Effect of *Monascus* sp. as an Adjunct Starter on Physicochemical Properties and Proteolysis in Semi-hard Cheeses during Ripening

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Abstract Two kinds of semi-hard cheeses, with *Monascus purpureus* and without *M. purpureus*, were manufactured, and effects of *M. purpureus* on physicochemical properties and proteolysis were evaluated during 36 days of ripening. Addition of *M. purpureus* changed the microbial survival and showed no significant effect on physicochemical properties of the cheeses, including dry matter and pH. Regardless on the rind or in the core, the indices of proteolysis had no significant difference ($p > 0.05$), whereas there were significant differences of total free amino acid (FAA) and individual FAA between cheeses; this indicated that *M. purpureus* had no significant effect on the primary proteolysis, but affected the content and ratio of individual FAAs during maturation. Electrophoretic analysis showed strong degradation of α_{s1} -casein in the core and on the rind of cheeses, while β -casein was highly degraded on the rind but less in the core. Thus, *Monascus* spp. might have a potential application in the manufacture of cheeses.

Keywords: semi-hard cheese, *Monascus purpureus*, proteolysis, ripening

Introduction

During cheese ripening, proteolysis is the most complex and, in most varieties, the most important of the three primary biochemical events, since it contributes to textural changes of the cheese matrix due to breakdown of the protein network, decrease in a_w through water binding by liberated carboxyl and amino groups, and increase in pH. It also contributes to flavor, and perhaps off-flavor, of cheese directly through the formation of peptides and free amino acids (FAAs) and indirectly through the liberation of substrates (amino acids) for other flavor-generating reactions, as well as by facilitating the release of sapid compounds from the cheese matrix during mastication (1–3).

In most ripened cheeses, the progress of proteolysis can be divided into two phases as follows: primary proteolysis, which serves as the initial hydrolysis of caseins caused by residual coagulant and, to a lesser extent, by plasmin and perhaps cathepsin D and other somatic cell proteinases, resulting in the formation of large- and intermediate-sized peptides, and secondary proteolysis, which is the subsequent degradation of the products of primary proteolysis catalyzed by the coagulant and proteinases from the starter, non-starter, and secondary microflora of the cheese, producing small peptides and FAAs (1,2). Additionally, the general outline of the

proteolysis can differ markedly between cheese varieties due to different proteolytic agents occurring in different types of cheeses. Since the extent and pattern of proteolysis in cheese is used as an index of cheese maturity and quality, the extent and pattern of proteolysis for many types of cheeses has been extensively studied, such as Cheddar (4), Camembert (5–7), blue-veined cheese (8,9), and other cheeses (10,11). For surface-ripened cheeses, the secondary cultures, including *Penicillium camemberti* and *Penicillium roqueforti* make a major contribution to proteolysis due to their endopeptidase and exopeptidase activities and are recognized as being as important as the primary cultures (12).

Monascus spp. have been widely used in foods and medicines in the Orient for over 1,000 years, especially in China (13). Up to now, *Monascus* spp. are also employed in rice-wine brewing and food fermentation due to the enzymes secreted by them for the breakdown of carbohydrates and proteins (14). Moreover, many results suggest that *Monascus* fermentation products can be effective for reduction of levels of blood cholesterol; lowering of blood pressure; prevention of cancer development; and the management of diabetes, obesity, and Alzheimer's disease, because they contain numerous active constituents, such as monacolin K, γ -aminobutyric acid (GABA), and dimerumic acid (15,16). Therefore, *Monascus* fermentation products can be used as dietary supplements for medical therapy (16). Until

now, molds such as *P. camemberti* and *P. roqueforti* have been extensively studied as secondary starters in cheeses. However, there has been little information about *Monascus* spp. as the secondary starter employed in the manufacture of cheese. Recently, *Monascus* sp. was used as the secondary starter for producing the novel *Monascus*-fermented cheese and thus has the potential for producing this new kind of cheese (17).

In this study, semi-hard cheeses inoculated with *P. candidum*, *G. candidum*, and *Monascus* spp. but without *Monascus* spp. as the secondary starter were manufactured and the effects of addition of *Monascus* spp. on the physicochemical properties and proteolysis of semi-hard cheeses during ripening were evaluated.

