Application of red pigment producing edible fungi for development of a novel type of functional cheese

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Abstract

To develop a novel type of functional cheese, 9 strains of edible fungi, *Monascus* sp. were cultured on whey protein-based solid medium and screened in terms of secondary metabolite production including red pigments, lovastatin and citrinin. The amount of these metabolites in culture products depended not only on the strain, but also on the pH and temperature environment. The strain of *M. ruber* NBRC 32318 was selected because it produced red pigments and lovastatin with negligible amount of citrinin. After incubation at 25ºC for 10 d with initial pH condition of 4.0, the culture products were mixed with cheese curds, followed by ripening for 3 months at 11.5ºC. Higher level of water-soluble nitrogen was recognized in the culture products containing cheese compared to control cheese.

Practical applications

For cheese making, diverse microorganisms have been used. However, there have been limited trials on the development of dairy products using red pigment producing *Monascus* species used for fermented rice in the Eastern Asia for more than 1,000 years. Using whey protein base solid substrate, we selected suitable *Monascus* strain and defined the culture condition that provided nephrotoxin citrinin-free and functional metabolite of lovastatin-containing culture products, which were mixed with fresh cheese curds for ripening. In the resulting red mould cheese, proteolysis was accelerated with no bitter flavour and rancid. Although further assessments concerning health benefits by animal studies and cheese quality by sensory evaluation are necessary to be commercialised as a novel type of functional cheese, this study provides fundamental information regarding screening of suitable strains with its appropriate culture condition on whey protein solid substrate, focusing on biotechnological and chemical respects to ensure food safety.

Key Words : *Monascus*, cheese; whey protein; citrinin; lovastatin

1 INTRODUCTION

One of the most representative micro-organisms responsible for fermented foods is a group of the lactic acid bacteria. Although it is not exceptional for fermented dairy products, additional starter micro-organisms such as *Propionibacterium* sp., *Brevibacterium* sp. and filamentous fungi are used for cheese making. Mycelium fungi, used for cheese making, belong to genus *Penicillium*; *P. camemberti* grows on the surface of the curd showing white mycelium, while *P. roqueforti* is present inside the cheese showing blue veined color. Edible use of the two species is exclusive in cheese making.

Although limited information is available concerning its dairy use (Yu *et al*. 2016), *Monascus* species have been used for fermented rice in Eastern Asia, especially in China for more than 1,000 years (Feng *et al*. 2012). *Monascus* sp. is known to produce red, orange and/or yellow pigments. Additionally, diverse secondary metabolites responsible for health benefits including hypolipidemia, antihypertensive, antioxidant and antihyperglycemic effects and prevention of obesity and diabetes development have been recognized in *Monascus*-fermented rice (Burke, 2015; Chen *et al*. 2008; Feng *et al*. 2012; Lee and Pan, 2012; Wang & Lin, 2007). *Monascus* sp. can be cultured in liquid culture medium; however, it is considered that the solid-phase media is more suitable for enhancing pigment and functional secondary metabolite production. Accordingly, recent studies are designed to enhance the functional activity using other solid-phase media including dioscorea, soybean adlay and garlic (Lee *et al*. 2010; Lee and Pan, 2012; Lee *et al*. 2015; Pyo and Seong, 2009; Srianta *et al*. 2014). Lovastatin, monascin and ankaflavin are known as the functional metabolites; however, strain and culture condition should be carefully selected because some strains of *Monascus* sp. produce nephrotoxin, citrinin.

Since it is of interest to apply this species for development of a novel type of functional cheese along with red colored appearance, we tried producing red-mold cheese according to the protocol for manufacturing Camembert-type cheese by substitution of *Monascus* for *P. camemberti* as a preliminary trial. However, growth of this fungus was poor under this circumstance where nutrients were available from casein, milk fat globule, limited amount of carbohydrate and components from lactic starter in curd matrix. Additionally, as the optimal growth temperature of *Monascus* sp. is higher than temperatures appropriate for cheese ripening, alternative protocol should be introduced.

