2011; Mazorra-Manzano et al., 2013; Nicosia, Puglisi, Pino, Baglieri, et al., 2022). However, the high proteolytic activity of plant derived enzymes, like kiwifruit extract, could led bitter taste to the final product.

Lactic acid bacteria possess complex systems of exopeptidases (aminopeptidases and carboxypeptidases) capable of hydrolyzing milk caseins to satisfy their needs in amino acids and small peptides able to generate pleasant flavour compounds especially in traditional cheeses (Caggia et al., 2015; Carpino et al., 2017; Guarcello et al., 2016; Pino et al., 2018; Randazzo et al., 2008, 2010). These exopeptidases can be used to debitter protein hydrolysates reducing the Q values of peptides (Raksakulthai & Haard, 2003). Among exopeptidases, both the aminopeptidase N (PepN) and the X-Prolyl-dipeptidyl peptidase (PepX), which act synergistically, are involved in the debittering process. In detail, the PepN is capable of hydrolyzing hydrophobic amino acids from the N-terminal of peptides with a reduced rate of hydrolysis in the presence of proline (Stressler et al., 2013). Differently, the X-prolyl dipeptidase (PepX) has a high specificity of hydrolysis for peptides that contain proline, thus compensating for the lack of Pep N (Habibi-Najafi, Lee and Law 1996). Both PepN and PepX activities were extensively studied in lactic acid bacteria of food origin and, as previously reported by Nicosia et al. (2023), the *Lactococcus lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain showed a marked PepX and PepN activities (Christensen et al., 1999; González et al., 2010; Moslehishad et al., 2013; Psoni et al., 2007).

According to that, the aim of the present work was to setup an experimental cheese clotted by using a kiwifruit enzymatic extract and inoculated with the *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain as debittering adjunct culture.

2. Materials and methods

2.1. Preparation of the kiwifruit enzymatic extract

The kiwifruit enzymatic extract (KEE) was obtained from *Actinidia deliciosa* cv. Hayward fruits purchased in a local market in Viçosa (Minas Gerais, Brazil). In detail, kiwifruits were pressed using a hydraulic press (ABF Technologies, Cormeilles) and, after centrifugation at 8500 rpm for 20 min, the obtained kiwifruit aqueous extract was subjected to salting out technique as follow: kiwifruit aqueous extract was saturated at 45% through the gradual addition of NaCl (w/v), then centrifugated at 9000 rpm for 20 min at 4 °C. After discarding the supernatant, the resulting precipitate was used as KEE in cheesemaking tests.

2.2. Laboratory-scale coagulation tests

Laboratory-scale coagulation tests were performed to evaluate the coagulation features of the KEE. In detail, whole cow milk was heated at 37 °C and inoculated with different concentrations of the KEE (3, 1.5, 0.7, 0.25 g/L). The milk-clotting activity of the KEE was performed as reported by Benheddi and Hellal (2019).

2.3. Experimental cheesemaking

Two different experimental cheesemaking trials (A and B) were carried out using whole cow milk (three repetitions of each trial were conducted at different days) at the Laboratório de Pesquisa em Leites e Derivados (InovaLeite, Federal University of Viçosa, Minas Gerais, Brazil). Delvo Cheese CT-111 (DSM Food Specialists, the Netherlands), a blend composed of *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Streptococcus thermophilus* strains, was used as starter, whereas the *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain, previously isolated from Pará cheese (Fusieger et al., 2020; Martins et al., 2020) and characterized for aminopeptidase activity (Nicosia et al., 2023) was used as debittering adjunct culture. Q5C6 strain was revitalized twice in reconstituted skim milk (10 g × 100 mL^{-1} autoclaved for 10 min at 115 °C) and incubated at 37 °C for 24 h, then used at final cell density of 10^8 CFU × mL^{-1} in cheesemaking.

Fig. 1 shows the flowchart of the experimental cheesemaking trial A. In detail, the milk (30 L) was heat treated at 65 °C for 30 min, cooled to 37 °C then inoculated with the starter culture (1% v/v). Three fermentation batches (AKD, AK, and AC), each one consisting of 10 L of milk, were setup. In detail, the AKD and AK samples, with and without the addition of the Q5C6 debittering strain, were clotted using the KEE (0.7 g/L) and maintained at 37 °C for 90 min. The AC sample, without the inoculum of the Q5C6 debittering strain, was clotted using commercial rennet (0.05 mL/L) at 37 °C for 40 min and used as control sample. After incubation, the curd, obtained from each fermentation batch, was cut into small granules (4–6 mm), then transferred into cheese molds and pressed to facilitate the purging of the whey. Samples were immersed in brine (15% w/v) then stored, for 7 days, under controlled conditions of temperature and humidity (15 °C, 90–92% RH).

The flowchart of the experimental cheesemaking B is reported in Fig. 2. The milk (40 L) was pretreated as previously reported and, after starter culture addition, four fermentation batches (BKD1, BKD2, BKD3, and BC), each one consisting of 10 L of milk, were setup. The BKD1, BKD2, and BKD3 samples were clotted using the KEE (0.7 g/L) and inoculated with 1%, 2%, and 3% (v/v) of the Q5C6 debittering strain, respectively. After 90 min at 37 °C, the curd was processed as previously described. The BC sample, used as control, was obtained following the same procedure applied for the production of the AC sample, as described above.

