Formate producing capacity provided by reducing ability of Streptococcus thermophilus NADH oxidase determines yogurt acidification rate

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ABSTRACT

Yogurt is made by fermenting milk with 2 lactic acid bacteria, Lactobacillus delbrueckii ssp. bulgaricus (L. bulgaricus) and Streptococcus thermophilus (S. thermophilus). To comprehensively understand the protocooperation mechanism between S. thermophilus and L. bulgaricus in yogurt fermentation, we examined 24 combinations of cocultures comprising 7 fast- or slow-acidifying S. thermophilus strains with 6 fast- or slow-acidifying L. bulgaricus strains. Furthermore, 3 NADH oxidase deficient mutants (Δnox) and one pyruvate formate-lyase deficient mutant (ΔpflB) of S. thermophilus were used to evaluate the factor that determines the acidification rate of S. thermophilus. The results revealed that the acidification rate of S. thermophilus monoculture determined the yogurt fermentation rates, despite the co-existence of L. bulgaricus, whose acidification rate was either fast or slow. Significant correlation was found between the acidification rate of S. thermophilus monoculture and the amount of formate production. Result using ΔpflB showed that the formate was indispensable for the acidification of S. thermophilus. Moreover, results of the Δnox experiments revealed that formate production required Nox activity, which not only regulated dissolved oxygen (DO), but also the redox potential. NADH oxidase provided the large decrease in redox potential required by pyruvate formate lyase to produce formate. A highly significant correlation was found between formate accumulation and NADH oxidase activity in S. thermophilus. In conclusion, the formate production ability provided by the action of NADH oxidase activity determines the acidification rate of S. thermophilus, and consequently regulates yogurt coculture fermentation.

Key words: yogurt fermentation rate, Streptococcus thermophilus, NADH oxidase mutants, formate, redox potential

INTRODUCTION

Yogurt is one of the most popular fermented dairy products worldwide, dating back to 6000 B.C. (Weerathilake et al., 2014). Yogurt is made by fermenting milk with 2 lactic acid bacteria, Lactobacillus delbrueckii ssp. bulgaricus (L. bulgaricus) and Streptococcus thermophilus (S. thermophilus). (Food and Drug Administration (FDA), 2017)

There is a protocooperation between S. thermophilus and L. bulgaricus (Tamime and Robinson, 2007), which results in a higher acidification rate, a lower pH at the end of fermentation, and the production of exopolysaccharides and aromatic compounds when compared with that of monocultures (de Bok et al., 2011; Hervé-Jimenez et al., 2009; Siewerts, 2016). Fast acidification in yogurt fermentation is very important for stable production, cost reduction, and the prevention of contamination from environmental microorganisms (Stanojević-Nikolić et al., 2016, Sanlıbaba and Gucer, 2015, Shelef, 1994). Clarifying the actual compounds and the state of protocooperation that results in rapid yogurt fermentation is crucial. Various protocooperation factors were reviewed by Tamime et.al (2007) and particularly formate gained more attention as a provided substance from S. thermophilus to L. bulgaricus (Suzuki et al., 1986; Zourari et al., 1992, Trimbigno et al., 2020). Similarly, formate that stimulates the growth of S. thermophilus and/or L. bulgaricus was reported with the application of mixed-culture transcriptome profiling by Siewerts et.al (2010) as a post genomic research. Additionally, folate (Rao et al., 1984., Crichtenden et al., 2003; Sybesma et al., 2003), CO2 (Galesloot et al., 1968; Driessen et al., 1982; Yamauchi et al., 2019), and fumaric acid (Yamamoto et al., 2021) produced by S. thermophilus are provided to L. bulgaricus; on the other hand, amino acid and peptides produced by L.
bulgaricus (its protease and peptidase) are supplied to S. thermophilus (Rajagopal & Sandine, 1990; Thomas & Pritchard, 1987; Zourari et al., 1992).

We have previously reported that yogurt fermentation of S. thermophilus ST1131 and L. bulgaricus LB2038 began after the dissolved oxygen (DO) concentration in milk medium decreased to almost 0 ppm (Horiuchi, 2009) and that the suppression of acid production in coculture by DO in milk was caused by the suppression of formate production in S. thermophilus (Horiuchi and Sasaki, 2011). The results using an H2O-suppressed formate production in S. thermophilus (Horiuchi, 2009) and that the suppression of acidification rate of S. thermophilus ST1131 revealed that Nox is the major oxygen-consuming enzyme of the bacterium (Sasaki et al., 2014). Yogurt fermentation with the S. thermophilus Δnox mutant and L. bulgaricus LB2038 was significantly slower than with S. thermophilus ST1131 and L. bulgaricus LB2038, and the DO concentrations of the mixed culture did not decrease to less than 2 mg/kg within 3 h. These findings suggest that Nox of S. thermophilus ST1131 contributes greatly to yogurt fermentation, presumably by removing DO from milk.

Formate is a well-known co-factor in yogurt fermentation which accelerates the growth of L. bulgaricus in the absence of pyruvate formate-lyase (PFL) (Derezelle, 2005). PFL is an oxygen-sensitive enzyme that catalyzes the production of formate and acetyl-CoA from pyruvate and CoA (EC 2.3.1.54) (Sawers and Watson, 1998), and PFL requires activation by pyruvate formate-lyase activating enzyme (Crain et al., 2014). PFL of Escherichia coli and Streptococcus mutants are inactivated by oxygen (Knappe et al., 1974; Yamada et al., 1985), but the relation between formate production by PFL and Nox in S. thermophilus is not completely understood. Formate is also required for monoculture of S. thermophilus in milk and is used as a substrate not only for formyltetrahydrofolate synthetase in purine biosynthesis (Derezelle et al., 2005), but also for anaerobic ribonucleotide reductase (RNR) (class III NrdD), which uses formate as the reductant (Levitz et al., 2022).

