Regulation of CcpA on the growth and organic acid production characteristics of ruminal Streptococcus bovis at different pH

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Abstract

Background: Catabolite control protein A (CcpA) regulates the transcription of lactate dehydrogenase and pyruvate formate-lyase in Streptococcus bovis, but knowledge of its role in response to different pH is still limited. In this study, a ccpA-knockout strain of S. bovis S1 was constructed and then used to examine the effects of ccpA gene deletion on the growth and fermentation characteristics of S. bovis S1 at pH 5.5 or 6.5.

Results: There was a significant interaction between strain and pH for the maximum specific growth rate (μmax) and growth lag period (λ), which caused a lowest μmax and a longest λ in ccpA-knockout strain at pH 5.5. Deletion of ccpA decreased the concentration and molar percentage of lactic acid, while increased those of formic acid. Strains at pH 5.5 had decreased concentrations of lactic acid and formic acid compared to pH 6.5. The significant interaction between strain and pH caused the highest production of total organic acids and acetic acid in ccpA-knockout strain at pH 6.5. The activities of α-amylase and lactate dehydrogenase decreased in ccpA-knockout strain compared to the wild-type strain, and increased at pH 5.5 compared to pH 6.5. There was a significant interaction between strain and pH for the activity of acetate kinase, which was the highest in the ccpA-knockout strain at pH 6.5. The expression of pyruvate formate-lyase and acetate kinase was higher in the ccpA-knockout strain compared to wild-type strain. The lower pH improved the relative expression of pyruvate formate-lyase, while had no effect on the relative expression of acetate kinase. The strain × pH interaction was significant for the relative expression of lactate dehydrogenase and α-amylase, both of which were highest in the wild-type strain at pH 5.5 and lowest in the ccpA-knockout strain at pH 6.5.

Conclusions: Overall, low pH inhibited the growth of S. bovis S1, but did not affect the fermentation pattern. CcpA regulated S. bovis S1 growth and organic acid fermentation pattern. Moreover, there seemed to be an interaction effect between pH and ccpA deletion on regulating the growth and organic acids production of S. bovis S1.

Keywords: Streptococcus bovis, Catabolite control protein A (CcpA), pH, Organic acids, Fermentation pattern

Introduction

Generally, high-concentrate diets based on grains are applied in modern intensive ruminant production to increase production. However, ruminants fed on high-concentrate diets display increased yields of volatile fatty acids (VFA) and lactic acid in the rumen and decreased rumen pH, which may cause subacute ruminal acidosis [1, 2]. In this process, the concentration of lactic acid is much lower than VFA because there is a...
balance between the production of lactic acid and its conversion into VFA. Once the balance is broken, lactic acid begins to accumulate in the rumen. Because lactic acid is a stronger acid than VFA (pKa 3.9 vs 4.7–4.9), its accumulation often brings about a downward spiral in ruminal pH, which may induce rumen lactic acidosis [3]. Previous studies demonstrated that an initial overgrowth of Streptococcus bovis is the major cause of increasing lactic acid and declining ruminal pH, which will result in the inability of most ruminal bacteria, and the acid-tolerant Lactobacilli becoming predominant [4, 5]. Therefore, S. bovis is considered the major etiologic agent of rumen lactic acidosis, and suppressing its overgrowth and overproduction of lactic acid is vital to prevent rumen lactic acidosis when animals are supplied with a high grain diet.

