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Soft Cheese: Mushroom Milk Clotting Enzyme Substitute

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ABSTRACT

Background and Objective: The study addresses the need for sustainable and ethical alternatives to traditional calf rennet, commonly used in cheese production. With growing concerns about the environmental impact, ethical considerations of animal-derived products and the rising demand for plant-based and microbial alternatives, there is a clear knowledge gap regarding the effectiveness of fungal milk-clotting enzymes (MCE) as substitutes. This research explores the technological and nutritional suitability of MCE derived from oyster mushrooms. **Materials and Methods:** The potential of the milk-clotting enzyme (MCE) derived from the edible oyster mushroom *Pleurotus florida* 14 MICC as a substitute for calf rennet in soft cheese production was evaluated. Its performance was compared to that of commercial calf rennet and microbial coagulants. The study also examined the cheese samples' chemical, textural, sensory and nutritional properties over a 4-week storage period at 5°C. **Results:** The study found no significant ($p \leq 0.05$) differences in moisture, fat, protein, texture, or sensory attributes of cheese made with various coagulants, though mushroom crude MCE cheese had the highest ash, acidity and WSN/TN. Amino acid profiling revealed variations, with calf rennet cheese having the highest lysine content (78.49 mg/g protein) and microbial coagulant cheese excelling in leucine (38.06 mg/g protein). While microbial coagulant cheese achieved the highest PER and BV, partially purified mushroom MCE improved the leucine chemical score, making it comparable to commercial coagulants. **Conclusion:** Cheese made with different coagulants showed no significant differences in basic composition, texture, microstructure or sensory attributes. However, mushroom crude and partially purified MCE enhanced specific amino acid profiles, raising the nutritional value and chemical score, making them promising alternatives to commercial coagulants.

KEYWORDS

Cheese, mushroom, fungal milk clotting enzyme, calf rennet substitute, enzymatic coagulation

INTRODUCTION

Cheese is a dairy product produced in a wide range of flavors, textures and forms by coagulation of the milk protein casein. The utilization of calf rennet enzyme extracted from the fourth stomach of suckling calves primarily contains chymosin along with pepsin in cheese production[1,2] and has become increasingly restricted due to factors such as dietary preferences and consumer concerns regarding animal-based products[3].

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Since cheese is a popular dairy product worldwide, the demand for milk coagulants has rapidly increased alongside the growth in cheese production[4]. Thus, significant research efforts have been focused on discovering alternative sources of calf rennet for cheese making, particularly from microbial origins, in an attempt to meet the growing demand for milk coagulants in cheese production fungi are part of microbial diversity and their extracellular enzymes can be easily recovered through bioprocessing[5]. Among fungi, mushrooms are macrofungi belonging to the basidiomycetes and ascomycetes groups. The oyster mushroom is one of the most widely cultivated edible fungi worldwide[6].

Thus, MCE derived from molds has been developed as a substitute for calf rennet. Regarding mushroom species, Nakamura *et al*.[7] explored MCE-producing strains among commonly consumed mushrooms, including *Hericium erinaceum* MAFF 435060, MAFF 430234 and NBRC 100328, which were utilized in cheese-making[8]. Identified the edible basidial mushroom *Coprinopsis lagopides* as a novel active producer of MCE, making it a potential calf rennet substitute in cheese production. Also Sato *et al*.[9] found that MCE from *Hericium erinaceus* MAFF 435060 effectively coagulates heat-treated milk at both 66°C for 30 min and 130°C for 2 sec.

Furthermore, recent studies have mentioned that *Mucor thermohyalospora*, *Rhizopus azygosporus*[10], *Mucor circinelloides*[11] and *Aspergillus oryzae*[12] could serve as sources of milk-clotting protease in cheese production.

Thus, microorganisms as sources of enzymes have many advantages due to their natural features, the possibility of rapidly creating new features, the possibility of genetic manipulation and low production costs[13,14]. Hence, many fungal and bacterial proteases are widely used for cheese production[1,15].

The scientific interest in the nutritional value and medicinal properties of the edible oyster mushroom has opened a new area of research into its biologically active substances. While it is increasingly recognized as a source of MCE, studies on its biochemical characteristics remain limited. Further research is needed to investigate its technological suitability for cheese production.

Hence, the present study aimed to examine the suitability of edible oyster mushroom (*Pleurotus florida* 14 MICC) MCE as a calf rennet alternative in soft cheese production. Also, the influence of such mushroom MCE on the chemical, textural, sensorial and nutritional characteristics of soft cheese was investigated in comparison to both commercial calf rennet and microbial coagulant.

MATERIALS AND METHODS

Raw materials and chemicals: The dried fruiting bodies of the oyster mushroom (*Pleurotus florida* 14 MICC) were sourced and identified at the Central Laboratory for Agricultural Climate, Agricultural Research Center, Giza, Egypt in July, 2022. These samples were dried, ground into a fine powder using a laboratory mill and stored in sealed containers at 4°C for subsequent analysis. Skim milk powder was acquired from BIELMLEK Spółdzielnia Mleczarska, Poland. Fresh buffalo and cow milk were collected from the Animal Production Research Institute, Dokki, Egypt. Microbial rennet powder (RENIPLUS), derived from *Mucor miehei*, was procured from Gaglio Star, Spain.

