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Metabolic Mechanism and Regulation of Acid Production by Streptococcus bovis in the Rumen: Postprint

Authors: Chen Lianmin, Shen Yizhao, Wang Hongrong

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Abstract

Streptococcus bovis (S. bovis) is a predominant lactic acid-producing bacterium in the rumen that plays a crucial role in the development of rumen lactic acidosis induced by high-concentrate diets. Previous studies have demonstrated that S. bovis acid production through carbohydrate metabolism is primarily regulated by glucose transport mechanisms, enzymes in the glycolytic pathway, and intermediate metabolites. Furthermore, research has also revealed that environmental pH, growth phase, and catabolite control protein (CcpA) exert significant effects on its acid production rate and pattern. This review summarizes recent research on the metabolic pathways and influencing factors of acid production from dietary carbohydrate fermentation by S. bovis, providing insights into the mechanisms of rumen lactic acidosis from a microbial metabolic perspective.

Full Text

Metabolic Mechanism of Acid Production by *Streptococcus bovis* in the Rumen and Its Regulation

CHEN Lianmin, SHEN Yizhao, WANG Hongrong*
College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China

Abstract: *Streptococcus bovis* (*S. bovis*) is a predominant lactate-producing bacterium in the rumen that plays a critical role in the development of ruminal lactic acidosis when ruminants are fed high-concentrate diets. Previous research has demonstrated that carbohydrate metabolism and acid production in *S. bovis* are primarily regulated by glucose transport mechanisms, as well as by enzymes and intermediate metabolites in the glycolytic pathway. Additionally, environmental pH, growth stage, and the catabolite control protein A (CcpA) have

been shown to significantly influence the rate and pattern of acid production. This review synthesizes recent studies on the metabolic pathways and regulatory factors involved in carbohydrate fermentation and acid production by *S. bovis*, providing a reference for understanding the mechanisms of ruminal lactic acidosis from a microbial metabolic perspective.

Keywords: *Streptococcus bovis*; lactic acid; rumen acidosis; metabolic pathway

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Modern intensive livestock production practices that rely on high-concentrate diets rich in starch can trigger rapid proliferation of *S. bovis* in the rumen. This bacterium ferments readily digestible carbohydrates to produce large quantities of lactic acid, leading to lactic acid accumulation and accelerating the progression of ruminal acidosis. Consequently, controlling the growth and metabolic acid production of *S. bovis* to maintain lactic acid at appropriate levels represents a potential strategy for preventing ruminal lactic acidosis.

Numerous studies have shown that *S. bovis* primarily produces acids through glycolysis or the Embden-Meyerhof-Parnas (EMP) pathway. Researchers have increasingly focused on regulating the glycolytic flux of glucose derived from dietary carbohydrates within *S. bovis* cells as a means to prevent ruminal lactic acidosis. The glucose transport system, glycolytic pathways, enzymes and intermediate metabolites in these pathways, environmental pH, different growth stages, and catabolite control protein A (CcpA) all significantly influence carbohydrate metabolism and acid production in *S. bovis*. This review examines recent advances in these areas to provide insights into the mechanisms of ruminal lactic acidosis from a microbial metabolic perspective.

1. Effect of Transmembrane Glucose Uptake Mechanisms on Acid Production by *S. bovis*

Starch from feed is hydrolyzed by amylases into glucose and other sugars in the rumen, which *S. bovis* then assimilates and ferments to obtain energy for growth and acid production. Current research indicates that glucose transport across the membrane in *S. bovis* occurs primarily through two pathways: the phosphotransferase system (PTS) and facilitated diffusion.

In facilitated diffusion, extracellular glucose moves down its concentration gradient into the cell through membrane carrier proteins or channels without energy consumption. In contrast, the PTS specifically transports glucose across the membrane into the cytoplasm via active transport. This system exhibits higher affinity for glucose and serves as the main mechanism for sugar transport against concentration gradients, though its transport capacity is lower than that of facilitated diffusion.