Previously, we revealed that urease plays a crucial role in yogurt acidification and its deficiency inhibits the fermentative acceleration of protocooperation by examining 5 combinations of 3 fast- or slow-acidifying strains of S. thermophilus with 2 CO₂-responsive or CO₂-unresponsive strains of L. bulgaricus (Yamauchi et al., 2019). However, there was a large difference in the fermentation time regardless of the difference in urease activity, thereby indicating that urease activity was not the main factor determining the fermentation time.

As stated previously, there are numerous reports of protocooperation factors associated with metabolites and oxygen related to yogurt acidification; however, these effects on protocooperation vary depending on the specific strains and their different combinations. In this study, we evaluated 24 combinations of cocultures of 7 fast- or slow-acidifying S. thermophilus strains with 6 fast- or slow-acidifying L. bulgaricus strains. Furthermore, 3 nox-deficient mutants (Δnox) and one pflB-deficient mutant (ΔpflB) of S. thermophilus were used to evaluate the factor that determines the acidification rate of S. thermophilus. The aim of this study is to provide comprehensive information regarding the protocooperation between S. thermophilus and L. bulgaricus.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The strains used in this study are listed in Table 1. Strains other than ATCC 19258T and ATCC 11842T were obtained from stock culture strains of the Food Research and Development Center, Meiji Corporation (Tokyo, Japan).

Each L. bulgaricus and S. thermophilus strain stored at −80°C was pre-cultured at 37°C for 16 h in skim milk and yeast extract (SMY) medium, which consisted of 10% (wt/wt) skim milk and 0.05% (wt/wt) yeast extract (first preculture). The SMY medium was sterilized for use by autoclaving (121°C, 7 min), and then immediately cooled to 4°C. The first precultures of both L. bulgaricus and S. thermophilus were inoculated (1%; vol/vol) into fresh SMY media, incubated at 37°C to reach an acidity of 0.7%, and then cooled immediately to 4°C (second preculture). These second precultures were used as the inoculating seed for various milk media described below. Three S. thermophilus strains (WT and mutants) were cultured and precultured in 1/2 M17 broth supplemented with 1% lactose to determine NADH oxidase activities, DO consumption, and Eh, as shown in Figure 6 (A)-(E).

To examine the effect of formate or adenine supplementation on the acidification rate of S. thermophilus monocultures, 0.5 mM sodium formate (Wako Pure Chemical Industries, Ltd., Japan) or 50 µg/mL adenine hydrochloride (Wako Pure Chemical Industries, Ltd., Japan) was added to 10% skim milk medium, which contained 0.1% (wt/wt) peptides as described above. For the monoculture and coculture media, 10% (wt/wt) skim milk was prepared by heating it to 95°C and then immediately cooling it to 43°C to prevent formate production from lactose thermal decomposition. Formate is produced from the thermal decomposition of lactose during the milk sterilization process at 99°C and 105°C (Trimigno et al., 2020). The above treatment could minimize the formate accumulation in milk and

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provide insight on the behaviors of \textit{S. thermophilus} and \textit{L. bulgaricus} and their protocooperation in milk.

The second preculture of \textit{S. thermophilus} and \textit{L. bulgaricus} was inoculated at 1% (vol/vol) into the skim milk medium. For \textit{L. bulgaricus} and \textit{S. thermophilus} mono-culture experiments, the second preculture was inoculated at 2%, and either 1 mM sodium formate (Wako Pure Chemical Industries, Ltd., Japan) or 0.1% (wt/wt) peptides generated from the digestion of casein (CE90GMM, Nippon Shinyaku Co., Ltd., Japan) was added to the skim milk medium prepared as described above. The second precultures of \textit{S. thermophilus} and \textit{L. bulgaricus} were then inoculated at 2% (vol/vol) into each medium. Monocultures and cocultures were incubated at 43°C.

**Sequencing ST500 pflB gene**

The recombinant techniques used were performed according to the standard methods (Maniatis, 1982). A DNA fragment carrying the \textit{pflB} coding sequence from ST500 chromosome was amplified using primers LAB045 (5′-GCATGGCTTCTTGCCCTCCT-3′) and LAB046C (5′-CGCTATTGCGAGCTCTTTAT-3′) (Eurofins Genomics, Tokyo, Japan), ST500 genomic DNA, and Takara ExTaq DNA polymerase (Takara Bio Inc., Kusatsu, Japan). DNA sequence of the amplified fragment was determined by ABI 3100-Avant Genetic Analyzer (Applied Biosystems).