In our previous study (Kumura *et al*. 2017), whey protein concentrate (WPC) was used as solid substrate for *Aspergillus oryzae* and the culture products were mixed with fresh cheese curd prepared under the procedure for traditional semi-hard type cheese making. In

subsequent 3 months' ripening, the flavor properties of the resulting products were modified by the addition of the culture products of *A. oryzae*. This protocol is advantageous because autoclaving of WPC solution readily leads to solidification due to the heat denaturation of whey protein. If necessary, the ingredients including acidic or alkaline solution for pH adjustment can be mixed with WPC solution prior to autoclaving. Accordingly, suitable culture condition can be selected to prepare adjunct fungal materials for cheese making under an aseptic handling. In this previous study (Kumura *et al*. 2017), the culture products were selected on the basis of the protease profile because the protease production was proved to be dependent on not only the strain, but also culture condition including temperature and initial pH value. This context encourages us for the application of *Monascus* sp. culture products as adjunctive materials for cheese preparation, if a culture condition, allowing citrinin-free, but abundant functional metabolites and brilliant red pigment, is successfully found.

In this study, several strains of *Monascus* sp. were incubated under different culture conditions, and citrinin and lovastatin production were compared. After selection of the appropriate strain and culture condition, the culture products were used for cheese making. Finally, chemical analysis was conducted to characterize the red mold cheese and the potential of *Monascus* sp. for dairy application is discussed.

2 MATERIALS AND METHODS

2.1 Strain and Growth Media

Strains used in this study were two strains of *M. pilosus* (AHU 9090 and NBRC 4520), four strains of *M. purpureus* (AHU 9085, AHU 9087, AHU 9451 and NBRC 4513) and three strains of *M. ruber* (NBRC 4532, NBRC 9203 and NBRC 32318). The strains AHU 9090, AHU 9085, AHU 9087 and AHU 9451 were obtained from the culture collection of Hokkaido University and other five strains were from Biological Resource Center (Chiba, Japan). These strains were grown on potato dextrose agar (PDA; Merck KGaA) at 30°C for 10 days. Spore suspensions were prepared by adding 9.0 g/L sodium chloride (NaCl) solution into grown culture on PDA and diluted to the concentration of 2.5×10^5 spores/ml.

WPC80 (Fonterra) was dissolved in 3-fold weight of deionized water and adjusted to pH 6.5 with lactic acid or tri-sodium phosphate. Subsequently, the WPC solution received beta-galactosidase solution (GODO-YNL, GODO SHUSEI CO., Japan) at a working concentration of 125 ppm and maintained at 5°C for 24 h. Using lactic acid or tri-sodium phosphate, the solution was adjusted to the desired pH value and divided (10 g) to 100 ml Erlenmeyer flask and autoclaved at 121°C for 15 min to prepare the solid-phase medium. The medium was inoculated with 50 µl of the spore suspension and cultivated at 25, if necessary

30 or 35°C for 10 days.

2.2 Extraction and analysis of secondary metabolites

The resulting culture (1 g) was ground and suspended in 10 ml ethyl acetate containing 35 mM hydrochloric acid. After heat treatment at 60°C for 30 min, supernatant was recovered by centrifugation at 15,000 × g and 25ºC for 10 min. After lactonization by adding 1 ml of trifluoroacetic acid (TFA) (Panda *et al*. 2009), the suspension was filtered through filter paper (Advantec No. 5C). The filtrate was evaporated under vacuum and re-dissolved in 1 ml of acetonitrile. The suspension was filtered by 1.0 μm syringe filter (Whatman) and then by 0.5 μm syringe filter (TOSOH). The sample was subjected to high performance liquid chromatography (HPLC) analysis.

2.3 HPLC

HPLC analysis was carried out as described by Wu *et al*. (2011) with some modifications. Citrinin and lovastatin were analyzed by HPLC on J-Pak Supero C_{18} column (250 mm×4.6 mm id, 5 μm particle size) by isocratic elution using 0.05% (v/v) TFA in acetonitrile-water (62.5:37.5, v/v) as mobile phase. The column oven was set at 40°C. The injection volume was 10 μl and the flow rate was set at 1.0 ml/min. Lovastatin was detected by UV detector with wavelength set at 234 nm and citrinin was detected by fluorescence detector with excitation and emission wavelength set at 330 and 500 nm, respectively.

