A new manufacture method for set yogurt with low-temperature reduced dissolved oxygen fermentation

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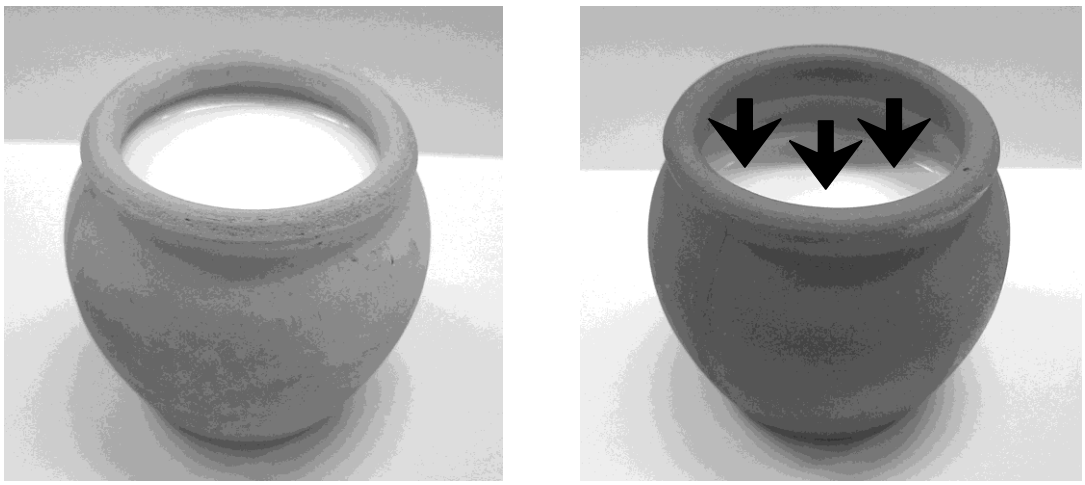
Contents

General introduction

Since ancient times, humans have attempted to control the fermentation process. Fermentation is one of the oldest methods for extending the shelf life of milk (1). Fermented milk contains many kinds of milk products and yogurt is one of these fermented milk products. The definition of yogurt given in the Food and Agriculture Organization of the United Nations/ World Health Organization standards (2) is as follows: "Yogurt is the coagulated milk product obtained by lactic acid fermentation through the action of *Streptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus delbureckii* subsp. *bulgaricus* (*L. bulgaricus*) from milk (cow's milk, sheep's milk, goat's milk, buffalo's milk, etc.) and milk products. The microorganisms (*S. thermophilus* and *L. bulgaricus*) in the final product must be viable and abundant." Such products are beneficial to human health (1). Michaylova et al. (3) suggested that *S. thermophilus* and *L. bulgaricus* strains widely used for commercial yogurt production may have originated from plants in Bulgaria. There is a traditional home-made yogurt which that is prepared in an unglazed pot in Bulgaria.

Bulgarian traditional home-made yogurt

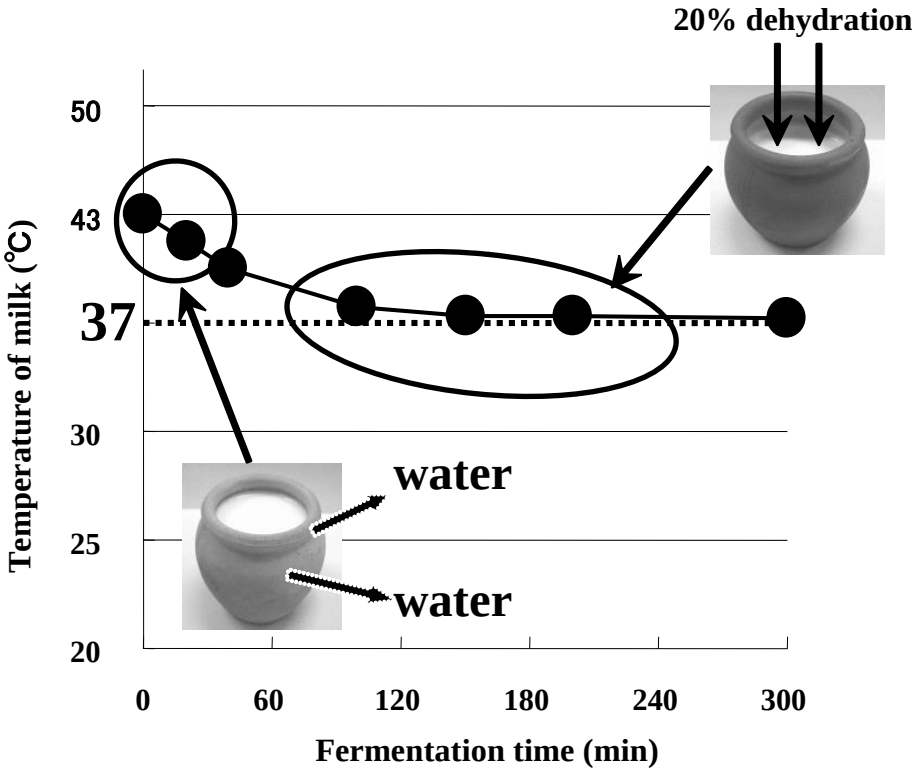
The features of this Bulgarian traditional home-made yogurt are as follows. During fermentation, about 20% of the water in the milk is absorbed into the pot. As a result, milk in the pot becomes roughly 1.2 times concentrated. The milk in the pot is gradually cooled by vaporization. As a result, fermentation proceeds at relatively low temperatures. Low temperature fermentation creates a smooth and creamy yogurt. The author tried to commercially produce “Bulgarian traditional home-made yogurt”. It was thought that the most important feature of this production method was related to the low-temperature fermentation. However, there was a problem in reproducing the low-temperature fermentation. This took much longer than anticipated and therefore became a barrier to commercial production. Attempt to shorten the fermentation time of low-temperature fermentation was focus of this study.



Before fermentation

After fermentation
20% dehydration

Comparison of before fermentation and after fermentation of Bulgarian traditional home-made yogurt prepared in an unglazed pot



Variation of temperature of milk during traditional fermentation in an unglazed pot

The combination *S. thermophilus* and *L. bulgaricus* is traditionally used for the manufacture of yogurt. When both bacteria exist in a mutually beneficial relationship, this association is known proto-cooperation. The proto-cooperation between *S. thermophilus* and *L. bulgaricus* had been described in terms of nutritional exchanges, with *L. bulgaricus* supplying peptides and amino acid and carbon dioxide, which stimulate the *L. bulgaricus* growth (4). Driessen et al. (5) suggested that in a mixed culture, acid production is much larger than the sum of a single culture and in yogurt the growth of *S. thermophilus* is stimulated by free amino acids and peptides liberated from the milk proteins by *L. bulgaricus*. Veringa et al. (6) suggested that the two bacterial strains in yogurt, *S. thermophilus* and *L. bulgaricus*, stimulate each other during their

associative growth and the substance which stimulates *L. bulgaricus* and which is produced by *S. thermophilus* in yogurt milk is formic acid. Suzuki et al. (7) reported that formic acid was the only effective substance for the active growth of *L. bulgaricus* among purine ring precursors. Galesloot et al. (8) concluded that proteolytic, *L. bulgaricus* liberates certain amino acids in milk, which stimulate *S. thermophilus*. Pablo et al. (9) suggested that the symbiotic relationship between *S. thermophilus* and *L. bulgaricus* is very important for yogurt and cheese production because it reduces fermentation times. The recent completion of the genome sequences of *S. thermophilus* (10) and *L. bulgaricus* (11), have allowed for examination of the proto-cooperation between these bacteria at a molecular level. Herve-Jimenez et al. (4) revealed specific physiological changes in *S. thermophilus* during growth stimulation due to the presence of *L. bulgaricus* and they reported that the combination of transcriptomic and proteomic analyses not only revealed undocumented nutritional effects on the BCAA, Arg, and purine metabolisms with their regulators but also found other unexpected effects, like the adaptation to H_2O_2, indicating that this bacterial proto-cooperation is more complex and less straight forward than previously reported.