**Construction of nox and pflB knockout mutants of S. thermophilus**

The \textit{nox} knockout mutants of ST501 and ATCC 19258T were obtained in the same way and the same vector was used to have ST1131 \textit{Dnox} as described previously (2014, Sasaki). The \textit{nox} sequence was referred to the accession numbers of CP000023.1 and it was used as an insertion fragment to construct \textit{nox} knockout vector. The result confirming the construction of \textit{nox} deficient strain is shown in Figure S1. (Supplementary Data; https://figshare.com/s/a31994f8b76fbb1d874a)

The \textit{pflB} knockout mutant was constructed as follows. The \textit{pflB} sequence was referred to the accession numbers of CP000023.1 and it was used as an insertion fragment to construct \textit{pflB} knockout vector. A 2275 bp structural gene of the \textit{pflB} was amplified by PCR using the primer pairs PYY0030 (5′-TCT-AGAGTTGAAAAAGCGTGGGAAGGC-3′) and PYY0033 (5′-CTCGAGCCCATAGCATCGTCCGTTGA-3′), which contained a XhoI site (Eurofins Genomics, Tokyo, Japan), ST1131 genomic DNA, and Takara ExTaq DNA polymerase (Takara Bio Inc., Kusatsu, Japan), and were cloned into pGEM-T (Promega K.K., Tokyo, Japan) to obtain pGEM-T-\textit{pfl}. Then, inverse PCR was performed using the primer pairs PYY0031 (5′-ACCGATGATACTCCAGCGGCGA-3′) and PYY0032 (5′-GCTTTACACTCTCGTCTTCG-3′), and pGEM-T-\textit{pflB} as template. To the amplified fragment, the spectinomycin resistance gene, obtained by treating pSPC1 (Higuchi et al., 1999) with BamHI and the Kle-nox fragment of \textit{E. coli} DNA polymerase I (Takara Bio Inc., Japan), was ligated to obtain pGEM-T-\textit{pflB::spc}). The \textit{pflB::spc} fragment of the plasmid was cut out with \textit{XhoI} and introduced into the \textit{XhoI} site of pG7-host6

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**Table 1. Bacterial strains**

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<tr>
<th>Strains</th>
<th>Relevant phenotype</th>
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<tr>
<td>\textit{Streptococcus thermophilus}</td>
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<tr>
<td>ATCC19258T\textsuperscript{f}</td>
<td>Slow-acidifying strain</td>
<td>Mora et al. (2004)</td>
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<td>ST1131</td>
<td>Fast-acidifying strain</td>
<td>Sasaki et al. (2014)</td>
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<td>ST499</td>
<td>Fast-acidifying strain</td>
<td>Yamauchi et al. (2019)</td>
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<td>ST501</td>
<td>Fast-acidifying strain</td>
<td>This study</td>
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<tr>
<td>ST502</td>
<td>Slow-acidifying strain</td>
<td>This study</td>
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<tr>
<td>ST503</td>
<td>Slow-acidifying strain</td>
<td>This study</td>
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<tr>
<td>ST1131\textit{Δpfl}</td>
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<td>This study</td>
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<tr>
<td>ATCC19258\textit{Δnox}</td>
<td>\textit{nox} deletion mutant</td>
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<tr>
<td>ST501\textit{Δnox}</td>
<td>\textit{nox} deletion mutant</td>
<td>This study</td>
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<tr>
<td>\textit{Lactobacillus delbrueckii ssp. bulgaricus}</td>
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<tr>
<td>ATCC11842\textsuperscript{f}</td>
<td>Slow-acidifying strain</td>
<td>Mora et al. (2004)</td>
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<td>LB2038</td>
<td>Fast-acidifying strain</td>
<td>Sasaki et al. (2014)</td>
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<td>Slow-acidifying strain</td>
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<td>LB497</td>
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(Appligene, Heidelberg, Germany), a temperature-sensitive plasmid of Gram-positive bacteria. The resulting plasmid was designated as pG\textsuperscript{+}host6-pflB::spc and the ST1131 strain was transformed with the plasmid. After transformation, the pflB knockout mutant was generated as described previously using double crossover homologous recombination (2014, Sasaki). The genotype of the knockout mutant was confirmed by colony-direct PCR using the primer pairs PYY0030 and PYY0033, followed by digestion with a restriction enzyme as shown in Figure S2. (Supplementary Data; https://figshare.com/s/a31994f8b76fbb1d874a).

### Measurement of acidity, fermentation time, and lactate during fermentation

Acidity was measured according to the previously described method by Yamauchi et al. (2019). Fermentation time was defined as the time taken to reach 0.6% acidity with a few exceptions. The acidities used to calculate the fermentation time in each experiment are described in the Figure legends. To measure D- and L-lactate levels in the skim milk medium, protein was precipitated according to the method described by Yamauchi et al. (2019). D- and L-lactate levels were measured using high-performance liquid chromatography (HPLC) and a SUMICHIRAL OA-5000 column (Sumika Chemical Analysis Service, Ltd., Osaka, Japan) (Iwamoto, 2022).

### Measurement of DO

The DO levels were measured using optical oxygen sensors (VisiFerm DO ARC 225, Hamilton, Reno, NV, USA). The DO consumption rate (µM/min) was determined using the following formula:

\[
\text{DO consumption rate} (\mu \text{M} / \text{min}) = \frac{\text{Initial DO concentration} (\mu \text{M})}{\text{time} (\text{min}) \text{to reach DO of} 0 \mu \text{M}}.
\]

### Measurement of formate concentration

Formate levels in the skim milk medium were measured using an F-kit (Roche Diagnostics K.K., Tokyo, Japan). Measurements were carried out following the manufacturer’s instructions.

### Measurement of NADH oxidase activity

Nox activity was measured according to the method described by Sasaki et al. (2014). One unit of activity equaled 1 µmol of NADH oxidized to NAD per min.

