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Microbiological, chemical, and artificial sensory assessment of Sicilian cheeses made using different milk-clotting enzymes

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ABSTRACT

In the present study, cheeses coagulated with different milk-clotting enzymes were investigated. In detail, calf rennet, microbial coagulant, and a kiwifruit enzymatic extract were used in three independent cheesemaking processes. Microbiological, chemical, and sensory profiles, by using E-Eye, E-Nose, and E-Tongue platforms, were evaluated. Based on microbiological data, mesophilic bacteria, Lactobacilli, and Lactococci showed similar viable count in cheeses clotted with animal rennet and with vegetable coagulant. Differently, higher value of mesophilic bacteria and lower count of both Lactobacilli and Lactococci were detected in samples obtained using microbial coagulant. Additionally, as revealed by the fatty acid composition, the kiwifruit enzymatic extract allowed to obtain a final product with improved health properties. Concerning the artificial senses, E-Eye, E-Nose, and E-Tongue, a similar sensory profile was revealed among cheeses clotted using the kiwifruit enzymatic extract and the calf rennet whereas a different sensory profile was observed in microbial clotted cheeses. This work demonstrated that the kiwifruit extract is a valid coagulant alternative to calf rennet and microbial coagulant.

1. Introduction

Animal rennet, obtained from the abomasum of suckling ruminants, has been used in cheesemaking for centuries and is the milk-clotting enzyme mainly used in the cheese industry (Liu et al., 2021). Nowadays, due to economic reasons as well as factors related to dietary habits, ethical and religious reasons, the use of animal rennet, for cheese production, is limited (Liu et al., 2021; Manuelian et al., 2020; Nicosia, Puglisi, Pino, Caggia, & Randazzo, 2022). According to that, dairy companies have highlighted the need to find alternative milk-clotting enzymes suitable to replace animal rennet in cheesemaking process (Ben Amira et al., 2017; Liu et al., 2021). In this context, recombinant, microbial, and plant-derived proteases have attracted great interest as animal rennet substitutes (Egito et al., 2007; Nicosia, Puglisi, Pino, Caggia, & Randazzo, 2022). Several microbial milk-clotting enzymes, principally produced by fungi and bacteria, have been characterized (Liu et al., 2021). However, due to the high proteolytic activity and the low specificity, which lead to both low cheese yield and bitter taste in the final product, only few of them are suitable for cheese production (Harboe et al., 2010; Liu et al., 2021; Nicosia, Puglisi, Pino, Caggia, & Randazzo, 2022). Among them mucorpepsin (EC 3.4.23.23), is an aspartic protease produced by the filamentous fungi ascribed to *Rhizomucor miehei* and *Rhizomucor pusillus* species, and the endothia pepsin (EC 3.4.23.22), which is produced by *Cryphonectria parastica* (Nicosia, Puglisi, Pino, Caggia, & Randazzo, 2022). Concerning plant-derived proteases, more than 40 cultivars were investigated as sources of milk-clotting enzymes but most of them are not suitable for cheese production due to poor cheese yield and the development of a bitter taste during cheese ripening (Liu et al., 2021). In this context, the kiwifruit (*Actinidia deliciosa*), thanks to the presence of actinidin (EC. 3.4.22.14), was considered a promising milk-clotting agent in cheesemaking (Puglisi, Petrone, & Lo Piero, 2013), hydrolyzing milk casein (Lo Piero et al., 2011). According to Mazorra-Manzano et al. (2013), which compared the milk-clotting properties of fresh kiwifruit (*Actinidia deliciosa*), melon (*Cucumis melo*), and ginger rhizomes (*Zingiber officinale*), the kiwi extract showed the best performance, allowing to obtain a curd

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with characteristics similar to those obtained using commercial chymosin (Mazorra-Manzano et al., 2013). In addition, Fguiri et al. (2021) evaluated the suitability of ginger, pineapple, and kiwi extracts in camel milk cheesemaking, in comparison to commercial rennet. The authors reported that kiwi proteases exhibited the best performances in relation to yield, and textural properties of curds. Although the purification of kiwifruit extracts is a complex and time-consuming process, Nicosia and co-workers (2022b) recently developed a fast, cheap, chemical-free, and eco-friendly technology to set up a kiwifruit (*Actinidia deliciosa*) aqueous extract. One of the main disadvantages associated with the use of the kiwifruit enzymatic extract is related to the high proteolytic activity which can determine the development of a bitter taste in the final product. For this reason, the use of aminopeptidases positive strains, able to hydrolyze bitter peptides, can lead to obtain a final product with appreciate sensory profile (Nicosia, Pino, Maciel, et al., 2023; Nicosia, Pino, Maciel, et al., 2023). This aspect is very important because the consumers' acceptability arises from specific sensory characteristics, including aroma and flavor. In this context, the sensory evaluation is a key step in assessing the ability of vegetable enzymatic extracts to successfully replace animal rennet (Short et al., 2021; Chiofalo et al., 2020; Amira et al., 2017). Conventionally, panelists consider odor, taste, and color characteristics of cheese; however, this can have drawbacks, such as time-consuming, human objectivity, and thus non-repetitive analysis requiring professional tasters (Sinija & Mishra, 2009). In this regard, the electronic eye, electronic nose, and electronic tongue can be considered promising tools to evaluate and differentiate cheeses as they allow for objective and measurable data. E-Eye, E-Nose, and E-Tongue are three analytical systems usually used to assess the quality of both food and feed (Di Rosa & Leone, 2018a; Oteri et al., 2021) as well as to study the changes in color, odor, and taste of raw milk (Ciosek & Wroblewski, 2008; Di Rosa et al., 2018b; Lo Presti et al., 2023), fish (Oteri et al., 2022; Trabelsi et al., 2021), honey (Di Rosa et al., 2018c, 2018d, 2019; Russo et al., 2023), meat and meat products (Larrain et al., 2008; Valous et al., 2009). However, these instruments must be properly used under optimal working conditions, since improper use could limit the amount of useful information obtainable from samples (Haddi et al., 2014). The aim of this study was to evaluate the sensory, microbiological, and chemical profile of Sicilian cheeses, clotted using a kiwifruit enzymatic extract, compared to cheeses clotted by calf rennet and a microbial coagulant. The color, the odor, and the taste of the experimental cheeses were evaluated by using artificial senses (E-Eye, E-Nose, and E-Tongue).

