# Original article

## Characterization of *Streptococcus oligofermentans* sucrose metabolism demonstrates reduced pyruvic and lactic acid production

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***Keywords***: *Streptococcus oligofermentans; lactate dehydrogenase; pyruvic acid; lactic acid*

**Background** *Streptococcus (S.) oligofermentans* is a newly identified bacteria with a yet to be defined mechanism of sucrose metabolism that results in acid production. This study aimed to investigate the biochemical mechanisms of *S. oligoferm-entans* glucose metaolism.

**Methods** The *S. oligofermentans* LMG21532, *Lactobacillus (L.) fermentum* 38 and the *S. mutans* UA140 were used to characterize sucrose metabolism by measuring lactate dehydrogenase (LDH) activity and lactic acid production. Continuous dynamics and high performance capillary electrophoresis were used to determine LDH activity and lactic acid production, respectively, from bacteria collected at 0, 10 and 30 minutes after cultured in 10% sucrose.

**Results** These analyses demonstrated that LDH activity of the three bacterial strains examined remained stable but significantly different throughout the sucrose fermentation process. The *S. oligofermentans* LDH activity ((0.61±0.05) U/mg) was significantly lower than that of *L. fermentum* ((52.91±8.97) U/mg). In addition, the *S. oligofermentans* total lactate production ((0.048±0.021) mmol/L) was also significantly lower than that of *L. fermentum* ((0.958±0.201) mmol/L). Although the *S. oligofermentans* LDH production was almost double of that produced by *S. mutans* ((0.32±0.07) U/mg), lactic acid production was approximately one sixth that of *S. mutans* ((0.296±0.058) mmol/L). Additional tests examining pyruvic acid production (the LDH substrate) demonstrated that lactic acid concentrations correlated with pyruvic acid production. That is, pyruvic acid production by *S. oligofermentans* was undetectable following sucrose incubation, however, (0.074±0.024) and (0.175±0.098) mmol/L pyruvic acid were produced by *S. mutans* and *L. fermentum,* respectively.

**Conclusion** *S. oligofermentans* is incapable of fermenting carbohydrates to produce enough pyruvic acid, which results in reduced lactic acid production.

*Chin Med J 2011;124(21):3499-3503*

Recently our research group isolated a novel Streptococcus species from dental plaque and saliva of caries-free humans belonging to the mitis group of oral streptococci that produced reduced levels of lactic acid and therefore named *Streptococcus (S.) oligofermentans*.[1] Glucose metabolism is the primary metabolic pathway utilized by microorganisms to generate energy and bacteria associated with oral plaque typically produce lactic acid that results in caries formation via glycolysis. It is interesting that *S. oligofermentans* can also utilize lactate oxidase to convert lactic acid into $H_2O_2$ thereby inhibiting the growth of *S. mutans*.[2] *S. oligofermentans* possesses various different biological characteristics and its mechanism of glucose metabolism remains unclear. Understanding the mechanism(s) associated with the glucose metabolism pathways of this organism can be used to develop novel dental caries prevention and treatment methods.

Pyruvic acid plays a critical role in metabolic pathways and is generated by microbes via glucose metabolism and subsequently converted into various end products depending on the respective metabolic pathways utilized. In the presence of oxygen, pyruvic acid is oxidized to acetyl coenzyme A by pyruvate dehydrogenase prior to entering the tricarboxylic acid (TCA) cycle. Due to the relatively anaerobic environment of dental plaques, bacteria primarily generate lactic acid via glycolysis.[3,4] Lactate dehydrogenase (LDH) is an enzyme associated with the terminal stages of glycolysis and reduces the glycolytic pyruvic acid intermediate into lactic acid. LDH exists in two forms: 1,6-biphosphate dependent LDH (FBP-LDH) that is specifically activated by FBP and FBP-independent LDH (iFBP-LDH) which likely functions independently of FBP.[5] LDH is necessary for

DOI: 10.3760/cma.j.issn.0366-6999.2011.21.012
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This study was supported by grants from 115-National Key Technologies R&D Programme, the Ministry of Science and Technology of China (No. 2007BAI18B01), National Natural Science Foundation of China (No. 8107088), and Beijing Municipal Natural Science Foundation (No. 7102163).
Conflict of interests: None.

bacteria to produce lactic acid and for cariogenisis.[6-8] In the presence of adequate substrates, bacterial LDH activity may be indicative of glucose metabolism, lactic acid production and potentially cariogenic activity.[9] In this study, the FBP-dependent and -independent LDH activities of streptococci were characterized in addition to assessing pyruvic and lactic acid production to characterize the biochemical mechanisms of *S. oligofermentans* glucose metabolism.