Citrinin (Enzo life Sci. Switzerland) and lovastatin (Tokyo Chemical Industry Co., Ltd.) were dissolved in ethanol to prepare stock solution. Standard solutions of citrinin and lovastatin were prepared by dilution of the stock solution with ethanol in a concentration range of 0.02-20 μg/ml, and 0.1-200 μg/ml, respectively.

2.4 Preparation of adjunct materials for cheese making

Beta-galactosidase treated WPC80 solution received lactic acid to adjust to the pH value of 4.0 and aliquoted (10 g) to 100 ml Erlenmeyer flask followed by autoclaving. *M. ruber* NBRC 32318 was cultured at 25°C for 10 d and the pooled culture products were treated with a food processor to obtain fine particles. As the control, un-inoculated solid WPC80 medium was prepared in the same manner.

2.5 Cheese making

Three batches of cheese making were carried out in February 2017 for lots No. 1 and No. 2, and in August 2017 for lot No. 3. Holstein-Friesian raw milk of was obtained from

the experimental farm in the Field Science Center for Northern Biosphere, Hokkaido University. Composition of the whole milk, in February, was 41.5 g/L and 88.3 g/L for fat and solid non-fat (SNF), respectively and that in August was 35.7 g/L and 85.1 g/L for fat and SNF, respectively. The whole milk was standardised with skim milk and the resulting fat content of the cheese milk was found to be 31.3±1.7 g/L. The milk (120 kg) was heated at 72ºC for 15 sec, and after cooling it to 31ºC, it was transferred to a vat with 120 ml of 1.5 M calcium chloride solution and 2.4 L of bulk starter (BD culture CH N-01; Chr. Hansen, Denmark) prepared in sterilised skim milk. After incubation for 60 min, 6 g of calf rennet (Chr. Hansen, Denmark) dissolved in 120 ml of 0.1 M NaCl solution was added to induce coagulation of the milk. After curd formation, it was cut into cubes 10 mm^3 in size and left for 15 min. Subsequently, gentle stirring was performed at 31ºC for 30 min and 45 kg of whey was discarded. Then, the same volume of hot water (60ºC) was added to reach 40ºC, followed by 15 min of agitation at this temperature. After the drainage, the curds were recovered and mixed with fine particles of adjunct materials uniformly by hand to prepare experimental cheese. The ratio of the curds and adjunct materials (w:w) was 19:1 and transferred to 1 kg size of mould for Gouda type cheese. As the control cheese, un-inoculated solid WPC medium was used. These two types of cheese was prepared triplicate for each lot. Following brief pressure (1.0 kg/cm^2 for 10 min.), inversion and a second stage of pressure (1.5 kg/cm^2 for 50 min.) were performed. The curd was cooled in the water overnight and immersed in 0.25 kg/kg NaCl solution for 15 h. The curds were matured at 11.5ºC for 13 wk with a relative humidity of 90%. After 15 d of manufacture, cheese surface was coated with wax (Paramelt, Netherland).

2.6 Analysis of cheeses

All assays were performed in triplicate.

The moisture was determined according to the IDF recommendation (2004). Fat, protein, and salt calculated from chloride content were measured by the AOAC method (1995a-c).

Water-soluble nitrogen (WSN) was determined according to the method of Ardö (1999) with some modifications: a sample (5 g) was added to 25 ml of deionized water and after treatment with a stomacher for 5 min, maintained at 40ºC for 1 h. The sample was centrifuged and the supernatant was passed through glass wool to entrap lipid. The resulting filtrate was subjected to micro Kjeldahl method (AOAC, 1995b).