2.4. Physico chemical analysis

Physico chemical analyses of cheese samples were performed after 7 days of storage at 15 ± 1 °C. The moisture content was determined gravimetrically by drying 5 g of samples at 105 ± 2 °C until a constant mass was obtained (ISO 5534:2004). Fat content was measured by Gerber-van Gulik method (ISO 3432:2008). Protein was calculated by determination of total nitrogen by the Kjeldahl method, using a conversion factor of 6.38 (ISO 8968-1:2014). The total content of ash was determined gravimetrically by the incineration method at 550 °C (IDF 27:1964). For pH, 10 g of sample were homogenized in 40 mL of distilled water. After 5 min, the mixture was filtered through cotton wool and the pH was measured (at a temperature of 25 ± 1 °C) using a pH meter (Kasvi K39-1014B, São José dos Pinhais, PR, Brazil) (Pino et al., 2021; Randazzo et al., 2021). Each analysis was performed in triplicate and results are reported as mean and standard deviation.

2.5. Microbiological analysis

One-week old cheese samples were subjected to culture-dependent analysis. In detail, 25 g of sample were diluted using 225 ml of NaCl solution (0.90% w/v) and homogenized using a stomacher (BagMixer® 400, Interscience, Saint Nom, France) for 90 s. Decimal dilutions were obtained and plated using the following agar media and conditions: Plate Count Agar (PCA) (Kasvi, São José dos Pinhais, PR, Brazil), aerobically incubated at 30 °C for 48–72 h, for total mesophilic count; MacConkey agar (Merck, Darmstadt, Germany) anaerobically incubated at 36 °C for 18 h for Enterobacteria enumeration; M17 agar (HiMedia, Mumbai, MH, India), incubated at 30 °C for 24–48 h, for lactococci count; Potato Dextrose Agar (K25-1022, Kasvi) supplemented with 1.5% of tartaric acid solution (10% w/v) incubated at 25 °C for 3–5 days for yeasts; Mannitol Salt Agar (MSA) (Kasvi), incubated at 32 °C for 48 h for staphylococci detection; de Man Rogosa and Sharpe agar (MRS), incubated at 32 °C for 72 h, under microaerophilic condition, for lactobacilli (Kasvi) (Randazzo et al., 2009). The presence of *Listeria monocytogenes* and *Salmonella* spp. was evaluated following the method previously described by Pino et al. (2018) and Carpino et al. (2017). Results are expressed ad mean log cfu/g and standard deviation of three replicates.

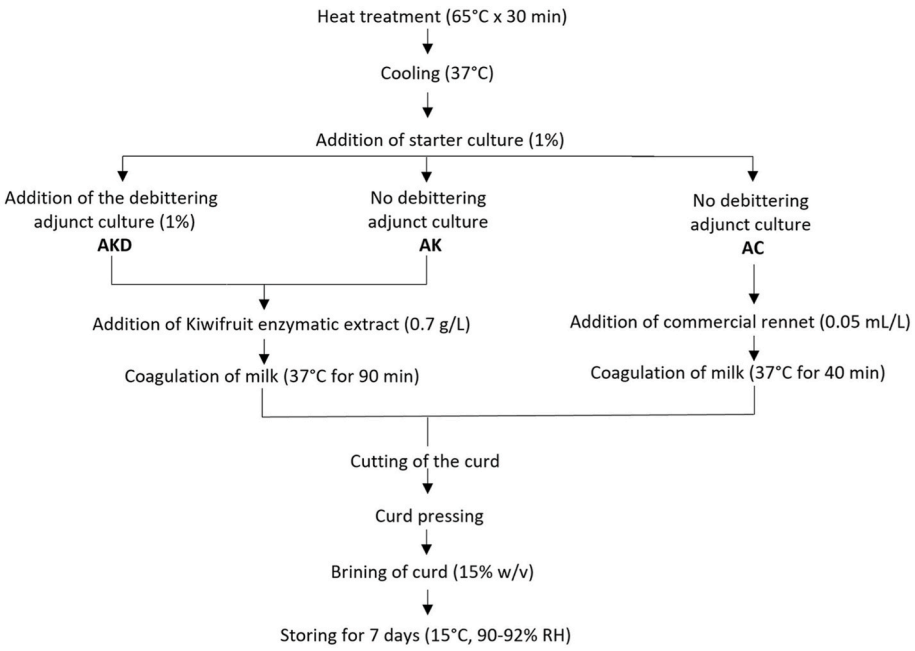

Fig. 1. Flow chart of the experimental cheesemaking A using the KEE (kiwifruit enzymatic extract) and the *L. lactis* subsp. lactis bv. diacetylactis Q5C6 strain as debittering strain. AKD, clotted with KEE and with the addition of the Q5C6 debittering strain; AK, clotted with KEE and without the addition of the Q5C6 debittering strain; AC, clotted using commercial rennet and without the addition of the Q5C6 debittering strain.

