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**Abstract**

In recent years, biocontrol of foodborne pathogens has become a concern in the food industry, owing to safety issues. *Listeria monocytogenes* is one of the foodborne pathogens that causes listeriosis. The major concern in the control of *L. monocytogenes* is its viability as it can survive in a wide range of environments. The purpose of this study was to isolate lactic acid bacteria with antimicrobial activity, evaluate their applicability as a cheese starter, and evaluate their inhibitory effects on *L. monocytogenes*.

*Lactococcus lactis* strain with antibacterial activity was isolated from raw milk. The isolated strain was a low acidifier, making it a suitable candidate as an adjunct starter culture. The commercial starter culture TCC-3 was used as a primary starter in this study. Fresh cheese was produced using TCC-3 and *La. lactis* CAU2013 at a laboratory scale. Growth of *L. monocytogenes* (5 log CFU/g) in the cheese inoculated with it was monitored during the storage at 4°C and 10°C for 5 days. The count of *L. monocytogenes* was 1 log unit lower in the cheese produced using the lactic acid bacteria strain compared to that in the cheese produced using the commercial starter.

The use of bacteriocin-producing lactic acid bacteria as a starter culture efficiently inhibited the growth of *L. monocytogenes*. Therefore, *La. lactis* can be used as a protective adjunct starter culture for cheese production and can improve the safety of the product leading to an increase in its shelf-life.

**Keywords**: *Lactococcus lactis*, bacteriocin, *Listeria monocytogenes*, cheese starter culture, foodborne pathogen

## Introduction

Listeriosis is a foodborne disease caused by *Listeria monocytogenes*. It can lead to sepsis, meningitis, encephalitis, and even death (de Noordhout *et al*., 2014). Despite its low incidence compared with that of other foodborne illnesses, listeriosis is one of the major issues in the food industry because of its high fatality rate. *L. monocytogenes* is found in dairy products, particularly in ready-to-eat cheese products. As *L. monocytogenes* survives in various environments such as a those with a wide range of temperature (0–45°C) and pH (4.1–9.6), it can contaminate cheese at several stages of production; therefore, its growth is difficult to control (Lungu *et al*., 2008; Melo *et al*., 2014).

Several methods have been used to control the growth of *L. monocytogenes* in cheese, including using bacteriocin or bacteriocin-producing lactic acid bacteria (LAB). Bacteriocins are peptides or proteins, ribosomally synthesized by bacteria, which have antimicrobial ability against closely related species. The application of bacteriocin-producing bacteria is advantageous as they are stable, cost-effective, and safe. Anti-listerial activity of LAB in cheese have also been reported (Coelho *et al*., 2014; Dal Bello *et al*., 2012; Kondrotiene *et al*., 2018).

In the present study, we aimed to determine the effects of bacteriocin-producing LAB isolated from raw milk on the growth of *L. monocytogenes* in milk broth and cheese.

## Materials and Methods

### Isolation of bacteriocin-producing lactic acid bacteria

Potential bacteriocin-producing LAB were isolated from raw bovine milk, obtained from a Chung-Ang University-affiliated farm (Anseong, Republic of Korea). The sample was serially diluted ten-fold and plated on MRS agar (BD Difco, USA). The plates were incubated at 37℃ for 24–48 h, and a total of 90 well-isolated colonies were collected. Each colony was inoculated into MRS broth for 24 h at 37℃.

To screen for antimicrobial activity, the cell-free supernatant (CFS) was obtained after neutralization with 1N NaOH, centrifugation at 13,000 rpm for 10 min at 4℃, and filtered through 0.45 μm filters to remove bacterial cells. Then, each supernatant was spotted on the tryptic soy agar (TSA, BD Difco, USA) plate inoculated with a lawn of *Listeria monocytogenes* ATCC 19115 as an indicator strain. The plates were incubated at 30℃ for 12 h, and antibacterial activity was confirmed with the presence of inhibition zone. The strains with antibacterial activity were routinely cultured in MRS broth at 37℃ overnight and were preserved in 10% skim milk supplemented with 25% (v/v) glycerol, stored at -80℃ for further use.

### Identification of bacteriocin-producing strain

The bacteriocin-producing strains were identified by Gram staining, carbohydrate fermentation profile (analytical profile index (API) test), and 16S rRNA gene sequencing analysis. Gram staining and API analysis was performed using a Gram-stain kit (BD Difco, USA) and API 50 CHL kit (Biomérieux, France), respectively, according to the manufacturer's instructions.