After extraction of fat according to the AOAC protocol (AOAC, 1995a), free fatty acids (FFA) were extracted by phenol-red method (Saito 1979). Oleic acid was used as the standard

and converted to content of oleic acid (mmol) in 100 g of cheese.

Extraction of secondary metabolites from cheese was performed as described above; however, after evaporation of the filtrate, samples were re-dissolved in 3 ml of acetonitrile and passed through glass wool to entrap milk lipid. The resulting materials were filtered by 1.0 μm and 0.5 μm syringe filter to be subjected to HPLC analysis.

2.7 Determination of recovery efficiency of secondary metabolites from the culture products and cheese

Known amount of citrinin (10, 15 and 20 μg) or lovastatin (50, 100, 150 and 200 μg) was added to the 0.5 g of *Monascus* culture product or 1.0 g of the control cheese. The values were subtracted with those obtained from corresponding samples, which had received no citrinin or lovastatin. The recovery was expressed as the percentage of the obtained values versus added amount of the secondary metabolites.

2.8 Statistical analysis

Percentage of WSN in total nitrogen, FFA and lovastatin content in cheeses were analyzed by Tukey-Kramer's multiple comparison test using JMP software (version 11.0; SAS Institute, Inc., Tokyo, Japan). Differences were considered to be statistically significant at $P < 0.05$.

3 RESULTS

3.1 Selection of the strain and growth media

As shown in Figure 1, growth and pigment production depended on the strain. In terms of red pigment production on this medium, *M. purpureus* AHU 9085 and AHU 9087 and *M. ruber* NBRC 9203 and NBRC 32318 were selected for the following studies.

Figure 2 shows the production of citrinin and lovastatin. Since recovery of citrinin and lovastatin from the culture products was 85 and 72%, respectively, converted values were shown in Fig. 2. There were two strains of *M. purpureus* produced citrinin with undetectable amount of lovastatin. In contrast, two strains of *M. ruber* produced lovastatin with undetectable amount of citrinin. These metabolites were abundantly produced when incubation of these fungi was initiated in acidic pH circumstances. Capability of lovastatin production in NBRC 32318 was higher than that in NBRC 9203. However, no lovastatin and citrinin were detected when NBRC 32318 was incubated at 30 or 35ºC for 10 d despite red color pigmentation being recognized (data not shown). Accordingly, strain NBRC 32318 was incubated on the WPC medium pH 4.0 at 25ºC for 10 d to prepare the culture products for

cheese making.

3.2 Cheese composition

Figure 3 shows the appearance of red mold cheese after 3 months of ripening and Table 1 shows the composition of the experimental cheese from the three batches.

Although WSN ratio in the total nitrogen and FFA content showed insignificant difference, when all the data were subjected to statistical analysis; the former parameter in red mold cheese was increased compared to that in corresponding preparation of control cheese. No bitterness and rancid were noted by brief tasting in the two types of cheese. Since known amount of citrinin or lovastatin was added to cheese and determination was performed according to our experimental procedure, recovery of citrinin and lovastatin was 76 and 68%, respectively; converted values were shown in Table 1. The red mold cheese was assumed to contain 1.5~2.3 mg/100 g of lovastatin with undetectable amount of citrinin.

DISCUSSION

Since most of *Monascus* sp. strains are devoid of lactose consuming capability (Shi and Pan 2010; Tseng *et al*. 2000), pre-treatment with beta-galactosidase was necessary when WPC was used as a solid substrate for favorable growth of the tested strains. Referring to the previous studies on the red pigment and lovastatin productivity (Ahn *et al*. 2006; Jirasatid *et al*. 2013), incubation temperature was defined at 25ºC. Among 9 strains, *M*. *purpureus* AHU 9085 and 9087 and *M*. *ruber* NBRC 9203 and 32318 showed red pigment production on WPC medium, which was apparently independent of the initial culture pH condition. As red pigment production of other strains belonging to the same species, namely AHU 9451, NBRC 4513 and 4532, was undetectable, red pigment producing capability was due to the traits of the strain, not nutritional respect in WPC medium. However, some nutritional components in WPC medium might affect citrinin production. Tsukahara *et al*. (2009) compared citrinin, lovastatin and pigment production using 29 strains of *Monascus* sp. on moistened rice as the substrate and provided culture temperature history comprising 30ºC for 4 d followed by 25ºC for 17 d. They reported production of citrinin by *M*. *ruber* NBRC 9203 and NBRC 32318, which was inconsistent with our results. This difference could be due to the nutritional and/or temperature environment, because we confirmed appearance of a peak corresponding to the retention time of citrinin in HPLC chromatogram when the extract of moistened rice culture product of NBRC 32318 was evaluated (Takahashi unpublished result). Further studies will be needed to elucidate influence of nutritional components in WPC medium on the gene

