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Milk Clotting and Proteolytic Activity of *Auricularia Auricula* Fungus and Its Potential in the Production of Feta Cheese

Mahdieh Raoofi Asl Soofiani [1], Negin Noori[1*], Hassan Gandomi[1], Ali Khanjari[1], Nafiseh Alighazi[2]

1. Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
2. Department of Food Hygiene, Karaj Branch, Islamic Azad University, Karaj, Iran.

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ABSTRACT

Introduction: This study's objective was to evaluate the *Auricularia auricula* fungus as a coagulant of bovine milk and its potential in feta cheese production. The *Auricularia auricula* fungus displays high levels of milk-clotting activity, presumably due to clotting proteolytic enzymes.

Methods: Feta cheese was manufactured from bovine milk, coagulated with *Auricularia auricula* and the proteolytic properties of the fungus were determined.

Results: on polyacrylamide gel, substrates in the presence of the fungus showed higher proteolysis activity than control samples. In comparison, cheeses made with bovine rennet had more distinct protein bands than those produced with fungi. An initial evaluation of the cheese's flavor indicated a lack of bitterness and yielded positive results.

Conclusion: Due to its properties and availability, the *Auricularia auricula* fungus might be a suitable candidate for replacing calf rennet. More research is needed to evaluate its use as a coagulant in large-scale cheese production in the future.

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Introduction

Cheese is one of the most important dairy products, with its protein rich in essential amino acids. Feta is a type of cheese with a relatively salty, slightly acidic taste and a pleasant flavor that enjoys universal acceptance (1). Feta is white, with a soft texture. It is kept in salt water as it matures. Since ancient times, sheep's milk, goat's milk, or a mixture of both have been used to produce feta cheese (2). One of the main steps in cheese processing is solidifying the casein micelles in milk and converting them into a three-dimensional gel matrix. The enzyme chymosin, which is very active, is used in the preparation of animal rennet. The primary role of chymosin in making cheese is associated with the hydrolysis of the phenylalanine-methionine bond in milk casein, leading to its coagulation (3). Milk coagulation can occur in various ways. Most cheese forms a coagulum through the action of milk-clotting enzymes (4). Various types of milk-clotting enzymes, including animal, microbial, and plant-derived enzymes, have been introduced. Calf rennet, obtained from animal stomachs (rennin), was the first and foremost reported standard milk-clotting enzyme. However, due to the short shelf life of rennin, the incidence of bovine spongiform encephalopathy (BSE), and restrictions for religious reasons (e.g., Judaism and Islam), dietary preferences (e.g., vegetarianism), or objections to genetically engineered foods (e.g., Germany and the Netherlands forbid the use of recombinant calf rennet), the supply and demand for bovine rennet are limited (5). Another type of milk-clotting enzyme is a plant coagulant, which has been used for a long time as a traditional rennet. However, due to their strong proteolytic activity and poor textural characteristics, many plant coagulants are not suitable for industrial applications (3). Therefore, the search for suitable plant proteases is ongoing (6). Other proteases that can be introduced as substitutes for rennet include microbial origin proteases such as *Rhizomucor miehei*, *Rhizomucor pusillus*, and *Endotypalariatica*, which are heavily involved in cheese production. A study of the proteolytic effects in fungal rennet cheeses

* *Corresponding authors:* Negin Noori, Associate Professer, Department of Food Hygiene, Faculty of Veterinary Medicine, University of Tehran, Qareeb Street, Azadi Avenue, Tehran 14155-6453, Iran. Tel: +98-21 61117067, Fax: +98-21 66933222 Email: nnoori@ut.ac.ir.