Calf rennet was purchased from Mifad, Misr food additives, Egypt. The starter culture used in the cheese manufacture consisted of a mixture of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* were obtained from Egyptian microbial culture collection, (MIRCEN), Ain Shams University. All other reagents and chemicals were used in analytical grade.

Soft cheese manufacture: Fresh soft cheese made from mixed cow's and buffalo's milk (1:1) which contains 3.2% protein, 4.5% fat, 12.16% total solids and 0.71% ash according to Salih and Abdalla[16]. The mixed milk heated to 72°C for ~15 sec, then cooled to 37°C and inoculated with 2% active cheese starter culture. Also, calcium chloride was added at the level of 0.02 and 3% salt was added to pre-cheese milk and mixed well. Thereafter, the resulting mixed milk was divided into four portions as follows: The first portion was coagulated with calf rennet (T1), the second batch was made with microbial coagulant (T2), while the third and fourth portions were coagulated with crude (T3) and partial purified MCE (T4) from oyster (*Pleurotus florida* 14 MICC) mushroom which prepared as mentioned by Bakr *et al*.[17]. Fresh and stored cheese samples for 1, 2, 3 and 4 weeks in the refrigerator at 5°C were taken for analysis. The whole experiment was repeated in duplicate and each analysis in duplicate and average results were tabulated.

Chemical characterization of cheese: The chemical composition of the cheese samples was analyzed following the procedures described by AOAC and Helrich[18]. The analysis included moisture, ash, total solids, fat, total protein and titratable acidity (expressed as lactic acid) using 1/9 N NaOH. The pH was measured with a pH meter and water-soluble nitrogen (WSN) was determined using the method outlined by Ling[19].

Texture profile analysis (TPA) of cheese: The texture profile analysis (TPA) of soft cheese during its storage was conducted using a Universal Testing Machine (Co metech, B type, Taiwan) equipped with a 25 mm diameter conical-shaped probe. The force (N) versus time (s) data were recorded and key textural parameters, including hardness, chewiness, cohesiveness, gumminess and springiness, were calculated based on the force-time curve according to Gunasekaran and Ak[20].

Amino acids profile of cheese: The amino acids profile of soft cheese made with different coagulants was determined using the HPLC-Pico-Tag method according to Heinrikson and Meredith[21] and White *et al*.[22].

Calculation of chemical score, protein efficiency ratio and biological value of cheese: The chemical score of selected soft cheese made with different coagulants based on their amino acid content was calculated according to Bhanu *et al*.[23], as follows:

$$\text{Chemical score} = \frac{\text{mg of amino acid in 1 g test protein}}{\text{mg of amino acid in 1 g reference protein}} \times 100$$

The protein efficiency ratio (PER) of selected soft cheese made with different coagulants based on their amino acid content was calculated using the equation, as follows:

$$\text{PER} = -0.468 + 0.454\ (\text{leucine}) - 0.105\ (\text{tyrosine})$$

The biological value (BV) of selected soft cheese made with different coagulants based on their amino acid content was calculated using the equation suggested by Oser[24], as follows:

$$\text{BV} = 49.09 + 10.53\ (\text{PER})$$

Microstructure profile of cheese: Scanning Electron Microscopy (SEM) was performed for cheese samples prepared with different coagulants. Cheese samples were freeze-dried using a laboratory scale freeze-drying unit (Alpha 1-4LDplus, CHRIST, Germany) and then gold-palladium coated in a vacuum using a sputter coater device (Polaron 5100 Edwards S150A). Microstructures of cheese samples were examined with a Quanta FEG 250 (FEG Company, Netherlands) scanning electron microscope using a large-field detector (LFD) and operating at 25 kV and at ×2000 magnification.

Sensory properties evaluation: The sensorial attributes of cheese samples were evaluated by regular score panels chosen from ten of the staff members of the National Research Centre and Animal Production Research Institute. Cheese samples were evaluated for body and texture (40 points), flavor (50 points) and color (10 points) according to Pappas *et al.*[25].

Statistical analysis: The statistical analysis of all results was performed by SAS statistical software[26] using the ANOVA procedure for analysis of variance. The results were expressed as Mean±Standard Error and the differences between means were tested for significance using Duncan's multiple ranges at $p \leq 0.05$.

RESULTS

Impact of oyster mushroom MCE on soft cheese: The technological suitability of MCE from edible oyster mushroom (*Pleurotus florida* 14 MICC) as a calf rennet substitute in soft cheese production was examined in comparison of available commercial animal calf rennet and microbial coagulant (Fig. 1). Thus, the chemical, textural, sensorial and nutritional characteristics of the resulted cheese were investigated during its storage period at 5°C for 4 weeks.

Chemical characterization of cheese: The chemical changes of soft cheese during their storage period which was coagulated with mushroom (*Pleurotus florida* 14 MICC) in both crude and partially purified MCE forms compared to commercial coagulants were shown in Table 1.