For 16S rRNA analysis, the genomic DNA was extracted using QIAamp PowerFecal DNA Kit (Qiagen, Germany) and amplified using 2X H-star Taq PCR Master Mix (BioFACT, Republic of Korea). Polymerase chain reaction (PCR) was performed using the universal bacterial primers 27F (5′-AGAGTTTGATCMTGG CTCAG-3′), 1492R (5′-TACGGYTACCTTGTTACGACTT-3′), 785F (5′-GGATTAGA TACCCTGGTA-3′), and 805R (5′-GACTACCAGGGTATCTAATC-3′). The PCR products were purified using a PCR purification kit (Qiagen, Germany) and sequenced by SolGent Co. Ltd. (Daejon, Republic of Korea).

The analyzed sequences were confirmed using the EzTaxon-e server (www.ezbiocloud.net/) (Kim *et al*., 2012) and NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al*., 1990).

**Antibacterial activity of bacteriocin**

Bacteriocin activity was assessed using a spot-on-lawn method as described previously (Phumisantiphong *et al*., 2017) with minor modifications. Briefly, each indicator strain was inoculated to 4 mL of molten TSA and overlaid on the base TSA plate. After solidification, 20 µL of the neutralized CFS of LAB strains was spotted onto the indicator lawn. After incubation at 30℃ for 12 h, a clear inhibition zone was observed. The foodborne pathogens used as indicator strains were cultured in tryptic soy broth (TSB, BD Difco, USA) at 37℃ overnight before use. The experiment was conducted in triplicates.

**Evaluation of acid production**

The acid production of the *La. lactis* CAU2013 was evaluated and compared with that of the commercial starter TCC-3 (Chr. Hansen, Denmark), which consisted of *Lactobacillus*

*delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. The cultures were grown in MRS broth at 37℃ overnight. Then, individual cultures and mixture of *La. lactis* CAU2013 and TCC-3 (1:1 ratio) were inoculated in 10% skim milk broth (Harrington and Hill, 1991) and whole milk. The pH and titratable acidity (TA) were measured every three hours for 12 h while incubating at 30℃. To determine TA, 0.1% phenolphthalein was used as an indicator, and 0.1 N sodium hydroxide (NaOH) for titration.

**Anti-listerial activity of strain CAU2013 as an adjunct starter in milk**

To determine the anti-listerial properties of strain CAU2013 when used as an adjunct starter in milk, 10% skim milk broth and whole milk media were inoculated with an overnight culture of CAU2013 and 1:1 ratio of CAU2013 and TCC-3 starter (final concentration of 7 log CFU/mL). Additionally, the overnight culture of *L. monocytogenes* ATCC 19115 was inoculated to each setup (final concentration of 5 log CFU/mL). The inoculated milk media were incubated at 30℃ for 12 h. The viable cell count of *L. monocytogenes* was determined every three hours. Samples were diluted serially in ten-fold increments using 1×phosphate-buffered saline (PBS, pH7.5) and plated on Oxford agar (BD Difco, USA).

**Manufacture of laboratory-scale cheese**

The lab-scale cheese was manufactured following the methods of Mills *et al*.(2011) with some modifications. TCC-3 was used as the primary starter and *La. lactis* CAU2013 as an adjunct culture. The starter cultures were initially grown in MRS broth at 37℃ for 24 h before inoculation into 10% skim milk broth and incubated for 18 h at 37℃ before use. Additionally, *L. monocytogenes* ATCC19115 was cultured in TSB for 18 h at 37℃ before use.

Milk (400 mL) (Seoul milk, Republic of Korea) was heated to 31℃ before the inoculation of starter culture. The starter cultures were inoculated as follows: TCC-3 and *La. lactis* CAU2013

and TCC-3 (1:1 ratio), both at a final concentration of 7 log CFU/mL. Subsequently, 0.01% *L. monocytogenes* at a level of 5 log CFU/mL was inoculated into both treatments. After 30 min, 0.2 g/L of rennet was added, and the mixture was stirred for 2 min. Once coagulum formed firmly, the curd was cut into cubes, and the mixture was stirred for 10 min. Then, the mixture was heated to 36°C for 10 min and stirred for 20 min. The whey was drained off, and curd was distributed into the sterile dish. The samples were stored at 4°C and 10°C for 5 days. The procedure of cheese production is illustrated in Figure 1.