Modern industrial yogurt is classified into stirred yogurt, drinking yogurt, and set yogurt types. Stirred yogurt and drinking yogurt are produced by fermentation of yogurt in a tank, and then packaging the yogurt mix is packaged into individual containers. In the case of stirred yogurt with fruit and drinking yogurt with fruit, after the yogurt is mixed with fruit jam, juice, or nectar, the mixture is packaged into individual containers. Set yogurt is produced by packaging the yogurt mix into individual containers before fermentation. As a commercial product, it is important for the set yogurt to have curd with sufficient hardness to stand up to the impacts caused by the shaking which occurs during transport by truck over long-distances to market. In general, industrial yogurt fermentation is carried out at 40 to 45°C, because it is evident that this results in the fastest rate of acid production and curd with the desired firmness (23). However, it is well known that yogurt made by low-temperature (less than 40°C) fermentation with *S. thermophilus* and *L. bulgaricus* has a much smoother texture than yogurt fermented at 40 to 45°C. Cho-Ah-Ying et al. (12) suggested that set yogurt incubated at 38°C is smoother than yogurt incubated at 43°C. Driessen (13) suggested that the starter culture might produce more polysaccharides under low-temperature fermentation, resulting in a smoother perceived texture. However, there were 2 problems with low-temperature fermentation. The low-temperature fermentation takes a much longer time, which decreases productivity, and the resulting set yogurt curd is weak.

In this study, the author focused on set yogurt and found that reducing dissolved

oxygen (DO) concentration in the yogurt mix with LB81 composed of *L. bulgaricus* 2038 and *S. thermophilus* 1131 to nearly 0 mg/kg reduced the fermentation time and resulted in a new set yogurt fermentation method that involves reducing the DO concentration in the yogurt mix to nearly 0 mg/kg prior to incubating the mix at a lower temperature. The combination of reduced DO and lower temperature fermentation (LT-ROF) brought about a better set yogurt with a smoother texture, strong curd structure, increased rich taste, mild acidity, reduced whey syneresis and rapid fermentation. The author has also successfully developed excellent fat free, set yogurt without using sugar, stabilizers or thickeners by LT-ROF.

In this study, the author investigated the influence of oxygen on the symbiosis between *L. bulgaricus* 2038 and *S. thermophilus* 1131. The yogurt starter cultures *L. bulgaricus* and *S. thermophilus*, are facultatively anaerobic, so the fermentation of yogurt with these bacteria progresses well in the presence of oxygen. From a previous study, it was found that acid production by a culture of LB81 was suppressed greatly at 1 mg/kg or more DO in the yogurt mix and was promoted at 0 mg/kg, however acid production by *L. bulgaricus* 2038 or *S. thermophilus* 1131 alone was neither suppressed nor enhanced by the DO concentration in the yogurt mix (14). Therefore it was assumed that DO would greatly affect yogurt fermentation, but the mechanisms for DO affecting yogurt fermentation have not been elucidated thoroughly. Therefore, the author attempted to find out more about the mechanisms underlying the effects of DO on yogurt fermentation with respect to proto-cooperation in this study. For this purpose, experimental conditions the same as, or similar to, the yogurt mix was essential and a skim milk medium was used throughout this study. It was thought that DO consumption by *S. thermophilus* was important for yogurt fermentation, therefore an NADH oxidase gene, *nox*, of *S. thermophilus* 1131 was selected as the oxygen-consuming gene and a knockout strain was constructed. The results obtained by this knockout strain suggested that the NADH oxidase of *S. thermophilus* 1131 is very important for yogurt fermentation. (15)

Strategy of this study

Chapter 1

A manufacture method for superior set yogurt under reduced oxygen conditions

・An acid production by LB81 was promoted by reducing dissolved oxygen (DO) concentration in the yogurt mix nearly to 0 mg/kg.
(Reduced DO fermentation: ROF)

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・The combination of ROF and low temperature (LT-ROF) brought about a better set yogurt with a better set yogurt with smooth texture, strong curd structure and increased rich taste.

Chapter 2

Effect of oxygen on symbiosis between *L. bulgaricus* 2038 and *S. thermophilus* 1131

・Two bacterial strains in yogurt, *L. bulgaricus* and *S. thermophilus*, stimulate each other (symbiosis).
・Stimulation of *L. bulgaricus* is mainly caused by production of formate by *S. thermophilus*.

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・Stimulation of *S. thermophilus* is mainly stimulated by free amino acids and peptides liberated from the milk proteins by *L. bulgaricus*.
・DO did not interfere with the growth of single cultures *L. bulgaricus* 2038 and *S. thermophilus* 1131.
・DO inhibit to the symbiotic relationship between *L. bulgaricus* 2038 and *S. thermophilus* 1131.
・Reduced DO accelerated acid production of LB81.

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・Acceleration of acid production of LB81 by reduced DO would be depended on acceleration of formate production.
・Suppression of acid production of LB81 by DO would be depended on suppression of formate production.

Chapter 3

NADH oxidase of *S. thermophilus* 1131 is important for yogurt fermentation with *L. bulgaricus* 2038

- ROF brought about faster cell growth of *S. thermophilus* 1131 and earlier l-lactate and formate accumulation in milk medium, which is favorable to industrial yogurt production.
- *S. thermophilus* 1131 was found to work mainly to remove DO in yogurt fermentation.
- The results using H_2O-forming NADH oxidase (nox-2) defective mutant (Δ*nox*) of *S. thermophilus* 1131 revealed that Nox-2 played an important role for DO reduction during yogurt fermentation.
- Yogurt fermentation by a starter composed of Δ*nox* and *L. bulgaricus* 2038 was significantly slow, presumably because this starter could not reduce DO concentration to less than 2 mg/kg.

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- It was suggested that Nox-2 of Strep. thermophilus 1131 contributes greatly to yogurt fermentation.

Chapter 4

Development of superior fat free set yogurt with LT-ROF

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- Fat free set yogurt product prepared by LT-ROF had almost the same fatty taste, smooth texture, and sufficient hardness to stand up to the impact of shaking during transport as normal fat set yogurt product containing 3.0% (wt/wt) milk fat prepared by control fermentation.
- Fat free set yogurt product prepared by LT-ROF had been sold as commercial production of Meiji Bulgaria Yogurt LB81 Zero Fat Plain in Japan since 2009.

Chapter 1
A manufacture method for superior set yogurt under reduced oxygen conditions

1.1 Introduction

There was a traditional home-made yogurt which has been prepared in an unglazed pot in Bulgaria. The features of this traditional home-made yogurt are as follows. During fermentation, water in the milk is absorbed into the pot. As a result, milk in the pot becomes concentrated. The milk in the pot is gradually cooled by vaporization. As a result, fermentation proceeds at relatively low temperatures. Low temperature fermentation creates smooth and creamy yogurt. This practice, however, is rarely applied at present.