The PTS comprises three enzyme components: phosphoenolpyruvate-dependent protein kinase enzyme I (EI), heat-stable histidine-phosphoryl protein (HPr), and enzyme II (EII). EI and HPr are non-specific soluble cytoplasmic proteins

shared by different sugar PTS transport systems. EII is sugar-specific and highly conserved at the structural domain level, typically containing three domains (EIIA, EIIB, and EIIC) that may be organized within a single protein fused by linker sequences or separated into 2–4 protein components during evolution, with transport activity requiring their association. EIIA and EIIB are hydrophilic phosphotransferase domains oriented toward the cytoplasm, while EIIC generally forms a hydrophobic membrane-bound channel domain.

When glucose is abundant extracellularly, intracellular phosphoenolpyruvate (PEP) serves as a phosphate group donor. EI accepts a phosphate group from PEP to form EI-P, which then transfers the phosphate to the histidine residue at position 15 of HPr, forming histidyl-phosphorylated HPr (HPr-[His-P]). The phosphate is further transferred through the EIIA-EIIB pathway, resulting in phosphorylated EIIB (EIIB-P). Phosphorylated EIIB can activate EIIC, which specifically recognizes glucose, phosphorylates it to glucose-6-phosphate (G-6-P), and transports it into the cytoplasm for entry into the glycolytic pathway [Figure 1: see original paper].

Research has shown that Gram-positive bacteria can also transfer phosphate to the serine residue at position 46 of HPr, forming seryl-phosphorylated HPr (HPr-[Ser-P]) through an ATP-dependent HPr kinase. HPr-[Ser-P] participates not only in glucose transport but also in the transcriptional regulation of multiple genes in Gram-positive bacteria such as *Bacillus*, *Streptococcus*, and *Lactobacillus*. In transcriptional regulation, HPr-[Ser-P] exhibits extremely high affinity for CcpA. After binding to CcpA to form a complex, it targets the catabolite-responsive element (CRE) located at the 5' end of operons or upstream regions, ultimately activating or repressing gene transcription.

Additionally, HPr-[Ser-P] can inhibit both PTS and non-PTS sugar transport through an inducer exclusion mechanism by activating sugar-phosphate phosphatases, which dephosphorylate sugar phosphates and cause efflux of glucose that has already entered the cytoplasm. Cook et al. proposed that fructose-1,6-bisphosphate (FDP) might activate protein kinases that contribute to the inducer exclusion effect of HPr-[Ser-P], and subsequent studies confirmed that FDP can activate HPr kinase, thereby inhibiting sugar transport. Asanuma et al. investigated the effects of HPr phosphorylation on *S. bovis* growth and acid production, finding that HPr-[Ser-P] levels decreased as proliferation rates declined, while HPr-[His-P] and intracellular phosphate concentrations increased. This study suggested that phosphate concentration could regulate the relative predominance of HPr-[His-P] versus HPr-[Ser-P] through modulation of HPr kinase activity, thereby controlling *S. bovis* proliferation and acid production.

2.1. Metabolic Pathways for Acid Production from Glucose in *S. bovis*

Starch and other carbohydrates are ultimately degraded to glucose by amylases and other enzymes. Under anaerobic conditions, one molecule of glucose un-

dergoes ten enzymatic reactions to yield two molecules of pyruvate with energy production, a process known as glycolysis. This pathway represents the initial enzymatic degradation of glucose in both eukaryotic cells and bacteria, serving as the common route for glucose catabolism. In *S. bovis* cells in the rumen, pyruvate produced from glycolysis has four main fates: (1) conversion to lactate by lactate dehydrogenase (LDH); (2) conversion to formate by pyruvate formate-lyase (PFL); (3) conversion to ethanol by pyruvate decarboxylase and alcohol dehydrogenase (ADHE); and (4) conversion to acetyl-CoA, which can then be converted to acetate by phosphotransacetylase and acetate kinase, to ethanol by ADHE, or to citrate for biosynthesis via condensation with oxaloacetate. Additionally, PEP produced from glycolysis can generate oxaloacetate through the action of phosphoenolpyruvate carboxykinase (PCK) [Figure 2: see original paper].

2.2. Effects of Metabolic Enzymes and Intermediate Metabolites on Acid Production by *S. bovis*

Research has identified key intermediate metabolites such as FDP and triose phosphates, and enzymes including fructose-1,6-bisphosphate aldolase (FBA), LDH, and PFL as critical regulators of acid production patterns in *S. bovis*. These metabolites and enzymes interact to modulate the rate and pattern of glycolytic acid production.