**Microbial analysis of laboratory-scale fresh cheese**

The viable cell counts of LAB and *L. monocytogenes* in the lab-produced cheese were determined in duplicate every day during storage at 4°C and 10°C. For microbial analysis, 1 g of cheese was homogenized in 9 mL of PBS buffer and were serially diluted ten-fold in the same buffer and plated on the appropriate agar plate. The LABs were enumerated on MRS agar after incubation at 37°C for 3 days, and *L. monocytogenes* on Oxford agar after incubation at 37°C for 24 h. All of the experiments were conducted in triplicates.

## Results and discussion

### Isolation and identification of bacteriocin-producing strains

Among the 90 colonies isolated from raw milk, one isolate exhibited antibacterial activity against *L. monocytogenes*. The strain CAU2013 was characterized as a gram-positive, coccus-shaped bacterium. The biochemical characteristics determined using the API 50 CHL kit are described in Table 1. 16S rRNA gene sequence analysis revealed that strain CAU2013 is most likely a strain of *Lactococcus lactis* (Table 2), which commonly produce nisin (Shin *et al*., 2016). Neighbor-joining (NJ) phylogenetic tree of the strain CAU 2013 and related type strains based on 16S rRNA gene sequences also clearly show that this strain belongs to *Lactococcus* lactis (Supplementary Figure 1). *La. lactis* strains are historically used in the fermentation and preservation of food and are generally recognized as safe (GRAS) (Cook *et al*., 2018). Therefore, *La. lactis* CAU2013 was selected for downstream applications in the study.

*L. monocytogenes* ATCC 19115 was used as an indicator strain for all experiments because it belongs to the serotype 4b, which causes most cases of listeriosis.

### Antibacterial activity of bacteriocin

The bacteriocin produced by *La. lactis* CAU2013 had antibacterial activity against all *Listeria* strains as well as *Staphylococcus aureus*, which are common foodborne pathogens (Yoon, 2020). However, no antibacterial activity was observed against other gram-positive foodborne pathogens, such as *Salmonella enteritidis* and *Escherichia coli* (Table 3). Generally, nisin is highly effective against gram-positive bacteria by binding to lipid II, which leads to the inhibition of cell wall biosynthesis or pore formation in the membrane. However, nisin cannot

bind to its target lipid II in gram-negative bacteria, because of the presence of the outer membrane (Li *et al.,* 2018).

**Characterization of acid production**

The changes in pH and TA values in 10% skim milk broth and in whole milk are presented in Figure 2. *La. lactis* CAU2013 reduced the pH of skim milk broth from 6.41 to 5.77 and that of whole milk from 6.65 to 6.20. Additionally, TA value increased to 0.25 in both broths. Ayad *et al*. (2004) described fast, medium, or slow-acidifying strains as $\triangle pH(=pH_{at\ time}-pH_{zero\ time})$ of 0.4 U achieved after 3 h, 3–5 h, and > 5 h, respectively. Also, Raquib *et al*. (2003) classified strains with titratable acidity as low, moderate, or fast when the TA values were < 0.5, between 0.5 and 0.6, and > 0.6, respectively. Therefore, *La. lactis* CAU2013 can be classified as a low acidifier strain. This result is consistent with other studies that reported poor acid production from *La. lactis* strains (Ayad *et al*., 2004; Coelho *et al*., 2014).

The pH values measured corresponded with the calculated TA and were generally similar for skim milk broth and whole milk. The mixed starter, consisting of TCC-3 and *La. lactis* CAU2013, accelerated the acidification in milk. Nevertheless, bacteriocin-producing strains delay acidification (Garde *et al*., 1997); however, the strain CAU2013 did not show similar properties. The accelerated acidification might be because of the interaction between the strains; however, the underlying mechanisms need further research. Ávila *et al*. (2005) observed that enterocin-producing adjunct starter enterococci enhanced milk acidification, which may be stimulated by the low-molecular-weight nitrogen compounds produced by primary starter, *Lactobacillus helveticus* LH92.