### Measurement of redox potential

The measured redox potential (E\textsubscript{h}) was monitored every 30 min using a portable HM-31P pH meter (DKK-TOA Corporation, Tokyo, Japan) and DM\textsubscript{32}P DO/pH meter (DKK-TOA Corporation) and converted to E\textsubscript{h} according to the method previously described (Tachon et al., 2010). The E\textsubscript{h} value was transformed to E\textsubscript{h7} the redox potential at pH 7, using the Lieistner-Mirna equation:

\[
E_{h7} = E_{h} - \alpha \times (\text{pH } 7)
\]

where the \( \alpha \) constants determined for milk and casein supplemented milk were 41 and 46, respectively.

### Statistical analysis

The GraphPad Prism\textsuperscript{6} software (GraphPad, San Diego, CA, USA) was used to perform statistical analysis. The statistical difference between groups was determined by 2-sided Student’s t-test. One-way ANOVA (ANOVA) with post-hoc Tukey’s post-hoc test was used for the comparison of more than 2 groups. The correlation between the fermentation rate and formate accumulation, and between formate accumulation and NADH oxidase activity was investigated by Pearson correlation coefficient test. \( p \) values less than 0.05 were considered statistically significant.

### RESULTS & DISCUSSION

#### Monoculture fermentation time of L. bulgaricus and S. thermophilus

First, we estimated monoculture acidification rate of 6 \textit{L. bulgaricus} and 7 \textit{S. thermophilus} strains in the culture conditions (given below) and sorted the fast-/slow-acidification strain as shown in Table 1.

It was fallible to assess the monoculture acidification rate of \textit{L. bulgaricus} or \textit{S. thermophilus} in milk without reconstitution since it was difficult to grow their monocultures normally in milk. To overcome these deficiencies, each strain was supplemented with cofactors: 1 mM sodium formate (provided by \textit{S. thermophilus} in cocultures) was added to \textit{L. bulgaricus} monocultures, whereas 0.1% (vol/vol) CP (peptides and amino acids that result from the digestion of casein) (provided by \textit{L.}}
bulgaricus in cocultures) was added to S. thermophilus monocultures. Supplementing these cultures enabled the comparison of the monoculture acidification rates of L. bulgaricus and S. thermophilus.

There was a large difference in the fermentation period of the 6 L. bulgaricus and 7 S. thermophilus monocultures varied between strains from 4 to over 35 h (Figures 1A and B). Three L. bulgaricus strains (LB2038, LB494, and LB497) and 3 S. thermophilus strains (ST501, ST499, and ST1131), which exhibited significantly shorter fermentation periods than the other strains, were categorized as “fast-acidifying strains.” Accordingly, 3 L. bulgaricus strains (LB496, ATCC 11842T, and LB495) and 4 S. thermophilus strains (ST503, ST502, ATCC 19258T, and ST500) with longer fermentation periods were categorized as “slow-acidifying strains.” (Table 1)

Yogurt fermentation (coculture) rates depended on the acidification rate of S. thermophilus monoculture

We evaluated the fermentation period of cocultures of S. thermophilus ST1131 (a fast-acidifying strain) with 6 L. bulgaricus fast- and slow-acidifying strains in skim milk medium (Figure 1C). The coculture fermentation period was almost the same when ST1131 was cultured with any of the 6 L. bulgaricus strains (Figure 1C). As expected, the cocultures of fast-acidifying S. thermophilus ST1131 and fast-acidifying L. bulgaricus (LB2038, LB494, or LB497) showed rapid fermentation (4~6 h). Interestingly, the coculture of fast-acidifying S. thermophilus ST1131 with slow-acidifying L. bulgaricus (LB496 or LB495) also exhibited a short fermentation time. The fermentation time of LB495 was greatly shortened by coculturing with ST1131, from 21.2 to 5.7 h (Figure 1A, C). In the case of slow-acidifying ATCC 11842T, the coculture fermentation time was significantly longer when compared with fast-acidifying L. bulgaricus LB2038 or LB497, although it was shortened by coculture with ST1131 from 14.9 to 8.3 h (Figure 1C). Since there was a large difference from 3.8 to 21.2 h in the monoculture fermentation time of 6 L. bulgaricus, coculture with fast-acidifying S. thermophilus ST1131 reduced the time gap between them.

However, this gap reduction between the fermentation time was not observed when the fast-acidifying L. bulgaricus strain LB2038 was cultured with the 7 different S. thermophilus strains (Figure 1D).

The fermentation time of the coculture of fast-acidifying L. bulgaricus LB2038 with slow-acidifying S. thermophilus (ST503, ST502, ATCC 19258T or ST500) was 19–30 h, which was significantly longer than that of L. bulgaricus LB2038 with fast-acidifying S. thermophilus (ST501, ST499 or ST1131), their fermentation times were 5 - 8 h. The same trend was observed when the slow-acidifying L. bulgaricus LB495 or ATCC 11842T was fermented with the 7 strains of fast-acidifying and slow-acidifying S. thermophilus (Figure 1D). Figure 1B (the fermentation time of S. thermophilus monocultures) and Figure 1D (the fermentation time of cocultures) showed nearly identical patterns.

The results of 24 cocultures of 7 fast- or slow-acidifying S. thermophilus strains with 6 fast- or slow-acidifying L. bulgaricus strains (Figures 1C, D) showed that these strains of cocultures made progress at a similar rate to that of S. thermophilus monoculture. These observations suggested that the yogurt fermentation rates correspond mainly to the acidification rate of S. thermophilus monoculture irrespective of the fast-/slow-acidifying L. bulgaricus strains combinations considered.

