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Screening of adjunct cultures and their application in ester formation in Camembert-type cheese

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ABSTRACT

The ethanol content and esterase and alcohol acyltransferase activities are the limiting factors in the synthesis of ethyl esters in Camembert-type cheeses. This study aimed to investigate the effects of alcohol, esterase and alcohol acyltransferase activities on ethyl ester formation in Camembert-type cheeses. Five experimental cheeses were prepared with three adjunct cultures with different enzyme activities and two levels of ethanol content (400 or 800 μg/g). The cheeses were aged for 4 weeks and analysed weekly for basic physicochemical, textural, volatile and sensory properties. The results showed that both the enzyme activity and ethanol content were limiting factors in the synthesis of ethyl esters in the Camembert-type cheeses. Variation in the esterase synthesis activity was observed among lactic acid bacteria, and the starter culture *Lactococcus lactis* MA 14 LYO distinguished itself through its high acidifying and esterase hydrolysis abilities. The addition of CCFM 12, a lactic acid bacteria strain with high esterase and alcohol acyltransferase activity, along with 400 or 800 μg/g of ethanol, notably enhanced the generation of ethyl esters and the corresponding fruity flavour, without causing dramatic changes in the basic physicochemical indices and microbial profile. In addition, cohesiveness was influenced by the addition of 400 and 800 μg/g of ethanol, and more resilience with 800 μg/g of ethanol had been found. The results showed that the addition of CCFM12 with 400 and 800 μg/g of ethanol may be applied in the production of Camembert cheese to enhance its fruity flavour.

1. Introduction

Esters, especially ethyl esters such as ethyl acetate and ethyl butanoate, are considered important flavour compounds that impart a pleasant fruity flavour to beverages, yogurt, cheese, etc. (Blades et al., 2009). The major pathways for the biosynthesis of esters in fermented dairy foods are esterification (the reaction of alcohols and acids catalysed by esterase) and alcoholysis (the reaction of glycerides and alcohols catalysed by alcohol acyltransferase [AAT]) (Liu et al., 2004). One of the key elements in the development of the fruity flavour in many hard cheeses is the presence of ethanol, which is known to be the limiting factor for ethyl esters formation in Cheddar cheese (Liu et al., 2004) and Swiss cheese (Richoux et al., 2008; Thierry et al., 2006). In Swiss cheese, the addition of ethanol enhances the concentration of ethyl esters and imparts a powerful fruity aroma (Richoux et al., 2008; Thierry et al., 2006). In soft cheese, in which abundant alcohol is detected, the determining factor for ethyl ester formation is rather the enzyme activities of the bacterial strains involved in the biosynthesis of esters such as esterase or ATT (Mukdsi et al., 2009) instead of ethanol (Molimard and Spinnler, 1996; Sablé and Cottenceau, 1999).

Variation has been reported in the enzymatic activities of starter cultures and adjunct cultures related to glycolysis, proteolysis and lipolysis (Hassan et al., 2012; Settanni and Moschetti, 2010). Mesophilic starter cultures *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* were associated with fruity flavours in Cheddar cheese due to the presence of esterase in crude cell extracts (Liu et al., 2004). Strains of *Streptococcus thermophilus* (Liu et al., 2003) and *Lactobacillus fermentum* (Crow et al., 2002) were also shown to possess a strong ability to synthesise ethyl butanoate.

However, previous studies showed that only limited amounts of

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esters were detected in mould-ripened Camembert cheese (Pionnier et al., 2002) indicating the potential to improve the formation of esters in such cheese. In a previous study, the addition of 400, 800 or 1200 μg g^{-1} of ethanol prior to coagulation appeared to be effective in generating ethyl esters and enhancing the fruity flavour in Camembert cheese (Hong et al., 2017). Therefore, in the present study, 12 lactic acid bacteria (LAB) strains were screened as adjunct cultures for esterase and AAT activities, and the effects of esterase and AAT activities on ethyl ester formation in Camembert-type cheese along with ethanol were investigated to provide a potential approach to improve the fruity flavour of Camembert cheese.

2. Materials and methods

2.1. Bacterial strains

The 12 LAB strains used for screening in this study were obtained from the Culture Collections of Food Microbiology, Jiangnan University (Wuxi, China) and are listed in Table 1. Starter cultures of *Lc. lactis* MA 14 LYO, *Penicillium camemberti* PC 12 LYO 20 D and *Geotrichum candidum* GEO17 LYO 2 D were obtained from Danisco Co., Ltd. (Shanghai, China).

2.2. Chemicals

Calf rennet extract with 2200 international milk-clotting units (IMCU)/g was obtained from Danisco Co., Ltd. (Shanghai, China). Ethanol (99.7%) was obtained from Dongguan Zhenxin Science and Technology Co., Ltd. (Guangzhou, China). *p*-nitrophenyl butanoate and decanoate, heptadecanoic acid were purchased from Sigma (Shanghai, China).