The rapid decline in pH during the initial stage of cheese production is crucial for curd

178 formation and prevention of the growth of undesirable microorganisms. Therefore, the fast-
179 acidifying strains can be used as primary starters, while the slow-acidifying bacteria can be used
180 as adjunct starters. As the strain CAU2013 has antibacterial property but has low acid
181 production ability, it is better suited as an adjunct starter culture.
182
183 **Anti-listerial activity of strain CAU2013 as an adjunct starter in milk**
184 The growth of *L. monocytogenes* was monitored in skim milk broth and whole milk during
185 incubation at 30℃. In skim milk broth with *La. lactis* CAU2013, the concentration of *L.*
186 *monocytogenes* count was reduced by 3 log units more compared with that of other samples
187 after 3 h and not detected following 6 h of fermentation (Figure 3a). In the whole milk with the
188 strain CAU2013, *L. monocytogenes* count was reduced by 0.5 log unit after 6 h, and 1 log unit
189 after 9 h compared with that of other samples (Figure 3b).
190 The results support the findings from several studies that reported that the addition of
191 bacteriocin affects the biocontrol of spoilage bacteria. Muñoz *et al*. (2007) investigated that *E.*
192 *faecalis*-produced enterocin in milk and found that it could control the growth of
193 *Staphylococcus aureus*. In addition, according to Arqués *et al*. (2011), the addition of nisin in
194 milk decreased *L. monocytogenes* count by 3 log units after 4 h.
195 The efficiency of the combined starter cultures in the inhibition of *L. monocytogenes* was
196 lower in whole milk than in skim milk. The difference in the composition between the two milk
197 media could be a factor responsible for the difference. In addition, Muñoz *et al*. (2007) stated
198 that low effectiveness in foods could be attributed to higher retention of the bacteriocin
199 molecules by milk components, resulting in slower diffusion. However, in both cases, inhibition
200 of *L. monocytogenes* growth was observed. The results suggest the potential application of *La.*
201 *lactis* CAU2013 in various food systems to control *L. monocytogenes* growth.

## Inhibition of *L. monocytogenes* in laboratory-scale fresh cheese

The cell count of the starter cultures was determined during the storage at 4℃ and 10℃ (Figure 4). In both the cases, LAB reached a final concentration of 9 log CFU/g during cheese manufacture.

During the storage at 4℃, the cheese treated with TCC-3 starter culture maintained *L. monocytogenes* count at 7.5 to 7.7 log CFU/g. In contrast, the cheese treated with TCC-3 and *La. lactis* CAU2013 had less *L. monocytogenes* count, approximately 0.5 log unit at 0 h and 1 log unit after 5 days with a final concentration of 6.4 log CFU/g. Besides, during the storage at 10℃, the cheese treated with TCC-3 starter culture maintained the bacterial count between 6.86 and 7.31 log CFU/g (Figure 4a). within contrast, the cheese treated with TCC-3 and CAU2013 had less *L. monocytogenes* count, approximately 1 log unit at 0 h and 1.5 log unit after 5 days, with a final concentration of 5.76 log CFU/g (Figure 4b). This result is consistent with a study that reported that 2 log unit reduction was observed in cheese with *La. lactis* strain (Coelho *et al*., 2014). Moreover, Kondrotiene *et al*. (2018) showed that nisin-producing *La. lactis* strains decreased the growth of *L. monocytogenes* in fresh cheese during 7 days of storage at 4℃.

Therefore, the results support that manufacturing cheese using a bacteriocin-producing starter reinforced the inhibition of growth of *L. monocytogenes*, and it would be effective in controlling contamination during cheese production. Additionally, after storage at temperatures of 4℃ and 10℃, *L. monocytogenes* count was reduced, which may confirm the potential of LAB in controlling the growth of *L. monocytogenes* during storage at refrigeration temperature.

## Conflicts of interest

The authors declare no potential conflicts of interest.

## Author contributions

Conceptualization: Kim GB. Data curation: Yoon SH, Kim GB. Investigation: Yoon SH, Kim GB. Writing - original draft: Yoon SH. Writing - review & editing: Yoon SH, Kim GB.

## Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

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294 **Table 1.** Carbohydrate fermentation patterns of the two isolated bacteriocin-producing lactic
295 acid bacteria. The test was performed with API 50 CHL kit and all data are from this study. +,
296 positive; -, negative

| Carbohydrates | | Carbohydrates | |
|---|---|---|---|
| Glycerol | - | Salicin | + |
| Erythritol | - | D-cellobiose | + |
| D-arabinose. | - | D-maltose | + |
| L-arabinose | + | D-lactose (bovine origin) | + |
| D-ribose | + | D-melibiose | - |
| D-xylose | + | D-saccharose (sucrose) | + |
| L-xylose | - | D-trehalose | + |
| D-xylose | - | Inulin | - |
| Methyl-beta-D-xylopyranoside | - | D-melezitose | + |
| D-galactose | + | D-raffinose | - |
| D-glucose | + | Amidon (starch) | + |
| D-fructose | + | Glycogen | - |
| D-mannose | + | Xylitol | - |
| L-sorbose | - | Gentiobiose | + |
| L-rhamnose | - | D-turanose | - |
| Dulcitol | - | D-lyxose | - |
| Inositol | - | D-tagatose | + |
| D-mannitol | + | D-fucose | - |
| D-sorbitol | - | L-fucose | - |
| Methyl-alpha-D-mannopyranoside | - | D-arabitol | - |
| Methyl-alpha-D-glucopyranoside | - | L-arabitol | - |
| N-acetylglucosamine | + | Potassium gluconate | - |
| Amygdalin | + | Potassium 2-ketogluconate | - |
| Arbutin | + | Potassium 5-ketogluconate | - |
| Esculin ferric citrate | + | | |

297

298 **Table 2.** Identification of bacteriocin-producing strains by BLAST and Ez-Taxon.

| | BLAST | | EzTaxon | |
|---|---|---|---|---|
| **Strain** | **Taxon name** | **Similarity (%)** | **Taxon name** | **Similarity (%)** |
| **CAU2013** | *Lactococcus lactis* subsp. *lactis* | 100 | *Lactococcus lactis* subsp. *lactis* | 100 |
| | *Lactococcus lactis* subsp. *hordniae* | 99.86 | *Lactococcus lactis* subsp. *hordniae* | 99.86 |
| | *Lactococcus lactis* subsp. *tructae* | 99.39 | *Lactococcus lactis* subsp. *tructae* | 99.39 |

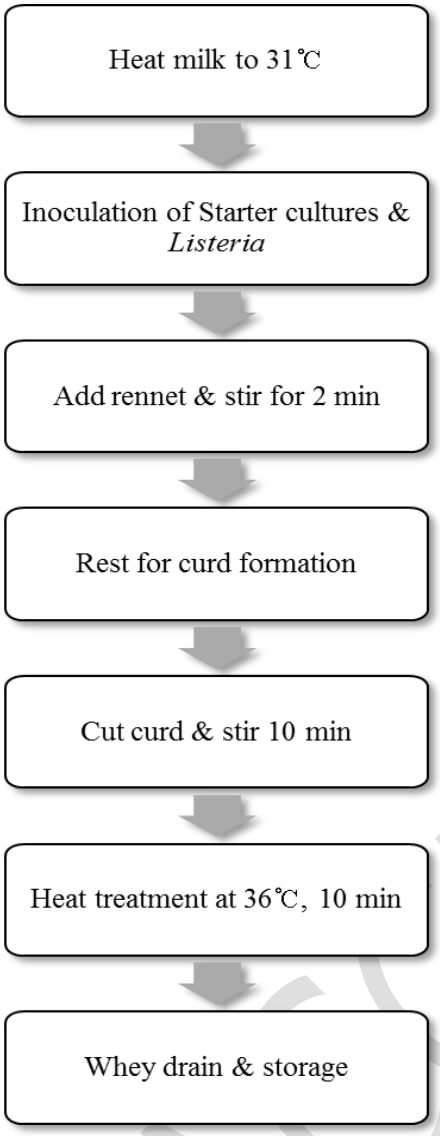
299

300

301

302 **Table 3.** Antimicrobial spectrum of bacteriocin from *L. lactis* CAU2013. +, < 10 mm; ++, >
303 10 mm; -, no inhibition zone.

| Indicator strain | Inhibition activity |
|---|---|
| Gram positive | |
| *L. monocytogenes* ATCC 15315 | + |
| *L. monocytogenes* ATCC 7644 | ++ |
| *L. monocytogenes* ATCC 19111 | + |
| *L. monocytogenes* ATCC 19114 | ++ |
| *L. monocytogenes* ATCC 19115 | ++ |
| *Staphylococcus aureus* RN6390 | + |
| Gram negative | |
| *Salmonella enteritidis* YHS 383 | - |
| *Escherichia coli* ATCC 25922 | - |




304 **Figure 1.** The procedure of lab-scale fresh cheese production.