2.2.1. Regulation of Enzymes by Intermediate Metabolites

Classical biochemistry holds that enzymes catalyzing irreversible reactions represent critical control points in metabolic pathways. In glycolysis, the reactions catalyzed by hexokinase, phosphofructokinase (PFK), and pyruvate kinase (PYK) are effectively irreversible, and their activities are influenced by various glycolytic products. Specifically: (1) hexokinase activity is inhibited by its product glucose-6-phosphate; when PFK activity is low, fructose-6-phosphate accumulates, and the relative balance between glucose-6-phosphate and fructose-6-phosphate leads to increased glucose-6-phosphate concentration. (2) PFK activity is inhibited by high concentrations of ATP and citrate because high ATP levels inhibit PFK binding to its substrate fructose-6-phosphate; additionally, high intracellular citrate indicates abundant biosynthetic precursors, making glucose degradation unnecessary, and citrate can enhance ATP' s inhibitory effect on PFK, slowing glycolysis. (3) PFK activity is activated by high concentrations of AMP, ADP, fructose-2,6-bisphosphate, and fructose-6-phosphate. Fructose-2,6-bisphosphate increases PFK' s affinity for fructose-6-phosphate while reducing ATP inhibition. Fructose-6-phosphate both accelerates the synthesis of fructose-2,6-bisphosphate and inhibits its hydrolysis. (4) PYK activity is activated by FDP; when energy stores are sufficient, high ATP concentrations allosterically inhibit PYK, slowing glycolysis. When blood glucose levels decrease, PYK phosphorylation in the liver is stimulated, reducing enzyme activity and slowing glycolysis to maintain blood glucose levels. Meanwhile, alanine is formed

from pyruvate by transamination, and increased alanine concentration indicates excess pyruvate precursor; alanine also allosterically inhibits PYK, slowing glycolysis.

Furthermore, FDP and triose phosphates [dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP)] play important regulatory roles in glycolytic acid production by *S. bovis*. Russell et al. found that high intracellular FDP concentrations in *S. bovis* correlated with increased lactate production and elevated LDH activity, suggesting that lactate production might be related to FDP activation of LDH—a phenomenon first demonstrated by Wolin in 1964. Asanuma et al. investigated the inhibitory effects of DHAP and GAP on PFL during *S. bovis* glycolysis, revealing dose-dependent inhibition. At 0.1 mmol/L DHAP, PFL activity decreased by 40% compared to its maximum, while at 0.1 mmol/L GAP, activity decreased by over 80%. Similar inhibitory effects of triose phosphates on PFL have been confirmed in *Lactococcus lactis*, *Streptococcus* species, and *Streptococcus mutans*, suggesting that large fluctuations in DHAP and GAP concentrations during glycolysis cause allosteric inhibition of PFL.

2.2.2. Regulation of Intermediate Metabolites by Metabolic Enzymes

Hexokinase, PFK, and PYK serve as key enzymes in glycolysis and importantly regulate fermentation acid production in *S. bovis*. Studies on PFK-overexpressing *S. bovis* strains found that PFK overexpression did not affect the ratio of formate to lactate production or growth rate, suggesting that PFK is not a major regulatory factor in *S. bovis* sugar metabolism. Similarly, research on PYK overexpression showed that although PYK activity was much higher in overexpressing strains, there were no significant differences in lactate and formate production or their ratios compared to normal strains, indicating that PYK overexpression does not affect the rate or pattern of glycolytic acid production in *S. bovis*—findings also confirmed in *Lactococcus lactis*. However, few studies have examined hexokinase regulation of glucose fermentation in *S. bovis*.

More extensive research indicates that FBA, LDH, and PFL play central regulatory roles in *S. bovis* glycolytic acid production. Asanuma et al. proposed that FBA overexpression in *S. bovis* could reduce lactate production when readily fermentable carbohydrates are abundant as substrates, while low FBA expression could increase lactate production when substrates are limited or less fermentable. Their studies on FBA-overexpressing strains revealed lower intracellular FDP levels, reduced LDH transcription compared to PFL, and decreased lactate production. However, DHAP and GAP concentrations were significantly higher than in normal strains, reaching 2.6 mmol/L and 0.49 mmol/L, respectively. Since FBA cleaves FDP into one molecule each of DHAP and GAP, overexpression should theoretically increase DHAP and GAP concentrations, inhibiting PFL activity and thereby increasing lactate production. However, the observed increase in DHAP and GAP actually correlated with decreased lactate and in-

creased formate production, likely because LDH activity is strongly dependent on FDP—FBA overexpression reduces FDP concentration, weakening LDH activity to a greater extent than the inhibitory effect of elevated DHAP and GAP on PFL.