Modern industrial yogurt is classified into 3 types. One is stirred yogurt, and the others are drinking yogurt, and set yogurt. The stirred yogurt and drinking yogurt are produced by fermentation of yogurt in a tank, and then packaging the yogurt mix into individual containers. The set yogurt is produced by packaging the yogurt mix into individual containers before fermentation. The traditional home-made yogurt in Bulgaria was set type. The sales of yogurt in Japan in 2012 were approximately ¥340 billions, approximately ¥160 billions of which were sales of set yogurt. While there is a great demand for set yogurt in Japan, stirred yogurt and drinking yogurt is very popular in Europe.

As a commercial product, it is important that the set yogurt have curd with sufficient hardness to stand up to the impact caused by shaking during transport by truck. Because the Japanese are strict with the appearance of food products, it is crucial that the set yogurt have curd with sufficient hardness. Nielsen (16) suggested that the texture of set yogurt should be firm enough to remove it from the container with a spoon. It is evident that fastest rate of acid production and the curd with desired firmness are obtained when the yogurt starter culture is incubated at 40 to 45°C (1), and in general, industrial yogurt fermentation is carried out at 40 to 45°C. A good set yogurt should have both hard curd and smooth texture. Mehanna (17) suggested that the required incubation time for making Zabadi (Egyptian yogurt with mixed culture of *S. thermophilus* and *L. bulgaricus*) increased with decreasing incubation temperature and that preparation of Zabadi at 30 or 35°C improved smoothness and minimized curd syneresis. With respect to sensory qualities, Cho-Ah-Ying et al. (12) suggested that a set yogurt incubated at 38°C was smoother than yogurt incubated at 43°C. Driessen (13)

suggested that the starter culture might produce more polysaccharides with low-temperature fermentation, which contributes to a smoother perceived texture. However, there were 2 problems with fermentation at 38 °C: fermentation takes a much longer time, which decreases productivity, and the set yogurt curd becomes weak.

The yogurt starter culture of *L. bulgaricus* and *S. thermophilus*, are facultatively anaerobic, so the fermentation of yogurt with these bacteria progresses well in the presence of oxygen. Shekar and Bhat (18) reported that rates of acid production in buffalo's milk by these lactic cultures increased with the decrease in initial oxygen content from 5.50 to 2.90 mg/kg, and that incorporating oxygen into milk to increase the initial oxygen content from 5.50 to 9.00 mg/kg strongly inhibited acid production.

Although some studies have been conducted on the influence of oxygen on lactic acid bacteria, only a small number of studies have reported on the influence of oxygen on yogurt fermentation with a mixed culture of *L. bulgaricus* and *S. thermophilus* in yogurt manufacture.

In this chapter, the author investigated the influence of oxygen on yogurt fermentation (yogurt production) with *L. bulgaricus* 2038 and *S. thermophilus* 1131. The author developed a rapid set yogurt fermentation method that involved reducing the dissolved oxygen (DO) concentration in the yogurt mix to approximately 0 mg/kg beforehand and then incubating the mix at a lower temperature. The combination of reduced DO and lower temperature brought about a better set yogurt product with smooth texture and strong curd structure to stand up to the impact caused by shaking during transport.

1.2 Materials and methods

1.2.1 Culture strains used

L. bulgaricus 2038 and *S. thermophilus* 1131, were used in this chapter. These strains were obtained from the culture collection of Research and Development Laboratories, Meiji Co., Ltd. The culture LB81, which contains *L. bulgaricus* 2038 and *S. thermophilus* 1131, was used in this chapter. This starter culture has been used in the commercial set yogurt production of Meiji Bulgaria Yogurt LB81 Plain in Japan since 1993.

1.2.2 Preparation of yogurt bulk starter of LB81

Each strain of *L. bulgaricus* 2038 or *S. thermophilus* 1131 stored at - 80°C was subcultured once at 37°C for 16 h in skim milk and yeast extract (SMY) medium, composed of 10% (wt/wt) skim milk supplemented with 0.1% (wt/wt) yeast extract

(preculture) after autoclaving (121°C, 7 min), and was cooled to 5°C. Both precultures of *L. bulgaricus* 2038 and *S. thermophilus* 1131 were inoculated (1 %; wt/wt) into fresh, sterilized SMY medium (95°C, 10 min), incubated at 37°C to reach an acidity of 0.7%, and cooled immediately to 5°C (yogurt bulk starter culture of LB81). The acidity was measured by titrating a 9-g sample against 0.1 *N* sodium hydroxide using phenolphthalein as the indicator. The yogurt starter culture was then stored at 5°C. The yogurt starter culture LB81, which was maintained at 5°C, was used until 3 d after preparation.

1.2.3 Preparation of yogurt with LB81

The method of yogurt preparation was based on a laboratory-scale manufacturing process commonly conducted at the Food Research and Development Center of Meiji Co., Ltd. The yogurt mix used in this study containing 3.0% (wt/wt) fat and 9.5% (wt/wt) SNF, was obtained by mixing raw milk [3.6% (wt/wt) fat], skim milk powder, butter [80.0% (wt/wt) fat], and water. These materials were supplied by the plants of Meiji Co., Ltd. After being homogenized at 15 MPa, the yogurt mix was heated to 95°C for 2 min and immediately cooled to 43 or 37°C. The yogurt bulk starter of LB81 (about 0.9% of acidity) was inoculated in the yogurt mix to a concentration of 2%. After mixing, 90 g of the mixture was placed into ten 100-ml polystyrene cups that were oxygen permeable. Fermentation was carried out at 43°C (normal fermentation temperature) or 37°C (lower fermentation temperature). The yogurt fermentation end point was set at 0.7% acidity. The yogurt fermentation time was the time required for the original acidity of 0.2% (from milk, milk product and bulk starter of LB81) to change to the end-point of 0.7%. Yogurt samples were stored at 5°C for 1 d before the analysis.

1.2.4 Fermentation method under reduced oxygen conditions

The DO reduction process was as follows. After yogurt starter culture inoculation, sterile nitrogen gas (filtered with a 0.45 μm cellulose acetate filter) was aseptically mixed and dispersed into the yogurt mix through a stainless steel pipe (about 3-mm bore) to reduce the DO concentration in the yogurt mix to nearly 0 mg/kg. Concentration of DO in the yogurt mix was measured with a DO meter (DO-24P, DKK-TOA Corp., Tokyo, Japan).

Yogurt fermentation was carried out at 43 or 37°C both after the DO reduction treatment and without prior treatment. Yogurt fermentation at 43 and that at 37°C without prior treatment are referred to as the control fermentation at 43°C and the

control fermentation at 37°C, respectively. Yogurt fermentation after reduced DO treatment at 43 and that at 37°C are referred to as reduced DO fermentation (ROF) and low-temperature ROF (LT-ROF), respectively.

1.2.5 Physical characterization of yogurt

The properties of yogurt samples were measured after 24 h storage at 5°C. The pH was measured with a pH meter (HM-50V, DKK-TOA Corp.).