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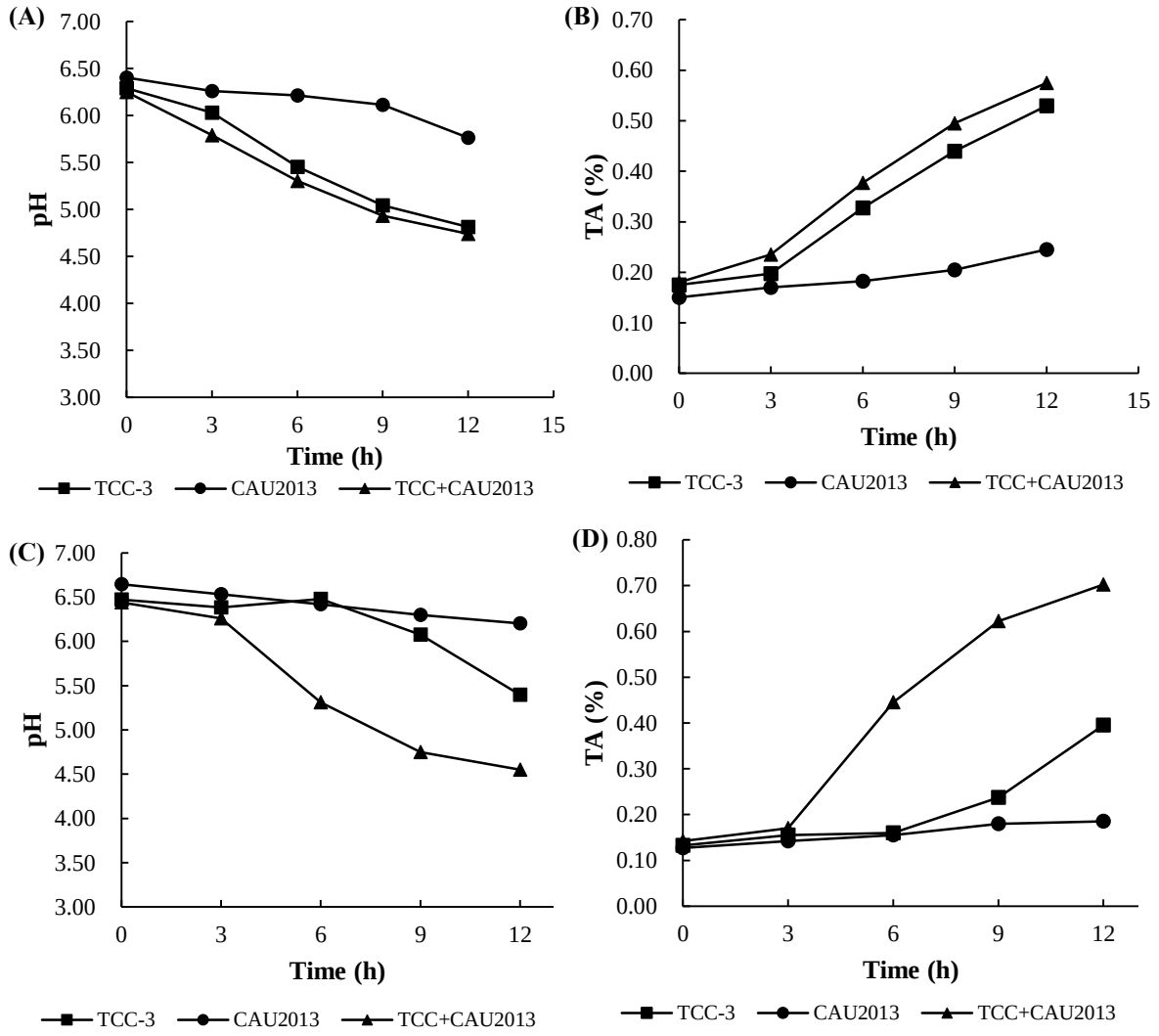


306

307 **Figure 2.** The values of pH and titratable acidity (TA) of strains grown in 10% skim milk and
308 whole milk at 30℃. (A) pH values and (B) TA values in 10% skim milk, (C) pH values and
309 (D) TA values in whole milk. TCC-3 (■), *L. lactis* CAU2013 (●), and the combination of
310 TCC-3 with CAU2013 (▲).