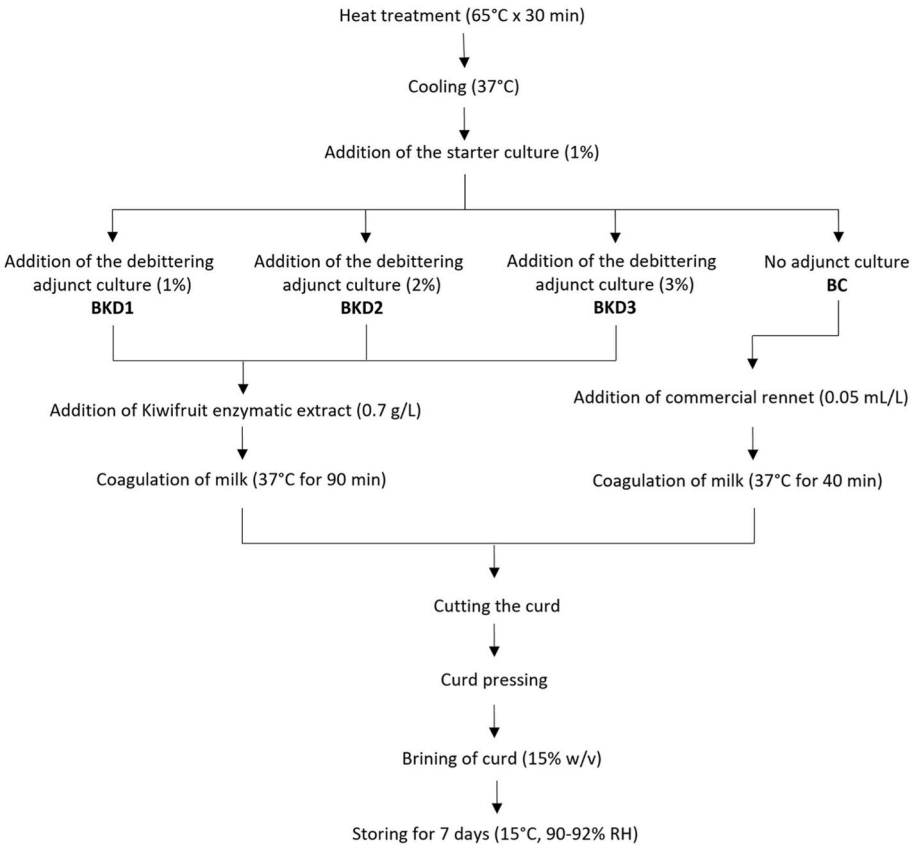

Fig. 2. Flow chart of experimental cheesemaking B using the KEE (kiwifruit enzymatic extract) and different concentrations of the *L. lactis* subsp. lactis bv. diacetylactis Q5C6 strain used as debittering strain. BKD1, cheese clotted using KEE with the addition of the Q5C6 debittering strain (1%); BKD2, cheese clotted using KEE with the addition of the Q5C6 debittering strain (2%); BKD3 cheese clotted using KEE with the addition of the Q5C6 debittering strain (3%); BC, clotted using commercial rennet without the addition of the Q5C6 debittering strain.

2.6. Sensory analysis

2.6.1. Ranking test

The 7-days old AKD, AK, and AC samples obtained from the experimental cheesemaking trial A, were subjected to ranking test for bitter taste. Forty (40) untrained panelists, 17 male and 23 female, aged between 18 and 51 years, participated to the sensory analysis. The test was conducted in individual booths, under white light, at the UFV's Technological Innovation Laboratory (Viçosa, Minas Gerais, Brazil). Cheese samples (approximately 10 g), at a temperature of 10 °C, were presented in black disposable plastic plates, coded with three random digits and served with toothpicks. Panelists were asked to taste randomized cheese samples, from left to right, and to score them as slightly (score 1), medium (score 2) or very (score 3) bitter.

Results, representing the sum of the obtained scores, were evaluated according to the Friedman test ($p < 0.05$). The research protocol followed the guidelines of the Helsinki declaration and all procedures involving human subjects were approved by the Committee on Ethics in Human Beings Research of the Universidade Federal de Viçosa (n° 3.516.953).

2.6.2. RATA test

The RATA test (Rate-all-that-apply) (Meyners et al., 2016) was applied to define the sensory profile of the 7 days-old cheese samples obtained from the experimental cheesemaking trial B (BKD1, BKD2, BKD3, and BC). Seventy (70) panelists (57% female, 43% male, ages: 18 to 62) were recruited among staff, students and visitors of the Federal University of Viçosa (Viçosa, MG, Brazil). Cheese samples (10 g at a temperature of 10 °C) were presented in black disposable plastic plates, coded with three random digits and served with toothpicks. Panelists were asked to evaluate the following descriptors: firmness, gumminess, ricotta consistency, pasty, cream cheese texture, brittleness, grittiness, creaminess, vegetable taste, milk taste, rancidity, astringent, fruity, bitterness, acid taste, salty taste, spicy, animal odor, biscuit odor, stickiness, moisture, and shiny appearance using a 6-point scale ranging from 0 (not applicable) to 5 (extremely applicable). In addition, panelists were asked to rate, through a 9-point scale, the overall acceptability. For each descriptor, the score attributed to each sample were subjected to one-way ANOVA followed by a pairwise multiple comparison test (Tukey HSD). Differences were considered statistically significant at $p < 0.05$. In addition, RATA test score was subjected to Principal Component Analysis (PCA). The research protocol followed the guidelines of the Helsinki declaration and all procedures involving human subjects were approved by the Committee on Ethics in Human Beings Research of the Universidade Federal de Viçosa (n° 3.516.953).