shows that bitter-flavored cheeses were produced with Endothia but not with the mucors. Using the relative staining intensity as a guide, the coagulants' proteolytic activities on the α and β fractions were rated (from least to most) as follows: α-casein - Endothia, Mucor, rennet; and β-casein - rennet, Mucor, Endothia. Rennet mainly attacked α-casein; Endothia predominantly attacked β-casein. The Mucors affected both fractions to about the same degree (7). Another fungal protease with milk coagulation potential is a beneficial fungus with the scientific name (*Auricularia. auricula. Judea*). *Auricularia auricula* fungus belongs to the Basidiomycota phylum and is of the species *Auricularia auricula*-judae. This fungus is known by various names, including wood ears, pork ears, Chinese mushrooms, tree ears, and black and white ears. It has been traditionally used as a medicinal and food supplement in China and Korea. *Auricularia auricula* is high in carbohydrates (nearly 630 g/kg in dried weight), minerals (P, Ca, and Fe), and protein, especially leucine and lysine amino acids. Research has shown that *Auricularia auricula*-judae β-D-glucan has biological activities and exhibits potent inhibition against acinar cell carcinoma (8). The methanol extract of *Auricularia auricula* inhibited lipid peroxidation and decreased liver damage in benzo[a]pyrene-treated mice (9). As a consequence, it is considered to be a healthful food. Researchers are paying more attention to its utilization. Further studies on its medicinal or functional food properties need to be conducted. *Auricularia auricula* fungus has a short harvest period each year and grows in a wet climate. This fungus is not a staple food; nevertheless, it is considered for use in creating new varieties of foods and expanding its role as a healthy food that promotes consumer health. In the 19th century, this fungus was used in traditional medicine. Today, this fungus is used as a food ingredient, especially in soups, and is also used in Chinese medicine. This fungus helps reduce blood sugar and cholesterol. This fungus is more scattered in temperate and tropical regions, but it is found throughout Europe, North America, Asia, Australia, South America, and Africa. *Auricularia auricula* has also been observed to be consumed in countries such as Ghana, Vietnam, Nepal, Mozambique, Indonesia, Poland, and Bolivia. Given the development of the cheese-making industry in recent years, the lack of animal rennet, the disadvantages of other milk-clotting enzymes, and the excellent potential of fungal proteases, research on the use of this fungus in the cheese industry and the descriptions of its characteristics seem necessary. *Auricularia auricula* fungus can be used as an alternative source of milk-clotting enzymes during the Feta cheese-making procedure. Moreover, the enzyme's dependence on pH and temperature and its stability profiles are entirely suitable for the chemical-physical conditions adopted during the cheese-making procedure fungus's can be used as an alternative source of milk clotting enzymes during the feta cheese-making procedure. Moreover, the enzyme's dependence on pH and temperature, along with its stability profiles, are entirely suitable for the chemical-physical conditions adopted during the cheese-making process (10). Considering all the above, the present research was conducted to determine the influences of a novel alternative source of milk-clotting enzymes (*Auricularia auricula*). To our knowledge, there is a gap in the recent literature regarding the investigation of the proteolytic activity of *Auricularia auricula*. This study focuses on determining the milk-clotting activity of this fungus and conducting electrophoretic analysis of the hydrolysis products after treating crude casein and milk, as well as its potential in Feta cheese production.

Materials & Methods

Preparation of Auricularia Auricula Fungus Powder

Five hundred grams of dried *Auricularia auricula* was prepared from northern Iran (Noor city) and authenticated by the Mycology Research Center at the University of Tehran. The mushrooms were then pulverized using a grinder (IKA M20 universal) and used as a replacement protease for rennet.

Milk-Clotting Activity

The milk-clotting activity was determined using the method of Uchikoba and Kaneda (1996). First, 10% solutions of skimmed milk powder in 67 mM NaH2PO4 at pH 6.8 and 30°C were prepared as a substrate. Different amounts of the fungal powder preparation (1, 5, 10, and 15 g) were added to a final volume of substrate (3 mL) both in the absence and presence of CaCl2 (5 mM). The endpoint, based on the advent of milk clots, was observed separately in the test tube.

The test was conducted in three replicates, and the average coagulation time was reported (11).

Proteolytic Activity Assay

The reaction mixture contained 2% (w/v) total casein (Sigma-Aldrich, USA), κ-casein (Sigma-Aldrich, USA), and two kinds of milk (pasteurized semi-skimmed and pasteurized whole milk) as substrates dissolved in 67 mM NaH2PO4 at pH 7.2 and 2.5 mM DTT, as explained in Lo Piero et al. (2011). The hydrolysis against different kinds of milk was estimated by incubating the milk with 20 mg of fungal powder, following the method described by Lowry et al. (1951). The assay mixture (1 mL) was incubated for 21 minutes at 55°C. The reaction was then terminated by adding 1.5 mL of 5% (w/v) TCA (trichloroacetic acid). Blanks were prepared by adding the fungal powder at the end of the incubation period, just before the addition of TCA and precipitation. The sample tubes were centrifuged at 9000 g for 10 minutes, and the absorbance of supernatants was evaluated at 280 nm using a Beckman Spectrophotometer, DU® 650 Model, and a Shimadzu UV-VIS 1240 spectrophotometer (Shimadzu Corporation, USA). An arbitrary fungal powder unit was determined as the amount of fungal powder that yields a 0.001 absorbance change at 280 nm per minute under the assay conditions. All tests were repeated three times (12, 10).