S. bovis is a gram-positive bacterium that utilizes starch and soluble sugars as substrate in the rumen, thereby producing lactic acid, formic acid, acetic acid and ethanol. Several previous studies have shown that the fermentation products of S. bovis are regulated by the pH, carbon source and substrate concentration [6–10]. Our previous work with S. bovis S1 demonstrated that the carbohydrate source (soluble starch vs. glucose) had a major effect on lactic acid production due to the transcriptional regulation of metabolic genes [10]. Furthermore, we evaluated the relative importance of pH and starch concentration in fermentation characteristics of S. bovis S1 and found that the fermentation of the strain was more sensitive to the pH changes [11]. In addition to the environmental factors, the fermentation pattern of S. bovis is also controlled by the catabolite control protein A (CcpA) [12], which is a pleiotropic regulatory protein in low-GC gram-positive bacteria involved in carbon and nitrogen metabolism, biofilm formation, toxic gene expression, and other physiological processes. Several studies have reported that CcpA activates or inhibits transcriptional expression of the target gene via binding to the specific sequence of its target gene, such as catabolite response element (cre) [13–15]. The regulation of target genes by CcpA is not only associated with heat-stable protein (HPr) [16–18] and small molecule compounds such as fructose-1,6-diphosphate (FDP) and 6-phosphate glucose [19], but also affected by the environmental factors, such as substrate type, oxygen presence and pH [20–22]. Based on the above literatures and our previous work, we hypothesized that CcpA transcriptionally regulates the fermentation pattern of S. bovis S1, which might be is affected by pH. To test this hypothesis, we constructed the ccpA gene knockout mutant of S. bovis S1 using homologous recombination technology, and used it to investigate the effect of ccpA gene deletion on the growth and fermentation characteristics of S. bovis S1 at different pH.

Materials and methods

Bacterial strains

Strain S. bovis S1 (CCTCC AB 2016240) used in this study was previously isolated from the rumen fluid of Saanen dairy goats (late lactation) in our laboratory [10]. Its ccpA-disrupted mutant was constructed by homologous recombination as follows. First, DNA fragments corresponding to the upstream (1053 bp fragment; primer pairs ccpA Up F/ccpA Up R, Table 1) and downstream (1101 bp fragment; primer pairs ccpA Down F/ccpA Down R, Table 1) sequences of ccpA were amplified by PCR using S. bovis S1 genomic DNA as a template. The erythromycin resistance gene erm was amplified with the primers erm F and erm R (Table 1). The PCR product was purified using a PCR purification kit (Qiagen, Beijing, China) according to the manufacturer’s instructions. The amplified fragments were respectively cloned into EcoRI, BamHI and Sacl restriction sites of pUC19 vector to generate pUC19-ccpA up-erm-ccpA down (pCE). The recombinant vector pCE was electroporated into S. bovis S1 cells using an electroporation system at 2.5kV, 200Ω, and 25μF. Finally, knockout mutants were selected on MRS plates containing 1μg/mL erythromycin at 37°C for 3–4 days.

The result of knockout was validated by PCR and DNA sequencing. Briefly, genomic DNA of S. bovis S1 wild-type strain and its mutant strain were extracted with the TIANamp Bacteria DNA Kit (DP302, TianGen Biotech Co. Ltd., Beijing, China) and used as the templates. Primers used for verification are shown in Table 1, which were designed based on the internal sequence of the ccpA gene (ccpA1-F/ccpA1-R) or across the upstream and downstream sequences of the ccpA gene (ccpA2-F/ccpA2-R). PCR analysis was performed using the 2× Taq PCR MasterMix II Kit (KT211–02, TianGen Biotech Co. Ltd., Beijing, China) according to the manufacturer’s instructions. The amplified PCR products were electrophoresed on a 1% (wt/vol) agarose gel. Sequence analysis for wt2 and ko2, which were the products of primer ccpA2 amplified from S. bovis S1 wild-type strain and the ccpA-knockout strain respectively, was performed by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. for further verification.