2.7. Statistical analysis

One-way ANOVA followed by Tukey post-hoc test ($p < 0.05$) was applied to physico-chemical and microbiological data using the XLSTAT® software (version 2022.4.1.1365, Addinsoft, New York) and to RATA test data using the Minitab 16.0 software (Minitab Inc., State College, PA, USA). Principal Component Analysis (PCA) was performed using the XLSTAT® software (version 2022.4.1.1365, Addinsoft, New York).

3. Results

3.1. Laboratory-scale coagulation tests

Table 1 shows the coagulation abilities of different concentration of the KEE (3, 1.5, 0.7 and 0.25 g/L) as well as the perception of the bitter taste. Among the tested concentration of the KEE, 0.07 g/L was selected allowing to obtain low bitter taste and acceptable clotting time.

Table 1
Coagulation features of different KEE concentrations and perception of the bitter taste.

Concentration of kiwifruit extract in milk (g/L)	Observations
3	Coagulation occurred very quickly (10 min). The increased proteolytic activity led to the development of an extremely strong bitter taste.
1.5	Coagulation time comparable to commercial rennet (50 min). Pronounced bitter taste
0.7	Slow coagulation (90 min) resulting in a firm clot. Slight aftertaste of bitterness
0.25	Very slow coagulation (160 min) resulting in a soft clot. Imperceptible bitter taste

3.2. Physico-chemical analysis of experimental cheeses

Tables 2 and 3 report moisture, fat content, ash, protein, and pH values of cheese samples obtained from the experimental cheesemaking trial A and B, respectively. Concerning cheese samples obtained from the experimental cheesemaking trial A, no statistically significant differences were recorded among samples for moisture, ash, and pH. The fat content was higher in cheeses coagulated with the KEE (AKD and AK) than cheese obtained using commercial rennet (AC). Regarding to protein content, AC and AK samples showed similar values whereas a low concentration of protein (16.36%) was registered in AKD sample (Table 2). Among cheese samples obtained from the experimental cheesemaking trial B, significant differences were observed for ash, protein, and pH (Table 3). Overall, samples obtained using the KEE (BKD1, BKD2, and BKD3) showed lower values, for all the parameters, than those observed in BC sample. In detail, the ash content of cheese coagulated with KEE ranging from 2.70 to 2.88% while the BC sample show statistical difference content with 3.20%. As regards the protein content, samples BKD1, BKD2 and BKD3 (21.54, 21.36 and 21.18% respectively) show statistical differences with sample BC which showed a value of 23.40%. Finally, the pH of the experimental samples was around 4 while the highest was recorded in the BC sample (5.28).

3.3. Microbiological analysis of experimental cheeses

Tables 4 and 5 show the mean values and standard deviations of the main microbial groups detected in cheese samples obtained from experimental cheesemaking trial A and B, respectively. Overall, *Listeria* and *Salmonella* were never detected in the analyzed samples. No statistically significant differences were recorded among cheeses obtained from the experimental cheesemaking trial A, for mesophilic bacteria, lactobacilli, Enterobacteriaceae, and staphylococci. The ADK sample showed the highest cell density of lactococci (7.56 ± 0.07 log cfu/g) whereas the highest count of yeasts was observed in the AC sample (3.31 ± 0.04 log cfu/g) (Table 4). Concerning cheese samples obtained from the experimental cheesemaking trial B, no differences were observed in

Table 2
Moisture, fat, ash, protein, and pH of cheese samples obtained from the experimental cheesemaking trial A.

	AC	AKD	AK	*p*-value
Moisture %	51.89 ± 0.14^{a}	49.9 ± 0.20^{a}	48.5 ± 0.23^{a}	0.192
Fat %	24.54 ± 0.20^{b}	30.51 ± 0.27^{a}	29.2 ± 0.01^{a}	0.013
Ash %	2.43 ± 0.03^{a}	2.41 ± 0.01^{a}	2.22 ± 0.04^{a}	0.468
Protein %	20.16 ± 0.20^{a}	16.36 ± 0.11^{b}	19.41 ± 0.19^{a}	0.011
pH	5.85 ± 0.02^{a}	5.46 ± 0.05^{a}	5.77 ± 0.05^{a}	0.121

Data are reported as average values and standard deviations of three replicates. AC, clotted using commercial rennet and without the addition of the Q5C6 debittering strain; AKD, clotted with KEE and with the addition of the Q5C6 debittering strain; AK, clotted with KEE and without the addition of the Q5C6 debittering strain. $^{a\text{-}b}$Different lowercase letters indicate statistically significant differences ($p < 0.05$) among samples.

Table 3
Moisture, fat, ash, protein, and pH of cheese samples obtained from the experimental cheesemaking trial B.