## METHODS

### Bacteria strains and culture conditions

*S. oligofermentans* LMG21532, the acid-producing *Lactobacillus (L.) fermentum* strain 38 and *S. mutans* UA140 (reference strain) were obtained from the Micro-Organism Institute of the Chinese Academy of Sciences. Bacteria were inoculated into BHI liquid media and cultured at anaerobically (10% $CO_2$) at 37°C. Growth was arrested at late logarithmic phase by placing cultures in an ice bath. Bacteria were collected by centrifugation (4 ×10 000 *g*) and washed twice with sterilized, deionized water to remove organic acids produced by growing bacteria. The respective bacterial strains were cultured for an additional 30 minutes to allow for the consumption of endogenous energy stores and starved bacteria collected by centrifugation as described above. Bacteria were then incubated with a 10% sucrose solution and LDH activity assessed at 0, 10 and 30 minutes after sucrose incubation.

### Sample preparation

Baseline LDH activity and organic acid production were obtained from the cell pellets and supernatants of starved bacteria. Bacteria were weighed and resuspended in sterilized, deionized water (100 mg wet cell weight/ml). The effect of sucrose on LDH activity was measured by incubating starved bacteria (100 mg wet cell weight/ml) in sterilized 10% sucrose solution anaerobically at 37°C (10% $CO_2$).

According to acid production profile in dental plaque,[10] 0 minute represents for baseline data before sugar challenge; 10 minutes, for highest acid accumulation time after sugar challenge; 30 minutes, for a general time plaque pH recovering after a sugar challenge. So here, bacteria were harvested by centrifugation 10 and 30 minutes later and supernatants and pellets assessed for organic acid production and LDH activity, respectively.

### Assessment of lactate dehydrogenase activity

Bacterial pellets (100 mg wet cell weight/ml) were resuspended in phosphate buffered saline (PBS, pH 7.0–7.4) containing 0.1% bovine serum albumin and 20 mmol/L dithiothreitol and then disrupted by ultrasonication at 0°C for 15 minutes using an ultrasonic disruptor (Branson-Digital sonifier 450, Branson, CT, USA). The suspension was then centrifuged (4 ×10 000 *g*) for 10 minutes and supernatants used for iFBP-LDH and FBP-LDH activity assessment. Specifically, LDH activity was assayed spectrophotometrically by recording the oxidation rate of reduced nicotinamide adenine dinucleotide (NADH) at 365 nm using an Hitachi 7060 automatic biochemical analyzer (Biotek ELX808, Japan).

The assay system contained 50 mmol Tris buffer (pH 7.4, containing 1.5 mmol/L pyruvic acid) and 0.8 mmol/L coenzyme NADH (Human COM, Wiesbaden, Germany). A total of 10 μl cell-free extract was suspended in 1 ml buffer, bathed in 37°C for 1 minute and reactions were initiated by adding 250 μl coenzyme NADH. The optical absorbance was recorded continuously for 1 minute every 17 seconds at 365 nm. One enzyme unit is the amount required to catalyze the oxidation of 1 μmol of NADH/min and specific activity is expressed as units per milligram of bacteria (U/mg). The 5 mmol/L FBP was added to the reaction mixture to assess the effect of FBP on LDH activity.

### Measurement of pyruvic and lactic acid production

High performance capillary electrophoresis using a Beckman Instruments Inc. model P/ACE 5000 (Brea, CA, USA) with a fused silica capillary (57 cm; i.d. 75 μm) was utilized for lactic and pyruvic acid analyses. Separations were carried out at 20 kV at 20°C. The electrolyte contained 10 mmol phthalic acid and 0.5 mmol tetradecyltrimethyl ammonium bromide (T-TAB). LiOH was used to adjust the pH to 5.7. A focus scanning UV detector was used with the detection wavelength set to 214 nm, 4 μl of sample was vacuum-injected for 10 seconds and the separation time was 6 minutes.

### Statistical analysis

Described experiments were repeated 3 times. Data were expressed as mean ± standard deviation (SD). Differences in the median production of organic acids by different bacteria were analyzed by Kruskal-Wallis test. The differences of LDH activities in different bacteria were analyzed by one-way analysis of variance (ANOVA). For the iFBP-LDH and FBP-LDH activity comparison, two-tailed tests were carried out. $P$ <0.05 was considered statistically significant.