2.3. Acidifying activity and autolytic ability

The acidifying activity of the strains was measured using a pH meter (Mettler Toledo, Shanghai, China) following the 24 h of incubation at 30 °C in 12% (w/v) skim milk. The autolytic ability was evaluated according to Bozoudi et al. (2015) with modifications. De Man Rogosa Sharpe (MRS) broth was inoculated (2% v/v) with the strains and the cells were harvested by centrifugation at 3186 × *g* for 15 min at 4 °C, washed twice in 0.05 M phosphate buffer solution (PBS) at pH 7.0, resuspended in 10 mL PBS and incubated at 37 °C. Optical density (OD) at 600 nm was measured prior to incubation (OD_1) and after 18 h (OD_2). The results were expressed with the following formula: autolysis (%) = (OD_1 − OD_2)/OD_1 × 100%. All the assays were performed in triplicate.

2.4. Cell-free extract preparation

The cell-free extract (CFE) was prepared as described by Pérez-Martín et al. (2013). Cells cultured in 400 mL of MRS broth were harvested after 16 h of incubation by centrifugation at 1147 × *g* for 10 min at 4 °C, followed by an adjustment of OD_{600} to 0.5. Then, 15 mL aliquots of the cell suspension were ultrasonically disrupted for 20 min at 5 s intervals using an Ultrasonic Processor (Sonics & Materials, Inc., Newtown, CT, USA) with the working power set at 40%. Cellular debris was removed by centrifugation (6000 × *g* for 5 min at 4 °C), and the supernatant was used as the CFE. The protein content was determined via the Coomassie brilliant blue method.

2.5. Hydrolytic and synthetic activity of esterase and alcohol acyltransferase activity

The hydrolytic activity of esterase was measured according to the method of Matthews et al. (2007) with modifications. The assays were performed with 860 μL of 0.1 M McIlvane buffer (0.1 M citric acid and 0.2 M K_2HPO_4) at pH 5.0, 100 μL of CFE (with the protein concentration in the range of 1–3 mg/mL) and 40 μL of 25 mM *p*-nitrophenyl acetate, butanoate or decanoate in ethanol as substrate. The reactions were incubated at 37 °C for 2 h, after which 100 μL of 0.5 M NaOH was added to stop the reaction and OD_{400} was determined using an ultraviolet spectrophotometer (Shimadzu, Tokyo, Japan). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute.

Synthetic activity of esterase was conducted according to the method of Mukdsi et al. (2009) with modifications. The mixture of 100 mM PBS, 5.5 mM free fatty acid (acetic acid, butanoic acid or decanoic acid), 5 M ethanol and CFE was prepared and incubated at 30 °C for 17 h. Then 1 mL sample was added to 2 mL *n*-hexane with 50 μL heptadecanoic acid as the internal standard. Extraction was performed by shaking vigorously for 2 min, followed by centrifugation at 900 × *g* for 3 min. Then, the upper layer was transferred to a gas chromatography vial. Controls without CFE were tested for the non-enzymatic formation of esters as described above.

Ester synthetic activity of AAT was conducted according to the method of Liu et al. (2003) with modifications. The assay mixture consisted of 100 mM PBS, 5.5 mM triglyceride (triacetin, tributyrin or tridecanoin), 5 M ethanol and CFE and was incubated at 30 °C for 17 h. Then 1 mL sample was analysed as described above.

Then the concentrations of ethyl esters produced by esterification or alcoholysis reactions were determined with gas chromatograph (SHIMADZU, Tokyo, Japan) equipped with a flame ionisation detector. A DB-WAX column (30 m × 0.32 mm inner diameter, 0.25 μm film thickness) was used. Sample injections of 1 μL were conducted in split mode at a 15:1 ratio, and the injection temperature was 220 °C. The initial column temperature was held at 40 °C for 3 min, and increased at 5 °C/min to 190 °C and sequentially increased to 220 °C at 4 °C/min and maintained for 2 min. The flow rate was 2.0 mL/min with nitrogen as the carrier gas. A unit of esterase or AAT was defined as the amount of ester formed per gram of enzyme. All the assays were performed in triplicate.

2.6. Cheese manufacture and sampling

Raw milk with a composition of 3.1% protein, 3.7% fat and 4.7% lactose was collected from a local farm and kept at 4 °C prior to the manufacture of the cheese. Camembert-type cheeses were manufactured following the methods described by Leclercq-Perlat et al. (2004) and Guizani et al. (2002) with modifications. The milk was pasteurised (65°C/30 min) and cooled to 32 °C before the inoculation of 2.5% commercial starter cultures of *Lc. lactis* MA 14 LYO, *Penicillium camemberti* PC 12 LYO 20 D and *Geotrichum candidum* GEO17 LYO 2 D. Calf rennet extract at a concentration of 0.20–0.25 g/10 kg was added to the milk after 45 min. After coagulation, the curd was cut into 2 × 2 × 2 cm blocks and left to heal for 1 h. Then, the curd was ladled into cheese hoops and turned every 15 min until the pH reached 5.4. The curd was brined in 21% NaCl solution for 1 h and then drained for 30 min. The cheese was then moved to a ripening chamber at 14 °C and 90% relative humidity. The blocks of cheese were turned every 2–3 days and wrapped with tinfoil at 14 days for further ripening. Each cheese was divided into sectors, and the cheese samples were taken with rind for triplicate analyses at days 1, 7, 14, 21 and 28.

Each batch of the cheese samples were manufactured in duplicate from the two batches of milk on different days. For the L1, L2 and L3 samples, *Lactobacilli* CCFM12, CCFM177 or CCFM410 were added (approximately 10^7 CFU/mL) as adjunct cultures together with the starter cultures described above. For the EL4 and EL5