	BC	BKD1	BKD2	BKD3	*p*-value
Moisture %	47.18 ± 0.08[a]	46.06 ± 0.03[a]	45.72 ± 0.07[a]	46.23 ± 0.11[a]	0.133
Fat %	25.30 ± 0.05[b]	29.05 ± 0.00[a]	29.55 ± 0.00[a]	29.00 ± 0.00[a]	0.010
Ash %	3.20 ± 0.10[a]	2.88 ± 0.06[b]	2.70 ± 0.04[b]	2.86 ± 0.02[b]	0.022
Protein %	23.40 ± 0.13[a]	21.54 ± 0.20[b]	21.36 ± 0.16[b]	21.18 ± 0.07[b]	0.018
pH	5.28 ± 0.09[a]	5.16 ± 0.06[a]	4.08 ± 0.01[b]	4.11 ± 0.05[b]	0.009

Data are reported as average values and standard deviations of three replicates. BC, clotted using commercial rennet without the addition of the Q5C6 debittering strain; BKD1, cheese clotted using KEE with the addition of the Q5C6 debittering strain (1%); BKD2, cheese clotted using KEE with the addition of the Q5C6 debittering strain (2%); BKD3 cheese clotted using KEE with the addition of the Q5C6 debittering strain (3%).
[a-b]Different lowercase letters indicate statistically significant differences ($p < 0.05$) among samples.

Table 4
Microbial counts expressed as log cfu/g and standard deviation of the main microbial groups detected on cheese samples obtained from the experimental cheesemaking trial A.

	AC	AKD	AK	*p*-value
Mesophilic bacteria	7.72 ± 0.12[a]	7.13 ± 0.08[a]	7.22 ± 0.06[a]	0.544
Lactobacilli	7.30 ± 0.11[a]	7.43 ± 0.07[a]	7.34 ± 0.08[a]	0.675
Enterobacteriaceae	2.78 ± 0.05[a]	2.51 ± 0.10[a]	2.65 ± 0.12[a]	0.128
Lactococci	6.53 ± 0.11[b]	7.56 ± 0.07[a]	6.85 ± 0.18[b]	0.012
Yeasts	3.31 ± 0.04[a]	2.56 ± 0.05[b]	2.49 ± 0.16[b]	0.011
Staphylococci	2.34 ± 0.08[a]	2.20 ± 0.04[a]	2.12 ± 0.05[a]	0.488
***Listeria* spp.**	<1	<1	<1	–
***Salmonella* spp.**	<1	<1	<1	–

Data are reported as average values and standard deviations of three replicates. AC, clotted using commercial rennet and without the addition of the Q5C6 debittering strain; AKD, clotted with KEE and with the addition of the Q5C6 debittering strain; AK, clotted with KEE and without the addition of the Q5C6 debittering strain. [a-b]Different lowercase letters indicate statistically significant differences ($p < 0.05$) among samples.

lactobacilli and Enterobacteriaceae cell densities. The lowest count of mesophilic bacteria was detected in BKD3 and BC samples, respectively. In addition, the BC sample showed the higher yeasts and staphylococci cell density.

3.4. Sensory analysis: ranking test

Results of the ranking test, applied to cheese samples obtained from the experimental cheesemaking trial A are reported in Table 6. Overall, all samples (AC, ADK, and AK) were differently scored by panelists ($p < 0.05$). In detail, the bitter taste was considered low in the AC sample (score of 42) whereas the ADK and AK samples were categorized as averagely and highly bitter, respectively (Table 6).

3.5. Sensory evaluation: RATA test

Fig. 3 shows the radar chart of the RATA test applied to cheese samples obtained from the experimental cheesemaking trial B (BC, BKD1, BKD2, and BKD3). Overall, out of the 22 selected descriptors, 13 (firmness, gumminess, ricotta consistency, pasty, cream cheese texture, brittleness, creaminess, rancidity, astringent, bitterness, acid taste, stickiness, and shiny appearance) were statistically significant different among samples ($p < 0.05$). In particular, firmness (mean score of 4.21),

Table 5
Microbial counts expressed as log cfu/g and standard deviation of the main microbial groups detected on cheese samples obtained from the experimental cheesemaking trial B.

	BC	BKD1	BKD2	BKD3	*p*-value
Mesophilic bacteria	7.36 ± 0.06[a]	7.65 ± 0.02[a]	7.53 ± 0.06[a]	6.54 ± 0.05[b]	0.010
Lactobacilli	7.04 ± 0.08[a]	7.51 ± 0.12[a]	7.43 ± 0.07[a]	7.81 ± 0.09[a]	0.118
Enterobacteriaceae	2.72 ± 0.12[a]	2.73 ± 0.06[a]	2.86 ± 0.10[a]	2.49 ± 0.03[a]	0.098
Lactococci	6.45 ± 0.18[b]	7.23 ± 0.11[a]	7.38 ± 0.12[a]	7.76 ± 0.02[a]	0.009
Yeasts	3.79 ± 0.06[a]	2.71 ± 0.04[b]	2.56 ± 0.15[b]	2.32 ± 0.02[b]	0.002
Staphylococci	3.04 ± 0.05[a]	2.34 ± 0.08[b]	2.15 ± 0.04[b]	2.64 ± 0.12[b]	0.023
***Listeria* spp.**	<1	<1	<1	<1	–
***Salmonella* spp.**	<1	<1	<1	<1	–

Data are reported as average values and standard deviations of three replicates. BC, clotted using commercial rennet without the addition of the Q5C6 debittering strain; BKD1, cheese clotted using KEE with the addition of the Q5C6 debittering strain (1%); BKD2, cheese clotted using KEE with the addition of the Q5C6 debittering strain (2%); BKD3 cheese clotted using KEE with the addition of the Q5C6 debittering strain (3%).
[a-b]Different lowercase letters indicate statistically significant differences ($p < 0.05$) among samples.