Electrophoretic Analysis

Whole casein (10 mg), milk proteins (Sigma-Aldrich, USA), pasteurized semi-skimmed milk, and pasteurized whole milk (10 mg of proteins) were dissolved in 67 mM NaH2PO4 at pH 7.2. Fungal powder (2% w/v) was added to the incubation of each protein solution (final volume 0.3 mL), and hydrolysis was carried out for 20 minutes at 55°C (10). The samples (17 µg) were prepared for SDS-PAGE (15 cm × 20 cm) by adding an equal volume of double-concentrated loading buffer (4 g/100 mL SDS, 30 g/100 mL sucrose, 125 mmol/L Tris-HCl, pH 8.0, 0.042 mmol/L DTT, 2 g/100 mL bromophenol blue). Immediately after finishing electrophoresis, the gels were separated from the plates and stained with 0.25 g/100 mL Coomassie Brilliant Blue G-250 in 50 mL/100 mL methanol containing 7.5 mL/100 mL acetic acid, and left for 8 hours. Destaining was accomplished using a solution of 15 mL/100 mL methanol, 7.5 mL/100 mL acetic acid, and 150 mL/100 mL water (2:1:10). As previously described, protein content analysis of whey and cheese curds was determined by SDS-PAGE (17 µg). The cheese curd was centrifuged at 13,000 g for 10 minutes in a Beckman J2-HS centrifuge, rotor JA-20 (Beckman Instruments, Fullerton, CA, USA) to drain the whey (Refrigerated centrifuge model 5810 R made in Germany). The resulting pellet was dissolved by heating at 55°C in 0.2 g/100 SDS with vigorous shaking for 30 minutes, and the supernatant was discarded. Lowry's method was used to evaluate the protein content of milk, curd, and whey (10).

Cheese Manufacture

Cheese samples were prepared using 5 kg of pasteurized whole bovine milk containing 3.4% protein, 3.70% fat, and 18.28 g/100 g in total solids. The preheated milk (35°C) was inoculated with 0.5% (v/v) starter cultures and supplemented with 0.1 g of CaCl2/kg of milk. The milk was kept at 35°C for approximately half an hour to allow the starter cultures to mature before adding the fungus powder. Then, treatments containing 5% (w/v) fungus powder were added to the milk. After one hour, the curd was sliced crosswise into cubes of 1-2 cm^3. To facilitate draining, the curd was compressed for 6 hours, with pressure gradually increasing to nearly 2000 Pa in the first 2 hours. The clot was then sliced into pieces measuring 6 × 8 × 12 cm and placed in 20% (w/v) sterilized salt for 15 days at 12-14°C. After the initial maturation period (green warehouse), the cheese samples were transferred to 8% brine and refrigerated at 4°C for 6 weeks for the ripening period (4). "A primary cheese tasting was conducted to identify any bitter taste in the recently produced Feta cheese. Tasters were asked to appraise the cheese for flavor and general acceptability using a 5-point hedonic scale, rating from 1 (very undesirable) to 5 (very desirable). This was achieved by comparing the features of Feta cheese produced using *Auricularia auricula* fungus as a coagulant with those of Feta cheese produced with calf rennet. The cheeses were evaluated organoleptically by a group of seven experienced and proficient panelists (four women and three men, aged 20 to 45 years old). Feta cheese samples were divided into seven equal parts, each about 3 cm × 3 cm × 2 cm in size, and placed on white plates. The samples were randomly coded to prevent bias. This test was performed at room temperature (20 ± 2°C) under regular lighting in a single area (14).

Statistical Analysis

Statistical analysis was conducted using Microsoft Excel 2007 (Microsoft, Redmond, WA, USA). The coagulation effect of *Auricularia auricula* fungus in Feta cheese production was assessed using SPSS version 20 software. The investigations were performed in triplicate on individually isolated preparations, and the results are presented as the mean and standard deviation (±SD) of these tests. Quantitative data analysis was done using one-way ANOVA, and the results were presented as mean and standard deviation. The Tukey test was used to compare the means, with significance set at P≤0.05. Sensory characteristics were analyzed using the Kruskal-Wallis statistical test.