2. Materials and methods

2.1. Vegetable coagulant preparation

The kiwifruit enzymatic extract, used as a vegetable coagulant, was obtained from *Actinidia deliciosa* cv. Hayward fruits purchased from a local market in Catania (Sicily, Italy). In detail, kiwi fruits were pressed using a manual press and, after centrifugation at 8500×*g* for 20 min, the obtained aqueous extract was subjected to the salting-out technique as described by Nicosia and co-workers (2023b). The precipitate, resulting after centrifugation at 9000×*g* for 20 min at 4 °C, was used as vegetable rennet in cheesemaking experiments.

2.2. Cheesemaking experiments

Three cheesemaking experiments (named C, H, and I) were carried out, in parallel, at the La Cava dairy farm (La Cava Srl, Randazzo, Italy) according to Nicosia and co-workers (2023b) with slight modifications. In detail, 90 L of whole cow's and sheep's milk, mixed in a 1:1 ratio, were heat treated at 72 °C for 20 s, then cooled down to 38 °C and split into three separate batches (30 L in each). After the inoculum of a commercial starter culture, kindly provided by Sacco System srl (Cadorago, Italy), the three batch of milk were clotted with calf rennet (0.02 g/L, Caglificio Clerici Spa, Cadorago, Italy), sample C; kiwifruit enzymatic extract (0.7 g/L), sample H; or microbial coagulant (0.02 g/L, Caglificio Clerici Spa), sample I, respectively. Sample H was inoculated with 1% of the *Lactococcus lactis* subsp. *lactis* bv. diacetylactis Q5C6 debittering strain, as reported by Nicosia, Pino, Vaccalluzzo, et al. (2023). The curd, obtained from each batch, was cut with a knife, sliced into small granules, measuring 5–6 mm in size, placed into cheese molds then pressed to pour out the whey. After immersion in a brine solution (saturated solution at 20 °Bé at 12 °C) for 15 min, the obtained cheese samples (C, H, and I) were ripened under controlled conditions (14 ± 1 °C, relative humidity of 90%) for 15 days. Ten (10) molds were obtained from each batch and, among these, three (3) samples were randomly selected and subjected to microbiological, and chemical artificial senses analyses.

2.3. Microbiological analysis

Fifteen-day old cheese samples (C, H, and I) were subjected to culture-dependent analysis following the method and using the culture media and condition reported by Pino et al. (2018). In addition, the protocol proposed by Carpino et al. (2017) was applied to investigate the presence of *Salmonella* spp. All media were purchased from Liofilchem (Roseto degli Abruzzi, Italy). The analyses were conducted in triplicate and results were expressed as $\log_{10}$ CFU/g and standard deviation.

2.4. Chemical analysis

Fifteen (15) days old cheese samples (C, H and I) were analyzed for moisture content using the gravimetric method (ISO 5534:2004). The Gerber-van Gulik method was used to determine the total lipid content (ISO 3432:2008). The protein content was determined by the Kjeldahl method (ISO 8968-1:2014). The pH was measured at a temperature of 25 ± 1 °C using a pH meter (Mettler-DL25, Mettler-Toledo International Inc., Columbus, OH, USA). The total polyphenol content was determined by spectrophotometry using Folin-Ciocalteu's reagent according to the method described by Shetty et al. (1995) and was assessed by measuring the absorbance at 760 nm using a UV–visible spectrophotometer (UV–2401PC Shimadzu, Milan, Italy), with 95% ethanol (Merck, Milan, Italy) as analytical blank. Sterols were extracted according to the method described in EU Regulation 1348/2013. Total lipids were saponified and unsaponifiable matter was extracted with diethyl ether. The sterol fraction was separated by chromatography on silica gel. This fraction was analyzed by gas chromatography, after derivatisation with BSTFA: TMCS 99:1 to obtain the corresponding trimethylsilyl ethers, using a Dani Master gas chromatograph (Dani Instrument, Milan, Italy) equipped with a fused silica capillary column (Zebron ZB-5MSi, L = 30 m, ID = 0. 25 mm, FT = 0.25 μm) from Phenomenex (Torrance, CA, USA) and a flame ionisation detector (FID) according to the analytical conditions of Martini et al. (2021). Total lipid extraction was performed by the Folch method (Folch et al., 1957). The lipid fraction was subjected to hot esterification and analyzed on a Dani Master gas chromatograph (Dani Instrument, Milan, Italy) equipped with a Supelco SLB-IL100 capillary column (length 30 m, internal diameter 0.25 mm, flm thickness 0.25 μm, Supelco, Sigma Aldrich, USA) and a flame ionisation detector (FID) according to the analytical conditions reported by Di Bella et al. (2022). α-, δ-, and γ-tocopherol were quantified by High-Performance Liquid Chromatography Coupled to a Fluorescence Detector (HPLC-FD) following the method proposed by Rotondo et al. (2021).