Table 1: Chemical characterization of soft cheese coagulated with mushroom (*Pleurotus florida*) MCE compared to commercial coagulants during storage at 5°C

		Cheese treatments			
Parameter	Storage period (week)	T1	T2	T3	T4
Moisture (%)	Fresh	62.23±0.0^{a}	62.95±0.2^{a}	61.42±0.6^{ab}	62.32±0.5^{a}
	1	61.52±0.5^{ab}	61.53±0.0^{ab}	61.45±0.2^{ab}	61.60±0.3^{ab}
	2	59.57±0.3^{bc}	59.57±0.5^{bc}	58.23±0.4^{cd}	59.50±0.2^{bc}
	3	58.44±0.3^{cd}	58.13±0.2^{cd}	57.70±0.6^{cd}	58.55±0.3^{cd}
	4	57.19±0.8^{d}	57.59±0.3^{cd}	56.52±0.5^{d}	57.09±0.2^{d}
Protein (%)	Fresh	12.00±0.08^{de}	11.58±0.49^{ef}	11.58±0.21^{ef}	11.15±0.41^{f}
	1	12.00±0.12^{de}	12.00±0.25^{de}	12.22±0.12^{d}	11.37±0.12^{f}
	2	12.43±0.25^{cd}	12.22±0.37^{d}	12.22±0.37^{d}	11.58±0.37^{ef}
	3	13.08±0.12^{b}	12.43±0.25^{cd}	12.43±0.25^{cd}	12.00±0.25^{de}
	4	13.82±0.06^{a}	12.86±0.12^{bc}	12.43±0.25^{cd}	12.00±0.25^{de}
Fat (%)	Fresh	20.00±0.58^{def}	20.00±0.58^{def}	19.50±0.29^{ef}	19.00±0.58^{f}
	1	20.50±0.29^{cde}	21.00±0.58^{bcd}	20.00±0.58^{def}	20.00±0.58^{def}
	2	21.00±0.58^{bcd}	21.50±0.29^{abc}	21.50±0.29^{abc}	20.50±0.29^{cde}
	3	22.00±0.58^{ab}	21.50±0.29^{abc}	22.00±0.58^{ab}	21.00±0.58^{bcd}
	4	22.50±0.29^{a}	21.75±0.43^{abc}	22.00±0.58^{ab}	21.00±0.58^{bcd}
Ash (%)	Fresh	3.78±0.01^{abc}	3.00±0.29^{c}	4.31±0.01^{abc}	4.26±0.06^{abc}
	1	3.89±0.01^{abc}	3.03±0.04^{bc}	4.71±0.03^{abc}	4.69±0.01^{abc}
	2	3.94±0.06^{abc}	3.77±0.04^{abc}	4.80±0.06^{abc}	4.72±0.03^{abc}
	3	4.04±0.01^{abc}	4.11±0.09^{abc}	5.09±0.08^{abc}	4.90±0.07^{abc}
	4	4.07±0.03^{abc}	4.37±0.03^{abc}	5.43±0.04^{a}	5.14±0.09^{ab}

T1: Liquid calf rennet, T2: Microbial rennet powder, T3: Crude MCE form mushroom and T4: Partial purified MCE form mushroom. All parameters are represented as the Mean of Replicates±Standard Error. Means with different superscript small letters in rows are significantly different at $p \leq 0.05$

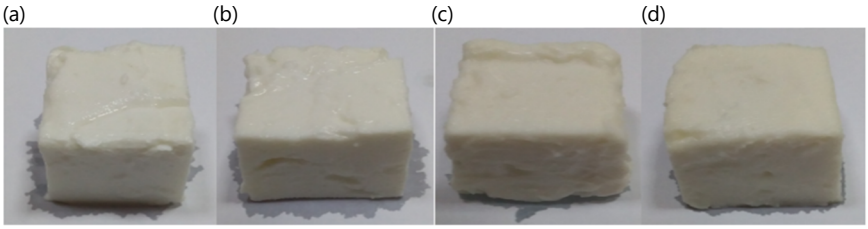

Fig. 1(a-d): Photo of soft cheese made with different coagulants at 5°C, (a) T1, (b) T2, (c) T3 and (d) T4
T1: Liquid calf rennet, T2: Microbial rennet powder, T3: Crude MCE form mushroom and T4: Partial purified MCE form mushroom

The results indicated that the moisture content of soft cheese coagulated with crude mushroom MCE was marginally lower compared to the cheese coagulated with calf rennet or microbial coagulant. However, no significant differences ($p \leq 0.05$) were observed during the storage period (Table 1).

Moreover, the moisture content of all resulted in cheese decreased gradually as the storage period progressed with significant ($p \leq 0.05$) differences after 2nd week of storage. These findings are by Amer *et al.*[27], who observed no significant differences on the soft cheese moisture content of cheese using fungal MCE from Mucor miehei NRRL 3420 compared to control cheese manufactured with calf rennet.

Also, Table 1 shows that the protein content of soft cheese coagulated with crude mushroom MCE was close to the results of cheese made with commercial coagulants without significant ($p \leq 0.05$) differences. Also, cheese coagulated with partially purified MCE from mushroom had the lowest protein content among resulted in cheese treatments which could be due to the higher proteolytic activity of partially purified form compared to crude extract of mushroom, Similar results concerning the cheese protein content were observed by Mamo *et al.*[12], who reported that the commercial rennet-made cheese had the highest protein content followed fungal MCE-made cheese. Also, Abou Ayana *et al.*[28] reported that Domiati cheese produced using calf rennet had higher total protein content than experimental Domiati cheese manufactured with Mucor mucedo KP736529 MCE.