## RESULTS

### Pyruvic and lactic acid production

Ten minutes after the addition of sucrose, lactic acid production by *S. oligofermentans* was minimal ((0.047±0.012) mmol/L), but no pyruvic acid was detected. Thirty minutes later, the lactic acid production leveled off ((0.048±0.021) mmol/L), but pyruvic acid was still undetectable.

By contrast, ten minutes after the sucrose exposure, lactic acid production by *S. mutans* was more than twice ((0.090±0.058) mmol/L) that observed for *S. oligofermentans* and pyruvic acid was present at detectable levels ((0.027±0.017) mmol/L) (Table 1). *L. fermentum* produced the highest amounts of lactic acid

**Table 1.** Effect of sucrose on lactic and pyruvic acid production* ($n$=3)

| Bacterial strains | Pyruvic acid (mmol/L) | | | Lactic acid (mmol/L) | | |
|---|---|---|---|---|---|---|
| | 0 minutes | 10 minutes | 30 minutes | 0 minute | 10 minutes | 30 minutes |
| *S. oligofermentans* | 0 | 0 | 0 | 0 | 0.047±0.012 | 0.048±0.021 |
| *S. mutans* | 0 | 0.027±0.017 | 0.074±0.024 | 0.029±0.029 | 0.090±0.058 | 0.296±0.058 |
| *L. fermentum* | 0 | 0.114±0.005 | 0.175±0.098 | 0.075±0.020 | 0.418±0.063 | 0.958±0.201 |
| $\chi^2$ values | – | 7.448 | 7.488 | – | 5.600 | 7.200 |
| *P* values | – | <0.05 | <0.05 | – | <0.05 | <0.05 |

*Acid concentrations at the 10 and 30 mmol/L subtracted from concentrations determined at 0 minute. $\chi^2$ and *P* values are the comparision results between any two groups.

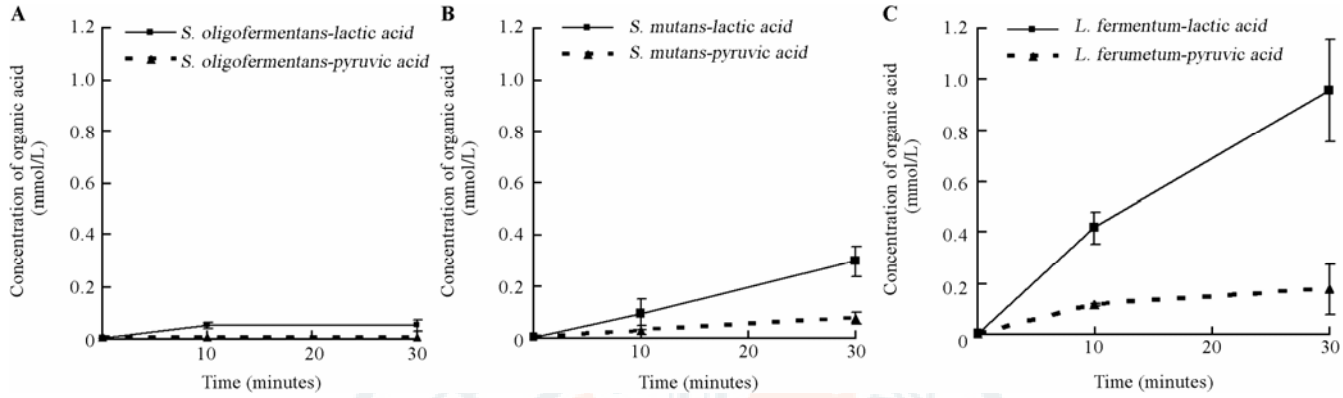


**Figure.** Correlation between lactic acid production and pyruvic acid synthesis. *S. oligofermentans* (**A**), *S. mutans*. (**B**) and *L. fermentum* (**C**) were grown in the presence of sucrose for 0, 10 or 30 minutes. At the respective time points, high performance capillary electrophoresis was used to determine lactic and pyruvic acid concentrations.

**Table 2.** Comparison of iFBP-LDH activity among bacterial species ($n$=3, U/mg)

| Time (minutes) | *S. oligofermentans* | *S. mutans* | *L. fermentum* | $F^*$ value | $P^*$ value |
|---|---|---|---|---|---|
| 0 | 0.60±0.03 | 0.34±0.07 | 56.12±6.52 | 731.12 | <0.01 |
| 10 | 0.61±0.08 | 0.29±0.08 | 50.76±14.84 | 147.53 | <0.01 |
| 30 | 0.61±0.06 | 0.32±0.02 | 51.87±5.92 | 262.57 | <0.01 |
| $F^\dagger$ values | 0.047 | 0.191 | 0.242 | – | – |
| $P^\dagger$ values | 0.954 | 0.831 | 0.793 | – | – |

*,†*F* and *P* values are the comparision results between any two groups.