Table 6
Sum of the ranking test scores.

n = 40	Samples			*p*-value
	AC	AKD	AK	
Rank sum	42[c]	86[b]	112[a]	<0.001

[a–c]Different lowercase letters indicate statistically significant differences ($p < 0.05$) among samples according to Friedman's test. n, number of panelists.
AC, clotted using commercial rennet and without the addition of the Q5C6 debittering strain; AKD, clotted with KEE and with the addition of the Q5C6 debittering strain; AK, clotted with KEE and without the addition of the Q5C6 debittering strain.

gumminess (mean score of 3.34), and shiny appearance (mean score of 2.31) were mainly perceived in the BC sample; the BKD1 sample received the highest score for both ricotta consistency (mean score of 2.63) and brittleness (mean score of 1.78); pasty (mean score 2.56), cream cheese texture (mean score 1.73), creaminess (mean score 3.20), rancidity (mean score 1.17), astringent (mean score 1.63), bitterness (mean score 2.27), acid taste (mean score 3.07), and stickiness (mean score 2.06) were mainly perceived in BKD2 sample.

3.6. Overall appreciation

Fig. 4 shows the results of the 9-point scale overall appreciation of cheesemaking trial B. No statistically significant differences were detected by panelist between the control sample (BC, score of 6.629) and the cheese clotted using KEE with the addition of the Q5C6 debittering strain (1%) (BKD1, score of 5.914). Lower appreciation scores were attributed to BKD2 and BKD3 samples. Fig. 5 shows the Principal Component Analysis (PCA) of the sensory profile of experimental cheese samples (cheesemaking B). PC1 and PC2 account for 99.43% variances in sensory descriptors showing that BC is associated with several descriptors, such as: gumminess, shiny appearance and firmness while the cheeses coagulated with vegetable enzyme were located at the opposite side along the first dimension, showing correlation with pasty, creaminess and acid taste attributes. Moreover, BKD2 and BKD3 samples cluster in the same quadrant of the graph associating themselves with attributes such as acid taste, bitterness and creaminess, and BKD1

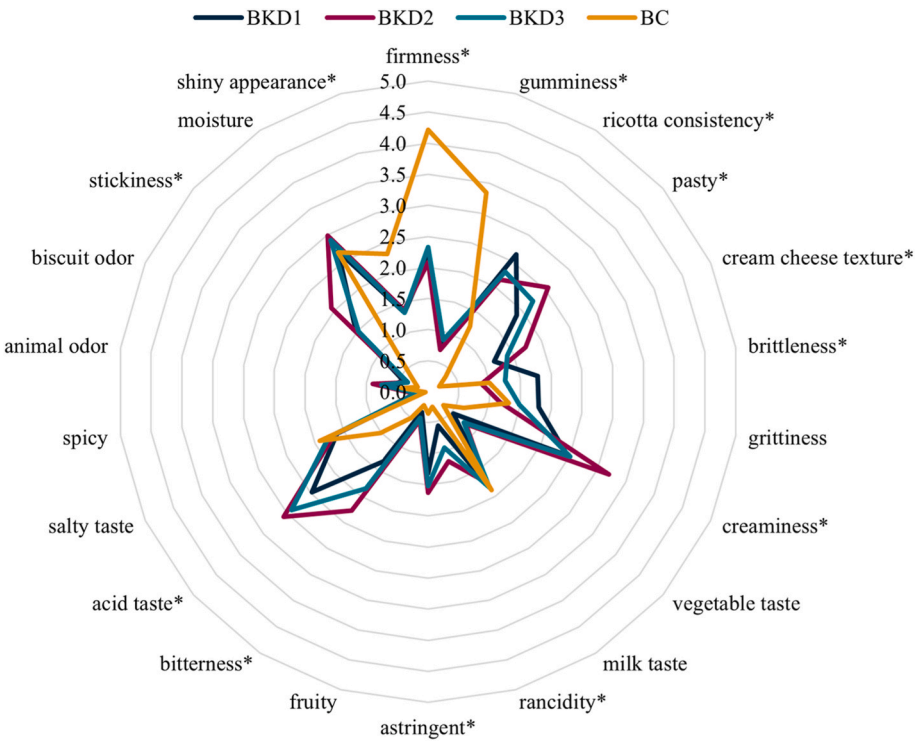

Fig. 3. Radar chart of the RATA test applied to cheese samples obtained from the experimental cheesemaking trail B (BC, BKD1, BKD2, and BKD3). Each descriptor was scored based on a 6 point scale (0, not applicable; 5, extremely applicable). * Statistical differences among samples at $p < 0.05$.

