applied sciences

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Exploring New Fruit- and Vegetable-Derived Rennet for Cheese Making

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Featured Application: Development of new vegetable-derived rennet for cheese making.

Abstract: Cheese production is an ancient practice to preserve a perishable food, such as milk, for a long time. The first step of cheese processing involves the addition of rennet, which contains the enzymes necessary for the hydrolysis and coagulation of the caseins present in milk. Typically, animal-derived rennet, such as calf rennet containing chymosin, are used as source of enzymes for cheese processing. Alternatively, microbial chymosin or recombinant chymosin is used. However, recently, plant-derived rennet such as the ones derived from thistle and bitter orange flowers and from artichoke (*Cynara cardunculus* var. *scolymus*) have also been demonstrated to be valid sources of enzymes for cheese processing. Therefore, herein, different plant and fruit extracts were tested and compared for their ability to coagulate milk caseins. In particular, beyond artichoke and cardoon (*Cynara cardunculus*) extracts, those from pineapple (*Ananas comosus* (L.) Merr.), papaya (*Carica papaya* L.), common fig (*Ficus carica* L.) milky sap, and oyster mushroom (*Pleurotus ostreatus* (Jacq. ex Fr.) P. Kumm.) were investigated for their proteolytic, esterase, and milk-clotting activities. The extracts were then exploited as vegetable and fruit rennet for the experimental production of cheeses, which were examined, after 30 days of maturation, for their moisture, fat, protein, and free fatty acid (FFA) content. Interestingly, the artichoke, cardoon, and thistle mushroom extracts showed high proteolytic activity compared to calf rennet, while the level of esterase activity appeared to be similar for all the extracts. The papaya extract showed the lowest proteolytic and esterase activity. Although the pH, moisture, fat, and protein contents were very similar to those of cheese made with calf rennet, the medium- and long-chain FFAs broadly differed among produced cheeses, with variations in the lipid quality indices.

Keywords: fruit- and plant-derived rennet; cheese making; milk-clotting activity; fatty acids

Citation: Pacifico, S.; Caputo, E.; Piccolella, S.; Mandrich, L. Exploring New Fruit- and Vegetable-Derived Rennet for Cheese Making. *Appl. Sci.* **2024**, *14*, 2257. https://doi.org/10.3390/app14062257

Academic Editors: Elena Curti and Roberto Anedda

Received: 8 February 2024
Revised: 4 March 2024
Accepted: 5 March 2024
Published: 7 March 2024

1. Introduction

Cheese making is one of the most important processes used for millennia to preserve milk, a fresh food, for a long time. Over time, cheese production has evolved to become one of the most important and widespread foods in the world. Nowadays, there are about 2000 different types of artisanal and industrial cheeses in the world, also depending on the milk origin. Dairy industries are present all over the world and reach an annual turnover of over EUR 250 billion. In Italy, one of the largest producers of high-quality cheeses, there are more than 3500 industries with an annual turnover of around EUR 15 billion.

Among the cheese production phases, the coagulation of milk is fundamental because the enzymes used in this phase influence the yield, consistency, and flavor of the cheese. In

fact, natural rennet is a mixture of different enzymes, such as chymosin or chymosin-like proteases (coagulation enzymes), lipase, esterase, lactase, and catalase [1]. Milk coagulation is characterized by the selective hydrolysis of κ-caseins, which leads to casein solidification and curd formation [2].

The main clotting enzyme used is chymosin, derived from calf rennet. The growing demand for calf rennet has led to the development of alternative rennet, among which the most used are those containing enzymes from *Rhizomucor miehei*, *Rhizomucor pusillus*, and *Aspergillus oryzae*. Alternatively, recombinant calf chymosin produced in *Escherichia coli*, *Bacillus subtilis*, and *Lactococcus lactis* is also used [1,2]. Some ancient artisanal cheeses produced in Italy, Spain, and Portugal use vegetable rennet derived from artichoke and its wild form, namely cardoon (*Cynara cardunculus*) [3]. Furthermore, in some areas of the Mediterranean, the milky sap from fig (*Ficus carica)* is also exploited [1]. The development of alternative rennet, and in particular those of vegetable origin, has become not only a commercial but also an ethical requirement, considering dietary styles, such as vegetarian or vegan ones, which do not involve the consumption of foods of animal origin, or all those who do not consume these products for religious reasons.

In general, the clotting activity of plant extracts is slower than that obtained from animal rennet, but plant-derived clotting enzymes are characterized by a high proteolytic activity against milk caseins, which generates peptides able to affect the cheese texture and contribute to the development of a bitter flavor [2]. During cheese production, one of the first steps is the solubilization of calcium phosphate by decreasing the pH of the milk. Calcium phosphate helps to stabilize casein micelles. When chymosin or a clotting enzyme are added to milk, the specific hydrolysis at the Phe_{105}–Met_{106} of κ-casein leads to the exposure of its hydrophobic surface, facilitating the aggregation of casein and the formation of clots [2].

Microbial rennet-like coagulants have properties like those of calf rennet; specifically, they are aspartic proteases (EC 3.4.23) able to hydrolyze bovine κ-casein at the Phe_{105}–Met_{106} bond, like calf rennet. Among plant proteolytic enzymes that show clotting activity are ficin, a cysteine endopeptidase, from the milky sap of *Ficus carica* (EC 3.4.22.3) [4] and papain from the latex *Carica papaya* (EC 3.4.22.2), a cysteine protease. The latter hydrolyzes proteins with broad specificity for peptide bonds, while preferring an amino acid bearing a large hydrophobic side chain at the P2 position and not accepting Val at P1 [4]. Furthermore, aspartic proteases from cardoon and artichoke show specific activity for the Phe_{105}–Met_{106} bond of bovine κ-casein [5]. In particular, the main proteases are cardosin A and cardosin B. The first enzyme is involved in the coagulation process, it has an activity like that of chymosin, which is responsible for the hydrolysis of κ-casein in milk, while cardosin B has an activity similar to that of pepsin and is responsible for specific proteolysis during cheese ripening [6].