**Table 3.** Characterization of FBP-LDH activity in different bacteria ($n$=3, U/mg)

| Time (minutes) | *S. oligofermentans* | *S. mutans* | *L. fermentum* | $F^*$ value | $P^*$ value |
|---|---|---|---|---|---|
| 0 | 0.64±0.04 | 0.33±0.10 | 52.96±8.23 | 411.05 | <0.01 |
| 10 | 0.63±0.07 | 0.31±0.10 | 53.93±9.98 | 345.98 | <0.01 |
| 30 | 0.64±0.05 | 0.29±0.02 | 54.26±5.99 | 580.78 | <0.01 |
| $F^\dagger$ values | 0.050 | 0.252 | 0.020 | – | – |
| $P^\dagger$ values | 0.952 | 0.785 | 0.980 | – | – |

*,†*F* and *P* values are the comparision results between any two groups.

(0.418±0.065 mmol/L, $P$ <0.01) and pyruvic acid ((0.114±0.005) mmol/L, $P$ <0.01) compared to the levels observed for either *S. oligofermentans* or *S. mutans* (Table 1). Thirty minutes after sucrose addition, low levels of lactic acid were detected in *S. oligofermentans* cultures ((0.048±0.021) mmol/L) and pyruvic acid was still undetectable. However, in the presence of sucrose at the same time point, *S. mutans* consistently produced lactic and pyruvic acids ((0.296±0.058) and (0.074±0.024) mmol/L, respectively) at significantly higher levels than those observed for *S. oligofermentans* (Table 1).

*L. fermentum* produced the highest levels of lactic ((0.958±0.201) mmol/L, $P$ <0.01) and pyruvic acid ((0.175±0.098) mmol/L, $P$ <0.01). Figure depicts the linear relationship between pyruvic and lactic acid production and these levels were elevated following incubation in the presence of sucrose.

### LDH activity

iFBP-LDH activities were not similar between the different bacterial strains examined and remained stable after the addition of sucrose. iFBP-LDH activity in *S. oligofermentans* was (0.61±0.05) U/mg compared to (0.32±0.07) U/mg for *S. mutans* and (52.91±8.97) U/mg ($P$ <0.01) for *L. fermentum* (Tables 2 and 3). Furthermore, following the addition of FBP, iFBP-LDH activity for each bacterial strain examined remained unaffected.

## DISCUSSION

Previously, we characterized *S. oligofermentans* as a new oral streptococcal species that fermented fewer carbohydrates and produced reduced levels of lactic acid.[1] Recently, Tong et al[2] also demonstrated that *S. oligofermentans* could produce hydrogen peroxide from lactic acid via a lactate oxidase pathway. Furthermore, co-cultures of *S. oligofermentans* and *S. mutans* significantly inhibited the growth of *S. mutans* and the acid production.[11] This study demonstrated that the lactic acid production capacity of *S. oligofermentans* was significantly reduced compared to the lactic acid levels produced by either *S. mutans* or *L. fermentum*. Interestingly, the LDH activity of *S. oligofermentans* was not diminished and was present at similar or slightly higher levels than those observed for *S. mutans*. It has been well established that LDH is critical for the generation of lactic acid as a by-product of glycolysis, resulting in the generation of pyruvic acid as an

intermediate. Pyruvic acid in turn is further reduced to lactic acid following the catalysis of LDH, releasing energy and completing the glycolytic cycle. Strains of *S. mutan*s deficient in *LDH* produced lactic acid at reduced levels that was associated with diminished cariogenic potential[6-8] or was lethal in some *S. mutans* strains.[12]