Results and Discussion

Milk-Clotting Activity

"The milk-clotting activity of *Auricularia auricula* fungus was tested, and the results of this investigation are shown in Table 1. The milk-clotting activity was associated with fungus powder content. As the amount of fungus increased, the milk-clotting time became notably faster. The first presence of the solid substance in the milk was recognized in 26 minutes using 1% (0.03 g) of fungus powder. The milk-clotting time was measured both in the absence and presence of CaCl2, and there was a significant difference in the milk-clotting time when using 1-10% of fungal powder in the absence and presence of this salt (P < 0.05) (Table 1).

Table 1. Milk clotting activity by *Auricularia. Auricula* in the presence and absence of 5 mM CaCl2. The values are means ± standard deviations (SD) of data from three experiments (*N* = 3). Values with the same letter do not differ significantly (P≤0.05).

Mushroom powder amounts (weight percentage)	Clotting time (CT) (min)	
	−CaCl2	+CaCl2
1	26.17±4.96^{a}	22.33±2.51^{a}
5	13.83±2.87^{b}	10.466±3.09^{b}
10	8.91±0.57^{bc}	6.18±0.75^{bc}
15	5.54±0.39^{c}	3.85±0.25^{c}

Proteolytic Activity

The proteolytic activity of *Auricularia auricula* fungus powder, assayed toward different substrates, is shown in Table 2. The *Auricularia auricula* fungus powder exhibited the highest degree of proteolysis on total casein, with 129.56 U/mg (Table 2). This amount, slightly higher than the activity obtained against κ-casein (123 U/mg), is presumably due to some lost target sites present in the total casein section but not in the κ-casein (10). However, the *Auricularia auricula* fungus powder's proteolytic activity toward whole casein and κ-casein is almost identical. There was no significant difference between them, presumably due to the fungus's high proteolytic activity affecting different caseins. The tendency toward proteolysis decreased when milk (whole and semi-skimmed) was used as a substrate. In other words, the protease's high proteolytic activity is usually regarded as an unfavorable characteristic of milk coagulants (14). The function of *Auricularia auricula* fungus powder was also measured using commercial milk as substrates (Table 2). Whole milk is the preferred substrate, displaying a specific activity amount equal to 30.9 U/mg. In contrast, the activity of *Auricularia auricula* fungus powder is slightly lower when semi-skimmed milk is used as the enzyme-substrate (25.1 U/mg). This characteristic is probably related to the lipolytic properties of the fungus.

Table 2. Hydrolysis of the total, k-casein, and two types of milk by *Auricularia. Auricula*. The values are means ± standard deviations (SD) of data from three experiments (*N* = 3). Values with the same letter do not differ significantly (P≤0.05).

Substrates	Specific activity (U/mg
Casein	129.56±2.6^{a}
k-Casein	123±16.78^{a}
Whole milk	30.9±0.1^{b}
semi-skimmed milk	25.1±0.34^{b}

Hydrolysis of Casein and Commercial Milk by Auricularia Auricula Fungus Powder Treatment

The SDS-PAGE pattern of the proteolytic particles achieved by treating either total casein or milk protein with *Auricularia auricula* fungus powder is displayed in Figure 1. The SDS-PAGE pattern of untreated whole casein shows three prominent bands corresponding to α, β, and κ-caseins, with estimated molecular weights of

33.9 kDa, 27.8 kDa, and 25.1 kDa, respectively (Figure 1, lane 3). The *Auricularia auricula* protease significantly digests total casein into a few large particles, yielding three bands with molecular weights of approximately 32.1 kDa, 21.8 kDa, and 15.8 kDa (Figure 1, lane 4) (10). The same pattern is observed when milk protein is the substrate (Figure 1, lanes 5 and 6).