All analyses were performed in triplicate and results are expressed as mean and standard deviation. All reagents and chemicals used were of analytical grade and were purchased from Merck (Darmstadt, Germany).

2.5. Artificial senses analysis

C, H, and I cheese samples, 15 days old, were subjected to

instrumental sensory analysis and evaluated for color, odor, and taste. For cheese color, a computer vision system (CVS), Iris Visual Analyzer 400 (Alpha M.O.S., Toulouse, France) was used. The computer vision, enclosing the automatic acquisition, processing, and analyses of digital images, is able to duplicate the human vision, obtaining useful information about physical objects. To perform the analyses, each sample was positioned inside the measurement chamber, guaranteeing controlled light conditions, and the images were acquired with a black background as described by Di Trana et al. (2023). Digital cameras register the color of any pixel from the image, using three color sensors per pixel. Hence, the most often used color model is the 'RGB model', in which each sensor captures the intensity of the light in the red (R), green (G) or blue (B) spectrum. For this reason, the colors extracted from each image were expressed in terms of R, G, and B amounts. The Threshold value selected were as follows: R = 73–186, G = 0–162 and B = 0–138.

An Electronic Nose (FOX 4000, Alpha M.O.S., Toulouse, France), equipped with 18 metal oxide sensors (P, T and LY type), was used to evaluate the odor profile of the analyzed samples (C, H, and I). For each sample, 10 replicates, each one consisting of 2 g of cheese placed into a vial of 10 mL, were prepared and analyzed using the following parameters: acquisition duration 120 s, acquisition time 1080 s, injection volume 500 μL, injection speed 500 μL/s, incubation time 1200 s, incubation temperature 60 °C, flushing time 120 s, syringe temperature 70 °C, fill speed 500 μL/s; agitation speed 500 rpm.

An Electronic Tongue (αAstree, Alpha MOS, Toulouse, France), equipped with 7 potentiometric sensors (ANS, PKS, CTS, NMS, CPS, ANS, SCS), was used to evaluate the taste profile. The E-Tongue was initially conditioned and calibrated with standard solutions (HCl and NaCl, Merck, Milan, Italy), then with a solution as similar as possible to the sample under analysis. Cheese samples were finely minced with a knife and divided into 4 g aliquots then analyzed according to Todaro et al. (2024). A beaker was prepared for each sample and 20 consecutive measurements were taken for each one. The sensors were rinsed with double-distilled water after each measurement. The last 10 s of signal acquisition related to the last 10 measurements were considered.

2.6. Statistical analysis

Microbiological and chemical data were subjected to One-way ANOVA followed by Tukey post-hoc test ($p < 0.05$), performed using the Minitab 16.0 software (Minitab Inc., State College, PA, USA). Sensor responses data, obtained from the individual instruments E-Eye, E-Nose, and E-Tongue, were analyzed by exploratory PCA performed using the Alpha Soft V12.4 (Alpha-MOS, Toulouse, France).

3. Results

3.1. Microbiological analysis

The mean values and standard deviations of the main microbial groups detected in C (clotted with calf rennet), H (clotted using a kiwifruit enzymatic extract), and I (obtained using a microbial coagulant) cheese samples are shown in Table 1. Overall, *Escherichia coli*, Listeria, and Salmonella were never detected in the analyzed samples. Mesophilic bacteria, Lactobacilli, and Lactococci showed similar viable count in both C and H samples. Differently, higher value of mesophilic bacteria and lower count of both Lactobacilli and Lactococci were detected in I samples. Similar yeast count was detected in all samples analyzed whereas, statistically significant differences were observed for Enterobacteriaceae and staphylococci count (Table 1).

3.2. Chemical analysis

The mean value and standard deviation of pH, moisture, total lipids, proteins, polyphenols, sterols, and tocopherols, detected in C, H, and I cheese samples, are shown in Table 2. The fatty acid profile, detected in the analyzed samples, is displayed in Table 3. Concerning chemical data, all samples showed different pH, the highest (6.40) and lowest (5.88) values were detected in I and H cheese samples, respectively. Moreover, C and I cheeses showed similar moisture, total lipids, proteins, and polyphenols content whereas the H sample exhibited higher moisture and polyphenols along with lower values of both total lipids and proteins (Table 2). Stigmasterol, campesterol, and β-sitosterol were detected only in H samples which also showed the lowest content of cholesterol. Similar concentration of α-, δ-, and γ-tocopherol was revealed in the analyzed samples. Concerning fatty acids composition,

Table 1
Microbial count, reported as $\log_{10}$ CFU/g and standard deviation, of the main microbial groups detected on cheese samples clotted using calf rennet (C), kiwifruit enzymatic extract (H), or microbial coagulant (I).

