The role of immobilized rennet on carbon cloth in flavor development during ripening of Gouda cheese

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Abstract Rennet-free Gouda (RFG) cheese was prepared to investigate the influence of rennet on the non-volatile and volatile profiles of cheese and was characterized by HPLC and GC/MS analyses. Chymosin, a major protease in rennet, was immobilized onto oxidized and chemically modified carbon cloth. The chymosin immobilization efficiency was 60.4%, and the milk-clotting activity used as an index of the stability of the immobilized chymosin decreased by around 20% in 2 weeks. However, the activity was maintained at 70–80% from 2 weeks to 32 weeks and was more stable than that of chymosin solution alone. Non-volatile (organic acids) and volatile profiles of the RFG cheese and rennet-containing normal Gouda cheese were not significantly different during ripening with a few exceptions. Therefore, it can be concluded that cheese flavor is developed by lactic acid fermentation, irrespective of the presence of rennet.

Keywords: carbon cloth, enzyme immobilization, chymosin, rennet, cheese flavor

Introduction

Cheese ripening is a slow and complicated process, involving a series of microbial, biochemical, and chemical reactions. Cheese flavor is known as a result of the balance and concentration of a various kind of volatile flavor compounds. The ripening of cheese involves 3 biochemical processes such as proteolysis, lipolysis, and glycolysis, the relative importance of which depends on the cheese variety (1). Proteolysis is directly involved in the development of consistency and flavor in the ripening cheese, two very important qualities (2). A gradual decomposition of casein takes place due to the combined action of various proteolytic enzymes present in the coagulant, milk, and lactic acid bacteria (starter and nonstarter). Rennet, composed of chymosin as a major enzyme, and starter bacteria are believed to be the main sources of proteolytic enzymes in cheese production (3). Thus, it is necessary to investigate the influences of rennet and starter bacteria on flavor development. In Cheddar cheese, the coagulant is responsible for the initial hydrolysis of caseins (1,4). The activity of the coagulant is largely restricted to α_{s1} -casein, with limited hydrolysis of β -casein and probably no effect on α_{s2} -casein. Indigenous milk and starter proteases are less important at the initial stages of proteolysis, however, starter bacteria enzymes are believed to be principally responsible for the production of small peptides and free amino acids. These products of proteolysis themselves are known to contribute to the flavor or act as precursors of flavor

components in the cheese. However, there is no clear evidence showing the direct contribution of rennet and starter bacteria to flavor development during cheese ripening. The contribution of rennet may be assessed by the use of a cheese in which acidulation is simulated by chemical acidification (5–7). The contribution of rennet to proteolysis may be hard to determine from starter-free cheeses since the resulting cheese contains no lactic acid originating from milk sugars at the renneting stage unlike standard cheese. Acidification is one of the main events in the manufacturing of most cheese varieties and usually involves the fermentation of lactose to lactic acid by selected lactic acid bacteria. There has been report of rennet-free cheese production using a modified method in which normal renneting progressed without clotting in the milk by reducing the Ca + Mg content, followed by pasteurization at 72°C and adjustment of the Ca + Mg content (2). Heat treatment may inactivate the coagulant and proteases of indigenous milk and starter, thereby changing caseins, peptides, and short-chain fatty acids in the milk. This method is not practically applicable, because the resulting rennet-free cheese is in different conditions from those of standard cheese at the start of ripening.

For a modified cheese to be acceptable, the cheese manufacturing method should be similar to the standard protocol. In this context, rennet-free cheese can be obtained if immobilized rennet (or chymosin) is employed. Milk can pass through the immobilized rennet column without leaving any coagulant in the hydrolyzed milk.

Typically, the milk is acidified and then the coagulant is added to cause clotting. In previous studies, to make Cheddar cheese using immobilized proteases with milk clotting activity (8,9), coagulation of milk in the column could be avoided by raising the pH of the milk to 8.0 and reversing the order of the lactic acid fermentation and hydrolysis. The ripening ratio and taste were similar to those of Cheddar cheese prepared by the traditional method, which is indicative of similar environments for the modified and standard cheeses.