Materials and Methods

Cheese manufacture and sampling Two types of semi-hard cheeses, experimental cheeses (HB cheese) and control cheese (B cheese), were repeated twice on 2 consecutive days, and were manufactured from whole cow milk. The B cheeses were manufactured without any *Monascus* sp. strains but with the commercial lactic starters; however, the HB cheeses were manufactured with the *Monascus* sp. strain plus the commercial lactic starters added in the B cheeses. Whole milk (250 L) was refrigerated, carried at 4°C to the pilot plant, pasteurized (75°C for 15 s) in a 400 L/H pasteurizer (SPX Corporation, Charlotte, NC, USA), and cooled to 30°C. The milk was then divided into two vats of 100 L each. The lactic acid starter containing *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Streptococcus salivarius* subsp. *thermophilus* (1.5 g/100 L, CHOOZIT™ RA 21; Danisco, Sassenage, France) was inoculated into each tank. At the same time, the *M. purpureus* BD-M-4 starter (1.7×10^3 CFU/mL, CGMCC No.9712), stored in China General Microbiological Culture Collection Center, Beijing, China, was inoculated into the tank for experimental cheeses. Then, the red yeast rice powder (0.2 g/L, Jiajie®; Shanghai Jiajie Natural Food Pigment Co., Ltd., Shanghai, China) was added into the tank for experimental cheeses, and the milk was gently stirred for 5 min in order to evenly mix in the red yeast rice powder. After 80–110 min, as pH of the milk became 6.3, rennet (1 g/100 L; Marzyme®; Danisco) was added. The progress of coagulation lasted about 25 min, and the curd was cut into cubes (1 cm³) after 40 min of hardening. The mixture of curd and whey was left standing for 10 min in the vat, and the whey was drained for 40 min at room temperature. Then, the curd was loaded into plastic molds, with a diameter of 105 mm and a height of 30 mm, producing cheeses weighing 220 ± 20 g. The cheeses were pressed for 4–8 h and pickled for 1 h in sterile (30 min at 121°C) supersaturated brine at $14 \pm 1^\circ\text{C}$ after the end of molding. Then, the cheeses were immersed into the solution containing *G. candidum* (6×10^3 spores per milliliter; CHOOZIT™ GEO 17 LYO 2D; Danisco) and *P. candidum* (1×10^6 spores per milliliter; CHOOZIT™ PC 12 LYO 20D; Danisco) for 1 h. After 1 h of drying to eliminate excess water, the

cheeses were transferred to ripening chambers for 4 days under a controlled condition (25°C and 90% relative humidity [RH]) and then for 17 days at 14°C and 90% RH. On day 5, the cheeses were turned. On day 21, they were wrapped and ripened at 4°C until day 36. At each ripening period (1, 8, 15, 22, 29, and 36 days), two cheese samples were taken randomly from the rind (5 mm thick all over the cheese surface) and the core (10 mm around the center of the cheese sample) for analysis.

Physicochemical analysis of cheeses For each part of the cheeses (the rind and the core of cheese mass), dry matter (DM), pH, and the content of nitrogen compounds in the soluble fractions were determined. DM was analyzed in triplicate by drying 3 ± 0.3 -g cheese samples at 150°C in an MB 45 moisture analyzer (Ohaus International Trading [Shanghai] Co., Ltd., Shanghai, China) until constant weight. Ground cheeses (2 ± 0.1 g) were mixed with 10-mL deionized water; then, the mixture was stirred for 10 min, and its pH was measured using a Delta 320 pH meter (Mettler-Toledo Ltd., Shanghai, China).

Nitrogen fractions and FAA measurements Proteolysis was assessed based on determination of total nitrogen (TN), pH 4.6 acid-soluble nitrogen (ASN), 12% trichloroacetic acid (TCA)-soluble nitrogen (NPN), and 5% phosphotungstic acid-soluble nitrogen (PTASN), according to the method presented by Leclercq-Perlat *et al.* (18). Proteolytic indexes, including the ratios of ASN/TN, NPN/TN, and PTASN/TN, were used as cheese-ripening indicators.