	C	H	I
Mesophilic bacteria	6.06 ± 0.40^{b}	6.05 ± 0.22^{b}	6.85 ± 0.12^{a}
Lactobacilli	6.87 ± 0.10^{a}	6.90 ± 0.21^{a}	6.14 ± 0.04^{b}
Lactococci	6.75 ± 0.40^{a}	6.78 ± 0.25^{a}	6.00 ± 0.10^{b}
Yeast	3.65 ± 0.20^{a}	3.71 ± 0.41^{a}	3.73 ± 0.12^{a}
Enterobacteriaceae	2.20 ± 0.04^{b}	1.55 ± 0.10^{c}	3.15 ± 0.11^{a}
Staphylococci	2.22 ± 0.05^{b}	1.51 ± 0.15^{c}	3.34 ± 0.24^{a}

Data are reported as average values and standard deviations of three replicates. [a-b] Different lowercase letters indicate statistically significant differences ($p < 0.05$) among samples. C, cheese clotted with animal rennet; H, cheese clotted with kiwifruit enzymatic extract; I, cheese clotted with microbial coagulant.

Table 2
Chemical profile of cheese samples clotted using calf rennet (C), kiwifruit enzymatic extract (H), or microbial coagulant (I).

	C	H	I
pH	6.10 ± 0.01^{b}	5.88 ± 0.05^{c}	6.40 ± 0.04^{a}
Moisture (g/100 g)	39.91 ± 0.08^{b}	47.84 ± 0.28^{a}	40.52 ± 0.13^{b}
Total lipids (g/100 g)	32.21 ± 0.44^{a}	28.90 ± 0.44^{b}	31.90 ± 0.22^{a}
Proteins (g/100 g)	24.90 ± 0.31^{a}	20.31 ± 0.59^{b}	25.55 ± 0.67^{a}
Polyphenols (mg/kg)	153.88 ± 0.02^{b}	422.90 ± 0.05^{a}	148.70 ± 0.15^{b}
Sterols (mg/100 g of cheese)			
Cholesterol	93.32 ± 0.60^{a}	55.41 ± 0.18^{b}	90.23 ± 0.14^{a}
Stigmasterol	nd	0.19 ± 0.05	nd
Campesterol	nd	0.16 ± 0.07	nd
β-sitosterol	nd	0.55 ± 0.15	nd
Tocopherols (mg/Kg)			
α-tocopherol	20.34 ± 0.48^{a}	20.83 ± 0.51^{a}	20.56 ± 0.44^{a}
δ-tocopherol	1.12 ± 0.21^{a}	1.06 ± 0.18^{a}	1.03 ± 0.14^{a}
γ-tocopherol	2.73 ± 0.14^{a}	2.85 ± 0.18^{a}	2.81 ± 0.16^{a}

Data are reported as average values and standard deviations of three replicates. Different letters indicate statistically significant differences ($p < 0.05$) among samples. C, cheese clotted with animal rennet; H, cheese clotted with kiwifruit coagulant; I, cheese clotted with microbial coagulant. nd, not detected.

Table 3
Fatty acid composition of cheese samples clotted using calf rennet (C), kiwifruit enzymatic extract (H), or microbial coagulant (I).

	C	H	I
SFA	69.20 ± 0.06^{b}	69.55 ± 0.10^{b}	71.22 ± 0.11^{a}
MUFA	23.39 ± 0.09^{a}	23.43 ± 0.07^{a}	23.40 ± 0.16^{a}
PUFA	4.00 ± 0.02^{a}	3.94 ± 0.05^{a}	3.11 ± 0.03^{b}
n3	1.24 ± 0.10^{a}	1.25 ± 0.03^{a}	1.21 ± 0.03^{a}
n6	2.41 ± 0.02^{a}	2.48 ± 0.04^{a}	2.38 ± 0.03^{a}
n6/n3	1.64 ± 0.14^{a}	1.65 ± 0.02^{a}	1.62 ± 0.02^{a}

Data are reported as percentage and standard deviation of three replicates. [a,b] Different lowercase letters indicate statistical significant differences ($p < 0.05$) among samples. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; n3, omega 3 fatty acid series; n6, omega 6 fatty acid series. C, cheese clotted with animal rennet; H, cheese clotted with kiwifruit coagulant; I, cheese clotted with microbial coagulant.

no statistically significant differences were observed for mono-unsaturated fatty acid (MUFA) as well as omega 3 fatty acid (n3) and omega 6 fatty acid (n6) series. Cheeses clotted using calf rennet (C) and using the kiwifruit enzymatic extract (H) are characterized by a similar content of both SFA and PUFA; both cheeses have a lower SFA content and a higher PUFA content compared to cheese I (Table 3).