Table 1 shows no significant ($p \leq 0.05$) differences in fat content for soft cheese made with mushroom MCE fractions in comparison of those coagulated with both calf rennet and microbial coagulant. The results in line with Mamo *et al.*[12], who observed no significant difference in the crude fat content of cheese coagulated with commercial rennet and partially purified MCE from *Aspergillus oryzae* DRDFS 13. However, the fat content of Prato cheese made with MCE from *Thermomucor* indicates N31[29], soft cheese manufactured using MCE from *Rhizomucor miehei* NRRL 2034[29] and fresh goat cheese coagulated with *M. miehei* MCE[30] were similar with calf rennet made-cheese. Also, fat content increased in all resulted in soft cheese treatments throughout their storage period which was mainly due to the loss of the moisture content of cheese during the storage period.

Also, Table 1 shows that the soft cheese coagulated with crude MCE of mushroom had the highest ash content among other cheese treatments with significant ($p \leq 0.05$) differences during all storage periods. It could be due to the mineral content of the added mushroom extracts especially in their crude form. These results were in harmony with those observed by Merheb-dini *et al.*[31] which Prato cheese made with *Thermomucor* indicate-sedative N31 enzyme had higher ash content than commercial enzyme-made Prato cheese. Moreover, the ash content of all resulting cheese was increased as their storage period progressed mainly due to the moisture reduction during storage.

Moreover, the WSN of cheese coagulated with partially purified MCE of a mushroom was higher than cheese coagulated with crude MCE extract from a mushroom with significant ($p \leq 0.05$) differences after 2 weeks of storage period which was mainly due to the higher proteolytic activity of partial purified MCE than crude extract of mushroom (Table 2). The results show that as the storage period increased, the WSN (%) of all treatments (T1, T2, T3 and T4) progressively rose. By week 4, the WSN values ranged from $0.51 \pm 0.02\%$ (T1) to $0.74 \pm 0.04\%$ (T4), with T4 showing the highest increase in WSN over the storage period.

These findings align with previous studies by Amer *et al.*[27] and Reps *et al.*[32], who reported higher levels of water-soluble nitrogen (WSN) in fungal-made cheese compared to control cheese made with calf rennet. Additionally, WSN levels in all types of soft cheese increased with prolonged storage, which is primarily attributed to moisture loss. However, during the early stages of proteolysis, WSN production occurs, which serves as an indicator of both the rate and extent of proteolysis. This process is crucial for the development of flavor and texture in cheese during its storage period[33,34].

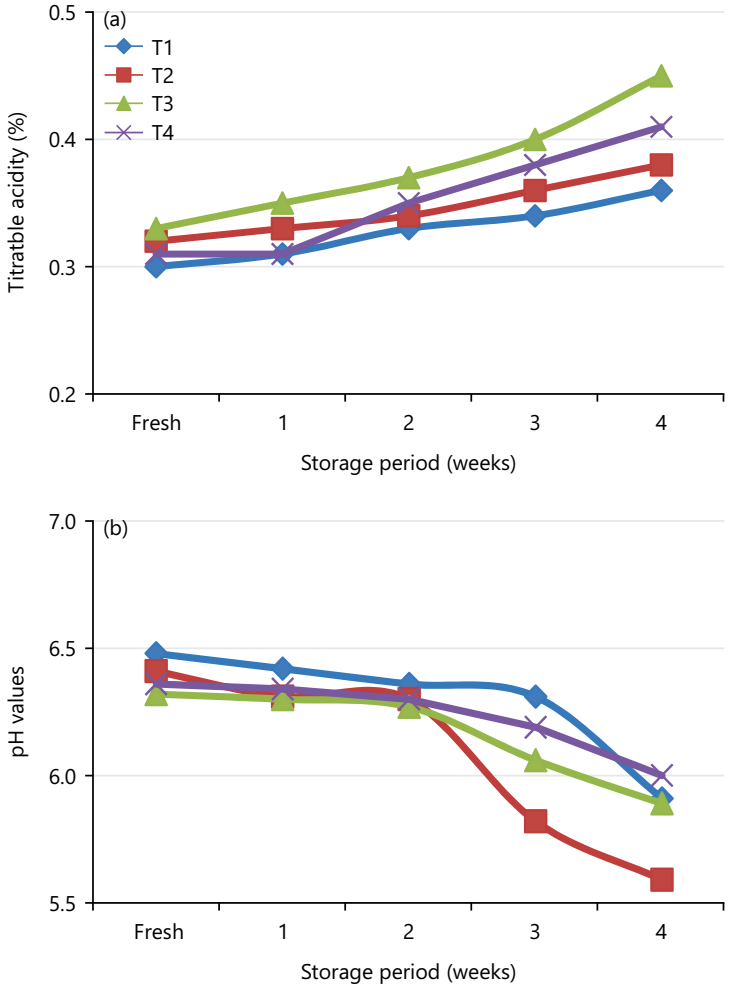

Fig. 2(a-b): Acidity and pH changes of soft cheese coagulated with mushroom (*Pleurotus florida*) MCE compared to commercial coagulants during storage at 5°C, (a) Acidity and (b) pH
T1: Liquid calf rennet, T2: Microbial rennet powder, T3: Crude MCE form mushroom and T4: Partial purified MCE form mushroom

Table 2: Water soluble nitrogen (WSN) content in soft cheese coagulated with mushroom (*Pleurotus florida*) MCE compared to commercial coagulants during storage at 5°C