Individual FAAs were determined in duplicate from PTASN filtrates. An aliquot of PTASN filtrate was filtered on a 0.22-μm pore diameter membrane after its pH was adjusted to 1.7–2.2 by adding 10 N NaOH solution. Then, its individual FAAs were analyzed on a high-speed amino acid analyzer (L-8900; Hitachi, Tokyo, Japan) using the method recommended by De Freitas *et al.* (19) with modification. All analysis was carried out in duplicate.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) SDS-PAGE analysis was performed using the gradient gel system (12% w/v) by Laemmli (20), as described by Zhang and Zhao (21), with some modifications. Two grams of pH 4.6-insoluble nitrogen fraction separated from pH 4.6-SN was washed three times using 6 mL of 1 mol/L sodium acetate buffer (pH 4.6). The residual fat was eliminated by washing with 4 mL of diethyl ether two times. The residue (~0.2 mg) was mixed with 2 mL Tris-buffer (0.06 mol/L, pH 6.8) containing glycerin (25% w/v), β-mercaptoethanol (5% v/v), SDS (2% w/v), and bromophenol blue (0.1% w/v). Then, the solution was diluted twenty times using Tris-buffer (0.06 mol/L, pH 6.8), mentioned above, in order to obtain the solution with protein concentration ranging from 5 to 10 mg/mL. After boiling and centrifuging at $10,000 \times g$ for 10 min, the supernatant was separated for SDS-PAGE analysis. The gradient gel was 1.5 mm thick, consisting of a 2-cm stacking gel and 10-cm running gel. A 10-μL aliquot of the supernatant

was loaded in the sample slots. The electrophoresis was carried out for 4 h at 120 V. As the electrophoresis ended, the gels were separated. The gels obtained were stained with Coomassie brilliant blue R-250 (0.25%) in methanol:water:acetic acid (5:5:1 v/v) and then de-stained in methanol:water:acetic acid (5:5:1 v/v). Seven kinds of standard protein markers and their molecular weights (Mws) (14.4–94.0 kDa) (MP102; Tiangen Biotech [Beijing] Co., Ltd., Beijing, China) were used. α_{s1} - and β -casein were identified using standard α - and β -casein from bovine milk (Sigma-Aldrich Co., St. Louis, MO, USA). The images were photographed using BIO-RAD Gel Doc™ XR+ Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

Microbiological analyses Microbiological analyses were carried out using the method performed by Leclercq-Perlat *et al.* (18). Total aerobic bacteria were determined on plate count agar (PCA; Shanghai Sheng Si Biochemical Technology Co., Ltd., Shanghai, China) and incubated at 30°C for 72 h. The counts of viable cells of *G. candidum* and *M. purpureus* were performed on YGC at 25°C for 5 days. The counts of viable cells of *P. candidum* were carried out by culturing on YGC (Qingdao Rishui Biological Technology Co., Ltd., Qingdao, China) containing 60-g NaCl per liter at 20°C for 8 days. All determinations were made in duplicate and expressed as log colony-forming units (CFU) per gram of DM of cheese samples.

Statistical analysis Mean values and standard error of mean of the data were reported. Differences between control and experimental cheeses were established by means of the one-way and two-way analysis of variance (ANOVA). A least significant difference (LSD) test was applied to compare the mean values of variables. Principal component analysis (PCA) was applied to the data of the individual

FAA concentrations for two types of cheeses at different ripening times. IBM statistical product and service solutions (SPSS) statistics software version 20 (IBM Corporation, Armonk, North Castle, NY, USA) was used in the statistical analysis.

Results and Discussion

Microbiological and physicochemical characteristics The change of the microorganisms on the rinds of the cheeses involved during the ripening is presented in Table 1. At day 1, the count of total microbiota on the rind of the B cheese was 3.2×10^7 CFU/g DM and then grew rapidly between days 1 and 8, reaching 1.1×10^9 CFU/g DM at day 8. After day 8, the total microbiota count decreased and remained about 4.2×10^8 CFU/g DM until the end. In addition, at day 1, the total microbiota count on the rind of the HB cheese was 4.2×10^7 CFU/g DM compared with that of the B cheese. After day 22, the total microbiota count increased to 1.9×10^9 CFU/g DM and then decreased. At the end of the ripening time, the count of total microbiota was about 1.5×10^9 CFU/g DM. The increase of total microbiota was mainly attributed to more fermentable carbohydrates and high ripening temperature suitable for their growth. Additionally, lack of fermentable carbohydrate and low temperature probably led to the decrease of the total microbiota. Ong *et al.* (22) reported that the starter (lactococci) counts in Cheddar cheese decreased during week 24 of ripening due to unfavorable conditions in the cheese, such as low pH and lack of fermentable carbohydrate.