The physical properties of smoothness and hardness of yogurt were measured, using a curd meter (ME-302; Iio denki Tokyo, Japan). However, there is no optimum method for evaluating the smoothness of set yogurt. The ME-302 curd meter is specially designed to evaluate the hardness of set yogurt and can also be used for evaluating smoothness. Specifically, the surface angle formed by pressure of a yogurt knife with a weight of 100 g was measured. Here, the weight at which the elastic surface curve was broken and there is penetration occurred was defined as hardness (g), whereas the angle of that curve (the penetration angle) was used as an indicator of smoothness (with the angle having a value from 0 to 90°, and with smaller values representing a smoother tissue). Three yogurt samples were analyzed at each trial and average readings were taken.

The degree of whey syneresis of yogurt was defined as the ratio (%) of the volume of the supernatant after centrifugation (2,150 × *g*, 10 min, 5°C) to yogurt sample. This method helped us to estimate rapidly and in advance the actual whey syneresis of yogurt after storage.

1.2.6 Microbiological analysis

To count the viable cells of yogurt bacteria, aliquots of the yogurt sample after 1 d of storage at 5°C were poured onto plates and mixed with 15 ml of bromocresol purple plate count agar (Eiken Chemical Co., Ltd, Tokyo Japan) that had been autoclaved and kept at 50°C. The plates were incubated at 37°C for 72 h. The colonies of *L. bulgaricus* 2038 and *S. thermophilus* 1131 were identified by their rough shape and smooth shape, respectively, in the agar plates.

1.2.7 Sensory Evaluation

Two yogurt samples in 100 ml cups were made either by the control fermentation method at 43°C or by the LT-ROF method and were stored at 5°C for 1 d before evaluation. The sensory evaluation was carried out using 200 consumers according to the following method. Yogurt consumers were recruited and 200 panelists were

selected. The panelists consisted of 50 males and 50 females who regularly consume yogurt 1 to 3 times a week and 50 males and 50 females who consume yogurt 4 times a week or more. Each panelist evaluated only one kind of yogurt at a session. Panelists evaluated both a control yogurt and a yogurt made by the LT-ROF method for smoothness, sourness and mildness at 2 sessions. Sessions were held at least 1 h apart. The absolute evaluation data (scored 1 through 5 for either sample) and the results were analyzed using *z*-tests (19).

1.3 Results

1.3.1 Reduction of DO concentration in the yogurt mix during fermentation

The change in DO concentration in the yogurt mix during fermentation was measured with the yogurt starter culture LB81, which was composed of *L. bulgaricus* 2038 and *S. thermophilus* 1131. Kinetics of the DO concentration in the yogurt mix with LB81 was shown in Figure1.1. As shown in this figure, the DO concentration in the yogurt mix was approximately 6 mg/kg at the beginning of fermentation at 43 °C, and was gradually reduced as the fermentation progressed. The time required for the DO concentration to be reduced from 6 to 0 mg/kg was approximately 60 min. The original acidity in the yogurt mix with bulk starter of LB81 was approximately 0.2%. The starter culture LB81 actively began to produce acid after the DO concentration in the yogurt mix was reduced to 0 mg/kg.

Next, the author examined the possibility that the yogurt mix without cultures (LB81 starter culture) could reduce the DO. The change in DO concentration in a yogurt mix was measured with a heat-killed (80°C, 10 min) culture of LB81. The results showed that the DO concentration in the yogurt mix was approximately 6 mg/kg both before and after incubation at 43°C for 5 h. From this experiment, the author considered that only the living starter culture bacteria could reduce the DO in the yogurt mix.

In addition, the change in DO concentration in the yogurt mix during fermentation with a single starter culture of *L. bulgaricus* 2038 or *S. thermophilus* 1131 was measured. The DO concentration in the yogurt mix decreased as fermentation progressed, as in the case of LB81 (data not shown).

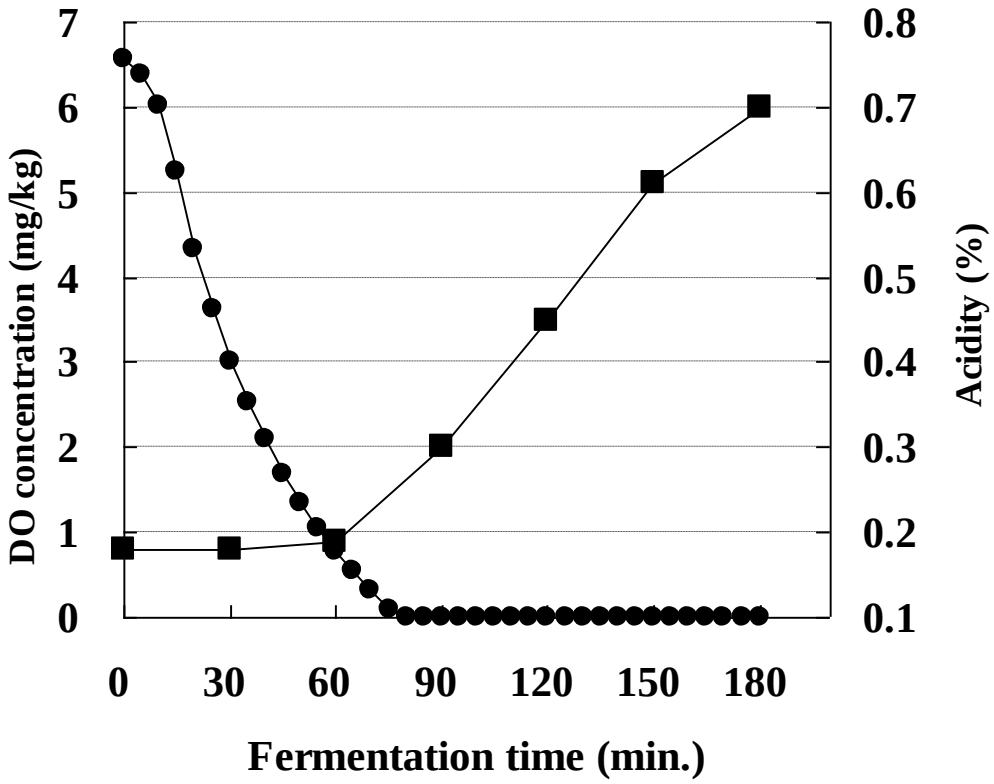


Figure 1.1

Acid production and variation of dissolved oxygen (DO) concentration kinetics of LB81. Incubation at 43 °C.

(●) DO concentration and (■) Acidity.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2009.92:4112-4121".

1.3.2 Acid production under constant DO concentration

From our results it was hypothesized that DO interferes with rapid acid production in yogurt manufacturing. Therefore, the author examined whether acid production was suppressed under a constant DO concentration using a jar fermenter.

Because acid production by the LB81 starter culture began after the DO concentration had decreased to almost 0 mg/kg, we assumed that DO in the yogurt mix had inhibited the acid production.

To evaluate the influence of DO on acid production precisely, cultivations under constant DO concentrations when using a jar fermenter was examined. The changes in acidity and pH are shown in Figure 1.2A and 1.2B. The acidity produced by LB81 after incubation at 43 °C for 150 min with DO concentration in the yogurt mix fixed at 4, 2, 1, and 0 mg/kg by adjusting the air or nitrogen gas bubbling into the yogurt mix was 0.2, 0.2, 0.3, and 0.7%, respectively. These experiments showed that the acid production by LB81 was suppressed greatly with 1mg/kg or more of DO in the yogurt mix and was promoted with 0 mg/kg. However, acid production by *L. bulgaricus* 2038 or *S. thermophilus* 1131 alone was neither suppressed nor advanced by the DO concentration in the yogurt mix (Figure1.3). The author observed that in the case of single culture *L. bulgaricus* 2038 or *S. thermophilus* 1131, the acid production rate under aerobic conditions (6 mg/kg) was almost the same as that under anaerobic conditions (0 mg/kg).