3.3. Artificial senses analysis

Results of E-Eye, E-Nose, and E-Tongue electronic instruments are shown in Figs. 1–3, respectively. As reported in Fig. 1, the first two components (PC1 and PC2) of the PCA plot of E-Eye data accounted for 97 percent of the total variance between the measurement of the samples. The PCA plot showed an overlap between C and H samples, which clustered in the left part of the graph and were associated to a great number of color codes, from the darkest to the lightest. Differently, I samples, positioned to the right of the graph, were characterized by the color codes 2181-2437-2438-2454. In the PCA plot of the E-Nose (Fig. 2) the first two components (PC1 and PC2) account for 100 percent of the total variance between the measurement of the samples. As for E-Eye, PCA also shows an overlap of group C and H, placing to the right of the graph. The volatile profile of the group H does not differ from the group C, however group H shows less internal variability. The I group diverges from C and H groups, along the PC1 axis, placing it to the left of the graph. For the taste profile, in the PCA plot of the E-Tongue (Fig. 3) the first two components (PC1 and PC2) account for 100 percent of the total variance among the measurement of the samples. The PCA plot shows a taste separation of the three groups; specifically, group I was placed on the left side of the graph, while group H on the right top and group C on the right and bottom of the graph. Although E-Tongue discriminated the three cheese groups, all the sensors were biased toward the group I.

To increase the probability of correct classification, a mid-level data fusion was implemented and the single matrix with data from all sensors was subjected to PCA. In addition, data of nutritional characteristics, fatty acid classes, total content polyphenols, sterols were included. In the PCA graph (Fig. 4) the first two components (PC1 and PC2), explained the 100 percent of the total variance between the measurement of the samples. Observing the PCA (Fig. 4), the I group was at the bottom left of the graph, the H group was at the top of the graph, and the C group was at the bottom right of the graph. Observing the direction of the sensors of the sensory instrumentation, the group I was characterized by sensors T40/1 (E-Nose), ANS and CTS (E-Tongue), and 2439 (E-Eye), the group H was characterized by sensors 2182 and 2166 (E-Eye), while sensors LY2/gCT and LY2/AA (E-Nose), and 2454 (E-Eye) were headed toward group C. As for chemical analysis, SFAs headed toward the group I. Toward the group H, the polyphenols, campesterol, stigmasterol, and β-sitosterol head. While PUFAs were between H and C groups. The α-tocopherol, γ-tocopherol, and δ-tocopherol did not direct to a specific group.

4. Discussion

In the present study, kiwifruit extract, calf rennet, and microbial coagulant were used in three independent cheesemaking experiments. The experimental cheeses were characterized for microbiological and chemical characteristics as well as for sensory profile by using an artificial senses platform consisting of E-Eye, E-Nose, and E-Tongue. Overall, a similar microbiological profile was observed between cheeses clotted using the kiwifruit enzymatic extract and the calf rennet. The two cheeses showed high Lactobacilli and Lactococci counts, which could exhibit the antimicrobial activity against pathogens, confirmed by the lower level of Enterobacteriaceae and staphylococci than the cheese samples obtained with microbial coagulant showed. Compared to Caciofiore cheeses, curdled using *Cynara cardunculus* and *Onopordum tauricum* crude extract (Rampanti et al., 2023), a lower cell density of Lactobacilli, Lactococci, Enterobacteriaceae, mesophilic bacteria, and yeast was revealed. Similar results were obtained in comparison to Queijo de Nisa PDO cheese produced using raw ewe's milk clotted with cardoon (Cynara cardunculus, L.) rennet (Cardinali et al., 2022). This result could be due to the heat treatment, performed at the beginning of the cheesemaking, which allowed to a reduction of the bacterial load. Based on chemical data, the type of coagulant significantly influenced the pH, fat, moisture, and protein content of the obtained cheeses. In particular, the lower pH value detected in H samples could be related to the high cell density of Lactobacilli and Lactococci, as well as to the inoculum of the *L. lactis* Q5C6 strain as adjunct culture, which contributed to a higher degree of curd acidification. In addition, the lower fat and protein content, reveled in H samples, beyond consequence of the higher moisture, could be related to the clotting features of the vegetable rennet. Similarly, Aworh and Muller (1987) reported, in cheeses clotted using a *Calotropis procera* leaves extract, lower content of protein and fat as well as higher moisture compared to cheeses coagulated using the animal rennet. Moreover, a higher content of polyphenols was detected in H samples than in C and I ones. This result agrees with Serra et al. (2020) who demonstrated an improvement in the microbiological quality and nutraceutical properties of kiwifruit clotted cheeses. In fact, both sheep and buffalo cheeses made using kiwifruit extract revealed higher polyphenols content compared to cheeses made with calf-rennet. It is noteworthy that polyphenols exhibit antimicrobial activity as well as positive effect on human health, like anti-cancer, anti-diabetic, anti-aging, anti-hypertensive, anti-asthmatic, and cardio-protective properties (Pandev & Rizvi, 2009). These benefits also support the utilization of kiwifruit in cheese manufacturing to improve the overall nutritional and functional profile of the final product. Interestingly, in