G. candidum is a common microorganism in the surface-ripened cheeses, which plays a crucial role in flavor production. At the beginning of ripening, the viable cell count of *G. candidum* on the rind of the B cheese was about 1.3×10^4 CFU/g DM. Between days 1

Table 1. Changes of microorganisms and physicochemical characteristics in semi-hard cheeses during ripening

Parameters	Type of cheeses	Location	Ripening time (day)					
			1	8	15	22	29	36
DM (% wt)	B	Rind	53.53±2.22a ¹⁾	57.38±0.01ab	59.40±3.01abc	62.57±2.76bc	62.94±3.27cd	63.99±0.74d
		Core	53.53±2.22a	55.08±0.04ab	55.8 ±0.57ab	56.50±0.40b	58.01±0.23b	59.53±0.64b
	HB	Rind	54.33±0.63a	58.35±2.45a	60.87±0.73b	61.75±1.17bc	62.11±1.02cd	63.33±2.07d
		Core	54.33±0.63a	54.42±0.16ab	55.63±0.13b	55.96±0.11b	56.58±0.01b	56.77±0.04b
pH	B	Rind	5.23±0e	6.07±0.03d	6.14±0.02d	6.44±0.08c	6.74±0.06b	7.58±0.07a
		Core	5.23±0d	5.22±0.01d	5.42±0.08c	5.43±0.08c	5.73±0.03b	5.91±0.06a
	HB	Rind	5.13±0.01d	6.03±0c	6.18±0.03c	6.35±0.16bc	6.65±0.32b	7.50±0a
		Core	5.13±0.01b	5.29±0.04b	5.32±0.03b	5.67±0.03a	5.80±0.06a	5.90±0.23a
Total microflora count (log CFU/g DM)	B	Rind	7.51±0.07c	9.05±0.05a	8.50±0b	8.62±0.60ab	8.89±0.12a	8.23±0b
	HB	Rind	7.62±0.03b	9.03±0.31a	9.28±0.31a	9.29±0.21a	8.82±0.16a	9.17±0a
<i>G. candidum</i> (log CFU/g DM)	B	Rind	4.13±0.12d	7.09±0.21c	7.26±0.03bc	7.92±0.17a	7.59±0.12ab	7.46±0.34abc
	HB	Rind	2.85±0.02d	6.08±0.12c	6.35±0.16b	6.52±0.12b	7.23±0a	7.19±0.03a
<i>P. candidum</i> (log CFU/g DM)	B	Rind	4.84±0.28d	6.96±0.17c	6.96±0.21c	7.65±0.21abc	7.71±0.05ab	7.90±0.52a
	HB	Rind	3.71±0.21c	7.10±0.27b	7.23±0.10b	7.29±0.21b	7.84±0a	7.99±0.25a
<i>Monascus</i> spp. (log CFU/g DM)	B	Rind	NA ²⁾	NA	NA	NA	NA	NA
	HB	Rind	4.40±0.26	4.60±0.55	4.58±0.60	4.59±0.64	NA	NA

¹⁾Values are expressed as means±standard deviation. Means with a row with the same letter are not significantly different ($p>0.05$).

²⁾NA, not analyzed

and 8, *G. candidum* grew rapidly and then grew more slowly from day 8 to day 22. After day 22, *G. candidum* counts remained constant at about 3.4×10^7 CFU/g DM, which was close to the finding obtained by Leclercq-Perlat *et al.* (7). However, at the beginning of ripening, *G. candidum* count on the rind of the HB cheese was about 7.1×10^2 CFU/g DM, which was lower than that of the B cheese, possibly due to the interaction with *Monascus* spp. As with the B cheese, *G. candidum* on the rind of the HB cheese grew rapidly between days 1 and 8 and then grew slowly from day 8 to day 29. Between day 29 and the end of ripening, the count of *G. candidum* spores on the rind of the HB cheese remained constant at about 1.6×10^7 CFU/g DM, compared with the count of *G. candidum* spores of the B cheese.

Because *P. candidum* is an aerobe, it is always found that it grows on the rinds of the cheeses. Table 1 shows that the viable cell counts of *P. candidum* on the rind of the B cheese were 6.9×10^4 CFU/g DM on day 1, which is close to the results reported by Chen *et al.* (5). Between days 1 and 8, *P. candidum* grew rapidly and then the growth became slow from day 8 to day 36. At the end of ripening, the counts became about 7.9×10^7 CFU/g DM. Additionally, the viable cell counts of *P. candidum* on the rind of the HB cheese on day 1 were 5.1×10^3 CFU/g DM, which were lower than those of the B cheese. However, it seemed that the growth was higher than that of the B cheese between days 1 and 8, which probably indicated that *Monascus* spp. did not have a negative effect on the growth of *P. candidum*. At the end of ripening, the *P. candidum* counts on the rind of the HB cheese were up to 9.8×10^7 CFU/g DM, close to those of the B cheese. Leclercq-Perlat *et al.* (7) reported that *P. camemberti* grew fast during the first 14 days of ripening on the rind of Camembert-type cheeses and remained 2×10^5 CFU/g DM.