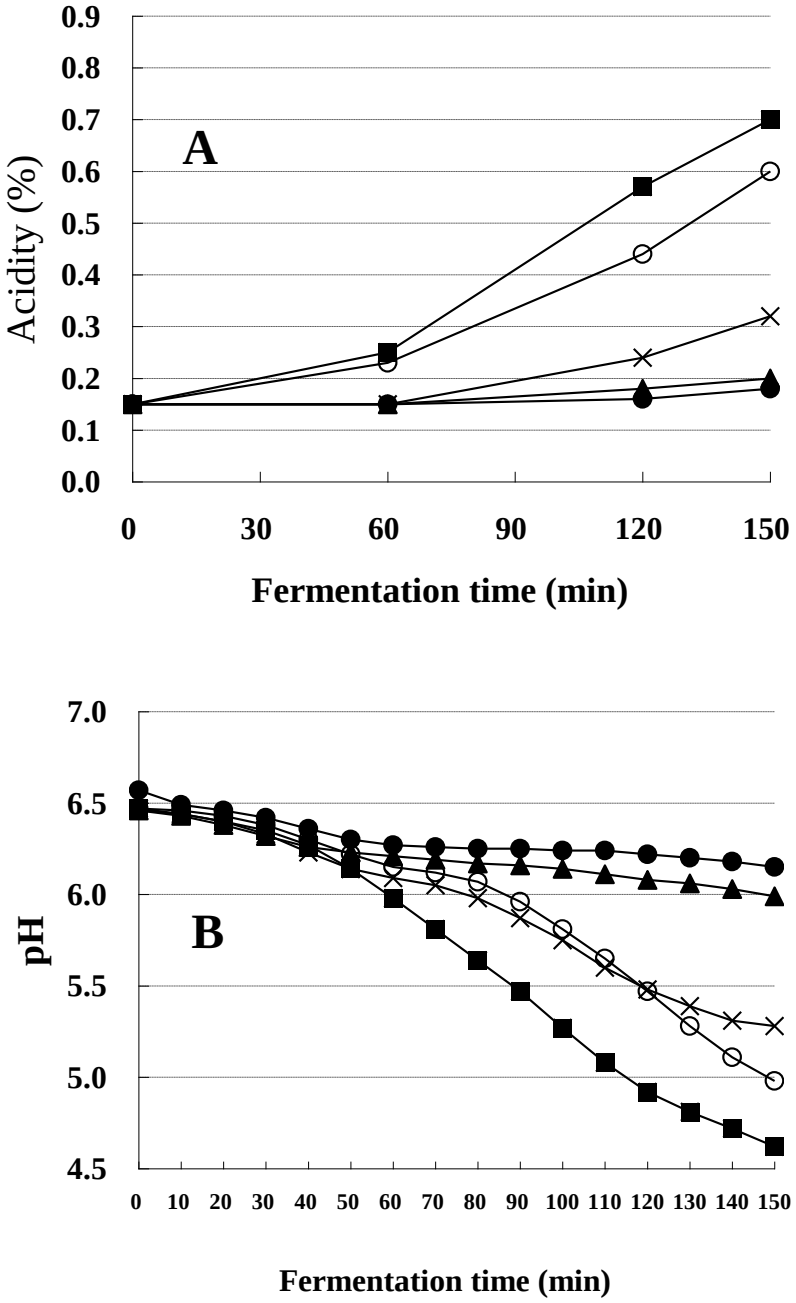


Figure 1.2

Influence of dissolved oxygen (DO) concentration on the acid production by yogurt cuture LB81 incubated at 43°C. Acid production was measured through changes in titratable acidity (A) or pH (B). Incubation of LB81 under DO concentration fixed at 4 (●), 2 (▲), 1 (×), and 0 mg/kg (■) and under normal air conditions (○).

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2009.92:4112-4121".

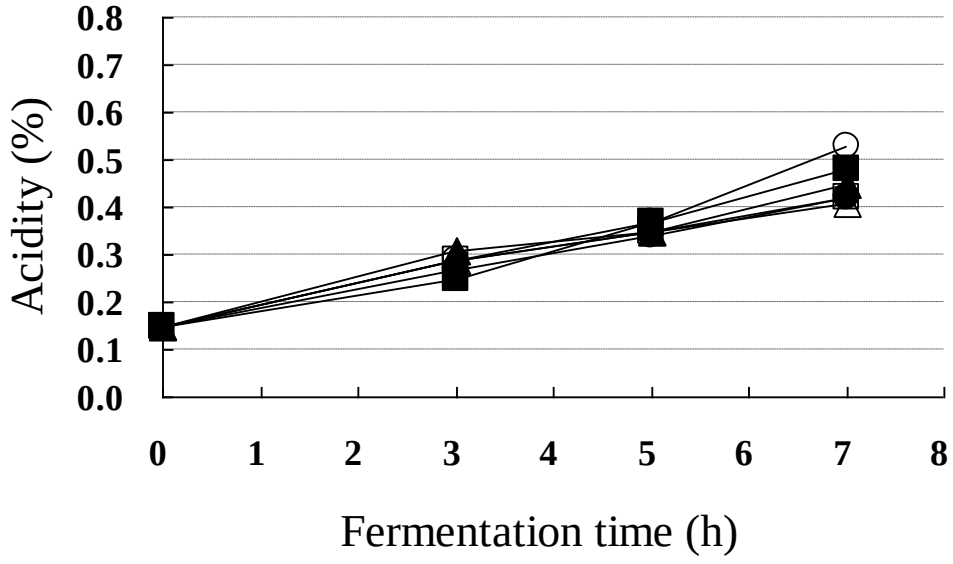


Figure 1.3

Influence of dissolved oxygen (DO) concentration on the acid production by the single yogurt culture *S. thermophilus* 1131 and *L. bulgaricus* 2038 incubated at 43°C. Acid production was measured through changes in titratable acidity. Each of cultures was incubated under a DO concentration fixed at 6 or 0 mg/kg. Symbols: (■) *S. thermophilus* 1131 control, (●) 6 mg/kg , (▲) 0 mg/kg . (□) *L. bulgaricus* 2038, (○) 6 mg/kg, and (△) 0 mg/kg.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2009.92:4112-4121".

1.3.3 Fermentation under reduced DO

From the results obtained above, the author assumed that acid production might be accelerated after the DO concentration in the yogurt mix had been reduced to nearly 0 mg/kg because there would be no suppression caused by the DO. The following experiments were examined.

The effect of reducing DO on acid production velocity was examined. As shown in Figure 1.4, the acid production by LB81 at 43°C was promoted by reducing the DO concentration in the yogurt mix nearly to 0 mg/kg. The time required for acidity in the yogurt mix to reach 0.7% was approximately 30 min less than in the control fermentation at 43°C. The acid production rate was almost the same, but the starting point of acid production in the fermentation with the reduced DO was 30 min earlier than in the control. This new fermentation method was referred to as ROF.