Acidities in coculture were composed different l-lactate/d-lactate ratio depending on combination.

Here we used the content of “acidity,” that is, the sum of L-lactate produced by S. thermophilus, and D-lactate produced by L. bulgaricus, and the proportion of L/D-lactate were different among combinations of their strains when their acidities were the same (Figure 1C and D). When there was a reduction in the difference of fermentation time by coculture with fast-acidifying S. thermophilus ST1131 (Figure 1D), the ratio of L-lactate to D-lactate with fast-acidifying L. bulgaricus LB2038 was 2.9 at 0.7% acidity. However, the L/D-lactate ratio increased to 19 at 0.7% acidity, when ST1131 was cocultured with a slow-acidifying L. bulgaricus strain ATCC 11842T (Supplementary Data S3; https://figshare.com/s/a3199f8b76fbb1d874a). Even when slow-acidifying S. thermophilus strains were cocultured with fast- or slow-acidifying L. bulgaricus strains, the fermentation time was close to that of the slow-acidifying S. thermophilus. In these cases, the L/D-lactate ratio was less than 1, thereby indicating that the cell number of L. bulgaricus was larger than that of S. thermophilus.

Correlation between acidification rate and formate accumulation in 7 S. thermophilus monocultures

Our previous work showed that the S. thermophilus ST1131 nox mutant could not produce formate and its acidification was severely delayed (Sasaki, 2014). Derzelle et. al. (2005) showed that formate supply improved S. thermophilus growth in milk. These results suggested that formate is important growth stimulating factor of S. thermophilus.

Monocultures of 7 S. thermophilus strains (ST501, ST499, ST1131, ST503, ST502, ATCC 19258T, and ST500) were performed to evaluate the correlation between the acidification rate and formate accumu-
Figure 1. Monoculture fermentation times of 6 *L. bulgaricus* strains (A) and 7 *S. thermophilus* strains (B). Coculture fermentation times of 6 *L. bulgaricus* strains with *S. thermophilus* ST1131 (C) and 7 *S. thermophilus* strains with 3 *L. bulgaricus* strains (LB2038 (black bar), LB495 (white bar), and ATCC 11842T (diagonal line bar) (D). Three *L. bulgaricus* strains (LB2038, LB494, and LB497) and 3 *S. thermophilus* strains (ST501, ST499, and ST1131) are “fast-acidifying strains.” Three *L. bulgaricus* strains (LB496, ATCC 11842T, and LB495) and 4 *S. thermophilus* strains (ST503, ST502, ATCC 19258T, and ST500) are “slow-acidifying strains.” Monocultures of *L. bulgaricus* or *S. thermophilus* were performed at 43°C in skim milk medium supplemented with 1 mM sodium formate or 0.1% peptides and amino acids that result from the digestion of casein (CP), respectively. Cocultures of *L. bulgaricus* with *S. thermophilus* were performed at 43°C in skim milk medium. Fermentation time (h) is the time required to reach 0.6% acidity, with the exception that monocultures of *S. thermophilus* ST500 and cocultures of *L. bulgaricus* ATCC 11842T with *S. thermophilus* ST500 or ST503 are only 0.5% acidity because their fermentations were too slow to reach 0.6% acidity. Error bars show the SD of the results from at least 3 independent experiments. Different lowercase letters indicate significant differences in each fermentation time ($P < 0.05$).
The formate accumulation of *S. thermophilus* strains ranged from 0 to 0.65 mM and differed among strains. In the fast-acidifying strains (ST501, ST499, and ST1131), formate accumulation was 0.45, 0.64, and 0.58 mM, respectively. However, in slow-acidifying strains, ST503, ST502, ATCC 19258T, and ST500, formate accumulation was 0.18, 0.21, 0.41, and 0 mM, respectively. The formate accumulation of the fast-acidifying strains was significantly higher than that of the slow-acidifying strains. A strong positive correlation (r = 0.838, P = 0.019) was observed between formate accumulation in milk and acidification rate in the *S. thermophilus* strains.

**Effect of formate supplementation on acidification of 7 *S. thermophilus* monocultures**

The fast-acidifying strains could accumulate more than 0.45 mM formate and slow-acidifying strains accumulated lesser than 0.41 mM formate (Figure 2). Then, we examined the effect of 0.5 mM formate supplementation on the acidification rate of *S. thermophilus* monocultures. Monocultures of 7 *S. thermophilus* strains (ST501, ST499, ST1131, ST503, ST502, ATCC 19258T, and ST500) were cultured in skim milk medium supplemented with or without 0.5 mM sodium formate to evaluate the effect of formate supplementation (Figure 3). The supplementation of 0.5 mM sodium formate significantly reduced fermentation time in 5 out of 7 strains (ST499, ST1131, ST503, ST502, and ST500). The time shortened by 0.5 mM sodium formate supplementation was 5 - 33 h in the slow-acidifying strains (ST503, ST502, and ST500) and 1 - 2 h in the fast-acidifying strains (ST499 and ST1131). The acidification of slow-acidifying ST500 was significantly accelerated by the supplementation of sodium formate. Since we could not detect formate accumulation in ST500, we performed a sequence analysis of the pfIB gene and identified a frameshift mutation at nucleotide position 471, leading to a truncated pfIB protein of 191 amino acid residues. The N-terminal 156 amino acid sequences of CNRZ1066 pyruvate formate-lyase (Accession number: CP000024) were identical to those of ST500 PfIB upstream of the frame shift mutation.