Viable *Monascus* spp. cell counts on the HB cheese surface were about 2.5×10^4 CFU/g DM at day 1. The growth was slow during the first 8 days ripening, and it seemed that the counts remained constant after 8 days, close to about 3.9×10^4 CFU/g DM.

The data on the change of the main physicochemical parameters, including DM and pH, on the rinds and in the centers of semi-hard cheeses during ripening are shown in Table 1. Regardless of the rinds and the cores of the two kinds of cheeses, DM increased gradually during the ripening period, which was attributed to surface evaporation and the exchange of volatile products (water, ammonia, fatty acids, etc.) between the cheeses and the environment around them (6). Moreover, there was a significant difference of DM between the rinds and the cores (except at day 1) during the whole ripening time ($p < 0.05$), which was mainly due to higher surface evaporation rate on the rind than in the core. However, there was no significant difference of DM on the rind between the two kinds of cheeses ($p > 0.05$), while there was significant difference of DM in the core.

pH of cheese increased continuously on the surface and in the core of all the cheeses during the whole ripening time, which was typical for mold-ripened cheeses. This could be ascribed to lactate consumption and alkaline product accumulation through proteolysis by mold (*G. candidum* and *P. candidum*) (7). At the same time, pH of

the rind increased more rapidly than that of the core, which was in agreement with findings of other researchers, who reported that deacidification was more pronounced on the surfaces than in the cores at each stage of ripening (6). It was also recognized that the increase in pH was correlated with fungal growth and with its proteolytic activity (7). At day 1, pH of the HB cheese was a little lower than that of the B cheese, resulting from low pH of the *Monascus* spp. fermentation solution added in the milk (approximately 3.7). However, there was no significant difference between the two kinds of cheeses at the same location ($p > 0.05$), except at day 1, meaning that pH of cheeses was not significantly affected by *Monascus* spp. as an adjunct starter during the whole ripening period.

Changes of extent of proteolysis in cheeses during ripening

Proteolysis in cheese during ripening plays a vital role in the development of texture and flavor. Therefore, the extent of proteolysis can be considered an index of cheese ripening. Proteolysis can be analyzed by quantifying the different soluble nitrogen fractions during cheese ripening (23). Generally, the proteolysis indices, including ASN, NPN, and PTASN, were widely employed for evaluating the extent of proteolysis (11). Table 2 showed that ASN/TN, NPN/TN, PTASN/TN, and total FAA of the B and HB cheeses in the cores and on the surfaces, changed with ripening time. For both cheeses, regardless of on the rind or in the core, ASN/TN significantly increased with increasing ripening time ($p < 0.05$), due to progressive proteolysis mainly catalyzed by residual rennet, indigenous milk proteinases, and the proteinases released by starter cultures, such as *P. candidum* and *G. candidum*. Additionally, during the ripening period, a net difference in the ASN index of both cheeses appeared between the rinds and the cores of the samples, which was typical for surface mold-ripened cheeses. It was mainly attributed to more extensive and rapid proteolysis of the rind than that in the core, resulting from the surface microbiota and higher pH (7). At the beginning of ripening, the ASN index for the HB cheese was 5.06%, a little lower than that of the B cheese (5.38%), which was probably due to lower pH of the HB cheese, far from an optimum pH of 5.4 for rennet. In the core, except on day 1, there was no significant difference ($p > 0.05$) for ASN index between the HB and B cheeses, meaning that addition of *Monascus* spp. had no significant effect on the primary proteolysis in the cores of the cheeses due to water-soluble or pH 4.6-soluble nitrogen in the cores of the cheeses, produced mainly through the primary proteolysis by the action of the coagulant (24). On the rind, there was also no significant difference ($p > 0.05$) for ASN index between the HB and B cheeses, which was possibly attributed to the primary proteolysis dominated by the action of coagulant and the proteinases secreted by *P. candidum* and *G. candidum*. It has been reported that the evolution of proteolysis was correlated with the growth of *G. candidum* and *P. camemberti* in Camembert-type cheeses known for their proteolytic and peptidolytic activities during the ripening (7).