It has been reported that the high proteolytic activity of plant-derived rennet causes lower cheese yields, bitter flavors, and texture defects when they are used for bovine milk, if the amounts of extract used are exceeded [4,7,8]. In some cases, plant extracts, such as those from cardoon and fig milky sap, have been used for making sheep and goat cheeses. It has been reported that the type of milk (bovine, sheep, or goat) and the milk composition influence the yield and quality of the cheese [4].

A study on an Italian artisanal cheese, obtained from raw cow's milk with an aqueous extract of *C. cardunculus* flowers, demonstrated that the curd forms slowly and that, after 30–45 days of maturation, the aroma of the cheese is intense with the scent of thistle and wild grass, while the flavor is slightly bitter [9]. Furthermore, it has been observed that cheese, obtained by using raw milk, contains lactic acid bacteria (*Lactococcus lactis* and *Leuconostoc mesenteroides*) up to two weeks of ripening, indicating that enzymes derived from *C. cardunculus* will not have a relevant effect on cheese ripening and in the release of volatile compounds that contribute to the cheese flavor [10].

Herein, artichoke, cardoon, fig latex, and papaya extracts were further investigated for valuable rennet for producing cheese, while other sources, such as pineapple (*Ananas*

comosus (L.) Merr.) and oyster mushroom (*Pleurotus ostreatus* (Jacq. ex Fr.) P. Kumm.), were of interest, and their clotting ability, as well as their milk coagulation activity, were innovatively analyzed and compared to that of calf rennet. Therefore, some physical parameters of the curds obtained from pasteurized whole milk were measured. In addition, the lipase/esterase activity of the extracts was measured since this activity influences the cheese flavor. The free fatty acid profile was further investigated, focusing on medium- and long-chain compounds, especially essential ω6- and ω-3 fatty acids (FAs), which can positively affect lipid quality indices and the nutritional value of FA-containing food products.

2. Materials and Methods

2.1. Chemicals and Raw Materials

All the reagents were purchased from Merck-Sigma-Aldrich (Darmstadt, Germany); rennet powder was purchased from Caglificio Clerici (Como, Italy), with 175 International Milk Clotting Units (IMCU/mL); and pasteurized whole milk was purchased at a market and was from a well-known Italian brand.

2.2. Plant, Fruit, and Mushroom Sources and Their Extraction

Artichokes (*Cynara cardunculus* var. *scolymus*), cardoons (*Cynara cardunculus*), and *Ficus carica* were cultivated without the use of chemicals and pesticides in an agricultural zone of the Acerra city (near Naples, Italy). Pineapple, papaya, and the fungus *Pleurotus ostreatus* were bought from organic crops. Plant, fruit, and mushroom samples were collected during their expected maturation time. Internal bracts of artichokes, whole oyster mushroom and cardoons, and the pulp of pineapple and papaya were utilized. The fresh materials were dried under a stream of air at 45 °C and stored at 4 °C until their use. Fig milky sap was taken and immediately used in mini-curd making.

The extracts were prepared following the procedure described by Liburdi et al. [4], with some modifications. The dried materials (10 g for each considered source) were incubated in 30 mL of tap water for 6 h under stirring at room temperature. After incubation, the supernatants were recovered by centrifugation (10 min at 9800× *g*, 4 °C) and freshly used for assays and mini-curd making. Fresh extracts were evaluated for their color and smell (see Table 1).

Table 1. Main characteristics of the vegetable and fruit rennet. The extract from vegetables and fruits were obtained in tap water starting from dried material. The extracts were tested for their pH, esterase activity, and protease activity (% of digested BSA). Milk-clotting activity (MCA) was determined and indicated as Standard Units (SUs); see Materials and Methods section. The fresh cheese curds obtained were weighted and observed for their color and odor. The yield of cheese curds was determined considering the amount obtained starting from 500 mL of milk.

	Calf Rennet	Internal Bracts of Artichoke	Cardoon	Pineapple	Papaya	Fig Milky Sap	Oyster Mushroom
pH	5.5	5.8	5.5	6.2	5.3	5.0	5.5
esterase activity (U/mL)	0.017 ± 0.001	0.010 ± 0.001	0.008 ± 0.001	0.013 ± 0.002	0.004 ± 0.001	0.012 ± 0.001	0.016 ± 0.002
protease activity § (% BSA digested)	46 ± 4	>99	>99	28 ± 5	18 ± 4	51 ± 4	98 ± 2
MCA (SU ◊)	8000	800	667	1067	593	8000	640
fresh c.p.* color	straw yellow	light greenish	light greenish	straw yellow	yellow	white	straw yellow
fresh c.p.* odor	fresh cheese	artichoke	hay	n.d.	n.d.	fig	fresh mushrooms
c.p.* yield from 500 mL milk (g)	72 ± 5	68 ± 3	70 ± 4	57 ± 3	67 ± 4	69 ± 3	51 ± 4

* c.p. = cheese curd; § the incubation time was 10 min; ◊ SU = Standard Unit (see M&M section); n.d. = not detectable.

2.3. Esterase Activity

The esterase activity was spectrophotometrically measured considering the enzymatic hydrolysis of the *p*NP-hexanoate substrate by the esterases in the studied extracts. The absorbance was measured at 410 nm due to the initial rates of *p*-nitrophenoxide release from the *p*NP-hexanoate hydrolysis using a Cary 100 spectrophotometer (Varian, Belrose, NSW, Australia). Assays were performed at 30 °C in a mixture containing 40 mM sodium phosphate buffer (pH 7.5), 4% acetonitrile, and 100 µM *p*NP-hexanoate and using 5 and 10 µL of each extract, or 1 and 2 µL of calf rennet. Assays were carried out in duplicate, and the results were expressed as the means of two independent preparations. The enzymatic activity was expressed as U/mL of an extract; one unit of activity is the amount of protein that released 1 mol of *p*-nitrophenoxide/min from *p*NP-hexanoate (at the temperatures used) [11]. The absorption coefficient used for *p*-nitrophenoxide was 14,000 M^{-1} cm^{-1} [11].