Experimental design

The experiment treatments were arranged as a 2×2 factorial design: two strains, which were S. bovis S1 wild-type strain and its ccpA-disrupted mutant respectively, were cultured in the media at pH 5.5...
or pH 6.5. Specific operations are as follows. First, the seed cultures of *S. bovis* S1 wild-type strain and its mutant strain were cultured in a modified MRS medium [10] in an anaerobic workstation (DG250, Don Whitley Scientific, England) at 37 °C. Next, the seed cultures of both strains (at exponential phase) were transferred with 1% (v/v) inoculum into 200 mL anaerobic serum bottles containing 100 mL basal medium, respectively. The basal medium was prepared according to Chen et al. [10], and it contained: 0.45 g/L KH₂PO₄, 0.45 g/L K₂HPO₄, 0.9 g/L NaCl, 0.9 g/L (NH₄)₂SO₄, 0.12 g/L CaCl₂·2H₂O, 0.19 g/L MgSO₄·7H₂O, 1.0 g/L tryptone, 1.0 g/L yeast extract, 0.6 g/L cysteine hydrochloride, and 3.0 g/L soluble starch (Cat#G8300, Solarbio, Beijing, China). The original pH of the medium was 4.2, which was adjusted to 5.5 and 6.5 using 10% NaOH (w/v) before sterilization. The cultivation was performed in an anaerobic thermostat shaker at 37 °C and 160 rpm, and the pH of the medium was monitored using a pH meter (SevenExcellence-S470, Mettler-Toledo, Switzerland) and adjusted to 5.5 or 6.5 by continuous titration with 10% NaOH (w/v) using an injection pump (TYD02–10, Leadfluid Technology Co., Ltd., Baoding, China). Three replicates were set for each treatment.

### Sample collection

Cell growth were monitored by measuring OD values at 600 nm every hour using SpectraMax M5 plate reader (Molecular Devices Corporation, USA). The cultures of each sample were harvested in duplicate when they reached the exponential growth phase. A portion of the samples was quickly placed in liquid nitrogen and stored at −80 °C for enzyme activity assay, while the remaining samples were centrifuged at 13,400 × g for 2 min at 4 °C. The obtained cell pellets were quickly frozen in liquid nitrogen for 15 min, then stored at −80 °C for further RNA isolation; the supernatant was filtered using a 0.22 μm filter membrane and stored at −80 °C for the determination of organic acids.

### Table 1 Primers used in this study

| Primer names | Primer sequences (5′-3′) | Purpose of use | Product length (bp) | Reference or source |
|--------------|--------------------------|----------------|--------------------|---------------------|
| ccpA up-F    | TGTAAAACGAGCTAGGCACTAATG| Construction of ccpA-knockout mutant | 1053 | This study         |
| ccpA up-R    | AAGCTCTAAGCTAGGCACTAATG| Construction of ccpA-knockout mutant | 1101 | This study         |
| ccpA down-F  | ATCTGATCAGCTGACAGCTAGA | Construction of ccpA-knockout mutant | 1053 | This study         |
| ccpA down-R  | GAGCTTGTCTGACAGCTGACAC | Construction of ccpA-knockout mutant | 1101 | This study         |
| erm-F        | CAAAGATCTAGGCACTAATG    | Construction of ccpA-knockout mutant | 1207 | This study         |
| erm-R        | GCTCATGATCAGCTGACAGCTG | Construction of ccpA-knockout mutant | 1207 | This study         |
| ccpA 1-F     | TGTAAAACGAGCTAGGCACTAATG| Verification of ccpA-knockout mutant | 426  | This study         |
| ccpA 1-R     | AAGCTCTAAGCTAGGCACTAATG| Verification of ccpA-knockout mutant | 426  | This study         |
| ccpA 2-F     | ATCTGATCAGCTGACAGCTGAC | Verification of ccpA-knockout mutant | 1827 | This study         |
| ccpA 2-R     | GAGCTTGTCTGACAGCTGACAC | Verification of ccpA-knockout mutant | 1827 | This study         |
| 16S-F        | GAAACCACGCTGGCGGA      | RT-qPCR          | 119  | Chen et al. [10]   |
| 16S-R        | CTCACTGTTTACGCGG      | RT-qPCR          | 119  | Chen et al. [10]   |
| pf-F         | GTTATCTGACGAGCTAATG   | RT-qPCR          | 190  | Chen et al. [10]   |
| pf-R         | TGCTGAGAAGAGTCTG      | RT-qPCR          | 190  | Chen et al. [10]   |
| ldh-F        | TGTAAAACGAGCTAGGCACTAATG| Construction of ccpA-knockout mutant | 109  | Chen et al. [10]   |
| ldh-R        | GCTACGCTTCTGACAGCTGAC | Construction of ccpA-knockout mutant | 109  | Chen et al. [10]   |
| α-amy-F      | TCAAGCTAGGCACTAATG    | RT-qPCR          | 134  | This study         |
| α-amy-R      | GTGCACGCTAGGCACTAATG  | RT-qPCR          | 134  | This study         |
| ack-F        | GGGCAGAAGAGCTGAGGAA   | RT-qPCR          | 134  | This study         |
| ack-R        | GTTGAAGACTGAGCTGAGGAA | RT-qPCR          | 134  | This study         |
Determination of organic acids and enzyme activity