Gouda cheese is one of the most popular cheese worldwide which was chosen as product for rennet-free cheese, since it can be easily prepared in general laboratory and it has relatively short ripening period. Therefore, in this study, rennet-free Gouda (RFG) cheese was prepared to investigate the factors that contribute to cheese flavor through covalent immobilization of rennet onto carbon cloth. Model testing of immobilization efficiency and stability was conducted with purified chymosin. Afterward, to test the application to cheese-making, rennet, which is a commercially available coagulant for cheese manufacture, was used. The organic acids and volatile compounds in the resulting rennet-containing normal Gouda (RCNG) cheese and RFG cheese were investigated to evaluate the differences in the cheese flavor profiles.

Materials and Methods

Materials Raw milk was kindly provided by a farm in Imsil-gun, Jeollabuk-Do, Korea and used for cheese making after pasteurization (43°C, 30 min). The carbon cloth (Panax 35 Fabrics; Zoltek Co., Bridgeton, MO, USA) was used as a support for immobilized rennet (or chymosin). FD-DVS FLORA-DANICA 50 U (*Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, CHR. HANSEN, Hoersholm, Denmark) and Naturen™ calf rennet (CHR. HANSEN, Hamilton, New Zealand) were used as the starter culture and the coagulant for cheese making, respectively. Chymosin (EC 3.4.23.4), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), and N-hydroxysulfosuccinimide (NHSS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 10% skim milk solution for measuring enzyme activity was prepared by mixing the desired amount of skim milk powder (Maeil Dairy Co., Seoul, Korea) in 10 mM CaCl₂ solution and used as reconstituted milk. For extraction of organic acids from cheese, a stomacher bag (BagPage®+, Interscience, Saint Nom, France) was used. All of the standard chemicals for the analysis of organic acids (acetic acid, acetoin, citric acid, lactic acid, propionic acid, and pyruvic acid) and volatile compounds (3-methyl-1-butyl acetate, 3-methylbutanal, 3-methylhexane, 2-phenylethyl acetate, and phenyl acetaldehyde) were purchased from Supelco Analytical (Bellefonte, PA, USA) and Sigma-Aldrich and used without further purification.

Activation of carbon cloth and covalent immobilization of rennet

The activation of carbon cloth and immobilization of the milk clotting enzyme rennet were basically conducted according to the method described by Miyawaki and Wingard (10) and Klibanov *et al.* (11). Carbon cloth with a thickness of 0.14 cm was cut into 15 cm×10 cm and was oxidized using two mineral acids, nitric and sulfuric acid, to give surface carboxylic groups. The carbon cloth cut was treated with 70% nitric acid at room temperature for 15 min and then rinsed with distilled water to remove acid. Thereafter, it was treated with 98% sulfuric acid at 170°C for 1 h, followed by rinsing with distilled water. Carboxylic acid groups on the carbon cloth surface after acid oxidation were reacted with 20 mM EDC and 5 mM NHSS at room temperature for 10 min, followed by rinsing 3 times with distilled water to remove excess EDC and NHSS. The chemically modified carbon cloth was incubated in 75 mL rennet at 25°C for 30 min. The rennet-immobilized carbon cloth was rinsed 3 times to remove the unimmobilized enzymes and was stored in distilled water at 4°C before use. A schematic diagram of the activation of the carbon cloth and enzyme immobilization is shown in Fig. 1.

Measurement of enzyme activity

Milk-clotting activity and chymosin immobilization efficiency: Five milliliters of reconstituted milk (10% skim milk containing 10 mM CaCl₂) was taken in a 30 mL test tube and incubated in a water bath at 37°C. Afterward, 0.5 mL chymosin solution was added to the test tube to measure the time until the formation of curd flecks. The chymosin solution (1 mg/mL), before and after immobilization onto the carbon cloth (1 cm×1 cm×0.14 cm) in the same manner as described for rennet immobilization, was diluted 250 times to measure the milk-clotting activity. One milk-clotting unit (MCU) is defined as the amount of enzyme required to clot 1 mL of reconstituted milk at 37°C in 40 min (12).

$$\text{Milk-clotting (U/mL)} = 2,400/t \times D$$

t: clotting time (s), *D*: dilution factor

The chymosin immobilization efficiency was obtained by calculating the MCU of the chymosin solution before and after immobilization. The chymosin immobilization efficiency is the percentage of chymosin immobilized onto the surface of carbon cloth, which is indicated by the MCU difference for chymosin solutions before (MCU_{BI}) and after immobilization (MCU_{AI}) (13).