expression related to citrinin production, which could provide advantageous information for use of WPC solid medium. In contrast, it was consistent with the previous results (Tsukahara *et al*. 2009) that among three strains of *M*. *ruber* NBRC 4532, 9203 and 32318, strain 4532 showed the lowest red pigment producing capability and strain 32318 showed twice as higher amount as lovastatin production compared with strain 9203. Although it was unsuccessful to induce lovastatin production of *M*. *ruber* NBRC 32318 higher than at 30ºC, it remains possible to increase lovastatin production if some nutrients are supplemented to the solid medium. Since lovastatin production of the two strains of *M*. *ruber* was preferred under acidic condition, it is of interest to initiate cultivation lower than at pH 4.0, although adjustment of pH value of WPC to lower than 4.0 caused browning during autoclaving due to Maillard reaction.

According to the definition of Tsukahara et al. (2009), *M*. *purpureus* and *M*. *kaoliang* were nominated as the high citrinin producer, and they showed poor lovastatin productivity. In contrast, *M*. *ruber* and *M*. *pilosus* strains produced lovastatin and were listed as the low citrinin producer because much lower amount of citrinin compared to *M*. *purpureus* and *M*. *kaoliang* was detectable. The phylogenetic analysis of the partial beta-tubulin gene showed that *M*. *purpureus* and *M*. *kaoliang* were placed in the same clade, whereas *M*. *ruber* and *M*. *pilosus* belonged to another same clade (Chen *et al*. 2008; Shi and Pan 2010). Additionally, *mafp1* gene, which encodes antifungal activity protein MAPF1, was recognized in *M*. *pilosus* and *M*. *ruber*; but not in *M*. *purpureus* and *M*. *kaoliang* (Tu *et al*. 2016). In spite of such close relationship, red pigment production of these two species on WPC medium was different. Tsukahara *et al*. (2009) focused on *M*. *pilosus* NBRC 4520 for functional fermented food inoculation due to its high lovastatin and low citrinin production with a deep-red color. In our study, NBRC 4520 showed limited growth with dot red pigmentation when incubation was initiated, near the neutral pH region. If an appropriate environment would be prepared such as nutritional supply, pH and/or temperature, desired features could be induced from NBRC 4520 on WPC substrate.

Extraction of secondary metabolites from grain culture products has been done using methanol, ethanol (>50%) or ethyl acetate (Ajdari *et al*. 2011; Panda *et al*. 2009; Pyo, and Seong 2009; Tsukahara *et al*. 2009; Wu *et al*. 2011). However, extraction efficiency of citrinin and lovastatin from WPC culture products using 75% (v/v) ethanol was less than 5%, using acidic ethyl acetate (data not shown). The possible reason of this poor recovery is hydrophobic binding of secondary metabolites to whey protein or peptides, which had been generated in the culture substrate during incubation. The protein content of this WPC medium is more abundant (ca. 20 g/100 g) compared to that of grains frequently used for the solid

substrates. The Monascus pigments such as yellowish citrinin are often associated with proteins or cell wall, forming complex pigments, difficult of being extracted (Vendruscolo *et al*. 2016). Finally, we established the extraction procedure of citrinin and lovastatin from *Monascus* culture products on WPC solid medium.