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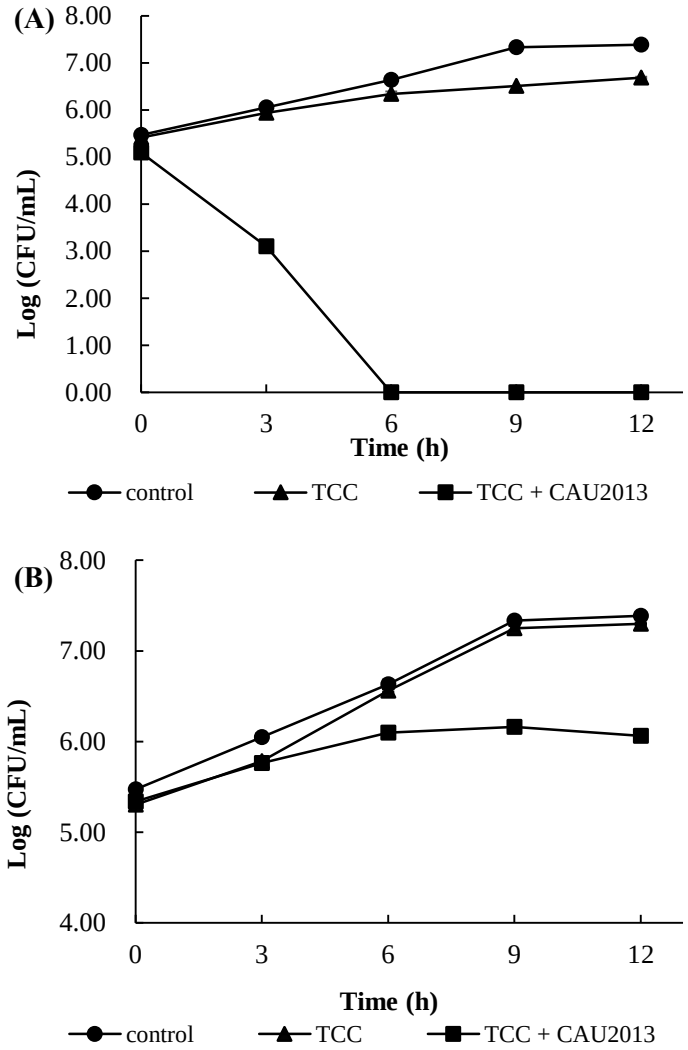
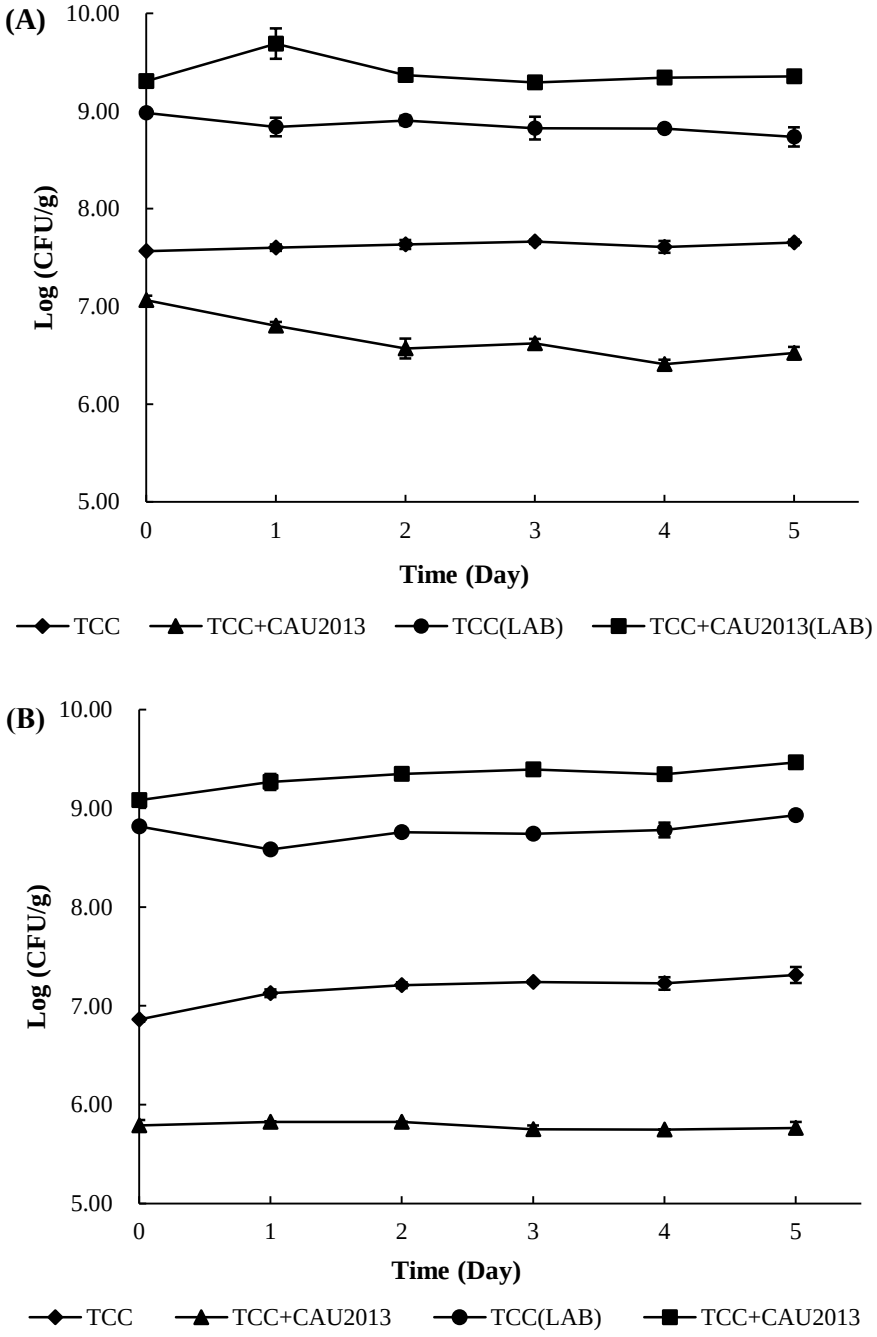
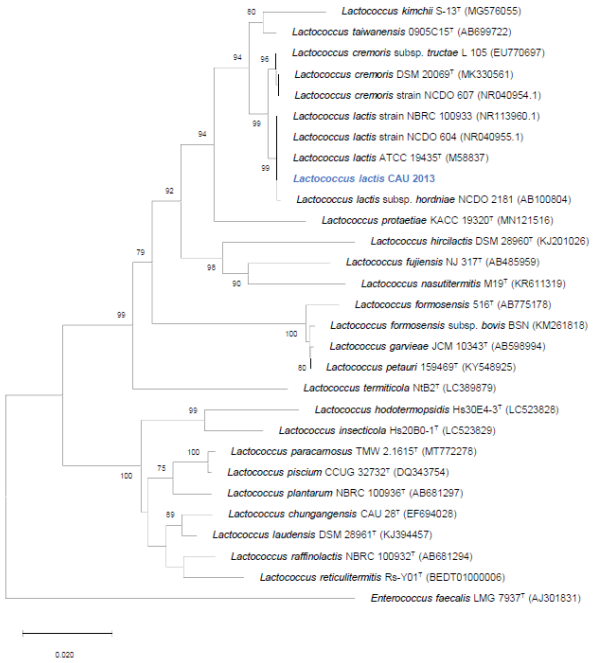