A high performance liquid chromatograph (HPLC, Shimadzu, Japan) equipped with an acclaim OA column (Sepax Carbomix H-NP) and a UV detector was used to determine concentrations of organic acids (lactic acid, formic acid, and acetic acid) in the supernatant. The column temperature was kept at 55°C, and the mobile phase was 2.5 mM H2SO4 with the flow rate set at 0.5 mL/min. Organic acids were then measured with a UV detector set at 210 nm. Commercial assay kits (Comin Biotechnology Co., Ltd., Suzhou, China) were used to measure the activities of α-amylase (Cat. No. DFMA-1-Y), lactate dehydrogenase (Cat. No. LDH-1-Y), and acetate kinase (Cat. No. ACK-1-Y). Before the measurement, 2 mL of bacterial fluid was centrifuged at 8000 × g for 10 min at 4°C, and the cell pellets were resuspended in the extracting solution from the kit. To lyse the bacterial cells, the suspension was then sonicated on an ice bath using a VCX-130 Sonicator (Sonics, USA) for 10 min with 30 s pulse on and 30 s pulse off, at 100 W. Unbroken cells were removed by centrifugation at 8000 × g for 10 min at 4°C, and the supernatant was collected for the determination of enzyme activities according to the corresponding kit instructions.

RNA extraction and RT-qPCR analysis

Before RNA extraction, 200 μL of bacterial fluid was centrifuged at 13,400 × g for 2 min at 4°C and the cell pellets were incubated with 180 μL of lysozyme solution (3 mg/mL) at room temperature for 10 min to break the cell walls. Total RNA was extracted from S. bovis S1 wild-type and mutant strains using the RNAprep Pure Bacteria Kit (DP430, Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer’s instructions. The quantity and quality of total RNA were evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and agarose gel electrophoresis, respectively. Next, total RNA was reverse-transcribed to cDNA in a 20 μL reaction mixture using the Primer-Script™ Reagent Kit (TaKaRa Biotechnology Co. Ltd., Dalian, China) according to the manufacturer’s instructions. The reverse transcriptase quantitative PCR (RT-qPCR) was performed with the ABI Step-One-Plus RT-PCR system (ABI 7500, Applied Biosystems, Foster City, CA) using the TB Green Premix Ex Taq™ II Kit (TaKaRa Biotechnology Co. Ltd., Dalian, China). The reaction was first performed in a 20 μL reaction solution containing 10 μL 2 × TB Green Premix Ex Taq II, 1.6 μL primer, 1 μL cDNA, 0.4 μL 50 × ROX, and 7.0 μL RNase-free water. The RT-qPCR conditions were as follows: 95°C for 30 s, followed by 40 cycles of the amplification at 95°C for 5 s and 60°C for 34 s. Primers of α-amy (gene encoding α-amylase), ldh (gene encoding lactate dehydrogenase), pfl (gene encoding pyruvate formate lyase), and ack (gene encoding acetate kinase) were designed using Primer-Blast and the 16S rRNA was used as an internal reference gene (Table 1). Relative gene expression was normalized to expression of the 16S rRNA gene and calculated using the 2−ΔΔCT method.