Although the stimulating effect of the formate supplementation on acidification rate could not be observed in ATCC 19258T, the supplementation of 50 µg/mL adenine shortened fermentation time from 22 to 8 h. In other strains whose fermentation time were shortened by the supplementation of sodium formate (ST499, ST1131, ST503, ST502, and ST500), 50 µg/mL adenine also shortened fermentation times similarly to sodium formate.
This result indicates that ATCC 19258T may have a deficiency in the purine biosynthesis pathway due to formate and suggests that fast acidification of *S. thermophilus* may not only require formate, but also an intact purine biosynthesis pathway.

It was observed that a low concentration of formate (0.5 mM) had a significantly stimulating effect on *S. thermophilus* acidification (Figure 3), because the milk medium must be almost formate or in purine starvation condition. Formate supplementation (data not shown). This result indicates that ATCC 19258T may have a deficiency in the purine biosynthesis pathway due to formate and suggests that fast acidification of *S. thermophilus* may not only require formate, but also an intact purine biosynthesis pathway.

Figure 3. Monoculture fermentation time of 7 *S. thermophilus* strains cultured at 43°C in skim milk medium supplemented with 0.1% CP without (black bar) or with 0.5 mM sodium formate (white bar). Fermentation time (h) is defined the time required to reach 0.6% acidity, with the exception that monoculture of *S. thermophilus* ST500 and ATCC19258T are 0.5% acidity because their fermentations were too slow to reach 0.6% acidity. Acidity (%) corresponds to the amount of acid produced by *S. thermophilus* in skim milk medium. Asterisks indicate significant differences (*P < 0.05, **P < 0.01, *** P < 0.005, **** P < 0.001). Error bars show SD of the results from at least 3 independent experiments.

**Effect of PfI deficiency on acidification of *S. thermophilus* ST1131**

*S. thermophilus* ST1131, a *pflB*-deficient mutant (Δ*pflB*), was constructed to evaluate the importance of formate production in skim milk fermentation. *S. thermophilus* ST1131 WT and Δ*pflB* strains were cultured in skim milk medium supplemented with 0.1% CP (Figure 4). PflB deficiency resulted in a significant delay in fermentation time and 42 h were required for Δ*pflB* to reach 0.6% acidity, whereas this was achieved within 4 h for the WT of ST1131. Sodium formate supplementation shortened the fermentation time of ST1131Δ*pflB* to that of the WT strain (Figure 4). The results suggested that a higher amount of formate production by PFL is essential for faster acidification of *S. thermophilus* (Figure 2, 3, and 4).

Formate has been considered as a protocooperation factor, which *S. thermophilus* provide to *L. bulgaricus*. However, formate producing ability of *S. thermophilus* was a key driver to determine the acidification rate of *S. thermophilus* monoculture.

**Formate function in the purine biosynthetic pathway and the ribonucleotides conversion in *S. thermophilus***

Derzelle et al. (2005) identified formate to be a substance of formyl-tetrahydrofolate (THF) synthetase for the synthesis of THF feeding the purine biosynthetic pathway in *S. thermophilus* (Derzelle et al., 2005).

Furthermore, formate plays another prominent role as a reductant in nucleic-acid metabolism, especially in *S. thermophilus*. Ribonucleoside-triphosphate reductases (RNRs, EC:1.1.98.6), which are essential enzymes in DNA biosynthesis that converts all 4 ribonucleotides...
into deoxyribonucleotides, are classified into 3 types: class I, class II, and class III. Bacteria such as *E. coli* and *Bacillus* have class I aerobic RNRs, while *S. thermophilus* have class II and class III RNRs, according to the Kyoto Encyclopedia of Genes and Genomes (KEGG). The class III RNRs of *S. thermophilus* are anaerobic and only use formate as a reductant (Levitz et al., 2022). Therefore, formate plays a critical role in DNA replication and repair of *S. thermophilus*. It was suggested that the formate-producing ability was involved in nucleic-acid metabolism and correlated with the proliferation and acidification rates of *S. thermophilus*.

Additionally, KEGG showed that 4 strains of *L. bulgaricus* have anaerobic ribonucleoside-triphosphate reductase, class III RNRs which require formate as a reductant (Levitz et al., 2022). Therefore, formate plays a critical role in DNA replication and repair of *S. thermophilus*. It was suggested that the formate-producing ability was involved in nucleic-acid metabolism and correlated with the proliferation and acidification rates of *S. thermophilus*.

Correlation between NADH oxidase activity and formate accumulation in 7 *S. thermophilus* monocultures

We previously found that Nox is required for formate production by *S. thermophilus* ST1131 in skim milk medium (Sasaki et al., 2014). Accordingly, the correlation between formate accumulation and NADH oxidase activities of 6 *S. thermophilus* strains (ST501, ST499, ST1131, ST503, ST502, and ATCC 19258T) was analyzed (Figure 5). Nox activities of 6 *S. thermophilus* strains ranged from 0.1 to 1.0 U/mg protein, and there was a strong positive correlation (r = 0.845, P = 0.034) between Nox activity and formate accumulation (Figure 5). This result suggested that NADH oxidase may be a factor that determines the amount of formate production in *S. thermophilus*.