2.4. Proteolytic Activity

The proteolytic activity was detected using Bovin Serum Albumin (BSA) in the assay. Briefly, in a solution of 15 µL phosphate buffer 10 mM, pH 7.0, containing 5 µg of BSA, 5 µL of each extract was added. The mixture was incubated 10 min at 37 °C; 1 µL of calf rennet was used. After the incubation, the reaction was stopped by adding 10 µL of O'Farrell solution. The samples were boiled and loaded onto sodium dodecyl sulfate-polyacryl-amide gel electrophoresis (SDS-PAGE; 12.5%), as described by Laemmli [12]. The electrophoretic analysis was performed using a Mini-Protean II cell unit (Bio-Rad, Hercules, CA, USA). After running, the gel was stained with Coomassie brilliant blue. The gel image was acquired and used to quantify the remaining fraction of undigested BSA (we used GelQuantNET software available at www.biochemlabsolutions.com/GelQuantNET.html (accessed on 3 March 2024)). The analysis was repeated two times.

2.5. Assay for Milk-Clotting Activity (MCA)

The MCA was determined following the procedure by Zhang et al. [13]. For each sample, 10 mL of whole pasteurized milk (about 3% of fat) was used, and its pH value was adjusted to 6.0 with citric acid (0.5 M; pH 2.0). A 50 mL test tube containing the milk was pre-incubated at 35 °C for 10 min; then, 0.5 mL of each investigated extract was added. Calf rennet and fig milky sap, being more concentrated, were added at lower amounts (0.1 mL). The samples were vortexed and incubated at 35 °C, and the time to the formation of the first visible clot was recorded. The enzymatic activity to clot 1 mL of milk in 40 min was defined as 1 Standard Unit (1 SU). The MCA of all the samples was calculated using the formula SU = (2400 × 10 × D)/(0.5 × T), where D = sample dilution (times diluted) and T = coagulation time (in seconds) [13].

2.6. Mini-Curd Making

Preliminarily, the ability of potential vegetable and fruit rennet to obtain cheese curd was tested. For this purpose, the pH of 50 mL of pasteurized whole milk (approximately 3% fat) was brought to 6.7 by adding citric acid (0.5 M; pH 2.0) and subsequently 2.5 mL of the plant extract. In the case of milky sap, 0.5 mL was added. The control cheese was obtained by adding 1 mL of diluted rennet powder (1 mg/mL, in 20 mM sodium phosphate buffer, pH 5.5, 860 IMCU/g; powdered rennet from Caglificio Clerici, Como, Italy). The standard process for producing semi-hard cheese was adapted. After this preliminary step, for the subsequent analysis, a scale up with 500 mL of whole milk was exploited to obtain semi-hard cheeses [14]. Two cheese-making tests were carried out. After cooling to 35 °C, vegetable rennet or calf rennet were added as a control; after 10 min, when the curd reached the appropriate strength, it was cut to the appropriate granulometry (8–10 mm), and the mixture of curd and whey was heated to 45 °C at a rate of 1 °C/min, under stirring. Then, the curd was placed in the molds, pressed to facilitate the expulsion of the whey for 20 h, and placed for 1 h in saturated brine (NaCl, 20%, *w*:*v*) at 25 °C. After 2 h of dripping, the

experimental cheeses were stored under vacuum at 4 °C for 30 days. At the end of this time, the mini-curds were used for analysis.

2.7. Global Composition of Cheeses

The cheeses were analyzed in three independent replicates for their physical features. pH was measured using the method of the American Public Health Association (APHA) [15]. Moisture [16] was removed through oven drying at 102 ± 1 °C (International Dairy Federation, 1982: 4A), while the fat [17] and protein [18] contents were determined using the Gerber–Van Gulik method (International Organization for Standardization, 1975) and the macro–Kjeldahl method (International Dairy Federation, 1993: 20B), respectively.

2.8. FFA Extraction and UHPLC-HRMS Quali-Quantitative Profile

To aliquots (1.0 g) of each cheese (1:20, *w*:*v*), 20 mL of 2-propanol was added. After vortexing for 3 min, each sample was sonicated at 40 KHz frequency for 30 min in an ultrasonic bath (Ultrasonics™ Bransonic™ M3800-E, Danbury, CT, USA). Then, the samples were centrifuged at 4800 rpm for 4 min in a Beckman GS-15R centrifuge (Beckman Coulter, Milano, Italy) equipped with an S4180 rotor and dried under vacuum (Heidolph Hei-VAP Advantage, Schwabach, Germany). The whole extraction procedure was repeated three times.

Before injection in the UHPLC-HRMS apparatus, the samples were re-dissolved with proper volumes of LC-MS-grade *n*-hexane and filtered on 0.2 µm RC membrane syringe filters (Millex, Phenomenex, Torrance, CA, USA). The UHPLC method was carried out on a NEXERA UHPLC system (Shimadzu, Tokyo, Japan), using a Luna® Omega C18 column (50 mm × 2.1 mm i.d., 1.6 µm particle size; Phenomenex, Torrance, CA, USA). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid. A linear elution gradient was optimized, starting from 55% B, held for 0.5 min; then, it ramped to 90% B in 7 min and was maintained for 1 min before restoring the initial conditions. The flow rate was 0.5 mL/min and the injection volume 2 µL.