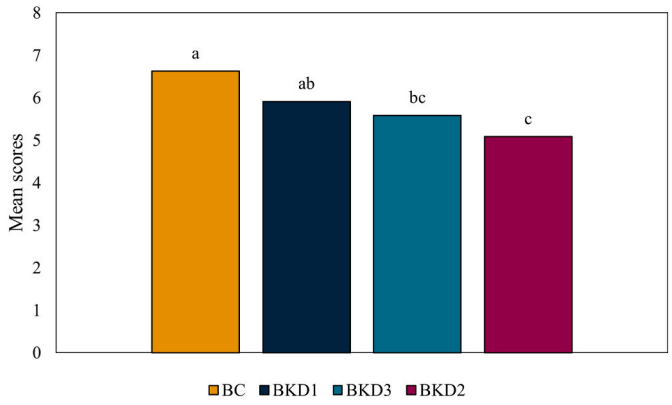

Fig. 4. Mean scores obtained by the experimental cheeses (BKD1, BKD2, BKD3 and BC) in the acceptance test (on a scale from 0 to 8, with 0 = "extremely disliked" to 8 = "extremely liked") according to the inoculum percentage of debittering *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain. Statistical differences among samples at $p < 0.05$.

sample differs from the other samples and from those attributes considered as "negative" by panelists.

4. Discussion

The global increase in cheese demand and the constant decrease in calf rennet supply, along with vegetarianism, religious restrictions (kosher and halal), and unfavorable consumer perceptions on the use of genetically modified microorganisms, contributed to increase the interest of dairy industries to use vegetal coagulants for cheese production (Bathmanathan et al., 2019; Jacob et al., 2011). Although some traditional cheeses in the Mediterranean area, Southern Europe, and West Africa are made using plant enzymes, the main challenge to use vegetable coagulants is represented by the bitter taste conferred to the final product (Ben Amira et al., 2017). According to that, the present study aimed to setup an experimental cheese clotted by using a kiwifruit enzymatic extract and inoculated with the *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain as debittering adjunct culture.

It is well known that the enzymatic extract of kiwifruit can be considered a promising substitute for chymosin due to the ability to form a good milk clot (Nicosia, Puglisi, Pino, Baglieri, et al., 2022). In this context, an optimal concentration of kiwifruit extract must be chosen in order to shorten the coagulation time and reduce the bitter taste caused by the secondary proteolysis carried out by the residual enzyme. To obtain this goal, in the present study, the use of 0.7 g/L of the kiwifruit enzymatic extract allowed to obtain acceptable coagulation time and slight bitter taste. A similar approach was applied by Benheddi and Hellal (2019) using the crude enzymatic extract of *Cynara cardunculus*. In particular, by testing different concentrations of the aforementioned extract, the percentage of 0.5 (v/v) was considered appropriate to obtain a cheese with acceptable sensory properties without affecting the coagulation process.

As previously reported, the use of vegetable coagulants could affect the physicochemical profile of the final product (Mazorra-Manzano et al., 2013). In particular, as observed in the present study, the experimental cheeses, obtained using the kiwifruit enzymatic extract, showed higher fat content than cheeses clotted using commercial rennet. The increase in fat content can be due to the greater ability of vegetable coagulant to retain fatty components in the curd. This finding agrees with Nuñez et al. (1991) who evaluated the chemical, rheological, microbiological, and sensory characteristics of the La Serena cheese obtained using a vegetable rennet from *Cynara cardunculus* L. as coagulant. Similarly, Sanjuán et al. (2002), by studying the effect of an aqueous extract of the thistle of *Cynara cardunculus* L. on physical and chemical characteristics of Los Pedroches cheese, highlighted a higher fat content in vegetable clotted cheeses compared to samples obtained using animal rennet as coagulant. Different results were obtained using apple leaves of *Calotropis procera* (Aworh & Muller, 1987) or ash gourd proteinase (Gupta & Eskin, 1977).

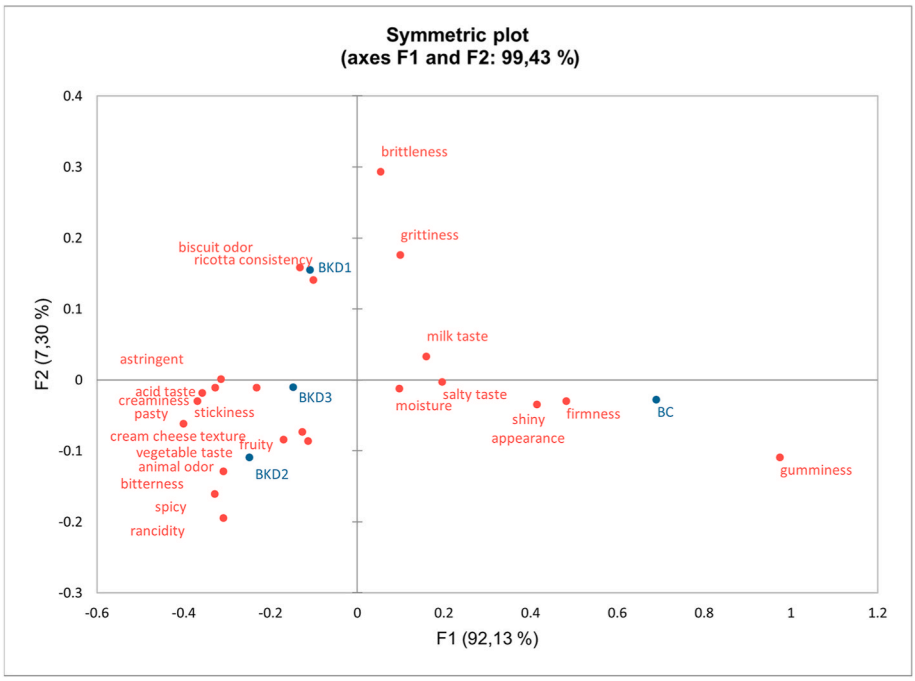

Fig. 5. Symmetric plot with dimensions 1 and 2 of Principal Component Analysis correspondence analysis (CA) of RATA (Rate-All-That-Apply) data from the sensory profiling of experimental cheeses (BKD1, BKD2, BKD3 and BC). The PCA was performed on the sum of scores given by all consumers to each term for describing each sample (RATA scoring).