Since LDH activity is heterogeneous among bacterial strains,[5] it is hypothesized that in the presence of appropriate substrates lactic acid production may be altered and its production correlated with LDH activity. In this study, lactic acid production and characterization of LDH activity were carried on simultaneously at respective time points. This type of analysis allowed us to correlate lactic acid production with LDH activity and demonstrated that LDH was constitutively expressed. The LDH activity of *L. fermentum* was significantly higher than that observed for *S. mutans* and *S. oligofermentans* that paralleled the ability of *L. fermentum* to produce elevated acid levels. In contrast, *S. oligofermentans* lactic acid production was significantly reduced compared to levels observed for *S. mutans*, however, the *S. mutans* intracellular LDH activity was slightly declined. This observation did not support the hypothesis that LDH activity was indicative of the acidogenic ability of bacteria. In the glycolytic pathway, lactic acid is generated by LDH catalysis using pyruvic acid as substrate. In addition to the catalytic role of LDH in this process, a catalytic substrate like pyruvic acid (key intermediate product in glucose metabolism pathway) is also necessary. These data suggested that the amount of pyruvic acid production plays a role in glucose metabolism. Before and after the addition of sucrose, production of pyruvic acid by *S. oligofermentans* was undetectable and lactic acid yields were low. However, both pyruvic and lactic acid production by *S. mutans* and *L. fermentum* increased over time.

For acidogenic bacteria, pyruvic acid is a key intermediate product of metabolism. During glucose metabolism, glucose is transformed into fructose 1,6-diphosphate, 3-phosphoglycerate and finally into pyruvic acid through a series of enzymatic reactions common to many organisms. Additional pyruvic acid metabolism requires oxygen as well as the appropriate amounts of substrate.[13,14] Micro-organisms associated with dental plaques survive in relatively anaerobic environments,[3,4] where pyruvic acid can not be further oxidized but is instead converted into lactic acid by LDH in the presence of sufficient glucose concentrations. Therefore, the amount of pyruvic acid produced by bacteria may influence the lactic acid production. Results presented in this report demonstrated that production of pyruvic acid by the respective bacteria examined was consistent with their ability to produce lactic acid. After exposure to sucrose, *L. fermentum* synthesized the highest levels of pyruvic and lactic acid. The reduced levels of pyruvic acid produced by *S. mutans* also paralleled the reduced levels of lactic acid production compared to *L. fermentum*. Pyruvic acid production by *S. oligofermentans* was undetectable during in the presence or absence of sucrose during the time points examined and lactic acid production was minimal. Since there was no pyruvic acid being detected in *S. oligofermentans*, the possible reasons for the production of lactic acid are: 1. the bacterium could not ferment sucrose effectively, and the limited pyruvic acid may have been reduced into lactic acid completely by high-efficiency LDH; 2. there was probably another metabolism pathway that partial pyruvic acid had been turned into formic acid and acetic acid with the catalysis of pyruvate formate-lyase.[15,16] It is likely that the pyruvic acid intermediate plays additional roles in lactic acid production that are yet to be defined. Due to the lack of catalytic substrate, *S. oligofermentans* was incapable of lactic acid production even though this bacterial strain had elevated LDH activity. Furthermore, some of the lactic acid produced by *S. oligofermentans* could have been converted into $H_2O_2$ by lactate oxidase.[2] These results suggested that *S. oligofermentans* may not exclusively obtain energy via glucose metabolism. More recently, novel L-amino acid oxidase activity was identified in *S. oligofermentans* that produced hydrogen peroxide from L-amino acids.[17] These two enzymatic activities conferred *S. oligofermentans* with the capacity to out-compete *S. mutans*. This metabolic characteristic of *S. oligofermentans* will change the micro-ecological environment of acid production by bacteria and will be conducive to inhibiting the growth of cariogenic bacteria thereby helping maintain dental health.

Depending on whether FBP is needed for catalytic activity, bacterial LDH can be divided into two types: FBP-LDH and iFBP-LDH.[5] LDH activity of most Streptococci and some *Lactobacillus sp.* is FBP-dependent.[5,18] In this study, the LDH activity of *S. oligofermentans* LMG21532, *S. mutans* and *L. fermentum* proceeded in the absence of FBP and the addition of FBP did not enhance the enzymatic activity, suggesting that the bacterial systems examined utilized iFBP-LDH. Moreover, the LDH activity of all three bacterial strains examined remained stable during gylcolysis before and after incubation in the presence of sucrose, further suggesting that LDH in these bacteria was constitutively expressed.

This study demonstrated that *S. oligofermentans* produced less lactic acid (than the other oral pathogens examined) in the presence of sucrose due to inefficient glucose metabolism resulting from an inability to synthesize pyruvic acid from carbohydrates. These data further demonstrated that *S. oligofermentans* could not produce significant levels of lactic acid, suggesting that it is unlikely for this organism to contribute to caries formation. Of course these results will need to be verified in follow up studies in order to clarify the mechanism of the pathogenesis of dental caries.

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*(Received April 26, 2011)*
*Edited by PAN Cheng*