$$\text{The chymosin immobilization efficiency} = \frac{(MCU_{BI} - MCU_{AI})}{MCU_{BI}} \times 100$$

Proteolysis activity: The proteolysis activity is defined as the amount of non-protein nitrogenous compounds soluble in trichloroacetic acid (TCA) upon proteolysis of the milk by enzyme (14). Pasteurized raw milk at pH 8 (adjusted by 0.1 M NaOH solution) was passed through the immobilized rennet column (2.5 cm ID×22 cm height,

Fig. 1. Schematic diagram of chymosin immobilization onto the carbon cloth. EDC; N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide, NHSS; N-hydroxysulfosuccinimide.

32°C) at a constant velocity (A). Five milliliters of proteolyzed milk was taken in a 30 mL test tube and an equal volume of 10% TCA was added to precipitate the milk protein. The precipitated milk protein was then removed by filtration, and the filtrate was measured by the Lowry method (15) to determine the non-protein nitrogenous compounds. One milliliter of the filtrate adjusted to pH 10 using 0.2 M NaOH solution was mixed with 5 mL of alkaline copper solution and kept at room temperature for 12 min. After that, 0.5 mL of 1 N Folin's reagent was rapidly mixed and kept at room temperature for 32 min. The absorbance of this solution at 750 nm was measured. The absorbance of raw milk, which was pasteurized and adjusted to pH 8.0 (B), was measured at 750 nm. The pasteurized raw milk (C) was treated as described above, without the TCA solution treatment, to measure the total protein content. The proteolysis activity of the immobilized rennet was calculated as follows.

$$\text{Proteolysis activity (\%)} = \frac{(\text{Abs}_{750 \text{ nm}} \text{ A} - \text{Abs}_{750 \text{ nm}} \text{ B})}{\text{Abs}_{750 \text{ nm}} \text{ C}} \times 100$$

Stability of immobilized chymosin The stability of the immobilized chymosin was evaluated by measuring the milk-clotting times. The chymosin was immobilized onto the carbon cloth (1 cm×1 cm×0.14 cm) in the same manner as described for rennet immobilization. Then the immobilized chymosin in water and the chymosin solution (diluted 250 times before immobilization) were stored at 4°C for 32 weeks. For a period of 10 weeks, the milk-clotting activities were measured at 2 week intervals, and the final MCU was measured at 32

weeks. However, in the case of the immobilized chymosin, the dimensional unit was converted from "centimeter" to "milliliter" considering the specific gravity (1.8) of the carbon cloth.

Cheese preparation and instrumental analysis

Rennet-containing normal Gouda cheese (RCNG cheese): The starter was added to 2.5 kg of pasteurized raw milk at 32°C and incubated for 45–50 min. Then rennet (10 times diluted) was added to the milk and left for 25 min to coagulate. The curd formed was cut into squares of 1 cm and stirred for 15–20 min before draining the whey through a gauze cloth. The curd was weighed, placed into the mold, and left undisturbed overnight. The curd blocks were immersed in 20% brine solution for curing, followed by drying in a refrigerator (11°C) overnight. The cheese obtained was trimmed to a suitable size for further analysis and was coated with paraffin wax, followed by ripening at 11°C.

Rennet-free Gouda cheese (RFG cheese): The pasteurized raw milk at pH 8.0 was passed through the immobilized rennet packed column at 9 mL/min. This procedure was repeated twice. The subsequent steps were the same as described for the RCNG cheese preparation.

Composition, organic acids and volatiles analysis: The moisture, fat, and protein contents of RCNG cheese and RFG cheese were determined using the AOAC Official method of analysis (16). Organic acids and their compositions in cheese were determined by HPLC analysis (17). For this, 5 g of cheese and 25 mL of 6.5 mM H_2SO_4 solution were added into a stomacher bag and mixed for 10 min

using a Bagmixer (Interscience). The cheese extract obtained was centrifuged at $7,000\times g$ for 5 min, and the supernatant was filtered through a $0.20\ \mu\text{m}$ syringe filter (Millipore, Temecula, CA, USA). The filtrate was analyzed for organic acids using the HPLC (LC-20AD, Shimadzu Co., Kyoto, Japan). The analysis was performed in a cation exchange column ($300\ \text{mm}\times 7.8\ \text{mm}$, Aminex HPX-87H, Bio-Rad, Hercules, CA, USA) at 65°C with a mobile phase of $6.5\ \text{mM}\ \text{H}_2\text{SO}_4$ at a flow rate of $0.8\ \text{mL/min}$. The detector was set to 210 and 290 nm. The peaks were identified by comparison of retention times with the standards (acetic acid, acetoin, citric acid, lactic acid, propionic acid, and pyruvic acid).