HRMS analyses were performed using an AB SCIEX TripleTOF® 4600 spectrometer (AB Sciex, Concord, ON, Canada), equipped with a DuoSpray™ ion source operating in negative electrospray (ESI) ion mode. The APCI probe was used for automated mass calibration in all scan functions using the Calibrant Delivery System (CDS). An untargeted approach was developed, consisting of a full scan TOF survey in the mass range 100–600 Da with an accumulation time of 250 ms and eight Information-Dependent Acquisition (IDA) MS/MS scans in the mass range 80–500 Da with an accumulation time of 100 ms. The applied source parameters were the following: curtain gas (CUR) 35 psi, nebulizer gas (GS 1) 60 psi, heated gas (GS 2) 60 psi, ion spray voltage (ISVF) −4.5 kV, interface heater temperature (TEM) 600 °C, declustering potential (DP) 60 V, and collision energy (CE) 45 V with a CE spread of 15 V. The instrument was controlled by Analyst® TF 1.7 software, while data processing was carried out using PeakView® software version 2.2.

Oleic, linoleic, and palmitic acids were used as standard compounds to build up calibration curves to quantify monounsaturated (MUFAs), polyunsaturated (PUFAs), and saturated fatty acids (SFAs), respectively. The results were expressed as a mean weight percentage of total FFAs ± SD.

Lipid quality indices were calculated according to Ulbricht and Southgate [19], Osmari et al. [20], Medeiros et al. [21], and Ivanova and Hadzhinikolova [22].

2.9. Statistical Analysis

All the results' values are expressed as mean values ± standard deviation (SD), derived from measurements carried out on two samples (plant, fruit, and mushroom matrices or cheeses therefrom) in two or three independent replicates. The number of replicates performed was properly specified in the paragraphs related to each analysis. The data for the principal component analysis were managed using the OriginPro 2015 software.

3. Results

In this study, the milk coagulation activity of different vegetable and fruit extracts was analyzed and compared to that of the calf rennet. Then, the extracts were utilized to produce experimental cheese, with the aim of acquiring data on different parameters that characterize the cheeses. In particular, semi-hard cheeses were produced.

3.1. Esterase and Protease Activities

Before analyzing the coagulation capabilities of milk, the extracts were examined for their esterase and protease activities. To define the reference values for these activities, powdered calf rennet solubilized in water at 175 IMCU/mL was used. The vegetable and fruit rennet were obtained as reported in the Materials and Methods section, while the milky fig sap was used fresh and diluted 10 times in water. At first, the pH value of all the extracts was measured. The pH values were all in the range of 5.0–6.2 (Table 1), in line with the pH range of 5.0 and 6.5, which is the range values observed in curds. The pH value impacts the firmness and the water-holding capacity of the cheese [23]. When in vitro esterase activity was measured (Table 1), it was observed that the oyster mushroom extract showed an activity (0.016 U/mL) similar to that exhibited by calf rennet (0.016 U/mL), followed by the pineapple extract, which, in turn, appeared to be more active than fig milky sap. The least active extract was papaya, which showed only 25% of the esterase activity with respect to calf rennet (0.004 U/mL; Table 1).

An assessment of the protease activity of the presumed vegetable rennet was also carried out. Indeed, all the vegetable matrices used in this study were chosen for the presence of known protease activity. In fact, cardosins A and B are present in cardoon and artichoke, ficin in fig latex, papain in papaya, bromelain in pineapple, and several proteases in oyster mushroom [24]. To evaluate the protease activity, using BSA as a substrate, the level of hydrolysis of BSA was considered. A difference in the hydrolysis pattern is the parameter that allows the activity to be measured and reveals the effectiveness of the different types of proteases in the extracts based on the activities recorded for calf rennet.

The SDS-PAGE analysis showing the protease activity of the plant and fruit extracts is reported in Figure 1. Also reported in Table 1, this analysis revealed that calf rennet hydrolyzes approximately 46% of the BSA, while artichoke, thistle, and oyster mushroom more than 98%. A protease activity of approximately 50% is shown for the fig milky sap extract, while low hydrolysis rates were recorded for pineapple and papaya (28 and 18%, respectively). There are various products obtained from the BSA hydrolysis. With regard to calf rennet, there is a residual polypeptide of approximately 35.0 kDa; a similar polypeptide is also present for the artichoke plus other peptides that is approximately 20.0 kDa. The cardoon extract showed polypeptides that are approximately 20.0, 25.0, and 30.0 kDa, while the pineapple and papaya extracts allowed for short peptides of approximately 20.0 kDa to be released. A polypeptide of approximately 45.0 kDa was released by fig milky sap and oyster mushroom. The latter further showed polypeptides at approximately 60.0, 30.0, 25.0, and 20.0 kDa.

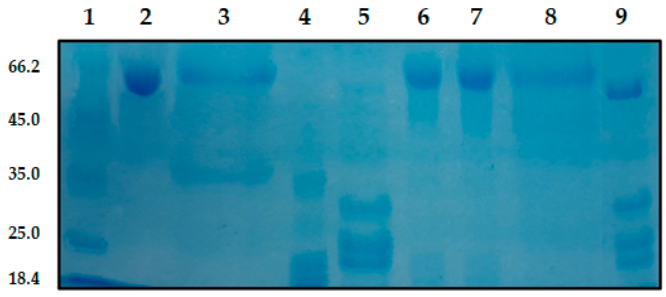


Figure 1. The 12.5% SDS-PAGE analysis. To evaluate the level of proteolytic activity of the vegetable and fruit rennet, 5 µg of BSA was used for each sample. (1) Molecular weight markers; (2) control, only 5 µg BSA; (3) 5 µg BSA + 2 µL calf rennet; (4) 5 µg BSA+ 5 µL artichoke; (5) 5 µg BSA+ 5 µL cardoon; (6) 5 µg BSA+ 5 µL pineapple; (7) 5 µg BSA+ 5 µL papaya; (8) 5 µg BSA+ 5 µL fig milky sap; (9) 5 µg BSA + 5 µL oyster mushroom.