Data analysis

Results were expressed as means and SEMs. All data were analyzed as a 2 × 2 factorial design by ANOVA using GLM procedures of SPSS (SPSS 25.0, IBM, USA) software. The statistical model included the effects of strain (wild-type strain or ccpA-knockout strain) and pH (6.5 or 5.5), as well as their interaction. Graphs were drawn using GraphPad Prism 8. The logistic function model [24] was used to non-linearly fit the growth curves of bacteria. The specific model is: “Ln(yt/y0) = D/ (1 + exp.((4 × μmax/D)(λ-t) + 2))”, where yt is OD value at time t, y0 is initial OD value, t is hour of the incubation, μmax is the maximum specific growth rate, D is the limit of ln (yt/y0), and λ is the growth lag period (h).

Results

Verification of ccpA gene knockout

The results of PCR verification of ccpA gene knockout are shown in Fig. 1. Figure 1A shows the expected sizes and targets of the PCR products, which was confirmed by the results of gel electrophoresis as shown in Fig. 1B (the full-length picture is provided in the Supplementary file). These results indicated that the ccpA gene was deleted successfully in the ccpA-knockout strain. In addition, the DNA sequencing results of wt2 and ko2 further confirmed that the ccpA deletion strain was constructed.

Growth characteristics

Figure 2 shows the growth curve of S. bovis S1 wild-type strain and ccpA deletion strain at different pH. When the pH was 6.5, the wild-type strain reached the stationary phase at 3 h and its OD value was 0.59, while the ccpA-deficient strain reached the stationary phase at 5 h and its OD value was 0.56. Under the pH of 5.5, the wild-type strain reached the stationary phase at 6 h and its OD value was 0.55, while the ccpA-deficient strain reached the stationary phase at 7 h and its OD value was 0.53.

Nonlinear fitting of the growth curves of S. bovis S1 wild-type strain and ccpA-deficient strain at different pH were constructed using the logistic function model. Results showed that the R² of the fitting equations were all above 0.99. The corresponding fitting parameters are shown in Table 2. There was a significant interaction...
between strain and pH for the growth lag period (λ) 
(P < 0.01) and maximum specific growth rate (μ_max) 
(P < 0.05). Compared with the wild-type strain at pH 6.5, 
the μ_max of other groups were reduced (P < 0.05), and 
the ccpA-deficient strain at pH 5.5 had the lowest μ_max 
(P < 0.05). The λ values of ccpA-deficient strain at both 
pH were increased compared with those of the wild-type 
strain, and the ccpA-deficient strain at pH 5.5 had the 
highest λ (P < 0.01).

Organic acids production
Table 3 shows the organic acid production characteristics of S. bovis S1 wild-type strain and ccpA deletion strain at different pH. Results indicated that both strains produced lactic acid, formic acid, and acetic acid at different pH. Among them, lactic acid made up the largest proportion of organic acids followed by formic acid, with acetic acid being the least abundant. The interaction between strain and pH affected the concentration of total organic acids (P < 0.05) and acetic acid (P < 0.01), and the acetic acid molar percentage to total organic acids (P < 0.05). Compared with the wild-type strain at pH 6.5, the total organic acids concentrations of ccpA-deficient strain at pH 6.5 were increased, but those of both strains at pH 5.5 were decreased. The acetic acid concentration and its molar percentage to total organic acids of ccpA-deficient strain were higher than those of wild-type strain at both pH 6.5 and pH 5.5, with the highest at pH 6.5. The significant strain × pH interaction was not observed for lactic acid and