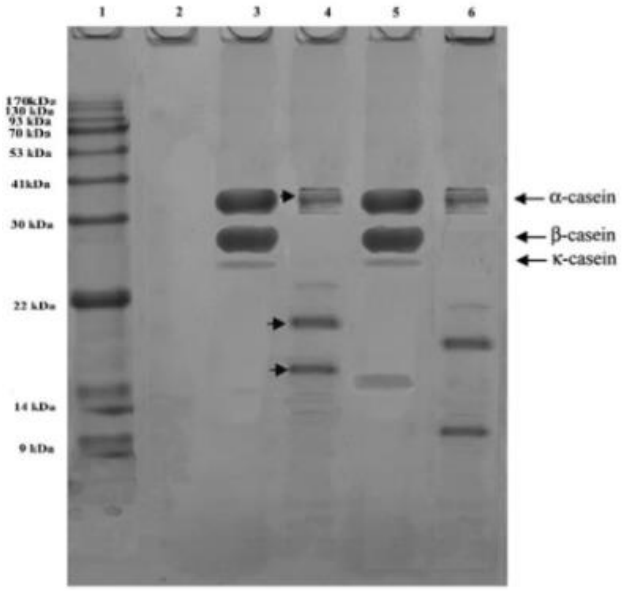

Figure 1. SDS-PAGE patterns of the whole casein and milk protein subjected to the powder of *Auricularia auricula* fungus treatment. 17 µg of each sample was loaded onto 12.5% slab gel. Lane 1: molecular weight standard; lane 2: powder of *Auricularia auricula* fungus (5 µg); lane 3: control total casein; lane 4: treated total casein; lane 5: control whole milk; lane 6: treated whole milk

The SDS-PAGE pattern obtained after fungus treatment of milk with different fat contents (whole milk and semi-skimmed milk) and their hydrolysates is displayed in Figure 2 (lanes 7-10) and reveals another level of hydrolysis on α-casein, β-casein, and κ-casein (Figure 2, lanes 8 and 10). The main digestion products detectable in both types of milk probably correspond to κ-casein hydrolysates with a visible molecular weight of 15.8 kDa (Figure 2, lanes 8 and 10).

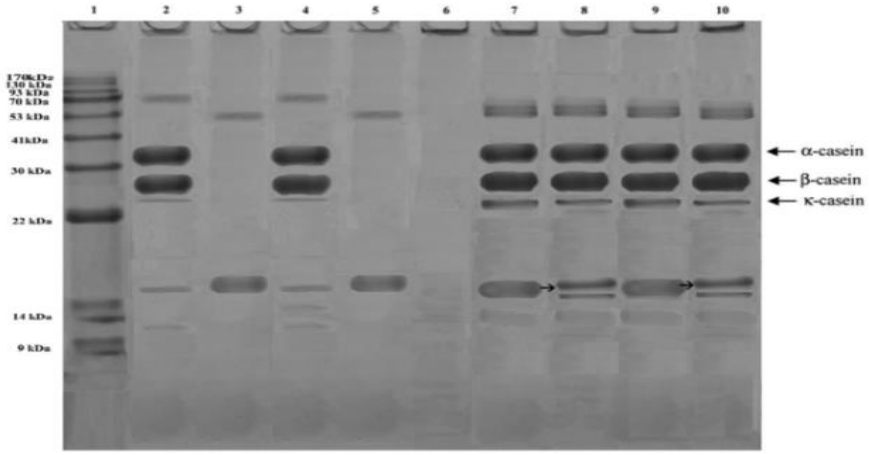

Figure 2. SDS-PAGE pattern of the curd cheese before salting. Samples corresponding to 5% in protein were loaded onto 12.5% slab gel. Lane 1: molecular weight standard, lane 2: treated curd cheese; lane 3: treated whey protein; lane 4: control curd cheese; lane 5: control whey protein; lane 6: powder of *Auricularia auricula* fungus (5 µg); lane 7: control whole milk; lane 8: treated whole milk; lane 9: control semi-skimmed milk; lane 10: treated semi-skimmed milk.

Comparing the SDS-PAGE patterns obtained after treating commercial milk (pasteurized whole milk and pasteurized semi-skimmed milk) with fungus powder to those obtained by treating total casein and milk protein shows significant digestion, likely corresponding to κ-casein hydrolysates, detectable in all samples. Other casein particles are degraded to a lesser extent. Although it was expected that the protective function of the milk fat, which might cover some of the casein target residues, would result in less hydrolysis of milk by the fungus compared to casein and milk protein, the hydrolysis levels were almost the same. (10).These results could be related to the lipolytic properties of this fungus, which can decompose milk fats and reduce their inhibitory effect on casein's target positions.