Additionally, Asanuma et al. investigated the effects of ADHE overexpression on acid production patterns in *S. bovis*, finding that despite a threefold increase in ADHE expression compared to normal strains, ethanol production showed no significant difference, and neither did PFL and LDH expression levels or lactate and formate production. This indicates that *S. bovis* glycolysis primarily favors lactate and formate production, with minimal influence of ADHE overexpression on the flux of pyruvate or acetyl-CoA toward ethanol. Asanuma et al. also noted that pyruvate conversion to lactate requires NADH oxidation to NAD+, and that GAP conversion to 1,3-bisphosphoglycerate by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) represents the sole source of NADH in *S. bovis* glycolysis. Therefore, GAPDH overexpression should provide more NADH for downstream lactate synthesis, increasing lactate production. However, studies using GAPDH-overexpressing *S. bovis* strains found that GAPDH overexpression did not alter the NADH/NAD+ ratio or the formate/lactate ratio, with acid production patterns still determined primarily by the relative predominance of LDH and PFL. Since these studies were conducted with *S. bovis* JB1 strains under pure culture conditions, the results may not be generalizable to other *S. bovis* strains in the rumen, where conditions are far more complex, and thus the regulatory roles of these enzymes cannot be dismissed.

3. Effect of pH on Acid Production by *S. bovis*

High-concentrate feeding first promotes proliferation of starch-degrading bacteria, which become dominant and produce large amounts of volatile fatty acids, causing pH decline. When pH drops to approximately 5.5, growth of most microorganisms is inhibited to some extent, while acid-tolerant *S. bovis* can proliferate extensively and produce lactate as the primary fermentation product. pH regulates glycolytic acid production in *S. bovis* mainly by differentially inhibiting or activating enzyme activities. Under continuous culture conditions at pH 6.7, *S. bovis* primarily produces formate and acetate with minimal lactate, whereas at pH 4.7, fermentation shifts to lactate production. This indicates that PFL activity is activated and LDH activity is inhibited at high pH, while low pH inhibits PFL and activates LDH. LDH and PFL activities peak at pH 5.5 and 7.5, respectively.

Asanuma et al. demonstrated that *S. bovis* FBA activity at pH 4.5 was much lower than at pH 7.0, with activity at pH 5.5 being only half that at pH 7.0. Since high FBA activity can reduce lactate production when readily fermentable carbohydrates are abundant, inhibition of FBA activity at low pH leads to increased FDP concentration, which further activates LDH and increases lactate production. Beyond affecting enzyme activity, pH also regulates LDH synthesis at the transcriptional level. Asanuma et al. found that LDH transcription was

much higher at pH 4.5 than at pH 6.9, though the signaling pathways or sensing mechanisms mediating these pH-induced changes in LDH gene transcription remain unknown and require further investigation.

Russell et al. examined the effects of dilution rate and pH on continuous co-culture of *S. bovis* and *Megasphaera elsdenii* (*M. elsdenii*) in vitro, finding that at higher pH (6.0–6.6), *S. bovis* had its greatest numerical advantage over *M. elsdenii* but produced little lactate. As pH decreased (5.4–6.0), the competitive advantage of *S. bovis* over *M. elsdenii* diminished while lactate production increased. When pH dropped below 5.4, *M. elsdenii* nearly disappeared and lactate accumulated extensively, demonstrating that environmental pH influences both acid production by *S. bovis* and its competitive relationship with *M. elsdenii*. However, other studies have found that once animals adapt to high-concentrate diets, *S. bovis* populations decrease to one ten-thousandth of their initial levels, comparable to populations in hay-fed animals, suggesting that *S. bovis* abundance is not solely related to pH decline and that microbial interactions may play important roles. Massive lactate production and accumulation further decrease pH and inhibit lactate-degrading bacteria, causing death and lysis of Gram-negative bacteria and release of endotoxins that disrupt rumen microbial balance and exacerbate metabolic acidosis. When pH falls below 5.0, *S. bovis* growth is inhibited and acid-tolerant lactobacilli gradually increase to become dominant, producing large amounts of lactate and releasing bacteriocins and other toxic substances that inhibit growth of *S. bovis* and other microbial groups.