Fig. 1 The result of PCR verification for wild-type strain (wt) and ccpA-knockout strain (ko) of S. bovis S1. A Diagram of PCR verification for genomic structure of wild-type strain (wt) and ccpA-knockout strain (ko). ccpA 1 was designed based on the internal sequence of the ccpA gene, with a size of about 426 bp; ccpA 2 was designed across the upstream and downstream sequences of the ccpA gene, with a size of about 1827 bp for wild-type strain and 1985 bp for ccpA-knockout strain. B The result of the PCR verification. wt1: a 426 bp PCR product of ccpA 1 amplified in wild-type strain; ko1: no PCR product of ccpA 1 amplified in ccpA-knockout strain; wt2: a 1827 bp PCR product of ccpA 2 amplified in wild-type strain; ko2: a 1985 bp PCR product of ccpA 2 amplified in ccpA-knockout strain

formic acid concentration, and their molar percentages to total organic acids. Deletion of ccpA gene decreased the lactic acid concentration (37.38 vs 40.15 mM) and its molar percentage to total organic acids (73.83% vs 81.66%), while increased the formic acid concentration (8.07 vs 5.46 mM) and molar percentage (15.93% vs 11.10%) compared to the wild-type strain (P < 0.01). Strains at pH 5.5 had decreased concentrations of lactic acid (37.59 vs 39.94 mM; P < 0.01) and formic acid (6.51 vs 7.01 mM; P < 0.01), while an increased lactic
Table 4  Effect of the absence of ccpA gene on enzymes activity in S. bovis S1 at different pH (n = 3)

| Items                  | Wild-type strain | ccpA-knockout strain | SEM1 | P-value2 |
|------------------------|------------------|----------------------|------|----------|
|                        | pH 6.5           | pH 5.5               |      |          |
|                        |                  |                      |      |          |
| Lactate dehydrogenase  | 20.46            | 23.66                |      |          |
| (U/L)                  |                  |                      |      |          |
| α-amyrase (U/L)        | 33.87            | 40.28                |      |          |
| Acetokinase (U/L)      | 2.34c            | 2.43c                |      |          |

1 SEM, standard error of mean
2 Strain, strain effect; pH, pH effect; Strain × pH, the interaction between strain and pH

Means with different superscripts differ significantly (P < 0.05)

acid molar percentage to total organic acids (78.06% vs 77.43%; P < 0.05) compared to those at pH 6.5.

**Enzyme activity and gene expression**

Table 4 shows the enzyme activities of S. bovis S1 wild-type strain and ccpA-deficient strain at different pH. The interaction between strain and pH did not affect the activities of α-amyrase and lactate dehydrogenase. Lower activities of α-amyrase (32.74 vs 37.07 U/L) and lactate dehydrogenase (18.75 vs 22.06 U/L) were observed for ccpA-deficient strain compared to the wild-type strain (P < 0.01), and higher activities of these two enzymes (38.67 vs 31.14 U/L and 21.65 vs 19.16 U/L) were observed at pH 5.5 than at pH 6.5 (P < 0.01). There was a significant interaction between strain and pH for the activity of acetate kinase, which was the highest in the ccpA-deficient strain at pH 6.5 (P < 0.05).

Figure 3 shows results of the relative expressions of α-amy, ldh, pfl, and ack of S. bovis S1 wild-type strain and ccpA-deficient strain at different pH. The strain × pH interaction was significant for the relative expression of ldh (P < 0.05) and α-amy (P < 0.01), both of which were the highest in the wild-type strain at pH 5.5 and the lowest in the ccpA-deficient strain at pH 6.5. The interaction between strain and pH did not affect the relative expression of pfl and ack. The relative expressions of pfl and ack in the ccpA-deficient strain were increased compared to the wild-type strain (P < 0.01). The lower pH improved the relative expression of pfl (P < 0.01), while did not affect the relative expression of ack.

**Discussion**

Ruminal pH generally fluctuates within a physiological range of about 5.5–7.0 in a 24-h period, which is driven by the amount of fermentable carbohydrate in each meal, innate buffering capacity of animals, and the absorption rate of organic acids [25, 26]. However, the rate of organic acids production is greater than absorption and the buffering capacity is also limited by inadequate salivary secretion when ruminants consume a large amount of rapidly fermentable (non-fiber) carbohydrates. As a result, ruminal pH will drop below its physiological level, which influences microbial composition and fermentation [3, 27]. It is worth noting that pH is an important environmental factor for bacteria, which not only affects the bacterial growth and fermentation rate, but also the final yield and purity of fermentation products [28]. In the rumen, the growth and organic acid fermentation pathways of most bacterial are affected by ruminal pH [27, 29], even for acid-tolerant species such as S. bovis and Lactobacillus [7].