Several properties of the yogurt samples were checked after 1 d of storage at 5°C. The viable cell counts of *L. bulgaricus* 2038 and *S. thermophilus* 1131 of the yogurt made by ROF were 1.2×10^8 and 4.7×10^8 cfu/g, and were almost the same of the yogurt made by the control fermentation at 43°C (1.1×10^8 and 4.5×10^8 cfu/g). There were also no differences in other properties (acidity, curd tension, and pH) between the yogurt made by ROF and control fermentation at 43°C (data not shown). These results indicate that the yogurt made by ROF had almost the same characteristics without the fermentation pace as the yogurt made by the control fermentation at 43°C; thus, ROF was a favorable faster yogurt production method.

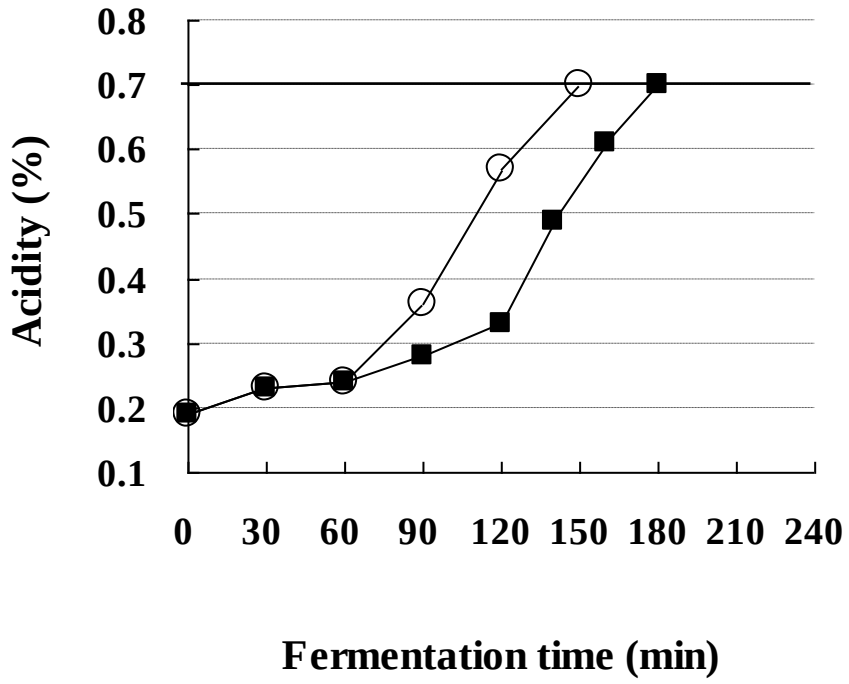


Figure 1.4

Effect of reduced dissolved oxygen (DO) fermentation (ROF) on the acid production by the yogurt culture LB81 when the DO concentration in the yogurt mix was regulated to approximately 0 mg/kg beforehand. Incubation was at 43 °C.
Symbols: (○) ROF at 43 °C and (■) the control fermentation at 43 °C.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2009.92:4112-4121".

1.3.4 LT-ROF

Encouraged by these results, the author applied this rapid fermentation method to the control fermentation at 37°C (low-temperature fermentation). It is well known that yogurt made by low-temperature fermentation has a smooth texture, but the fermentation process takes a much longer time than the control fermentation at 43°C (normal-temperature fermentation), and this longer time lowers the efficiency of yogurt manufacturing. Thus, experiments combining ROF and low-temperature fermentation at 37°C were carried out. As shown in Figure 1.5, the time required for acidity to reach 0.7% was 220 min with the control fermentation at 37°C, whereas it was 180 min with the LT-ROF. The time for the acidity of yogurt mix to reach 0.7 % at 37°C was decreased by approximately 40 min by reducing the DO.

1.3.5 Characteristics of yogurt prepared by LT-ROF

The fermentation time at 37°C was shortened by reducing the DO (LT-ROF), so next we compared the characteristics of the yogurt prepared by LT-ROF and by the control fermentation at 37°C. The viable cell counts of *L. bulgaricus* 2038 and *S. thermophilus* 1131 of the yogurt made by LT-ROF were 1.0×10^8 and 7.9×10^8 cfu/g, which were nearly the same as those by the control fermentation at 37°C. The acidity and pH of the yogurt stored at 5°C for 1 d after fermentation were essentially the same, but the appearance of these 2 yogurt samples was quite different. As shown in Figure7, when scooped up with a spoon, the yogurt made by the control fermentation at 37°C collapsed easily, but the yogurt made by LT-ROF was firm. The results of the sensory test with 200 consumers showed that the yogurt made by LT-ROF had a higher score with respect to “smooth texture” and “mild taste” compared with the yogurt made by the control fermentation at 43°C (Table 1.1).

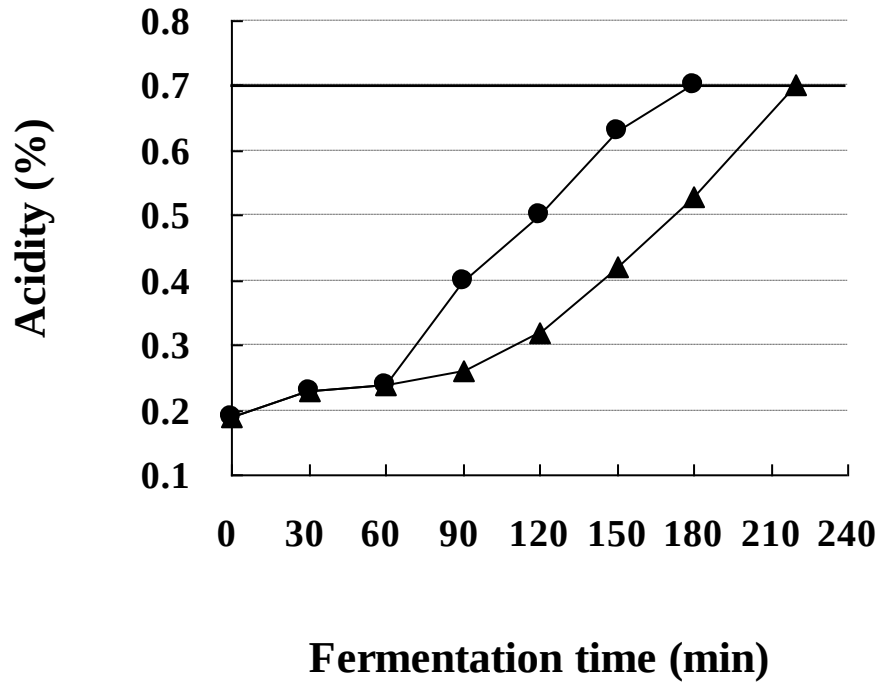
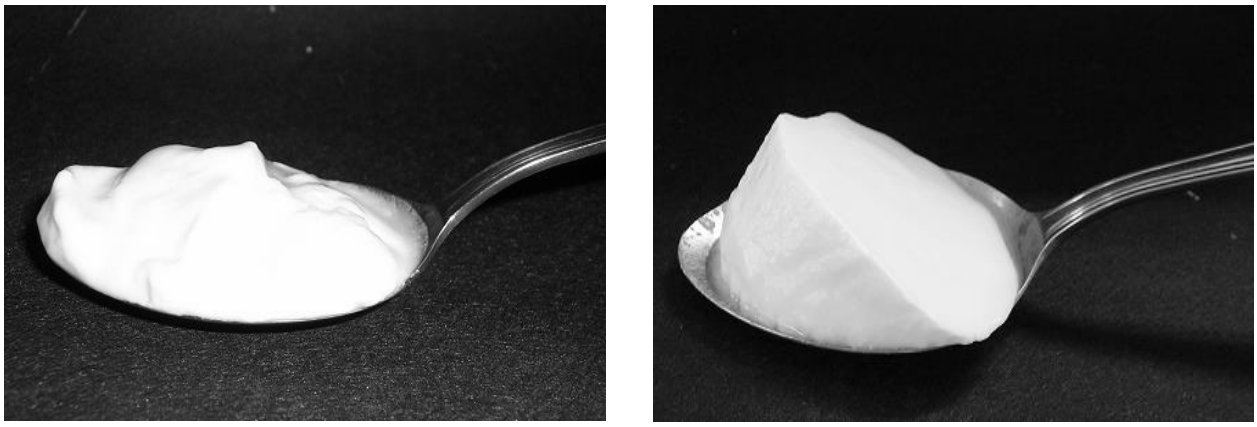