Concerning ash, which includes, along with salt added to the curd, natural mineral components of the milk, higher values were observed in vegetable clotted samples than in control ones. This observation is in line with data obtained by Sanjuán et al. (2002) in Los Pedroches cheese clotted by *Cynara cardunculus* L. extract.

Interestingly, the low protein content observed in cheese samples clotted by using the kiwifruit extract and inoculated with the debittering adjunct culture could be related to the proteolytic activity exerted by the *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain, as previously reported by Nicosia et al. (2023). In addition, the ability of the adjunct culture to produce lactic acid could explain the low pH value of cheeses.

The use of aminopeptidase positive strains to counteract the bitter taste is well documented (Christensen et al., 1999). In this context, Habibi-Najafi and Lee (2007) used a X-prolyl dipeptidyl peptidase, extracted from the *Lacticaseibacillus casei* spp. *casei* LLG strain, to reduce the bitterness in cheddar slurries supplemented with Neutrase® 0.5 L (neutral protease derived from *Bacillus subtilis*). The fast debittering, reported by the authors, was explained based on the ability of the enzyme to liberate an amino acid residue, containing proline at penultimate N-terminal position, from peptides with a Q value higher than 1400 (Habibi-Najafi & Lee, 2007). Similarly, Tan et al. (1993) used the aminopeptidase N, extracted from the *Lactococcus lactis* subsp. *cremoris* WG2 strain, to debitter a complex peptide mixture of a tryptic digest from bovine β-casein. Based on the obtained results, the ability of the studied aminopeptidases to reduce bitterness, by specific hydrolysis of high Q-value peptides, released from caseins, was demonstrated (Tan et al., 1993). Based on our knowledge, no previous studies were conducted to evaluate the ability of aminopeptidase positive strains to reduce the bitter taste in cheese clotted using vegetable enzymes. According to that, in the present study, the use of the *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain, able to exert both Pep N and Pep X aminopeptidase activities, determined a significant reduction of the perceived bitter taste by panelists. In detail, the results of the ranking test of cheesemaking A, demonstrated that the AKD sample inoculated with the debittering adjunct culture, obtained a lower bitter score than the AK cheese produced without the debittering strain. In addition, as demonstrated by sensory data, the BC sample obtained higher scores in firmness, shiny appearance, and gummines compared to the samples coagulated with KEE. These results are in agreement with Tejada et al. (2006) who compared Murcia al Vino cheeses coagulated with animal rennet or with the *Cynara cardunculus* extract. The observed differences are probably due to the extensive protein breakdown occurring in cheeses produced using vegetable coagulants that disrupts the casein network, leading to a more uniform structure. This effect determines the increase in creaminess and thus a softer texture of the cheese. Moreover, the percentage of the inoculated debittering adjunct culture significantly influenced both the sensory profile and the overall acceptability of the final product. These results are particularly interesting as the only sample that had an overall appreciation comparable to the BC control sample was the BKD1. More in depth, increased concentrations of the adjunct culture were associated to higher perception of descriptors usually associated to cheese defects (e.g. rancidity, acid taste, creaminess, stickiness, etc), determining in turn the reduction of the overall appreciation of the final product.

5. Conclusions

In conclusion, the present work confirmed the in vivo debittering activity of the *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain through cheesemaking trials. Moreover, results demonstrated that a combination of 0.7 g/L of kiwifruit enzymatic extract with 1% of the debittering *L. lactis* subsp. *lactis* bv. diacetylactis Q5C6 strain resulted in an appreciated cheese with overall acceptability comparable to those obtained using commercial rennet. Further studies will be conducted in order to evaluate the effect of the debittering adjunct culture on proteolysis and sensory profiles of vegetable coagulated cheeses produced at industrial scale and with different ripening times.

Author statement

All data, models, and code generated or used during the study appear in the submitted article.

Fabrizio Domenico Nicosia: Formal analysis, Data curation, Writing - original draft. **Alessandra Pino**: Data curation, Writing -

review & editing, Supervision. **Amanda Vaccalluzzo**: Data curation. **Vinícius Rodrigues Arruda Pinto**: Formal analysis, Data curation. **Andressa Fusieger**: Writing - review & editing. **Rosita La Cava**: Methodology, Validation. **Cinzia Caggia**: Writing - review & editing, Supervision. **Cinzia Randazzo**: Conceptualization, Supervision, Data curation, Writing - review & editing. **Antonio Fernandes de Carvalho**: Conceptualization, Supervision, Data curation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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