S. bovis can grow in the pH range of 4.5–6.7 with the highest growth rate at pH 6.4 [6]. In this study, the maximum specific growth rate of S. bovis S1 wild-type strain was greater at pH 6.5 than pH 5.5, indicating that the low pH inhibited the growth of S. bovis S1. This finding is consistent with the characteristic of this strain found by Chen et al. [11], and ccpA deletion does not alter this characteristic. The absence of ccpA, at both pH6.5 and 5.5, caused an extended lag phase and decreased maximum specific growth rate of S. bovis S1, which was consistent with the results obtained in other lactic acid-producing bacteria, such as Lactobacillus bulgaricus [21] and Lactobacillus casei [30]. However, the growth differences between the wild-type strain and ccpA-disrupted mutant of S. bovis 12U1 have not been previously observed [12], which is inconsistent with present findings. This discrepancy may be attributed to the different growth and metabolic functions of strains isolated from different environments, even if they belong to the same species [31]. Besides, the significant interaction between strain and pH was observed for λ and $\mu_{max}$ in this study, which caused a lowest maximum specific growth rate and a longest lag phase in the ccpA-deficient strain at pH 5.5. The finding suggests a possible synergic effect between low pH and ccpA deletion on suppressing the growth of S. bovis S1, which is probably because that both deletion of ccpA and low pH could inhibit its growth, and the gene expression of ccpA in S. bovis S1.
wild-type strain is influenced by the pH [11]. The results of present study imply that deletion ccpA gene would have a better effect on controlling the overgrowth of S. bovis S1 in rumen when ruminal pH is low, which need to be verified in the further study. We did not observe significant differences in maximum specific growth rate between wild-type strain at pH 5.5 and ccpA-disrupted mutant at pH 6.5, indicating that ccpA deletion inhibited the growth of S. bovis S1 only if the strain grown at the same pH condition.

The organic acid fermentation pattern of S. bovis is regulated by extracellular pH and growth rate [32, 33]. An earlier study found that at pH 6.7, lactic acid was the primary fermentation product when S. bovis JB1 was grown with a relatively fast growth rate, while it changed to formic acid, acetic acid and ethanol fermentation when the strain was grown slower in continuous culture [34]. As the pH dropped to 4.7, S. bovis JB1 mainly produced lactic acid even at slow growth rate, which is because the intracellular pH at this condition is similar to the optimal pH of S. bovis LDH [8, 34, 35]. However, the fermentation pattern of S. bovis S1 in the present study did not change when the extracellular pH dropped from 6.5 to 5.5, and the molar percentage of lactic acid is more than 80% of total organic acids at both pH, which is similar to the outcome previously observed in our lab [11]. The use of a batch culture and possibly insufficiently low extracellular pH of the current study may provide some explanation for the unaltered fermentation pattern of S. bovis S1 as extracellular pH dropped. Although the fermentation pattern was not affected by the extracellular pH in the present study, low pH reduced the final concentrations of lactic acid, formic acid and total organic acids of S. bovis S1 wild-type strain. Similarly, this pattern of changes was also found after ccpA knockout. The production of organic acids is associated with the specific activity and amount of enzymes. In Streptococcal, the LDH has a requirement for FDP [36], and the concentration of FDP produced by S. bovis S1 at pH 5.5 is lower than pH 6.5 [11], which may be the reason for the decreased lactic acid production of this study even though the activity of LDH and the ldh gene expression in both strains were significantly higher at pH 5.5 than pH 6.5.