The sample for volatile compounds analysis was prepared by the simultaneous distillation-extraction (SDE) method (18). Grated cheese (10 g each) with 100 mL of distilled water and 20 mL of *n*-pentane were loaded in the distillation flask and the solvent flask, respectively. The flasks were heated at 100°C for the sample flask and 50°C for the solvent flask. Each extraction was carried out for 30 min after the distilled water in the sample flask started to boil. The extracts were determined using a GC/MS (GCMS-QP2010, Shimadzu Co.). SPB-5 ($60\ \text{m}\times 0.25\ \text{mm}\times 0.25\ \mu\text{m}$, Supelco) was used as separation column, and helium was used as a carrier gas. The injector temperature and the interface temperature were set to 250°C . The column temperature was maintained at 40°C for 5 min and then heated at 4°C/min to 200°C , which was held for 34 min, followed by heating at 10°C/min to 230°C . Identification of volatile compounds was achieved by comparing their retention times with those of authentic samples, by comparing their mass spectra to those of mass spectral libraries (NIST, WILEY), and by comparison of Kovats indices (19) on DB-5 capillary column.

Statistical analysis: The moisture, fat, and protein contents of RCNG cheese and RFG cheese were analyzed using Statistical Analysis System (SAS, Ver.9, SAS Institute, Cary, NC, USA). Significant differences among means from triplicate analyses ($p<0.05$) were determined by Duncan's Multiple Range Test.

Results and Discussion

Activity and stability of immobilized chymosin The chymosin immobilization efficiency was calculated from the percentage of chymosin immobilized, which is the ratio of immobilized chymosin to the amount of chymosin used for immobilization. Since chymosin was immobilized on a solid structure (carbon cloth), a direct comparison of its activities to chymosin in a solution was hardly possible. Therefore, we determined the milk-clotting activities of the chymosin solution before and after immobilization. The milk-clotting units (MCUs, U/mL) before and after the immobilization of the chymosin solutions were found to be 1,405 and 557, respectively. The immobilization efficiency of chymosin onto carbon cloth was calculated to be 60.4%.

For evaluation of enzyme stability, the milk-clotting activity of the

Fig. 2. The milk-clotting activity of immobilized chymosin (CC-chymosin) and chymosin solution during storage.

Table 1. Moisture, fat, and protein contents in rennet-containing normal Gouda cheese and rennet-free Gouda cheese

Type of cheese	Average $\pm$ SD ¹⁾ (w/w %)		
	Moisture	Fat	Protein
Gouda	48.82 $\pm$ 0.15	20.31 $\pm$ 0.15	29.21 $\pm$ 0.79
Rennet-free Gouda	48.34 $\pm$ 0.14	18.64 $\pm$ 0.18	31.52 $\pm$ 0.64

¹⁾SD, standard deviation, and the values are the average of three experiments.

chymosin solution and the immobilized chymosin was determined for 32 weeks and the results are shown in Fig. 2. The milk-clotting activities of the chymosin solution gradually decreased during the entire storage period of 32 weeks and final activity was 60%. For the immobilized chymosin, the activity dropped by about 20% in the early stage of storage (2 weeks), but was maintained at 70–80% level from 2 weeks to 32 weeks. Therefore, the immobilized chymosin was more stable than the chymosin solution in long time storage.

Proteolysis activity of immobilized rennet Rennet was immobilized onto carbon cloth for cheese-making. After immobilizing rennet onto the carbon cloth as mentioned above, the proteolysis activity of immobilized rennet for the production of RFG cheese was determined. The extent of milk proteolysis is affected by the flow rate of the milk through the immobilized rennet column. In general, 3% protein hydrolysis causes milk coagulation. In this study, when pasteurized raw milk was passed twice through the immobilized rennet column at a flow rate of $9\ \text{mL/min}$, the propagation of milk proteolysis reached 3%.