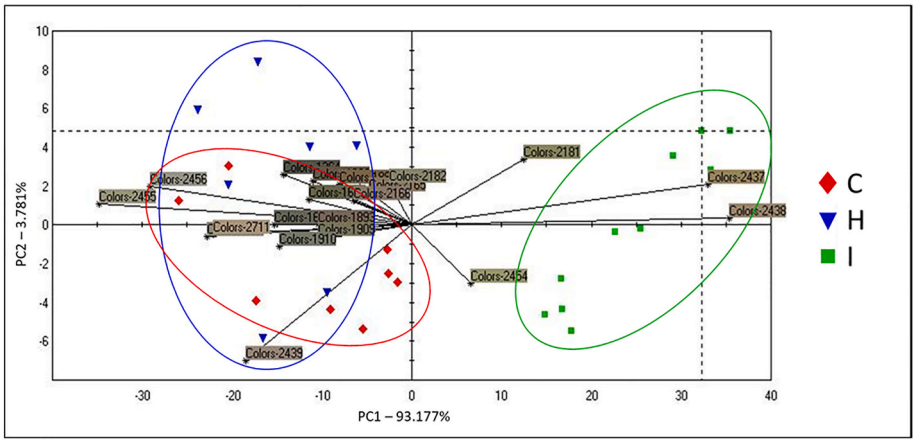


Fig. 1. Principal Component Analysis plot for the E-Eye (C, red; H, blue; I, green) and loading vectors.

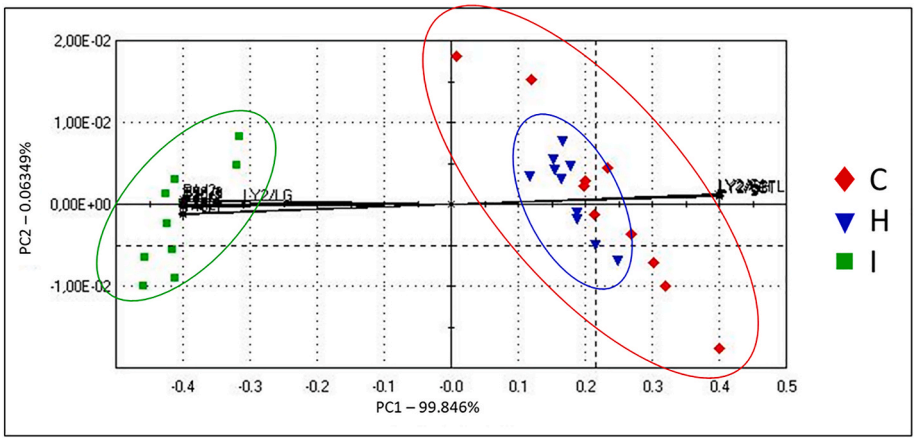


Fig. 2. Principal Component Analysis plot for the E-Nose (C, red; H, blue; I, green) and loading vectors.

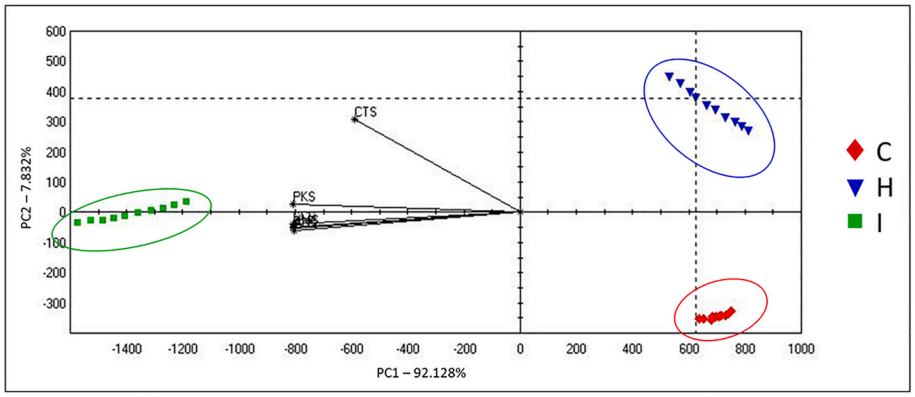


Fig. 3. Principal Component Analysis plot for the E-Tongue (C, red; H, blue; I, green) and loading vectors.

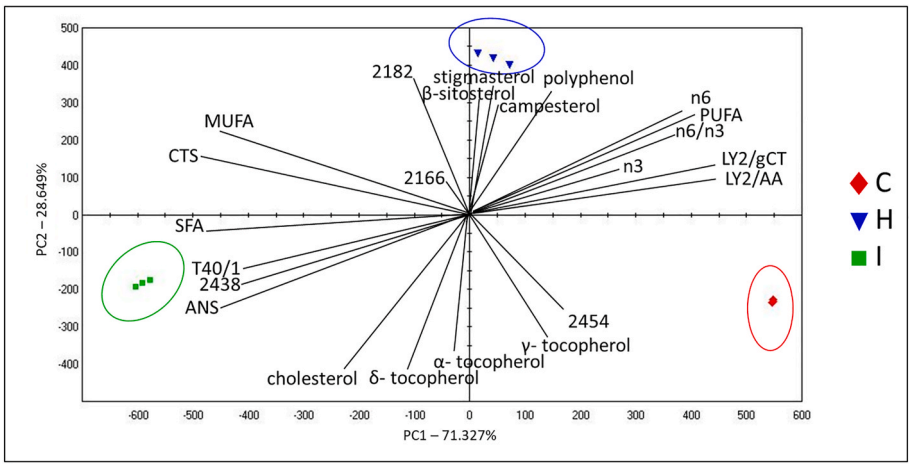


Fig. 4. Principal Component Analysis plot for the Medium fusion (C, red; H, blue; I, green) and loading vectors of selected variables.