3.2. Milk-Clotting Activity (MCA)

Milk coagulation is a fundamental step in cheese making. The clotting enzymes influence the cheese yield, texture, and flavor. For this reason, the MCA was measured for the vegetable and fruit extracts. The activity was measured according to Zhang et al. [13], by recording the time for the formation of the first visible clot. The milk-clotting activity (MCA) Standard Units (SU), which refer to the specificity of hydrolysis of the clotting enzyme towards κ-casein, are listed in Table 1, while Figure 2 depicts the samples experimentally investigated.

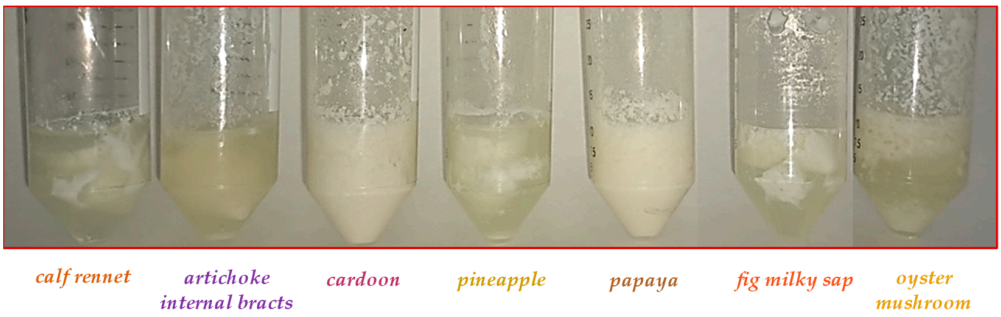


Figure 2. Milk-clotting activity of the investigated vegetable and fruit rennet, obtained using 10 mL of whole milk and 0.5 mL of the extracts except for fig milky sap (0.1 mL). Calf rennet was added in a volume equal to 0.1 mL.

The most efficient clotting enzymes were from calf rennet and fig milky sap, showing values of 8000 SU. Lower efficiencies were found for cardoon, papaya, and oyster mushroom, having MCAs of about 600 SU, whereas the extract from artichoke internal bracts was 10-fold less efficient than calf rennet (800 SU). Finally, the pineapple extract was about 8-fold less efficient than calf rennet, showing an MCA value equal to 1067 SU.

3.3. Mini-Curd Making

These enzymatic activity data were preliminarily acquired to study the use of vegetable and fruit rennet in cheese making. For this purpose, 500 mL of milk was used for each type of experimental cheese. A procedure to produce semi-hard cheese was followed. The curd was placed in molds, pressed for 20 h to facilitate the expulsion of the whey, and pickled. After 2 h of dripping, the obtained experimental cheeses were stored under vacuum at 4 °C for 30 days before they were analyzed (Figure 3).

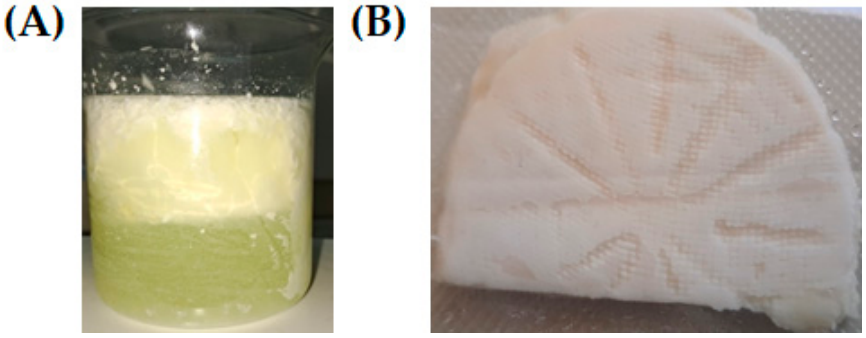


Figure 3. Mini-curd making. (**A**) For each rennet, 500 mL of whole milk was used and the procedure for semi-hard cheese preparation was followed. (**B**) At the end of the brine step, the cheese was preserved under vacuum at 4 °C for 30 days before the analysis. The mini-curd making were prepared in duplicate.

The first evaluation concerned the color and smell of the fresh pasta obtained. As expected, the colors ranged from yellow to light greenish (artichoke and thistle) (Table 1),

while the odor was influenced by the different extracts used, with typical odors of fresh cheese with calf rennet, artichoke with artichoke, hay with thistle, fig with milky fig sap, and fresh mushrooms with oyster mushroom and no particular odor detectable with papaya and pineapple (Table 1). Then, the yield expressed in grams of cheese obtained from 500 mL of milk was examined. As reported in Table 1, the best yield was observed with calf rennet (72 g of cheese curd per 500 mL of initial milk), while the lowest yield was observed with oyster mushroom (51 g). A good yield was observed for pineapple (58 g), whereas for the extracts from artichoke, thistle, papaya, and milky fig sap, the values were comparable with that from calf rennet (Table 1).

3.4. Cheese Chemical Composition

The chemical compositions of the control and experimental cheeses were also analyzed after 30 days of ripening. In particular, pH value, and the moisture, fat, and protein contents [25] were measured and are reported (as %) in Table 2.

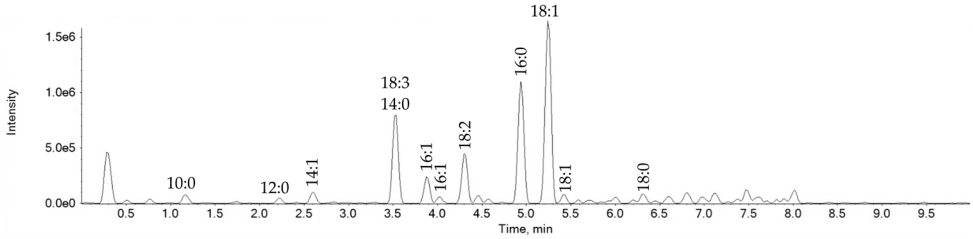
Table 2. Chemical composition of cheeses, determined at the end of ripening (30 days). Moisture, fat, and protein content are expressed as %. Results are mean of two independent experiments. Control cheese was obtained by using calf rennet.