316 **Figure 3.** Biocontrol of *L. monocytogenes* in 10% skim milk broth and whole milk. *L.*
317 *monocytogenes* was inoculated in milk broth (A) and whole milk (B), and incubated at 30℃
318 without starter (●), or with 1 % of TCC-3 (▲), or the combination of TCC-3 and CAU2013
319 (■).
320
321
322




323 **Figure 4.** The viable cell counts of *L. monocytogenes* and LAB in fresh cheese manufactured
324 with TCC-3 starter and combination of *L. lactis* CAU2013 and TCC-3 and then stored for 5
325 days. During the storage at 4℃ (A) and 10℃ (B), LAB in cheese produced with TCC-3 (●);
326 TCC-3 and CAU2013 (■) were measured. Also, *L. monocytogenes* was measured in cheese
327 with TCC-3 (◆) and cheese with mixed starter (▲).

328




**Supplementary Fig 1. Neighbor-joining (NJ) phylogenetic tree of *Lactococcus lactis* strain CAU 2013 and related type strains based on 16S rRNA gene sequences (GenBank accession numbers are enclosed in parenthesis).**
Numbers at nodes (value >70%) are bootstrap values based on 1000 resampling datasets. *Enterococcus faecalis* LMG 7937$^T$ was used as an outgroup.
Bar, 0.02 substitutions per nucleotide position.

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