Composition, organic acids and volatiles analysis of cheese The moisture, fat, and protein contents of RCNG cheese and RFG cheese are shown in Table 1. In general, the moisture content of cheese is affected by milk composition, cheese-making temperature, and curd cutting procedure (20). Although both cheeses were prepared under identical conditions except for rennet treatment, there was significant difference between the moisture contents ($p<0.05$). However, the difference was only less than 0.5%. The fat content of the RFG cheese was slightly lower than that of the RCNG cheese. This may be caused

Table 2. Organic acids found in rennet-containing normal Gouda cheese and rennet-free Gouda cheese during ripening period

Peak No. ¹⁾	RT (min)	Compound name	The percentage of the compounds during ripening period of cheese ²⁾ (%)							
			0 week		3 weeks		6 weeks		9 weeks	
			RCNG	RFG	RCNG	RFG	RCNG	RFG	RCNG	RFG
1	4.35	-	29.7	35.1	33.9	40.1	34.6	44.5	35.7	49.2
2	5.80	Citric acid	5.5	7.7	1.6	11.2	7.9	14.9	8.9	16.1
3	6.83	Pyruvic acid	2.4	2.6	3	1.9	2.1	2.5	3.3	1.5
4	7.11	-	1.4	2.1	1.9	2.3	2.4	2.7	1.9	3.1
5	7.99	-	0	2.8	0	2.7	0	3.4	0	3.2
6	8.99	Lactic acid	48.8	39.1	49.0	35.0	42.1	27.4	40.6	22.1
7	9.50	-	3.5	2.4	3.3	2.0	3.1	0.2	3.3	0.6
8	10.53	Acetic acid	3.0	3.7	2.7	1.8	2.8	1.4	1.5	1.3
9	12.08	Propionic acid	0	1.1	0	0.3	0	0.7	0	0.9
10	12.47	Acetoin	0.2	0	0.1	0	0	0	0	0
11	12.61	-	2.5	1.9	2.2	1.2	1.8	1.1	1.8	1.0
12	20.70	-	3.0	1.5	2.5	1.5	3.1	1.2	2.9	1.1

-, Unknown or not identified.

¹⁾Peak No.1 to 12 were detected at 210 nm except for No. 10, which was detected at 290 nm.

²⁾The percentage of each compound indicates the percentage of peak area for each compound when the sum of all peak areas is defined as 100%.

by the adsorption of fat onto the hydrophobic carbon cloth, when the milk was treated for RFG cheese preparation. The protein content of the RFG cheese was higher than that of the RCNG cheese. It was assumed that since the immobilization of rennet onto carbon cloth restricted the interaction of rennet with the protein in the milk, the concentration of the protein in the curd was high as a result. Organic acids from milk fat are known to contribute to the distinctive flavor of cheese during the ripening period, and lactic acid and free fatty acids are some of the most common organic acids related to the cheese flavor (21,22). The starter added to milk, meanwhile, influences the cheese flavor because it produces lactic acid, which is used as a substrate for many chemical reactions during cheese ripening (23). In addition, free fatty acids enhance the flavor of cheese in combination with other components at low concentrations (24,25). Thus, the organic acid profiles of the RCNG cheese and the RFG cheese during the ripening period were examined by HPLC analysis and the results are shown in Table 2. From the results of HPLC analysis, 4 simple organic acids (citric acid, pyruvic acid, lactic acid, and acetic acid) were identified in the RCNG cheese. Although quantitative differences were observed, all of these organic acids were also found in the RFG cheese.

On the other hand, lactic acid decreased during the ripening period in both cheeses, but peak no. 1, an unidentified compound, increased. Accordingly, there may be some kind of relationship between the two compounds. A small amount of propionic acid was detected in the RFG cheese, but not in the RCNG cheese. In previous research, it was reported that propionic acid was not found in the water soluble fraction of RCNG cheese (26). Therefore, it is assumed that the milk was influenced by propionic acid producing exogenous bacteria because of the prolonged exposure of milk to the air during RFG cheese preparation. Other than propionic acid, since RFG cheese has a similar organic acid composition as RCNG cheese, it can be

concluded that rennet in cheese barely influences the organic acid composition.