	Calf Rennet	Internal Bracts of Artichoke	Cardoon	Pineapple	Papaya	Fig Milky Sap	Oyster Mushroom
pH	5.05 ± 0.09	5.09 ± 0.07	5.04 ± 0.02	5.07 ± 0.03	5.05 ± 0.04	5.06 ± 0.03	5.08 ± 0.04
moisture	38.1 ± 0.2	49.2 ± 0.4	42.3 ± 0.3	45.7 ± 0.7	48.6 ± 0.6	43.1 ± 0.5	41.8 ± 0.3
fat	35.7 ± 0.3	29.2 ± 0.5	33.5 ± 0.2	32.2 ± 0.5	30.4 ± 0.5	32.8 ± 0.4	33.1 ± 0.2
protein	25.6 ± 0.3	21.4 ± 0.4	23.8 ± 0.6	24.0 ± 0.5	24.1 ± 0.4	23.6 ± 0.3	24.9 ± 0.3

While the pH values were similar for all cheese curds and were around 5.0, marked differences were observed for the other parameters considered. In particular, the moisture content was very different. In fact, the moisture in the cheese obtained with calf rennet was 38%. This value is similar to that recorded for the thistle and the thistle mushroom (which showed values around 42%). The highest percentage of humidity was determined for the cheese from artichoke rennet (49.2%), papaya (48.6%), pineapple (45.7%), and milky fig sap (43.1%). The data are reported in Table 2. A fat content between 32 and 33% characterized a large part of the cheeses (cardoon, pineapple, fig sap, and oyster mushroom), whereas it was lower in the artichoke and papaya rennet cheeses (about 30%). These fat content values were lower than that of cheese from calf rennet, which was equal to 35.7% (Table 2). The cheese curds produced exhibited a similar protein content in the 24–26% range. The only exception is represented by cheese curd obtained with artichoke rennet, for which a value of 21.4% was recorded (Table 2).

3.5. FFA Determination

Based on a UHPLC-MS analysis in negative ion mode, thirteen free fatty acids (FFAs) were identified and quantified. They were medium- and long-chain FFAs, whose qualitative profiles are reported in Figure 4. The total content of PUFAs in the control cheese, obtained using the calf rennet, was very similar to that calculated for the other samples, in which plant and fruit extracts were employed as milk-clotting agents. Linoleic acid (18:2, ω-6), whose identity was confirmed by injection of the pure standard compound, was the most abundant one in all the samples, followed by α-linolenic acid (18:3, ω-3). Their ratio ranged from 1.4 in the cardoon-based clotted cheese to 2.7 in the mushroom-based sample. A second octadecadienoic acid was tentatively identified as rumenic acid, whereas the less common octadecenoic acid was putatively vaccenic acid.



	FA's name	$[M–H]^-$ (theor. m/z)	error (ppm)
10:0	capric acid	171.1391	(0.9)
12:0	lauric acid	199.1704	(–0.3)
14:0	myristic acid	227.2017	(2.0)
16:0	palmitic acid *	255.2334	(–0.6)
18:0	stearic acid	283.2354	(–2.0)
14:1	myristoleic acid	225.1860	(0.0)
16:1	palmitoleic acid	253.2179	(–1.2)
16:1	hexadecenoic acid	253.2179	(0.4)
18:1	oleic acid *	281.2489	(–0.4)
18:1	octadecenoic acid	281.2489	(–0.4)
18:2	linoleic acid *	279.2334	(–0.6)
18:3	α-linolenic acid	277.2178	(–1.5)

* Confirmed by standard compounds

Figure 4. Representative BPC (base peak chromatogram) of medium- and long-chain FFAs identified in cheese samples. FA = Fatty Acid; Theor. m/z = mass-to-charge ratio, theoretical value.

Among the samples, slight differences were observed with regard to the content in the MUFAs and SFAs. In the first FFA subgroup, oleic acid (18:1; m/z 281.2489) contributed the most to the total percentage, especially in cheeses made with calf rennet, and milky sap from *F. carica* and *P. ostreatus* (about 21 and 20%, respectively). Finally, among the saturated FA, palmitic acid was the main constituent, with an estimated content equal to 22% in the calf rennet sample and variable percentages in the plant-based clotted cheese, ranging from a minimum of 12% in the case of cardoon to a maximum of 24% in the case of mushroom.

The relative quantitation of free fatty acids in the produced cheeses is reported in Figure 5A. The quantitative profile was obtained from the UHPLC-HRMS analyses, and each FFA content was expressed as mg/g of cheese. Figure 5B shows the plot of the data using a principal component analysis. The graph highlights that while a large part of the saturated and monounsaturated fatty acids do not discriminate the cheeses produced, the richness in palmitic, oleic, and linoleic acid differentiates the cheeses produced with fig rennet, oyrnet mushroom, and artichoke.

The relatively higher content of α-linolenic acid places cheeses made from thistle, pineapple, and papaya rennet in a common quadrant. Indeed, calculating the ω6/ω3 ratio, it was observed that cheeses from pineapple and papaya shared a value equal to 1.6, and cardoon rennet-based cheese showed a linoleic acid/α-linolenic acid ratio equal to 1.4. Cheeses from artichoke, fig milky sap, and oyrnet mushroom exhibited ω6/ω3 ratios equal to 2.1, 2.3, and 2.7, respectively. This finding highlights that the free fatty acid profile was positively affected by using vegetable rennet and allowed α-linolenic acid, the essential ω3 fatty acid, to be increased for a healthier diet.