**Effect of Nox deficiency on DO consumption rate and redox potential of *S. thermophilus* monocultures**

Although *S. thermophilus* ST1131 showed high Nox activity and formate accumulation, we selected 2 other strains which showed lower Nox activity and formate accumulation. We generated Nox deficient mutants (Δnox) for 2 *S. thermophilus* strains (ST501 and ATCC 19258T) based on the method of ST1131Δnox construction described by Sasaki et al. (2014) and confirmed that NADH oxidase activities were mostly inhibited not only in ST1131Δnox but also in ST501Δnox and ATCC 19258TΔnox (Figure 6A). The WT and the Δnox of 3 strains (ST1131, ST501, and ATCC 19258T) were cultured in skim milk medium supplemented with 0.1% CP to evaluate the effect of Nox deficiency on...
DO consumption rate (Figure 6B). NADH oxidase deficiency decreased DO consumption rates in ST501 and ATCC 19258T to the same extent as in ST1131, as previously reported (Sasaki et al., 2014). Their residual DO consumption activities were 34% (ST1131), 14% (ST501), and 28% (ATCC19258T) (Figure 6B). These results showed that NADH oxidase played a key role in DO consumption in *S. thermophilus* strains.

A major delay in the acidification rate was observed in all Δnox when compared with their WT counterparts (Figure 6C). The fermentation time required to reach 0.6% acidity increased over 3-fold in ST1131Δnox and 8-fold in ST501Δnox when compared with the WT, and in the case of ATCC 19258Δnox could not reach 0.6% and the final acidity was only 0.4% after 24 h of cultivation. Additionally, these acidification delays of Δnox strains could not be recovered under anaerobic nitrogen-saturated condition (Figure 6C).

We measured the redox potentials of Δnox and WT strains and in ST1131 and ST501 WT strains, the $E_{H7}$ decreased below 0 mV after 3 h of cultivation and subsequently reached −50 mV after 4 h (Figures 6D). As the $E_{H7}$ in ST1131Δnox and ST501Δnox gradually decreased to around 50 mV, they could not reach below 0 mV even after 6 h of cultivation (Figures 6D). However, in *S. thermophilus* ATCC 19258T WT, wherein the Nox activity is lower than that of ST1131 and ST501 (Figure 6A), the $E_{H7}$ reached around 0 mV after 6-7 h of cultivation but not below 0 mV even after 24 h of cultivation (Figure 6D). In ATCC 19258Δnox, $E_{H7}$ decreased extremely slowly when compared with WT, and reached to around 100 mV after 12 h of cultivation. The $E_{H7}$ values in all Δnox were unstable across multiple experiments and tended to show higher standard deviation. We hypothesized that the other plural mechanisms tried to complement NADH oxidase deficiency by reducing the redox potential, and the $E_{H7}$ values in all Δnox and became unstable.

These results showed that NADH oxidase deficiency induced the decrease of DO consumption ability (Figure 6B) and resulted in severe fermentation delay even under anaerobic conditions (Figure 6C) and inhibition, thereby reducing the redox potential ability (Figure 6D). Additionally, it was suggested that the fermentation of *S. thermophilus* required not only anaerobic conditions, but also the redox potential reducing condition produced by Nox.

Jeanson et al. (2009) reported that the redox potential of oxygen-free milk (saturated with nitrogen) was still positive and some of the enzymes that lower the re-
dox potential are required for formate accumulation in L. lactis. As Tachon et al. (2017) reported that L. lactis has a strong reducing ability and was able to decrease milk redox potential to −220 mV (Eh7), NADH oxidase (NoxE) and the respiratory chain components (NoxA, NoxB, MenC) were identified to contribute to the reduction of redox potential. NADH oxidase is responsible for reducing redox potential primarily by removing DO from milk, while the respiratory chain components are proposed to reduce the potential by reducing oxidizing compounds other than oxygen in milk. Unlike L. lactis, S. thermophilus does not have genes consisting of the respiratory chain, thus it is not surprising if S. thermophilus Nox has dual functions: the exclusion of DO and the reduction of oxidizing compounds in milk to achieve a low redox potential that facilitates formate production and acidification.

Effect of reducing reagent supplementation on formate production of S. thermophilus

To further analyze the function of NADH oxidase, the effect of 0.1% (vol/vol) ascorbic acid supplementation as a reducing reagent was examined on acidification rate, DO consumption rate, Eh7, and the formate accumulation of ST1131 WT (Figures 7A and B) and Δnox (Figures 7C and D). Both strains were cultured in skim milk medium supplemented with 0.1% CP.

By supplementing ST1131 WT with ascorbic acid (Figure 7A), the time required to reach 0.6% acidity was reduced by more than 1 h, and it took 30 min lesser to reach a concentration of 0 µM DO. With ascorbic acid supplementation, the Eh7 of WT could reach −50 mV approximately 2.5 h faster, and the formate concentration reached over 0.8 mM approximately 1.5 h faster than without ascorbic acid supplementation (Figure 7B).