		Treatments			
Parameter	Storage period (week)	T1	T2	T3	T4
WSN (%)	Fresh	0.17±0.02^{g}	0.17±0.02^{g}	0.24±0.02^{efg}	0.27±0.04^{def}
	1	0.20±0.04^{fg}	0.24±0.04^{efg}	0.31±0.02^{de}	0.34±0.04^{d}
	2	0.24±0.02^{efg}	0.20±0.04^{fg}	0.34±0.04^{d}	0.47±0.04^{c}
	3	0.34±0.04^{d}	0.24±0.02^{efg}	0.47±0.04^{c}	0.61±0.04^{b}
	4	0.51±0.02^{c}	0.47±0.04^{c}	0.64±0.02^{b}	0.74±0.04^{a}

T1: Liquid calf rennet, T2: Microbial rennet powder, T3: Crude MCE form mushroom and T4: Partial purified MCE form mushroom. All parameters are represented as the Mean of Replicates±Standard Error. Means with different superscript small letters in rows are significantly different at $p \leq 0.05$

Acidity and pH changes of cheese: It could be noted that the cheese acidity (Fig. 2a), with opposite trends in their pH values (Fig. 2b), made with partial purified MCE from mushroom (0.31-0.41% and 5.86-5.50 pH) were close to calf rennet (0.30-0.36% and 5.98-5.41 pH) and microbial coagulant (0.32-0.38% and 5.91-5.09 pH) without significantly ($p \leq 0.05$) differences during storage. It could be probably due to the proteolytic activity of mushroom MCE as mentioned in Fig. 2, combined with other acidic components resulting from the fermentation of residual lactose and degradation of cheese fat through their storage period[35,36].

Table 3: Amino acids content of soft cheese coagulated with mushroom *(Pleurotus florida)* MCE compared to commercial coagulants

	Cheese treatments			
Amino acids (mg/g protein)	T1	T2	T3	T4
Aspartic acid	94.43	101.33	119.49	110.90
Glutamic acid	176.49	182.56	186.73	176.19
Serine	82.56	71.73	70.02	67.66
Glycine	28.88	28.25	26.92	25.95
Histidine	37.57	27.28	23.90	24.88
Arginine	57.41	48.34	42.81	40.26
Threonine	73.53	72.18	57.20	57.02
Alanine	85.20	64.58	54.74	54.64
Proline	43.31	39.15	26.57	43.36
Tyrosine	52.59	52.98	60.44	52.82
Valine	43.86	40.10	43.16	36.37
Methionine	18.84	39.64	40.80	36.11
Cysteine	6.26	8.00	5.11	6.17
Isoleucine	35.56	36.32	38.42	31.95
Leucine	26.88	38.06	22.16	35.06
Phenylalnine	25.43	41.45	21.10	20.97
Lysine	78.49	56.78	59.84	70.92
Total EAA	340.16	351.81	306.58	313.28
Total amino acids	967.29	948.73	899.41	891.23
EAA/TAA ratio	0.352	0.371	0.341	0.352

T1: Liquid calf rennet, T2: Microbial rennet powder, T3: Crude MCE form mushroom, T4: Partial purified MCE form mushroom, EAA: Essential amino acids and TAA: Total amino acids

Amino acid profile of cheese: The amino acid content of soft cheese made with different coagulants are given in Table 3. The results indicated that all resulted from cheese contains most of the essential amino acids (EAA) with the highest content in calf rennet made-cheese (lysine, valine, threonine and histidine), followed by crude MCE from mushroom made-cheese (methionine and isoleucine) and microbial coagulant made-cheese (leucine and phenylalanine). Valine amino acid content of calf rennet made-cheese (43.86 mg/g protein) is close to both microbial (40.10 mg/g protein) and crude mushroom coagulants made-cheese (43.16 mg/g protein); while isoleucine of mushroom (crude MCE) made-cheese is close to commercial coagulants made-cheese. The lysine content of calf rennet-made cheese (78.49 mg/g protein) and leucine content of microbial coagulant made-cheese (38.06 mg/g protein) were close to partial purified MCE of mushroom made-cheese (70.92 and 35.06 mg/g protein, respectively).

Meanwhile, sulfur amino acids including methionine and cysteine recorded the highest content in cheese made with crude MCE of mushroom methionine content but cysteine content was recorded in microbial coagulant-made-cheese. Also, mushroom crude MCE-made made-cheese recorded the highest content of non-essential amino acids content including aspartic acid, glutamic acid and tyrosine.

Moreover, EAA and the total amino acids (TAA) content of cheese made with mushroom coagulant were lower than cheese made with calf rennet or microbial coagulant. Also, the EAA/TAA ratio of mushroom cheese was close to cheese made with commercial coagulants (Table 3).

It could be due to in the present study the same starter culture was used for cheese production. Hence, no difference was expected in terms of the release of the enzyme from the starter bacteria and/or the degree of starter autolysis in cheese. However, the liberation of amino acids in cheese is primarily controlled by starter peptidases and the degree of liberation of amino acids varies depending on the starter enzyme systems and degree of autolysis in cheese[36].