Figure 1.5

Effect of low-temperature reduced dissolved oxygen (DO) fermentation (LT-ROF) on the acid production by the yogurt culture LB81 when the DO concentration in the yogurt mix was regulated to approximately 0 mg/kg beforehand.
Incubation was at 37 °C.
Symbols: (●) LT-ROF and (▲) control fermentation at 37 °C.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2009.92:4112-4121".



Control fermentation at 37°C **LT-ROF**

Figure 1.7
Appearance of the yogurt made by low-temperature reduced dissolved oxygen (DO) fermentation (LT-ROF) and the control fermentation at 37°C,
when scooped up with a spoon.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2009.92:4112-4121".

Table 1.1 A sensory test with 200 yogurt consumers was carried out on the yogurt made by low-temperature reduced dissolved oxygen fermentation (LT-ROF; A) and the yogurt made by control fermentation method at 43°C (B), served in 100-ml cups

	Scores[1]	
Term	A	B
Smoothness	4.25*	3.91
Sourness	2.96	3.13
Mildness	3.94**	3.46

[1]The absolute evaluation data were scored 1 thorough 5 for both of the control and test yogurt for their smoothness (1 = not at all, 5 = very smooth), sourness (1 = weak, 5 = strong), and mildness (1 = not at all, 5 = very mild).

*P < 0.05; **P < 0.01.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2009.92:4112-4121".

Table 1.2 Mean values (± SD) of the physical properties of the yogurt made by low-temperature reduced dissolved oxygen fermentation (LT-ROF) and the yogurt made by control fermentation at 37°C or 43°C

	Physical properties of the yogurt[1]	
Yogurt production	Hardness (g)	Penetration angle (°)
Yogurt made by LT-ROF	50 ± 8	30 ± 2
Yogurt made by control fermentation at 37 °C	29 ± 2	30 ± 1
Yogurt made by control fermentation at 43 °C	50 ± 4	57 ± 3

[1]Physical properties of the yogurt are defined in the Materials and Methods section**.**

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2009.92:4112-4121".

The yogurt made by LT-ROF had nearly the same smooth texture as the yogurt made by the control fermentation at 37°C, but unexpectedly, had a firmer curd. To express these physical properties with numerical values, 2 methods were then used. First, the degree of whey syneresis of yogurt (%) was measured; this was defined as the ratio of the supernatants obtained by centrifugation (2,150 × *g*, 10 min, 5°C) of the yogurt sample. The degree of whey syneresis was in the following sequence: the yogurt made by LT-ROF (approximately 10%) = the yogurt made by the control fermentation at 37°C (approximately 10%) < the yogurt made by the control fermentation at 43°C (approximately 20%). This result indicates that the yogurt made by LT-ROF had almost the same close structure as the yogurt made by the control fermentation at 37°C. Second, the hardness and the penetration angle of the yogurt curd were determined with a 100-g yogurt knife and the ME-302 curd meter. In our experience, a hardness of 40 g and greater is sufficient to stand up to the impact of shaking during transport, whereas 30 g or less is not. The penetration angle could be a value up to 90°, with a smaller value indicating a smoother texture. As shown in Table1.2, the hardness and smoothness of the yogurt made by LT-ROF were 50 g and 30°, respectively, whereas those of the yogurt made by the control yogurt fermentation at 37°C were 30 g and 30°. This indicated that the yogurt made by LT-ROF had better physical properties for consumers than the latter (with the same hardness of control fermentation at 43°C and smooth texture of low-temperature fermentation at 37°C).

1.3.6 Application of LT-ROF to industrial yogurt manufacture

Application of LT-ROF was carried out on an industrial scale. A scale-up test of LT-ROF was carried out with 10 tons of yogurt mix in a yogurt plant of Meiji limited company. The time and characteristics of the laboratory-scale (450-ml) and the industrial-scale fermentation were compared. It took 180 min for the acidity of the yogurt mix to reach 0.7 % in the plant test, nearly the same as in the laboratory test. The viable cell counts of *L. bulgaricus* 2038 and *S. thermophilus* 1131, acidity, curd tension, and pH, and sensory properties of yogurt manufactured in the plant were nearly the same as for the yogurt made in the laboratory (data not shown).

Because the yogurt made in the plant distributed throughout the national market, it is important that the structure of the yogurt is sufficiently hard to stand up to the impact caused by shaking during transport. Thus, a long-distance (approximately 2,000 km) transport test by refrigerated truck was carried out with 1440 containers of yogurt manufactured by LT-ROF in some of plants of Meiji limited company. The results showed no yogurt broken up after the 2,000-km transport.

1.4 Discussion

1.4.1 Influence of DO on the yogurt fermentation progress

The DO in the yogurt mix was reduced as yogurt fermentation by the starter culture LB81 progressed. Because the DO concentration in the yogurt mix was not reduced either with heat-killed LB81 or without LB81, the author concluded that the starter culture of live bacteria reduced DO in the yogurt mix. The author found that the starter culture LB81 could not start active acid production before the DO concentration in the yogurt mix was reduced to 0 mg/kg. Moreover, when even 1 mg/kg of DO remained in the yogurt mix, the rate of acid production by LB81 was much lower, almost the same as the sum of acid produced by 2 single cultures of *L. bulgaricus* 2038 and *S. thermophilus* 1131 (data not shown). The author concluded that DO did not interfere with the growth of these single cultures, on the other hand, DO did stop rapid acid production because of the symbiosis of 2 single cultures of *L. bulgaricus* 2038 and *S. thermophilus* 1131.

1.4.2 Fermentation time abridgement by ROF

The discussion above explains why the DO in the yogurt mix is a barrier to acid production by the yogurt starter culture LB81. It was concluded that acid production by LB81 can be greatly accelerated by reducing DO in the yogurt mix before fermentation.

In our tests, the DO in the yogurt mix was reduced beforehand, which resulted in a 30-min reduction of the fermentation time needed for the acidity of the yogurt mix to reach to 0.7% when using LB81 as the starter culture. Galesloot et al. (8) suggested that the oxygen in the air suppresses acid production by the yogurt culture. Here, the author examined the suppression of acid production by DO quantitatively by fermentation under constant DO concentrations when using a jar fermenter and found that even 1 mg/kg of DO could suppress acid production by LB81.