The NPN index provided an index of secondary proteolysis, and

was related to enzymes secreted by microorganisms in the cheese (1). Change of the NPN index with maturation time showed the same trend with the ASN index. Correlation analysis showed that the ASN and NPN indices had a positive correlation with pH in the rinds of cheeses, which was in agreement with the finding by other authors (5,7).

The PTASN provided an indicator for production of FAA during cheese ripening and was therefore recognized as an appropriate index of secondary proteolysis. The PTASN index almost followed the similar dynamic with the ASN and NPN indices, but had a small difference. At day one, the PTASN index for the HB cheese was higher than that for the B cheese, mainly due to a higher extent of proteolysis catalyzed by proteinases or peptidases released by *M. purpureus* added in this experiment. However, as the ripening time increased, the proteolysis was dominated by the action of coagulant and proteinases secreted by *P. candidum* and *G. candidum*. Then, it was notable that there was no significant difference for the PTASN index between the two types of cheeses ($p>0.05$). The concentration of PTASN fraction for both kinds of cheeses on the rinds was significantly different from that in the cores throughout the whole maturation process ($p<0.05$).

Total FAAs in the B and HB cheeses during the ripening period are shown in Table 2. Change of total FAA content in both cheeses almost followed a similar dynamic to the PTASN index. The total FAA content of all cheeses increased significantly with increasing ripening time

($p<0.05$), and was also significantly affected by the location at the same cheese ($p<0.05$). Moreover, there was a significant difference between the two types of cheeses ($p<0.05$). On the rind, before 29 days, total FAA content of the HB cheese was significantly higher than that of the B cheese ($p<0.05$), and at day 36 there was no significant difference between the two types of cheeses ($p>0.05$). The change of total FAA content was probably attributed to additional extent of proteolysis catalyzed by more enzymes due to the growth of *M. purpureus*. And, at the end of ripening time, total FAA content for the HB cheese was comparable with that of the B cheese, which was in agreement with the growth of *G. candidum* and *P. candidum* during the maturation. In short, the results indicated that *M. purpureus* as the adjunct starter had no effect on the primary proteolysis, but affected the content and ratio of individual FAA during maturation in this study.

With regards to the effect of the ripening time, type of cheese, and the location of the nitrogen content of the various fractions, the ASN, NPN, and PTASN indices were significantly affected by two variables ($p<0.05$), including the ripening time and location, but there was no significant difference between the two types of cheeses ($p>0.05$). The values of different nitrogen fractions increased with increasing ripening time, meaning that a clear process of protein hydrolysis occurred during the ripening of the cheeses, which involved the degradation from large molecules (α , β , and γ -caseins), to small molecules (FAAs, small peptides, etc.).

Table 2. Changes in proteolysis indices of cheeses during ripening time

Variables	Ripening time/days	B cheeses		HB cheeses	
		Rind	Core	Rind	Core
ASN/TN/%	1	5.38±0.09aD ¹⁾	5.38±0.09aD	5.06±0.08bD	5.06±0.08bF
	8	20.31±0.27aC	13.25±0.21bCD	22.74±2.70aC	10.10±0.19bE
	15	20.66±5.62aC	14.05±0.09bBCD	23.53±5.23aC	14.00±1.92bD
	22	20.73±0.13bC	16.17±1.52cABC	24.61±1.67aC	18.17±1.11bcC
	29	38.74±0.07aB	22.95±0.38aAB	36.83±0.98aB	21.58±1.64bB
	36	53.18±3.57aA	24.69±9.35bA	52.96±7.52aA	25.89±1.23bA
NPN/TN/%	1	3.25±1.95aC	3.25±1.95aC	3.21±0.69aC	3.21±0.69aC
	8	11.83±2.00aB	7.17±2.46bcB	11.02±0.03bB	6.52±0.15cB
	15	11.91±3.17aB	7.60±1.00aB	14.54±3.95aB	11.00±0.82aB
	22	16.98±2.04aB	8.70±0.61cB	14.73±2.37abB	11.93±0.49bcB
	29	23.42±0.68aA	12.28±0.02cA	23.00±0.90aA	14.53±0.11bA
	36	23.94±2.78aA	12.52±0.19bA	24.54±0.53aA	14.16±0.38bA
PTASN/TN/%	1	1.08±0.04bD	1.08±0.04bB	1.71±0.09aB	1.71±0.09aB
	8	3.11±0.08aC	1.10±0.01aB	4.22±2.54aB	1.92±0.06aAB
	15	3.82±0.09abC	1.12±0.10cB	4.25±1.03aB	2.58±0.16bcAB
	22	4.12±1.82aC	1.39±0aB	4.30±1.26aB	2.92±2.05aAB
	29	6.42±0.36aB	3.43±0.67aA	5.09±1.77aB	3.42±1.08aAB
	36	9.60±0.80aA	4.02±0.28bA	8.74±0.51aA	4.19±0.85bA
FAA/mg/gDM	1	1.09±0.02bE	1.09±0.02bD	1.50±0.01aE	1.50±0.01aE
	8	7.74±1.66bD	2.46±0.02cC	13.29±0.87aD	3.94±0.40cD
	15	11.72±1.35bC	2.97±0.23cC	19.11±3.43aC	6.72±0.22bcC
	22	13.47±0.24bC	4.30±0.24dC	24.09±1.28aB	8.82±0.48cC
	29	18.61±1.13bB	8.29±1.46cB	25.11±1.51aB	9.54±0.74cB
	36	36.50±0.11aA	11.31±1.83cA	35.02±1.69aA	15.30±1.34bA