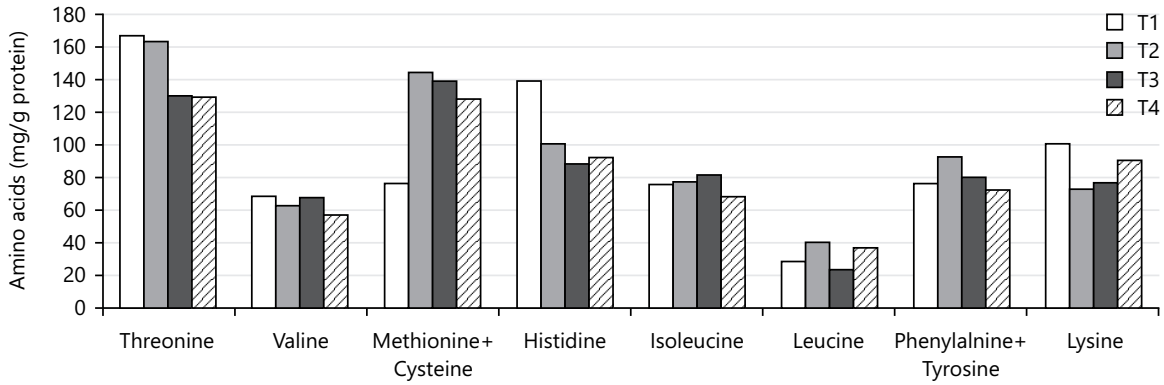

Fig. 3: Chemical score of soft cheese coagulated with mushroom (*Pleurotus florida*) MCE compared to commercial coagulants

T1: Liquid calf rennet, T2: Microbial rennet powder, T3: Crude MCE form mushroom and T4: Partial purified MCE form mushroom, Chemical score = mg of amino acid in 1 g test protein divided by mg of amino acid in 1 g reference protein×100, Reference protein used[37]

Table 4: PER and BV of soft cheese coagulated with mushroom (*Pleurotus florida*) MCE compared to commercial coagulants

	Cheese treatments			
Methods of evaluation	T1	T2	T3	T4
Protein efficiency ratio	6.21	11.25	3.25	9.90
Biological value	114.48	167.55	83.31	153.34

T1: Liquid calf rennet, T2: Microbial rennet powder, T3: Crude MCE form mushroom and T4: Partial purified MCE form mushroom

Chemical score, protein efficiency ratio and biological value of cheese: A chemical score is a comparison of the amount of the limiting amino acid in a food with the amount of that same amino acid in a reference food. There are two distinct uses of protein quality data: Assessment of a diet's ability to meet human protein and amino acid requirements and assessment of the protein adequacy for regulatory purposes of foods and food products sold to consumers. Figure 3 shows the chemical score of soft cheese made with different coagulants. It could be noted that leucine was the first limiting amino acid in cheese coagulated with crude MCE of mushroom but using partial purified MCE of mushroom as coagulant raised the chemical score of leucine which was close to cheese coagulated with commercial coagulants. Also, valine and isoleucine were the second limiting amino acids for the partially purified MCE of mushroom-made-cheese but using crude MCE of mushroom as a coagulant raised the chemical score of valine and isoleucine which is close to commercial coagulants made-cheese.

The protein efficiency ratio (PER) determines the effectiveness of protein in soft cheese made of different coagulants are presented in Table 4. The results indicated that cheese made with microbial coagulant had the highest PER followed by cheese coagulated with partially purified MCE of mushroom, calf rennet and crude extract of mushroom.

The biological value (BV) of a protein indicates the proportion of absorbed nitrogen that is retained and used for protein synthesis, reflecting the quality of the protein. This concept is valuable because it allows for the assessment of protein requirements from foods with varying qualities, as BV is directly related to the efficiency of protein utilization. Foods with higher BV values generally provide a better supply of essential amino acids. Table 4 demonstrates that incorporating partially purified MCE from mushrooms into cheese increased the BV of soft cheese compared to cheese made with calf rennet. This is particularly important for kidney patients who require protein with a high biological value.

Table 5: Texture profile analysis of soft cheese coagulated with mushroom (*Pleurotus florida*) MCE compared to commercial coagulants during storage at 5°C

		Cheese treatments			
Textural parameter	Storage period (week)	T1	T2	T3	T4
Hardness (N)	Fresh	1.30±0.12^{bB}	1.40±0.12^{abB}	1.60±0.12^{ab}	1.30±0.17^{bB}
	4	2.90±0.06^{bA}	3.39±0.58^{abA}	3.71±0.58^{aA}	3.00±0.58^{bA}
Cohesiveness (ratio)	Fresh	0.659±0.06^{aA}	0.637±0.06^{aA}	0.480±0.07^{bA}	0.557±0.06^{abA}
	4	0.677±0.06^{aA}	0.702±0.06^{aA}	0.613± 0.06^{aA}	0.613±0.06^{abA}
Springiness (mm)	Fresh	0.590±0.06^{abA}	0.623±0.06^{aA}	0.420±0.06^{bcA}	0.434±0.06^{bcA}
	4	0.413±0.06^{bcB}	0.511±0.06^{abcB}	0.381±0.06^{bB}	0.414±0.06^{bcB}
Gumminess (N)	Fresh	0.352±0.06^{eB}	0.382±0.06^{eB}	0.724±0.06^{dB}	0.857±0.06^{cdB}
	4	0.903±0.12^{dA}	1.595±0.06^{bA}	1.024±0.006^{cA}	1.839±0.006^{aA}
Chewiness (mm/N)	Fresh	0.225±0.06^{cB}	0.266±0.06^{cB}	0.359±0.006^{cB}	0.451±0.006^{cB}
	4	0.332±0.12^{cA}	0.815±0.006^{bA}	0.990±0.20^{bA}	1.497±0.06^{aA}