To support this statement, based on the FFA quantitative profile, lipid quality indices were also determined. It was found that the use of pineapple, cardoon, and papaya extracts as clotting agents guaranteed their improvement when compared to the control cheese. Indeed, hypocholesterolemic fatty acids (DFAs) were slightly enhanced, whereas hypercholesterolemic fatty acids (OFAs) decreased, giving rise to 1.01-, 1.16-, and 1.24-fold overall increases in their ratio, respectively (Figure 6A). Moreover, lower values of the atherogenicity (AI) and thrombogenicity (TI) indices were found for the same samples, with a more pronounced effect observed for cardoon (1.6 and 1.8-fold reductions, respectively) (Figure 6B).

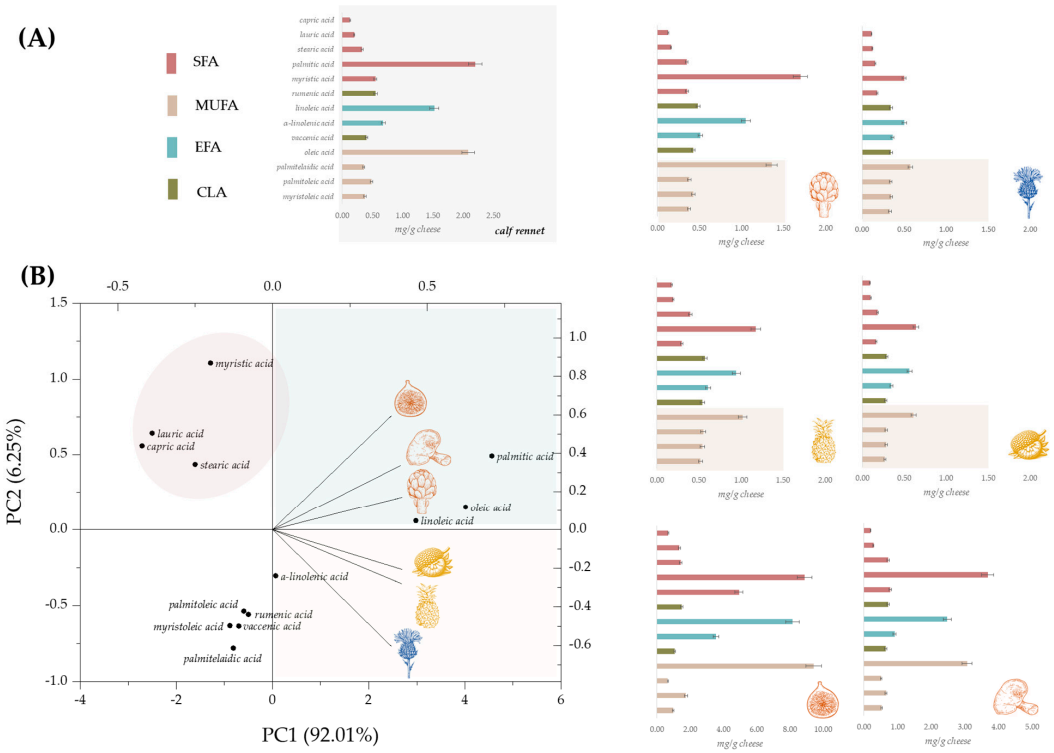


Figure 5. (**A**) Relative content (mg/g of cheese; mean value of independent measurements ± SD) of identified FFAs in cheeses obtained with different vegetable rennet. (**B**) Principal component analysis (PCA) biplot of FFA data in samples under investigation. Data were processed using OriginPro 2015 software.

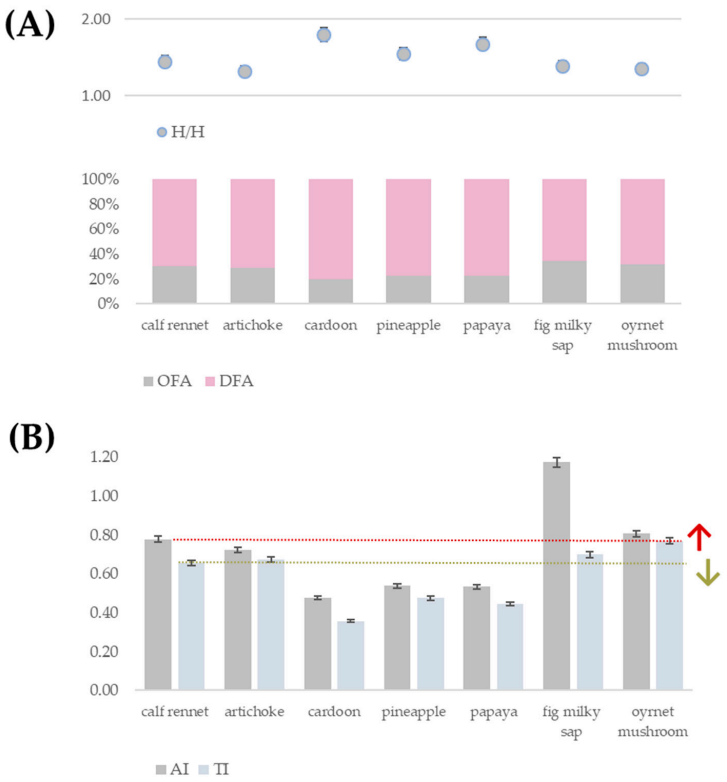


Figure 6. Lipid quality indices: (**A**) hypocholesterolemic fatty acids (DFAs), hypercholesterolemic fatty acids (OFAs), and their ratio of hypocholesterolemic and hypercholesterolemic fatty acids (H/H);

(**B**) index of atherogenicity (AI) and thrombogenicity (TI). The different color of the arrows are to highlight the upper values of AI or the down values of TI, respect to the control.

4. Discussion

Herein, a comparative study among calf rennet and vegetable/fruit extracts used as rennet in cheese making was carried out. Some artisanal cheeses were prepared with artichoke and cardoon [5], and it is known that the protease ficin, present in milky sap of figs, has clotting activity [4]. In this context, the putative clotting activities of the other three vegetables, namely papaya, pineapple, and the mushroom *Pleurotus ostreatus*, which are rich in proteases, were explored. In addition to the clotting activity, the esterase activity in these vegetable extracts was investigated since the hydrolysis of esters is critical in the development of cheese flavor.