¹⁾Values are expressed as means±standard deviation. Means with a row with the same letter are not significantly different ($p>0.05$)

Table 3. Correlation matrix established between physicochemical variables and second microorganism during the ripening of cheeses

	ASN	NPN	PTASN	pH	GEO	PEN	MO
ASN	1						
NPN	0.938** ¹⁾	1					
PTASN	0.980**	0.906**	1				
pH	0.976**	0.953**	0.974**	1			
GEO ²⁾	0.680*	0.826**	0.639*	0.763**	1		
PEN	0.769**	0.843**	0.723**	0.836**		1	
MO	-	-	-	-	-	-	1

¹⁾*Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level.

²⁾GEO, *G. candidum*; PEN, *P. candidum*; MO, *Monascus* spp.

Fig. 1. SDS-PAGE profile of pH 4.6-insoluble nitrogen fraction of the rind or the core in two kinds of cheeses at different ripening times. (A) the rind and (B) the core of the B cheese, (C) the rind and (D) the core of the HB cheese; lines 1-6 represent pH 4.6-insoluble nitrogen fraction obtained from cheeses at the ripening period of 1, 8, 15, 22, 29, and 36 days, respectively.

The correlation matrix between the changes of physicochemical variables and adjunct starters is presented in Table 3. It was notable that the proteolysis indices and pH were significantly related with the adjunct starters (*G. candidum* and *P. candidum*), but not with the adjunct starter *M. purpureus*. The same results were reported by Chen *et al.* (5), who found that the growth of *G. candidum* and *P. camemberti* in Camembert-type cheeses was positively correlated with ASN, NPN, and pH. Additionally, the adjunct starters had a

positive correlation with the proteolysis indices and pH, indicating the starters (*G. candidum* and *P. candidum*) played a role in promoting the proteolytic activity in this study. Moreover, pH was positively related with the proteolysis indices, including ASN, NPN, and PTASN, and also had a positive relation with the adjunct starters (*G. candidum* and *P. candidum*), which further verified the findings mentioned above. Leclercq-Perlat *et al.* (7) also reported that the correlation coefficients for NPN versus *G. candidum* and pH versus

Fig. 2. Changes of individual free amino acid (FAA) content with ripening time. (A) the core and (B) the rind of the B cheese, (C) the core and (D) the rind of the HB cheese.

ASN were 0.95 and 0.97, respectively. Fungal growth and its proteolytic and peptidolytic activities mainly contributed to the change of pH and nitrogen evolutions in surface mold-ripened cheeses (7).