T1: Liquid calf rennet, T2: Microbial rennet powder, T3: Crude MCE form mushroom, T4: Partial purified MCE form mushroom, N: Newton, mm: Millimeter and N/mm: Newton millimeter. All parameters are represented as the Mean of Replicates±Standard Error. Means with different superscript small letters in rows are significantly different at $p \leq 0.05$. Means with different superscript capital letters in columns are significantly different at $p \leq 0.05$

Texture profile of cheese: The TPA as an important feature of soft cheese made with different coagulants are presented in Table 5. It could be noted that no significant ($p \leq 0.05$) differences in hardness (1.3-1.6 N), springiness (0.420-0.623 mm) and cohesiveness (0.480-0.659 ratio) of the fresh resulted from cheese using different coagulants with the same trend after 4 weeks of storage. It may be mainly due to the similar moisture and fat contents of all resulting cheese made using both mushroom MCE and commercial coagulants without significant ($p \leq 0.05$) differences (Table 1) as described by Sandoval-Castilla *et al.*[38].

Also, gumminess of mushroom-made soft cheese (0.724-0.857 N) had the highest significantly ($p \leq 0.05$) values compared to both animal rennet (0.352 N) and microbial coagulant (0.382 N); while the chewiness of partial purified mushroom-made cheese (0.451 mm/N) had the highest significantly ($p \leq 0.05$) values among other coagulants, as well as crude mushroom-made cheese (0.359 mm/N) was close to microbial-made cheese (0.266 mm/N) in chewiness value. These findings are in agreement with those reported by Merheb-dini *et al.*[31], who indicated that the fresh goat cheese coagulated with *M. miehei* MCE had similar textural attributes (i.e., hardness, cohesiveness and springiness) with calf rennet made-cheese; whereas chewiness and gumminess were significantly higher than control cheese made with calf rennet.

Moreover, textural parameters (hardness and gumminess) of all resulting from soft cheese were significantly ($p \leq 0.05$) increased while no significantly ($p \leq 0.05$) difference in cohesiveness and springiness of cheese as the storage period prolonged which might be due to the reduction of their moisture content during storage.

Microstructure of cheese: Scanning Electron Microscope (SEM) micrographs of cheese texture provide a qualitative approach to following the changes observed in rheological indexes[39]. The microstructure of soft cheese coagulated with different coagulants was similar (Fig. 4) which could be due to the similar impact of experimental coagulant from a mushroom with animal calf rennet or microbial coagulant on the milk casein as the main fraction of the protein network in cheese, as well as the similar moisture and protein contents in all resulted from cheese which reflects to similar pore size, water retention and the number of casein linkages on cheese microstructure as mentioned by Sanjuán *et al.*[40]. These findings were confirmed with the textural (Table 5) and chemical attributes (Table 1) of cheese which indicated that no significantly ($p \leq 0.05$) differences among mushroom MCE made-cheese and cheese made using commercial coagulants. Thus, SEM micrographs of cheese support this hypothesis.

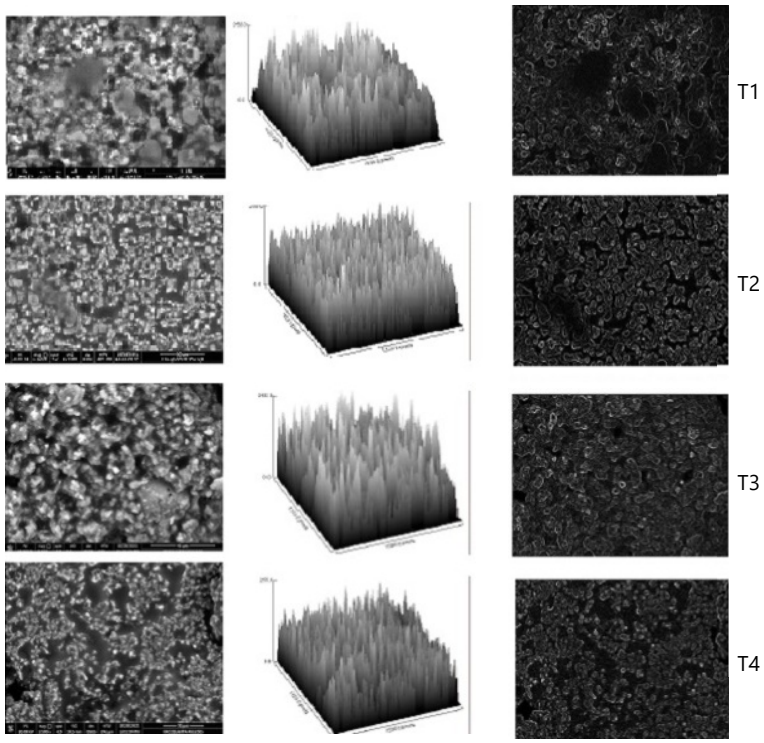

Fig. 4: Microstructure of soft cheese made with different coagulants
T1: Liquid calf rennet, T2: Microbial rennet powder, T3: Crude MCE form mushroom and T4: Partial purified MCE form mushroom