Fig. 3 Relative mRNA expressions of α-amylase, ldh, ack and pfl in wild-type strain (wt) and ccpA-knockout strain (ko) of S. bovis S1 at different pH. Values are the means (n = 3), with standard deviation indicated by vertical bars. α-amylase; ldh, lactate dehydrogenase; ack, acetokinase; pfl, pyruvate formate lyase
Inactivation of the *ccpA* gene significantly decreased lactic acid production, and increased the production of formic acid and acetic acid of *S. bovis* S1 growing at both pH 6.5 and pH 5.5. The findings are consistent with the results of Asanuma et al. [12]. Subsequently, we further analyzed the activity and gene expression of related enzymes involved in the organic acids production pathway. The activity and gene expression of enzyme for lactic acid production (LDH) were reduced, and enzymes for formic acid and acetic acid production (PFL and ACK) were increased in the mutant strain compared to the wild-type strain. This observation may explain the altered production of organic acids in *ccpA*-disrupted mutants. Similar results were found in other lactic acid bacteria. For example, Asanuma et al. [12] found that the ratio of formic acid to lactic acid in *S. bovis* 12 U1 increased significantly after *ccpA* gene deletion, and this change was directly associated with changes in related enzyme activities and gene expression. The transcription of the *las* operon encoding phosphofructokinase (*pfk*), pyruvate kinase (*pk*), and lactate dehydrogenase (*ldh*) in *L. lactis* was reduced by 75% in the *ccpA* deletion mutant strain compared to the wild-type strain, which increased ethanol and acetic acid and decreased lactic acid production [37]. Moreover, the activities of LDH, PK, and PFK in *L. bulgaricus* were significantly reduced after *ccpA* gene deletion, which resulted in reduced lactic acid yield [21]. In *S. mutans*, CcpA has been demonstrated to regulate the transcription of *ackA* (acetate kinase A) and *pta* (phosphotransacetylase) in response to pH [22]. In the present study, there was a significant interaction between strain and pH for the concentration of total organic acids and acetic acid production, which led to the highest total organic acids and acetic acid production in *ccpA*-disrupted mutant at pH 6.5. Moreover, a significant interaction between strain and pH was observed for the activity of acetate kinase and gene expressions of *ldh* and *α-amy*. The interaction effect between pH and *ccpA* gene deletion on these indicators is possibly caused by the fact that the expression of *ccpA* gene is regulated by extracellular pH in *S. bovis* S1 [11]. However, further experiment will be needed to verify this speculation.

**Conclusion**

Low pH inhibits the growth of *S. bovis* S1, and lowers the production of organic acids but does not alter the production pattern despite the increased activities of α-amylase and LDH. The deletion of *ccpA* gene inhibits the growth of *S. bovis* S1 and regulates the organic acid fermentation pattern towards lower lactic acid and higher formic acid and acetic acid, suggesting that CcpA is probably involved in the carbon metabolism of *S. bovis* S1. Moreover, there seems to be an additive effect between pH and *ccpA* deletion on regulating the growth and organic acids production of *S. bovis* S1. The mechanism underlying this interaction remains to be clarified.

**Abbreviations**

α-amy: α-amylase; ack: Acetic acid kinase; CcpA: Catabolite control protein A; cre: Catabolite response element; FDP: Fructose-1,6-diphosphate; HPr: Heat-stable protein; ldh: Lactate dehydrogenase; OD: Optical density; pfl: Pyruvate formate lyase; pta: Phosphotransacetylase; SEM: Standard error of mean.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12866-021-02404-x.

**Additional file 1.**

Acknowledgements

Not Applicable.

**Authors’ contributions**

YQJ and HRW designed the research; YQJ, YTF and YZ performed research; CW contributed analytic tools; YQJ and CW analyzed data; YQJ wrote the manuscript; CW and HRW provided critical comments; ME revised the language of the manuscript. All the authors read and approved the final manuscript.

**Funding**

This research was funded by the National Natural Science Foundation of China (NSFC No. 31872988, No. 31572429) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PADA).

**Availability of data and materials**

The sequence data during the current study are available in the [figshare] repository, [https://doi.org/10.6084/m9.figshare.14779206].

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Received**: 28 May 2021    **Accepted**: 26 November 2021

**Published online**: 15 December 2021
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