A significant alternation was observed when ascorbic acid supplementation was applied to ST1131 Δnox, since ascorbic acid supplementation showed stimulating effects on ST1131 WT fermentation. The acidification rate in Δnox was significantly slower when compared with WT (Figure 7A and C), the acidity of Δnox was 0.4% at 12 h, and could not reach 0.6% (Figure C). The redox potential for first 8 h was incapable of altering Δnox, and then for the next 4 h, Eh7 decreased by 200 mV. Formate could not be detected at all in Δnox for 12 h of cultivation (Figure 7D) and even after 30 h of cultivation (data not shown). However, with ascorbic acid supplementation, Eh7 began to decrease gradually for the first 4 h, reaching −50 mV after 5 h. In the treatment of ascorbic acid supplementation, formate could be detected after 6 h and gradually increased.
Figure 6 (Continued). (A) NADH oxidase activities of ST1131, ST501 and ATCC19258T, WT (black) and Δnox (White) using cell cultured at 37°C in M17 broth supplemented with 1% glucose with shaking. (B) Dissolved oxygen consumption rate (µM/min) of ST1131, ST501 and ATCC19258T, WT (black) and Δnox (White) in skim milk medium. DO consumption rate (µM/min) was obtained by dividing the initial DO concentration by the time which DO concentration reduced to 0 µM. (C) Acidification curves of WT and Δnox in ST1131, ST501 and ATCC19258T under aerobic / anaerobic condition in skim milk medium. The redox potential (Eh7) of S. thermophilus monocultures ST1131 (D), ST501 (E), and ATCC19258T (F) in skim milk medium. In Figure 6(A), 6(B), 6(D), 6(E), 6(F) experiments, preculture which cultured in 1/2 M17 broth supplemented with 1% lactose was used. Preculture in Figure 6(C) experiment, SMY was used. Main culture in (B)~(F) were incubated at 43°C in skim milk medium supplemented with 0.1% CP. Asterisks indicate significant differences (* < 0.05, ** < 0.01, *** < 0.005, **** < 0.001). Error bars show SD of the results from at least 3 independent experiments. Though an acidification curve of ST501Δnox was examined 2 times, SD couldn't show in Figure 6 (C).
Figure 6 (Continued). (A) NADH oxidase activities of ST1131, ST501 and ATCC19258T, WT (black) and Δnox (White) using cell cultured at 37°C in M17 broth supplemented with 1% glucose with shaking. (B) Dissolved oxygen consumption rate (µM/min) of ST1131, ST501 and ATCC19258T, WT (black) and Δnox (White) in skim milk medium. DO consumption rate (µM/min) was obtained by dividing the initial DO concentration by the time which DO concentration reduced to 0 µM. (C) Acidification curves of WT and Δnox in ST1131, ST501 and ATCC19258T under aerobic / anaerobic condition in skim milk medium. The redox potential (Eh) of S. thermophilus monocultures ST1131 (D), ST501 (E), and ATCC19258T (F) in skim milk medium. In Figure 6(A), 6(B), 6(D), 6(E), 6(F) experiments, preculture which cultured in 1/2 M17 broth supplemented with 1% lactose was used. Preculture in Figure 6(C) experiment, SMY was used. Main culture in (B)~(F) were incubated at 43°C in skim milk medium supplemented with 0.1% CP. Asterisks indicate significant differences (* < 0.05, ** < 0.01, *** < 0.005, **** < 0.001). Error bars show SD of the results from at least 3 independent experiments. Though an acidification curve of ST501Δnox was examined 2 times, SD couldn't show in Figure 6 (C).
It was observed that ascorbic acid supplementation increased the redox potential dropping, formate accumulation and the acidification rate even in wild type ST1131 (Figures 7A and B). The same stimulating effect of formate supplementation on the ST1131 acidification (Figure 3) was observed similar to ascorbic acid supplementation (Figure 7A).

ST1131 Δnox was incapable of formate accumulation without ascorbic acid supplementation and its acidification delayed deeply. The supplementation of ascorbic acid enabled ST1131 Δnox to decrease the redox potential and formate accumulation after 5 h of cultivation.

This observation showed that the formate production by PfIB requires not only an anaerobic environment, but also the reduction of the redox potential in skim milk medium, and NADH oxidase provides both conditions.

**Formate production complex consisting of formate-lyase activating enzyme, AdoMet and PfIB working under low redox potential conditions**

As described above, the reducing redox potential abilities of NADH oxidase may be an essential factor for activating PfIB. Formate was synthesized by PfIB, and this reaction required the formation of a specific glycyl radical on PfIB by the pyruvate Formate-lyase Activating Enzyme (PFL-AE), which is also oxygen-sensitive (Crain and Broderick, 2014). PFL-AE is an iron-sulfur protein, and its cofactor, S-adenosyl-L-methionine (AdoMet), was involved in the activation of PfIB. PFL-AE exists largely in combination with PfIB and AdoMet. A reduced [4Fe-4S]⁺ cluster, which provides the electron, was required for the reductive cleavage of AdoMet to generate the catalytically essential glycyl radical of PfIB (Broderick et al., 2019).
These sequential reactions may require the reducing reagents 
formate potential conditions (Bim D., 2020).

CONCLUSIONS

This study identified that the yogurt fermentation rate was mainly dependent on the acidification rate of S. thermophilus monoculture, determined by the capacity of formate production, irrespective of the combination of fast-/slow-acidifying L. bulgaricus strains. The Δpfb and the Δnox experiments revealed that formate may play a significant role in S. thermophilus acidification and proliferation, and formate production requires not only anaerobic conditions but also the reducing reduct potential conditions maintained by Nox. Although formate has been identified as an important protocell and a proliferative factor of L. bulgaricus heretofore, it also plays a role in determining the acidification rate of S. thermophilus and, consequently, yogurt fermentation. From the point of view of industrial yogurt manufacturing, it is important to select S. thermophilus strains with high NADH oxidase activities that can effectively remove oxygen and reduce the reduct potential of milk to produce higher amount of formate.

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