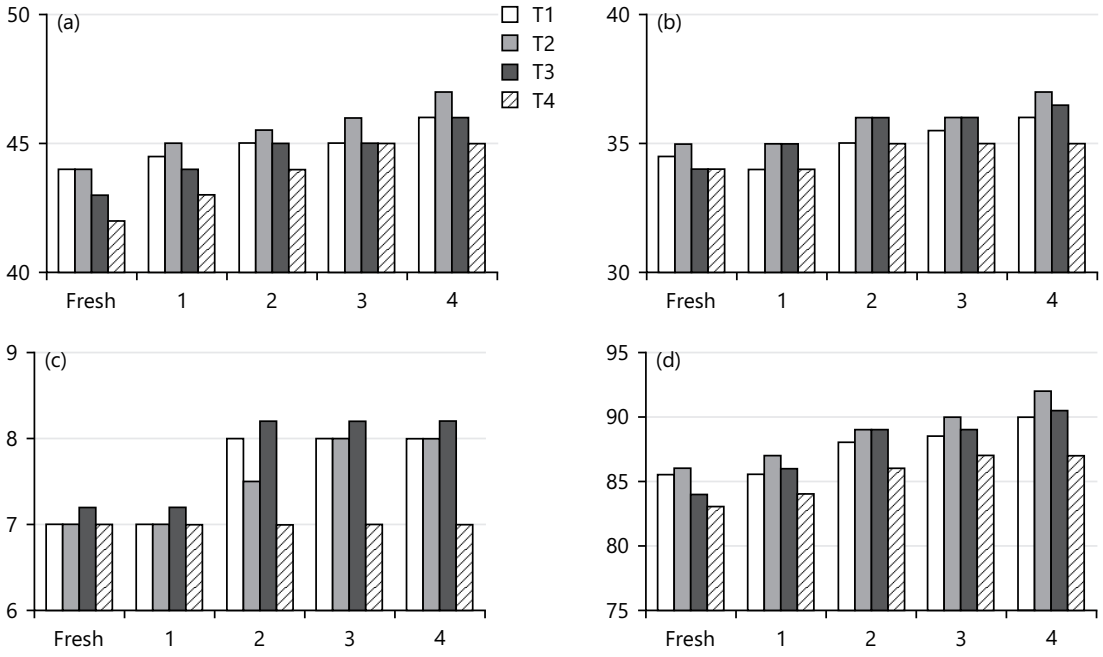

Fig. 5(a-d): Sensorial characteristics of soft cheese coagulated with mushroom (*Pleurotus florida*) MCE compared to commercial coagulants during storage at 5°C, (a) Flavor, (b) Body and texture, (c) Appearance and (d) Overall acceptability
T1: Liquid calf rennet, T2: Microbial rennet powder, T3: Crude MCE form mushroom and T4: Partial purified MCE form mushroom

Sensorial properties of cheese: Total sensorial attributes score including the collective scores of individual soft cheese properties such as flavor, appearance; body and texture which coagulated with different coagulants during its storage period for 4 weeks are presented in Fig. 5a-d. The sensorial

evaluation results showed no significant ($p \leq 0.05$) differences in all characteristics (Fig. 5a-c) and overall acceptability (Fig. 5d) of cheese made by different coagulants during storage with slightly higher scores for commercial coagulants compared to mushroom MCE fractions. It could be mainly due to the similar moisture content (Table 1) and TPA (Table 5) of all resulting cheese made using both mushroom MCE and commercial coagulants without significant ($p \leq 0.05$) differences.

The results of another study of Mamo *et al.*[12] indicated that the cheese made with fungal MCE from *Aspergillus oryzae* DRDFS 13 was slightly firm and acceptable as well and there was no significant difference in flavor, odor and texture scores which was similar to commercial rennet-made cheese. Moreover, soft cheese produced using MCE from *Mucor miehei* NRRL 3420[29], *Aspergillus niger* FFB1 MCE-made cheese[41] and fresh goat cheese made with *Mucor miehei* MCE[32] had similar organoleptic characteristics with control cheese manufactured using calf rennet. Hence, the results indicated that the mushroom MCE fractions in comparison to calf rennet or microbial coagulant could be used as a potential coagulant in soft cheese manufacture without any defects during its storage period at 5°C.

CONCLUSION

It could be concluded that MCE from oyster (*Pleurotus florida* 14 MICC) mushroom considers a good candidate as a calf rennet substitute in soft cheese production due to similar characteristics of the resulted cheese including chemical, nutritional, textural and sensorial properties in comparison of calf rennet and microbial coagulant made-cheese without any appeared defects during its storage period.

SIGNIFICANCE STATEMENT

Sustainable and ethical alternatives to traditional calf rennet which is commonly used in cheese production were addressed in the present study. Also, with growing concerns about the environmental impact, ethical considerations of animal-derived products and the rising demand for plant-based and microbial alternatives, there is a clear knowledge gap regarding the effectiveness of fungal milk-clotting enzymes (MCE) as substitutes. Hence, the technological and nutritional suitability of MCE derived from oyster mushrooms were explored. Cheese made with different coagulants showed no significant differences in basic composition, texture, microstructure, or sensory attributes. However, oyster mushrooms in both crude and partially purified MCE forms enhanced specific amino acid profiles, raising the nutritional value and chemical score, making them promising alternatives to commercial coagulants.

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