4. Effect of Growth Stage on Acid Production by *S. bovis*

Under batch culture conditions, as *S. bovis* transitions from logarithmic to stationary phase, the proliferation rate slows and LDH transcription levels decrease, with fermentation favoring formate production. Asanuma et al. suggested that different growth stages regulate acid production patterns at the transcriptional level. In contrast, during continuous culture of *S. bovis*, PFL transcription decreases while LDH transcription increases, with lactate as the primary fermentation product and bacteria maintaining high proliferation rates. This further demonstrates that growth stage regulates acid production. Studies on gene regulation of *S. bovis* in pure culture revealed that LuxS, encoded by the *luxS* gene, is a universal bacterial quorum-sensing regulator. LuxS expression correlates with *S. bovis* proliferation rate, being highest during logarithmic growth and rapidly decreasing upon entering stationary phase. LuxS expression is not affected by *S. bovis* cell density but can regulate cellular physiological functions and metabolism in the complex and dynamic rumen environment. The differential expression of LuxS at different proliferation rates shows consistency with changes in LDH expression during various growth stages, suggesting a potential regulatory role in *S. bovis* growth and metabolism that warrants further investigation.

5. Effect of CcpA on Acid Production by *S. bovis*

CcpA acts as a transcriptional repressor or activator that plays an important regulatory role in cellular metabolism. Studies using CcpA-deficient *S. bovis* strains revealed lower LDH transcription and higher PFL transcription compared to normal strains, with fermentation favoring formate production. Further research identified a CRE sequence in the CcpA promoter region, and showed that CcpA must bind to HPr-[Ser-P] to form a complex that can then bind to CRE and exert its regulatory function. Since CRE sequences are present upstream of both LDH and PFL genes, they represent potential binding sites for the CcpA-HPr-[Ser-P] complex, suggesting CcpA involvement in regulation of sugar metabolism and acid production in *S. bovis*. Beyond regulating LDH and PFL, studies have shown that CcpA may affect fermentation acid production by controlling other genes encoding metabolic enzymes in the glycolytic pathway. Asanuma et al. identified potential CcpA binding sites upstream of GAPDH, PYK, and PCK genes, and demonstrated that these three genes were downregulated in CcpA-deficient *S. bovis* strains under glucose fermentation conditions, with fermentation favoring formate production. These findings underscore the important regulatory role of CcpA in *S. bovis* metabolism and acid production.

Conclusion

S. bovis is closely associated with ruminal lactic acidosis, and studies on its metabolic acid production pathways and regulatory factors have partially elucidated the patterns of acid production in the rumen and provided references for understanding ruminal lactic acidosis at the microbial metabolic level. Current research has revealed the central regulatory roles of intermediate metabolites such as triose phosphates and FDP, key enzymes including FBA, LDH, and PFL, and environmental pH in *S. bovis* metabolic acid production. At the transcriptional level, genes such as *CcpA* and *LuxS* also play important regulatory roles, though research on transcriptional regulation remains in its early stages. Most studies have inferred regulatory functions based on transcriptional changes in only one or two genes encoding enzymes or regulatory factors, lacking systematic investigation and validation of metabolic pathways. Moreover, current research has focused excessively on pure culture studies of *S. bovis* under various environmental conditions, neglecting potential regulatory effects of microbial community succession, interactions, and harmful metabolites on *S. bovis* acid production during the development of ruminal lactic acidosis. Additionally, quantitative techniques for enzymes and genes remain limited to Western blot and Northern blot, which have low throughput, high costs, and are time-consuming. Future research should employ advanced high-throughput technologies such as metabolomics, proteomics, and transcriptomics to systematically explore *S. bovis* metabolic acid production patterns, and should investigate the effects of mixed rumen microbial communities on *S. bovis* acid production under various rumen environmental conditions to further elucidate the mechanisms of ruminal lactic acidosis.

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