the present study, cheeses clotted using the kiwifruit enzymatic extract, were characterized by the presence of stigmasterol, campesterol, and β-sitosterol, which represent an additional positive feature of cheeses obtained by kiwifruit, contributing to improve the overall nutritional and nutraceutical properties of the final product (Serra et al., 2020). Notoriously, tocopherols, fat-soluble microconstituents, are able to exert beneficial effects in humans including protection against cardiovascular disease, cancer, and age-related eye diseases (Borel & Desmarchelier, 2018; Reboul et al., 2006). In cheese, the concentration and composition of tocopherols is mainly related to genetic and physiological factors, forage diet, metabolism by the animal tissues, and composition of the milk (Lucas et al., 2006; Nozière et al., 2006; Rotondo et al., 2021) which can explain the absence of differences in the amount of α-tocopherol, γ-tocopherol, and δ-tocopherol detected in the analyzed samples.

In the present study, the sensory profile of the experimental cheeses was evaluated by applying an “electronic panel”. This approach, namely sensor fusion, allows to integrate different sensors as well as to combine

the obtained data improving the quality assessment. The sensor fusion approach is analogous to the cognitive process used by humans to continually integrate data generated from their senses, making inferences about the external world (Di Rosa et al., 2018c).

Data from E-Nose revealed how P- and T-type sensors go to characterize group I. P-type sensors are sensitive to ketones and T-type to alcohol compounds (Upadhyay et al., 2017). Ketones are formed by the enzymatic oxidation of fatty acids to β-keto acids and then their decarboxylation to methyl-ketones are compounds present at high concentrations in cheeses made with pasteurized milk and a specific starter culture (Castillo et al., 2007). Furthermore, the position of the T-type sensors could be explained by the formation of secondary alcohols, formed by enzymatic reduction of the respective methyl ketones (Delgado et al., 2011). The cheeses made with animal and vegetal coagulants (C and H group), were identified by the LY group sensors which, instead, respond to carboxylic acids, important components in the aroma of cheeses, and to aldehydes, deriving from the autoxidation of carboxylic acids with a role in the flavor development of cheese (Dunn & Lindsay, 1985; Muñoz N. et al., 2003). Moreover, the LY sensors respond to alcohols, probably the branched-chain primary alcohols formed by reduction of aldehydes derived from leucine (Delgado et al., 2011). In general, the substances that most affected the aroma of the cheeses were the same In the C and H group and could be related to the high presence of Lactobacilli and Lactococci. It is well known that the metabolism of free amino acids (FAAs) by LABs, produces aldehydes, alcohols, carboxylic acids, amines, and sulfur compounds (Kilcawley & O'Sullivan, 2017). Aromatic amino acids, branched-chain amino acids, methionine, and aspartic acid undergo a transamination reaction catalyzed by amino acid aminotransferase, converting them into α-keto acids. Subsequently, these α-keto acids are further metabolized into branched-chain and aromatic aldehydes, acyl-CoA, hydroxy acids, and methanethiol. The resulting organic acids can undergo esterification with alcohols, contributing to an enriched aroma of the cheeses (Belguesmia et al., 2014; Delavenne et al., 2012). Based on E-Eye, C and H samples showed a similar color profile whereas distinctive color codes were revealed in I samples. This evidence is in discordance with Garcia et al., (2012) who, using a CIE Lab system, did not found difference in color among cheeses produced with three types of coagulant (microbial, vegetable, and animal). In our study, groups C and H appeared to have the same colors even though group H had the darkest ones. These results, which could be due to the greater presence of polyphenols in cheeses made with kiwifruit coagulant (Han et al., 2011), are however not significant, contrary to what was found by Saraiva et al. (2019) in cheeses in which the use of Yerba mate have attributed light green shades.

An important contribution to cheese flavor is made by smaller peptides and free amino acids, as well as compounds derived from the catabolism of free fatty acids. It is well known how the use of vegetable rennet contributes to the formation of negative notes in taste, due to the higher proteolytic activity of vegetable coagulants compared to chymosin (Galan et al., 2012). The high proteolytic activity, leads to the production of short peptides, generating bitter and sour taste. The use of microbial coagulant also brings bitter taste in cheeses. In previous works (Ahmed et al., 2016; Vishwanatha et al., 2010), *B. stearothermophiluse* and fungal curds have been shown to cause bitter taste defects in several cheeses. In a study by Zhao et al. (2019), microbial coagulant isolated from glutinous rice wine was used for Cheddar cheese production and compared with a commercial rennet. Based on the sensory evaluation, the cheese with glutinous rice wine rennet showed no significant differences in terms of preference, compared to the cheese made with commercial rennet. Both cheeses showed good acceptability. These results are not in agreement with those obtained by E-Tongue. In fact, it was the only instrument that discriminates among the three groups of cheeses, highlighting different flavor notes induced by the different rennet used.