Protease and esterase activities were detected in vitro for all the vegetables and fruits. Different classes of proteases were revealed, which can act diversely. Moreover, artichoke, cardoon, and *Pleurotus ostreatus* showed high rates of BSA hydrolysis, being around 98–99%, whereas fig milky sap and calf rennet showed rates at about 50%, pineapple at 28%, and papaya at 18% (Figure 1, Table 1). The esterase activity was comparable for all the vegetable extracts with the calf rennet esterase activity. The papaya extract was the only one showing a low esterase activity (Table 1).

The milk-clotting activity of vegetable and fruit rennet was also evaluated since this parameter is linked to the ability to obtain curd. As reported in Table 1, all the extracts used showed milk coagulation activity, but only the milky fig sap was comparable to calf rennet; all the others had coagulant activities approximately 10 times lower than calf rennet. In particular, it was verified that by using a time interval similar to that of calf rennet, a lower curd yield was obtained, but by increasing the incubation time, a good curd yield was obtained. Larger quantities of plant and fruit extracts were not used to avoid them altering the final flavor.

The yield of the experimental cheeses, obtained starting from 500 mL of milk, was approximately 70 g of the curd with calf rennet, artichoke, thistle, papaya, and milky sap, while it was about 50 g with pineapple and mushrooms (Table 1). In terms of the chemical composition of the experimental cheeses, after 30 days of maturation, the moisture level was 10 to 30% higher than the calf rennet value (Table 2), whereas the protein content was comparable in all samples analyzed.

When the free fatty acids (FFAs) produced at the end of maturation were analyzed, important differences were observed between the cheeses produced with the different vegetable and fruit rennet. The data acquired underline an enrichment of the nutritional value of these products, which could positively impact the health of consumers. Although FFA profiling has long been achieved through gas chromatographic analysis after derivatization, the coupling of UHPLC with mass spectrometry [26] could be considered valuable, as it exploits the acidity of the carboxyl hydrogen atom for correct ionization in negative ion mode. In fact, in recent years, this latter approach has become very popular [27].

The content of FFAs in cheeses depends on several factors that contribute to fluctuations. The first is obviously related to the milk used, not only with regard to the animal species but also the animal feed, feed seasonality, breed, lactation stage, etc. [28]. Apart from that, it should be considered that the FFA profile in cheeses could differ from that of the milk used for production. In fact, cheese production technology and ripening times are pivotal in determining their relative amount in the final product. Although the content of SFAs found in the present work is lower if compared to the literature data, the ratio with MUFA total amount, expressed in percentage, was in accordance with previous data regarding the FFA fluctuations due to different ripening times [29]. Moreover, the hypothesis that the relative content could also be due to the plant matrix used as milk-clotting agents should not be discarded. Among the PUFAs, the only ω-6 and ω-3 acids detected were linoleic and α-linolenic acids, respectively, whose ratios ranged from 1.4 to 2.7. A second 18:2 PUFA was detected, although as a minor constituent. Its identity could be

ascribed to rumenic acid, the main conjugated linoleic acid (CLA), deriving from microbial metabolic processes occurring within the digestive tracts of ruminants and accumulating in the mammary gland [30]. Thus, it can be found in milk and dairy products, like yogurt or cheese, as well as vaccenic acid produced by the biohydrogenation processes in rumen, whose concentration is strongly influenced by cow feed.

It has been reported that maintaining an optimal balance between ω-6 and ω-3 fatty acids is essential for promoting a healthy lifestyle [31], in particular with a ω-6/ω-3 ratio > 4:1 [31]. Lower values than the control cheese were obtained for samples in which the pineapple, cardoon, and papaya extracts represented the milk-clotting agents. The results found using the cardoon extract were the most promising in this context and appeared lower than those recently reported in the literature [32], likely due to the different cheese-making processes and the direct use of dried flower instead of the extract in that case.

5. Conclusions

The use of vegetable rennet is of growing interest. In this context, the possibility of using enzymes extracted from plant and fruit sources to produce cheese was verified. For this purpose, in a preliminary phase, the coagulant capacity of the plant extracts and the esterase activity, indicated as a useful parameter for determining the flavor of cheeses, were verified. The data acquired are encouraging because they have highlighted the potential of these vegetable and fruit rennet. It is very important to balance the quantity of extracts and the coagulation time because the proteolytic enzymes of vegetables have a high protease activity and the excessive hydrolysis of caseins during cheese making gives bitter flavor notes. In conclusion, vegetable and fruit rennet can represent an alternative to the traditional use of animal rennet in cheese making. These new cheeses are compatible with vegetarian and vegan diets. The use of vegetable rennet is sustainable with the growing demand for rennet for cheese production, which represents an "inexhaustible" source. The fatty acid profile highlights interesting variations in the content of palmitic and oleic acid and in the ratio between linoleic and α-linolenic acid. This suggests that vegetable and fruit rennet can offer new health opportunities, promoting a pleasant intake of essential omega-3 fatty acids, increasingly indicated as a health promoting factor.

Author Contributions: Conceptualization, S.P. (Severina Pacifico), E.C. and L.M.; methodology, S.P. (Severina Pacifico) and L.M.; formal analysis, S.P. (Simona Piccolella) and L.M.; investigation, S.P. (Simona Piccolella) and E.C.; writing—original draft preparation, S.P. (Severina Pacifico), E.C. and L.M.; writing—review and editing, S.P. (Severina Pacifico), E.C. and L.M.; supervision, L.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by IGB-CNR and IRET-CNR, who covered the overhead costs.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Acknowledgments: We are grateful to Bernard Loeffler and Francesca Varrone for their excellent editing assistance.

Conflicts of Interest: The authors declare no conflicts of interest.

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