Although chemical composition of the experimental cheese depended on the preparation, ratio of water-soluble nitrogen to total nitrogen in red mold cheese was increased compared to that in corresponding preparation of control cheese in all batches, which could be understood as enrichment of ripening. Since ripening temperature was much lower than appropriate growth for *Monascus* sp., and cheese surface was coated during ripening to interfere with aeration, this increase was due to proteinases, which had been induced in the solid WPC medium during cultivation, rather than growth of micro-organism during the ripening period. In terms of proteinase from *Monascus* sp., Lakshman *et al*. (2011a, b) reported that extracellular acid proteinase from *M*. *purpureus* and *M*. *pilosus* reduced antigenicity of bovine milk whey protein. It is conceivable that *M*. *ruber* NBRC 32318 secreted proteinase responsible for whey protein as well during cultivation on WPC medium where whey protein was the major nitrogen source for fungal growth. Moreover, involvement of cellular binding proteases would be possible in the increase of WSN ratio because living mycelia was incorporated into cheese matrix.

Our previous study reported that a specific culture product from *A*. *oryzae* AHU 7139 on WPC solid medium to cheese curd caused intense rancid in three-month-aged cheese products (Kumura *et al*. 2017). In contrast, negligible difference of FFA content between control and red mold cheese was recognized, which demonstrated that influence of lipolytic system of *Monascus* could be excluded.

As shown in Figure 2, lovastatin content in the culture products for adjunctive materials was around 300 μg/g and its addition with cheese curd in our recipe will be about 1.5 mg lovastatin in 100 g of the cheese product theoretically. Accordingly, lovastatin content in cheese was maintained at as much amount as had been added to curd and it was rather increased in lot 1 that was estimated as 2.3 mg/100 g cheese. The reason of this enigmatic result is unclear, though it might be due to the action of the lovastatin synthetic enzymes during the ripening period. At least, it can be concluded that no degradation of lovastatin occurred during ripening. Lovastatin is known as cholesterol lowering agent due to the inhibition of hydroxymethylglutarate coenzyme A reductase and its daily administration ranges from 3 to 15 mg (Becker *et al*. 2008; Becker *et al*. 2009; D'Addato *et al.* 2017; European Food Safety Authority 2011). Although it might be requested to increase lovastatin content in red mold cheese, it is of interest to conduct in vivo study associated with lipid

metabolism using red mold cheese because health benefit including cholesterol reduction is found in Gouda-type cheese administration as well (Nilsen *et al*. 2015) and synergic effect of lovastatin and cheese on lipid metabolism could be expected.

In conclusion, *Monascus* sp. is possible to apply for development of a novel type of cheese. Further studies are required to estimate functionality of red mold cheese in terms of health benefits.

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Figure legend;

FIGURE 1 Growth and appearance of *Monascus* sp. on whey protein-based solid substrate at different initial culture pH circumstances. *M. pilosus* AHU 9090 and NBRC 4520, and *M. purpureus* AHU 9085, AHU 9087, AHU 9451 and NBRC 4513, and *M. ruber* NBRC 4532, NBRC 9203 and NBRC 32318 were cultured at 25ºC for 10 days. Initial culture pH values were also indicated.

FIGURE 2 Production of (a) citrinin and (b) lovastatin in *M. purpureus* AHU9085 (▨), *M. purpureus* AHU9087 (⁘), *M. ruber* NBRC9203 (□) and *M. ruber* NBRC32318 (■) cultivated on whey solid medium adjusted to the defined pH value at 25°C for 10 days. n=3
The values of citrinin and lovastatin were indicated after the calculation on the basis of the recovery efficiency of corresponding substances. Results represent the mean ± SE.

FIGURE 3 Appearance of red mold cheese.