For the E-Tongue, the AHS, CTS, NMS, ANS and SCS sensors specifically responded to sour, salty, umami, sweet and bitter tastes, respectively, while PKS and CPS function as universal (Zhu et al., 2022). Looking at the direction of the sensors, they point toward group I cheeses. We could hypothesize that group I is characterized by a set of tastes that distinguish cheeses made with microbial coagulant, according to Zhao et al. (2019), who found that the high level of FAA umami in cheese produced with microbial coagulant isolated from glutinous rice wine increase the intensity of other flavors, improving the taste of the cheese (Jinap & Hajeb, 2010). Results obtained from chemical and sensorial data fusion showed that cheeses made with kiwifruit coagulant are more similar to cheeses made with animal rennet than those made with microbial coagulant. However, group H differs from group C in the presence of the phytosterols Campesterol, Stigmasterol and β-sitosterol, which are the most abundant sterols in kiwifruit (Piironen et al., 2003). Sterols are produced by plants and have functions like those of cholesterol in humans. Dietary supplementation of plant sterols plays an important role in human metabolism, as they can attenuate the intestinal absorption of cholesterol (Gylling et al., 2014). In addition, polyphenols and their metabolites can act as activators or inhibitors of bacterial growth, and this depends on their chemical structure (Reguant et al., 2000).

In light with the results obtained from medium fusion, after skimming the irrelevant data, it was observed in more detail that the H group is characterized by light colors; in fact, color codes 2182 and 2166 correspond to a shade of yellow and a shade of blue-turquoise, respectively. Probably the color was influenced by the presence of polyphenols. Conversely, groups I and C were characterized by the color codes 2139 corresponding to a cyan-blue shade and 2454 corresponding to a medium-dark shade of turquoise, respectively. Therefore, groups I and C are characterized by darker color shades, but still of the same hue. Another interesting result is the sensory response of E-Nose. Sensor T40/1 characterizes group I; moreover, this group is characterized by a higher presence of saturated fatty acids (SFA) and a lower presence of polyunsaturated fatty acids (PUFA). In contrast, sensors representing short-chain fatty acids (LY) and polyunsaturated fatty acids (PUFA) are directed toward groups C and H. The presence of short-chain fatty acids in cheese contributes to its sensory qualities due to their lower threshold of perception compared to long-chain fatty acids (Laskaridis et al., 2013). Group I was produced with microbial-derived coagulant and has had its own market for years. Nevertheless, it is the one with high level of saturated fatty acids (SFAs). In the study conducted by Torres et al. (2022), Paipa cheese, obtained from the recombinant, microbial-derived enzyme chymosin EC 3.4.23.4, had a high content of saturated fatty acids and n3, n6, and n9. Differently, PUFAs are known for their potential health benefits, including protection from several diseases like osteoarthritis, cancer, and autoimmune disorders (Kapoor et al., 2021; Pino et al., 2020). PUFAs contribute to different cellular activities such as cell signaling, structural integrity and fluidity of cell membrane, as well as regulation of blood pressure and glucose level (Kapoor et al., 2021). According to that, foods rich in PUFAs should be incorporated to the routine diet and their presence in foods, alternative to vegetable and sea foods, can contribute to a good health and to the prevention of various diseases (Kapoor et al., 2021; Nguyen et al., 2019).

5. Conclusions

In conclusion, the present study provided a comprehensive characterization of cheeses obtained using different milk-clotting enzymes. Overall, compared with traditional animal rennet and microbial coagulant, the kiwifruit extract exhibited good nutritional and functional properties. In addition, based on sensory profile, cheeses clotted by using the kiwifruit enzymatic extract were similar to animal clotted cheeses. In conclusion, the kiwifruit enzymatic extract could be considered as a valid substitute of calf rennet.

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CRediT authorship contribution statement

Ambra Rita Di Rosa: Writing – original draft, Methodology, Formal analysis, Data curation. **Francesca Accetta:** Formal analysis, Data curation. **Fabrizio Domenico Nicosia:** Writing – original draft, Formal analysis, Data curation. **Federica Litrenta:** Writing – original draft, Formal analysis, Data curation. **Alessandra Pino:** Writing – review & editing, Validation, Methodology, Data curation. **Vincenzo Lopreiato:** Writing – original draft, Data curation. **Cinzia Caggia:** Writing – review & editing, Methodology. **Cinzia Lucia Randazzo:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alessandra Pino, Cinzia Caggia, and Cinzia Lucia Randazzo declare that they are members of ProBioEtna, a spinoff of the University of Catania, Italy. In addition, the authors declare that they do not have any personal, financial, professional, political, or legal interests with a significant chance of interfering with the performance of their ethical or legal duties.

Data availability

Data will be made available on request.

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