Feta Cheese Manufacture

This research utilized whole bovine milk (3.40% protein, 3.70% fat, and pH 6.7) for Feta cheese production using *Auricularia auricula* fungus powder as a coagulant. The SDS-PAGE pattern of the proteolytic particles obtained indicates that the coagulation observed from treating whole bovine milk with the fungus powder corresponds to κ-casein coagulum, and the composition of curd cheese before salting was investigated by SDS-PAGE (Figure 2). The main casein fractions reveal their complete digestion into three main particles with apparent molecular weights of 24 kDa and 15 kDa, respectively, and a smaller portion of about 11 kDa (Figure 2, lane 2). The same pattern is also observed when calf rennet is used as a milk coagulant to produce Feta cheese (Figure 2, lane 4). The whey proteins are separated from the casein clot, and the SDS-PAGE pattern is shown in Figure 2. By comparing these outcomes with those collected from the hydrolysis of milk (Figure 2) and data obtained from the SDS-PAGE pattern of calf rennet as a milk coagulant for producing Feta cheese (Figure 2), the aforementioned particles are likely hydrolysis products of caseins. The principal digestion product recognizable in treated milk and curd cheese is the original band, with a molecular weight of 15.8 kDa (Figure 2, lanes 8 and 10; Figure 2, lanes 2 and 4). The results of the preliminary analysis of cheese taste were conducted as described in Section 2.5. All evaluators stated that the cheese produced using *Auricularia auricula* fungus powder as a milk coagulant, compared with cheese production using calf rennet, did not have an undesirable or bitter taste, and no member of the taste panel noted any off-flavor. The appearance parameters (surface, texture, and form) were deemed acceptable by the taste panel. The texture and body were rated as satisfactory by the taste panel for cheeses made with the fungus. The overall acceptability on the hedonic scale was rated as 4 (desirable) and 5 (very desirable). Evaluators highlighted no differences between the sample and control Feta cheese prepared with calf rennet (Figure 3)

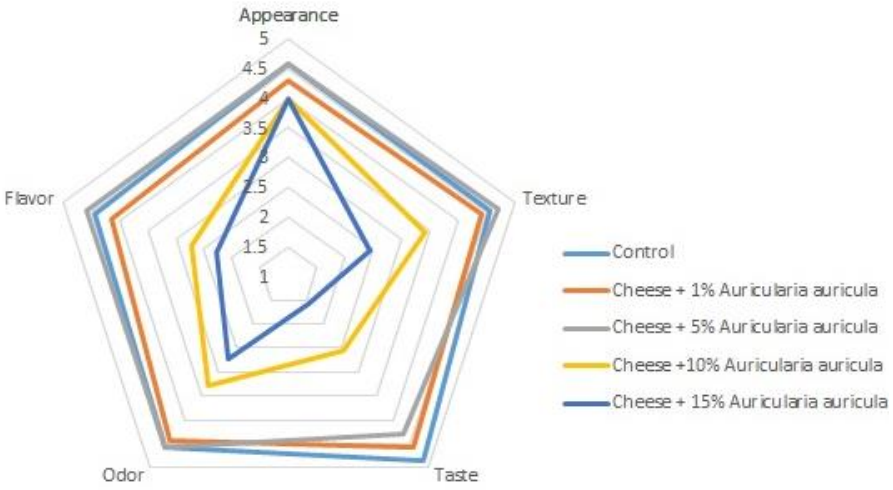

Figure 3. Sensory evaluation of Feta cheese produced using Auricularia auricula fungus as a coagulant.

Conclusion

"This study aimed to measure the applicability of the *Auricularia auricula* fungus as a fungal coagulant in Feta cheese manufacture and to introduce this fungus as a replacement for calf rennet. The focus was on its capability to clot milk and hydrolyze milk proteins and bovine casein. The data demonstrate that the milk-clotting and proteolytic activities of *Auricularia auricula* fungus were not affected by CaCl2, which is typically added to facilitate milk-clotting. During the cheesemaking procedure, the *Auricularia auricula* fungus powder acted as a coagulant, resulting in curd cheese where the casein coagulum was isolated from the whey proteins (Figure 2). The proteolytic activity of *Auricularia auricula* fungus is appropriate in whole and semi-skimmed milk, which is essential for enhancing the cheese's quality and sensory properties. The initial assessment of the Feta cheese's overall acceptability was very high, as considered by all tasters, especially regarding flavor, which was rated between desirable and very desirable. Due to the shortage of calf rennet, resulting from the slaughter of young calves and religious restrictions, the use of *Auricularia auricula* fungus can be an excellent alternative to calf rennet, making it unnecessary to import rennet. Due to the availability of *Auricularia auricula* fungus, its low exploitation costs, and its proper proteolytic activity, it could be used in the dairy industry to design new dietary products and obtain potentially bioactive peptides. The study's limitation was that *Auricularia auricula* is not cultivated in Iran, and we were obligated to gather it in its wild form.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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