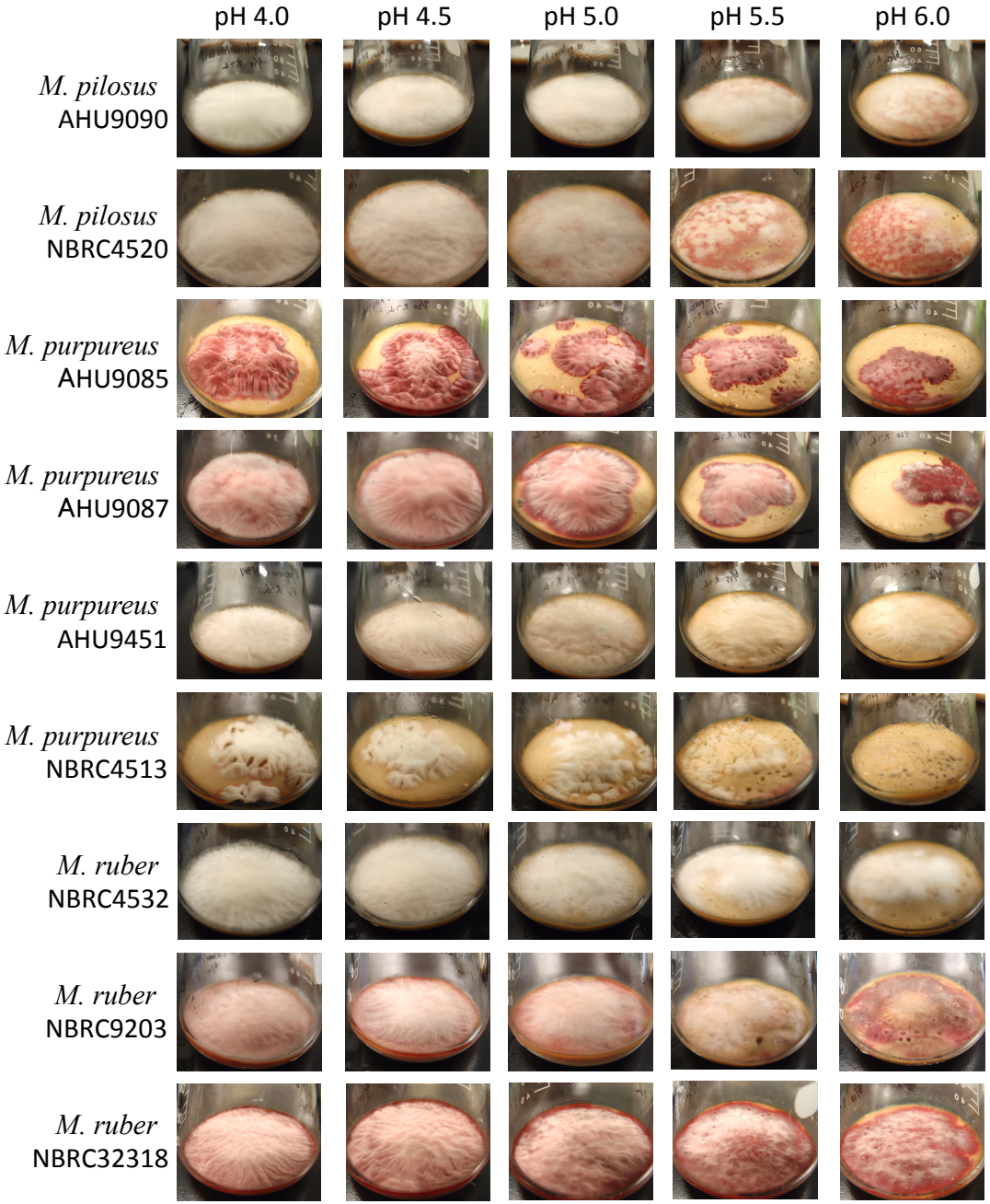
pH 4.0
pH 4.5
pH 5.0
pH 5.5
pH 6.0
M. pilosus AHU9090
M. pilosus NBRC4520
M. purpureus AHU9085
M. purpureus AHU9087
M. purpureus AHU9451
M. purpureus NBRC4513
M. ruber NBRC4532
M. ruber NBRC9203
M. ruber NBRC32318