In addition to the organic acids, various volatile compounds are also produced during cheese ripening, and it is known that these compounds affect the flavor of cheese. Aldehydes, ketones, ethyl esters, lactones, and alcohols are some of the most well-known volatile compounds in RCNG cheese (27). The volatile compounds produced during the ripening period were analyzed by GC/MS. The results of GC/MS analysis of volatile compounds on RCNG and RFG cheese ripened for 3 and 12 weeks are shown in Fig. 3 and the summarized form of the GC/MS analysis results is shown in Table 3. During 3 weeks of ripening, various volatile compounds were found in both cheeses and the composition of volatile compounds was very similar to each other. However, in Fig. 3C, a peak at the retention time of 36.11 min (peak no. 6) appeared in RFG cheese. This peak was identified as indole, which is known to have a putrid and musty odor at high concentrations, and a flowery smell at very low concentrations (27). Indole is generally produced from tryptophan metabolism by microbial enzymes (29). Although the starter used in this study did not contain indole producing bacteria, indole was detected in RFG cheese. Thus, production of indole may be due to airborne bacteria contamination during the process of milk proteolysis in RFG cheese preparation. Other than indole, fatty acids and their ester were mainly detected in the volatile compounds of both cheeses. Based on all the results of this study, we assumed that the volatile compounds in both cheeses do not depend on the presence of rennet. In order to better understand the quantitative differences in each volatile compound, further research needs to be done.

In the RCNG cheese, most of the volatile compound peaks disappeared after 12 weeks of ripening (Fig. 3B), and only high boiling compound peaks like ethyl palmitate remained. However, in

Fig. 3. Total ion current (TIC) chromatograms of rennet-containing normal Gouda cheese: 3 weeks (A), 12 weeks (B), and rennet-free Gouda cheese: 3 weeks (C), 12 weeks (D). Peaks: (1) 3-methylbutanal, (2) 3-methylhexane, (3) 3-methyl-1-butyl acetate, (4) phenyl acetaldehyde, (5) 2-phenylethyl acetate, (6) indole, (7) ethyl oleate isomers, (8) 9-oxo-nonanoic acid ethyl ester, (9) butyl hydroxy toluene, (10) dodecanoic acid, (11) ethyl stearate, (12) *n*-hexadecanoic acid, (13) dibutyl phthalate, (14) ethyl palmitate.

Table 3. Volatile compounds identified in rennet-containing normal Gouda cheese and rennet-free Gouda cheese during ripening and their flavor attributes

KI ¹⁾	ID ²⁾	Compound name	Flavor attribute ³⁾
654	A	3-Methylbutanal	Nutty, chocolate-like
671	A	3-Methylhexane	Solvent-like
876	A	3-Methyl-1-butyl acetate	Fruity, pear, banana-like
1043	A	Phenyl acetaldehyde	Honey, sweet, rose
1265	A	2-Phenylethyl acetate	Sweet, honey, fruity
1288	B	Indole	Fecal, putrid, musty
	C	Ethyl oleate isomers	Fatty
1470	B	9-Oxo-nonanoic acid ethyl ester	-
1504	B	Butyl hydroxy toluene	-
1568	B	Dodecanoic acid	Soapy, waxy
	C	Ethyl stearate	Waxy
1984	B	<i>n</i> -Hexadecanoic acid	Waxy, creamy
1922	B	Dibutyl phthalate	-
1993	B	Ethyl palmitate	Waxy

-, Unknown.

¹⁾Kovats retention index. KI is retention index on DB-5 capillary column.

²⁾Identification. The reliability of the identification proposal is indicated by the following: A, mass spectrum, retention time, and Kovats index compared with authentic standards; B, mass spectrum and Kovats index compared with NIST and Wiley mass spectral databases and literature data; C, mass spectrum compared with NIST and Wiley mass spectral databases.

³⁾Information about flavor attributes of volatile compounds mainly excerpted from Urbach (28).

the RFG cheese, the high boiling compound peaks disappeared after 12 weeks of ripening (Fig. 3D). Hypothetically, some bacteria in the RFG cheese may decompose fatty acids such as ethyl palmitate. From the results, the appearance or disappearance of some compounds such as indole and ethyl palmitate in RFG cheese may be caused by bacterial contamination with long-term exposure of milk to the atmospheric environment as it passes through the immobilized rennet column. Therefore, it can be concluded that the flavor determining factor of cheese during ripening is not rennet, but lactic acid fermentation (2,7,22,30).

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