The extent of proteolysis of cheese during maturation was also evaluated by SDS-PAGE, and the SDS-PAGE patterns from the precipitants of pH 4.6-insoluble nitrogen obtained from two kinds of cheeses are presented in Fig. 1. It could be easily observed that there appeared mainly two bands (α_{s1} - and β -casein) at the beginning of the ripening time (line 1), regardless of the rinds or the cores of two kinds of cheeses. As the ripening time increased, more caseins were hydrolyzed and the concentration of lower-Mw breakdown products of the caseins increased. At the same time, more breakdown products of caseins with Mw lower than 14.4 kDa were also observed. It was reported by Ong *et al.* (4) that several breakdown products with MW ranging from 7.4 to 30.2 kDa during ripening of Cheddar cheese, were obtained by SDS-PAGE. Additionally, it was notable that the extent of proteolysis was more extensive on the rinds of both kinds of cheeses than in the cores, which was in agreement with the results obtained from the ASN/TN. However, very little detectable differences for proteolysis were observed between the B and HB cheeses at the same location. It has been showed that there was more rapid hydrolysis of α_{s1} -casein than of β -casein for some blue cheeses owing to the occurrence of the metalloprotease of *P.*

roqueforti (9,25), whereas this phenomenon did not occur significantly in this paper (Fig. 1). However, at the end of ripening (day 36), most of α_{s1} - and β -casein on the rinds of two kinds of cheeses were hydrolyzed, while little β -casein in the core was hydrolyzed, since α_{s1} -casein was mainly degraded into α_{s1} -casein by rennet retained in the cheeses, while β -casein was mainly hydrolyzed by the extracellular endo- and exo-peptidases secreted by *P. candidum* due to the growth of *P. candidum* on the rind of the cheese (26,27).

Proteolysis is considered as the main contributor to the flavor of cheese through production of peptides and FAAs, because FAAs serve as precursor molecules of the catabolic reaction that generates the volatile flavor compounds (aldehydes, alcohols, carboxylic acids, thiols, etc.). In addition, different cheeses have their specific FAA composition, arising from the enzymatic degradation of peptides by various enzymes and also from amino acid interconversion and degradation (27). The concentrations of individual FAAs in a cheese were dependent on the manufacture technology, the extent and type of proteolysis, time and ripening conditions (28). Change of the concentrations of some individual FAAs in the B and HB cheeses with increasing ripening time is shown in Fig. 2. In this study, regardless of on the rinds or in the cores, all the FAAs of both cheeses showed a clear tendency of increase with the ripening time, with the exception of Lys, His, and Arg, since during proteolysis, these compounds were released by the proteolytic agent through the biochemical reactions

Fig. 3. Loadings of the FAA concentrations for the two first principal components.

that took place during the ripening period. At day 1, the concentrations of individual FAAs of the HB cheese were not less than those of the B cheese except Pro, which was in line with the result obtained from total FAAs. At the end of ripening, the concentrations of individual FAAs of the HB cheese in the core were not less than those of the B cheese, except Ser, while the concentrations of Asp, Thr, Glu, Met, Ile, Phe, Lys, and His on the rind of the HB cheese were higher than those of the B cheese due to the effects of *M. purpureus* on the production of FAAs. The major FAAs found on the rinds of two types of cheeses throughout the 36-day ripening period were Glu, Leu, Val, Phe, and Pro, while the major FAAs found in the cores were Glu, Leu, Val, and Phe. Similarly, Poveda *et al.* (29) and Pappa and Sotirakoglou (30) found that Glu, Val, Leu, Phe, and Lys were the major FAAs throughout the whole ripening time in Manchego cheeses and Teleme cheeses, respectively. Pino *et al.* (31) reported that the major FAAs found throughout the maturation of goat milk cheeses were Asp, Glu, Lys, Ileu, Phe, Val, Met, and GABA, representing more than 74% of total FAAs. The proteolysis of α_{s1} -casein, which has a high content of the amino acids Leu, Phe, and Val, occurs during the first days of ripening, which predominates over the proteolysis of β -casein (32). Differently, the high concentration of free Pro was found on the rinds of both types of cheeses, indicating the high degree of degradation undergone by β -casein, since Pro is one of the major amino acids in β -casein (29).

For each cheese, the concentrations of individual FAAs on the rind were higher than those in the core, which was in accordance with the indices of proteolysis, including ASN, NPN, PTASN, and total FAA. Irrespective of whether it was in the core or on the rind, the concentration of Glu of both cheeses dramatically increased during the maturation time, indicating that the longer the ripening time in these cheeses, the more umami the taste (33).

In order to establish the relationships between the different variables and to detect the most important causes of variability, PCA was applied to the individual FAAs' data for two types of cheeses at

different ripening times. The variability was explained by principal components (PC) 1 and 2, respectively, accounting for 96.9% of the total variance (TV). The loadings of the different variables for the two first principal components are shown in Fig. 3. PC1 explained 84.6% of the TV, while PC2 accounted for 12.3% of TV, and the FAAs that correlated best with PC2 included Lys and Arg.

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