Fig. 1

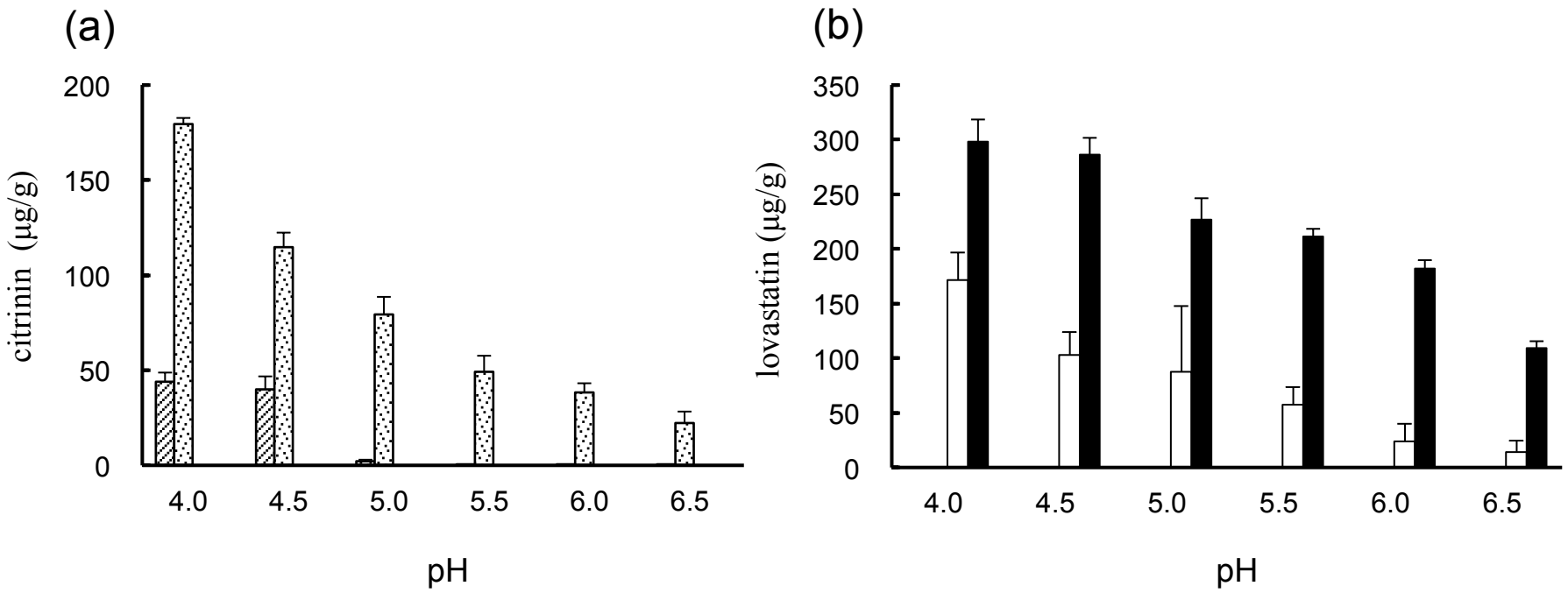
(a)
citrinin (μg/g)
200
150
100
50
0
4.0
4.5
5.0
5.5
6.0
6.5
pH
(b)
lovastatin (μg/g)
350
300
250
200
150
100
50
0
4.0
4.5
5.0
5.5
6.0
6.5
pH

Fig. 2

Fig. 3

Table 1. Chemical analysis of the experimental cheeses

lot		water (%)	lipid (%)	protein (%)	FFA (mmol) in 100 g cheese	Percentage of water soluble N in total N (%)	lovastatin (mg) in 100 g cheese
1	control	35.3	28.2	28.9	8.0	12.8	-
	red mold	33.4	30.5	28.3	8.3	16.8	2.3★
2	control	36.4	30.0	25.8	9.9	14.9	-
	red mold	35.2	30.0	27.5	10.1	19.5	1.5★
3	control	38.2	29.7	25.5	5.6	16.3	-
	red mold	37.0	30.4	24.8	6.0	24.5	1.5★

★ Indicates a significant difference vs control ($P < 0.001$)