It was concluded that the fermentation was accelerated by ROF because the time necessary for the starter culture LB81 to reduce the DO is shortened. By reducing DO beforehand, the symbiosis of the bacteria was promoted. The time required for the LB81 starter culture to reduce the DO reduction was approximately 60 min, but the yogurt fermentation time was nevertheless cut by only 30 min with ROF. This time lag might have been due to the time required for activation of the starter culture.

1.4.3 LT-ROF

Two problems are encountered in introducing the low-temperature fermentation (control fermentation at 37°C) into the industrial-scale manufacture of set yogurt. One is

the longer time of the fermentation process and the other is insufficient yogurt curd hardness.

Because the yogurt fermentation time was reduced with ROF, the author tried combining ROF with the control fermentation at 37°C (low-temperature fermentation). The author concluded that the texture of yogurt incubated at 37°C was smoother than the yogurt incubated at 43°C because the acid production at 37°C proceeded more slowly than at 43°C. The higher temperature of incubation might accelerate the aggregation of the casein micelles, resulting in a coarse protein network (19).

Surprisingly, it turned out that although acid production by LT-ROF was faster, it could make smoother yogurt. Our explanation for this is as follows. Our investigation revealed that yogurt curd formation began after the acidity reached approximately 0.4% for a yogurt mix that contained 9.5% SNF (wt/wt), and because the yogurt fermentation end point was set at 0.7% acidity, the curds formed in the time during which the acidity of the yogurt mix changing from 0.4 to 0.7%. This period is referred to as the yogurt curd formation time and it is presumed that the longer curd formation time makes the texture of yogurt smoother. As shown in Table 1.3, although the time of fermentation by LT-ROF was among the shortest of the 3 methods (180min: equal to the control fermentation at 43°C), its curd formation time was the longest. The curd formation time with LT-ROF was 90 min, whereas with the other methods it was 50 and 70 min. The author hypothesized that this was the reason the yogurt made by LT-ROF method was very smooth in spite of its short fermentation time.

In our experiments, indications of yogurt smoothness of the yogurt depended mainly on the sensory evaluation because no standard method exits for measuring the physical properties of set-type yogurt. The author proposed that the assessment of yogurt texture could be quantified based on 2 factors: one was the measurement of degree of whey syneresis, and the other was the penetration angle of yogurt knife with weight of 100 g into the yogurt curd (ME-302 curd meter).

Table 1.3 Comparison of yogurt curd formation times among 3 fermentation methods

Fermentation method	Yogurt curd formation time[1] (min)	Yogurt fermentation time[2](min)
LT-ROF[3]	90	180
Control fermentation at 37 °C	70	220
Control fermentation at 43 °C	50	180

[1]Yogurt curd formation time (min): fermentation began at 0.2% acidity and yogurt curd formation began from 0.4% acidity. Because the fermentation end point was set at 0.7% acidty, the yogurt curd formation time was the time for the acidty to increase from 0.4% to 0.7%. Yogurt curd formation times were measured by Figures 1.4 and 1.6.
[2]Yogurt fermentation time (min): The time for the acidty to increase from 0.2% to 0.7%.
[3]Low-temperature reduced dissolved oxygen fermentation

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2009.92:4112-4121".

1.4.4 Application of LT-ROF to the industrial yogurt manufacture

In the yogurt plant, all stages in yogurt manufacturing process are consecutive and are controlled by an automatic system so that the efficiency of manufacture is lowered considerably by extension or variation of fermentation time by even 10 min. In particular, the fermentation is the stage that takes the longest time. The control fermentation process at 37°C took 40 min longer than the control fermentation process at 43°C. This has prevented the introduction of low-temperature fermentation methods into commercial yogurt manufacture. This problem was solved by LT-ROF (Figure 1.6).

The set-type yogurt previously made by low-temperature fermentation had insufficient hardness to stand up to the impact of shaking during transport. This problem was also solved by using LT-ROF. The yogurt made by LT-ROF had sufficient hardness to stand up to the impact caused by shaking during transport (Table 1.2). The reason for this is not clear.

Moreover, the LT-ROF process can easily be introduced into ordinary yogurt plants because this requires only setting up a system for injecting sterile nitrogen gas into the yogurt mix to eliminate oxygen and adjusting the fermentation temperature. Meiji limited company have sold 4 kinds of yogurt products made by LT-ROF and the sales of these yogurt products would grossed approximately ¥15 billions in the Japanese

market in 2013.

1.5 Conclusion

Starter culture LB81, composed of *L. bulgaricus* 2038 and *S. thermophilus* 1131, reduced the DO in the yogurt mix before acid formation. The starter began to produce acid actively after the DO concentration in the yogurt mix had been reduced to nearly 0 mg/kg. Fermentation with LB81 was suppressed by the presence of more than 1 mg/kg of DO in the yogurt mix. The author concluded that DO interferes with the symbiotic relationship between *L. bulgaricus* 2038 and *S. thermophilus*1131. The author found that reducing DO concentration in the yogurt mix with LB81 to nearly 0 mg/kg reduced the fermentation time at 43°C compared with the control fermentation at 43°C.

The set yogurt made by the control fermentation at 37°C (low-temperature fermentation) had a smooth texture but had insufficient hardness to stand up to the impact of shaking during transport. Moreover, this low-temperature fermentation took a much longer time, which reduced the yogurt manufacturing efficiency and increased the risk of infection from various kinds of minor germs. Combining ROF with low-temperature fermentation (LT-ROF) reduced the time required for low-temperature fermentation, and the yogurt made by the LT-ROF method also had sufficient hardness.

The author believe that LT-ROF is a favorable method for industrial-scale manufacture of set yogurt that possesses a smooth texture and strong curd structure.

Chapter 2
Effect of oxygen on symbiosis between *Lactobacillus delbureckii* subsp. *bulgaricus* 2038 and *Streptococcus thermophilus* 1131

2.1 Introduction

Streptococcus thermophilus (*S. thermophilus*) and *Lactobacillus delbureckii* subsp. *bulgaricus* (*L. bulgaricus*) are traditionally used for the manufacture of yogurt (4). Driessen et al. (5) suggested that in a mixed culture, acid production is much larger than the sum of a single culture. They suggested that in yogurt the growth of *S. thermophilus* is stimulated by free amino acids and peptides liberated from the milk proteins by *L. bulgaricus*. Veringa et al. (6) suggested that the two bacterial strains in yogurt, *L. bulgaricus* and *S. thermophilus*, stimulate each other during their associative growth. They suggested that the substance which stimulates *L. bulgaricus* and which is produced by *S. thermophilus* in yogurt milk is formic acid. Suzuki et al. (7) suggested that formic acid was the only effective substance for the active growth of *L. bulgaricus* among purine ring precursors. Galesloot et al. (8) suggested that the more proteolytic, *L. bulgaricus* liberates in milk certain amino acids, which stimulates *S. thermophilus*. Pablo et al. (9) suggested that the symbiotic relationship between *S. thermophilus* and *L. bulgaricus* is very important for yogurt and cheese production because it reduces fermentation time. The stimulation of lactobacilli is caused mainly by the production of formate by streptococci.

The yogurt starter cultures of *L. bulgaricus* and *S. thermophilus*, are facultatively anaerobic, so the fermentation of yogurt with these bacteria progresses well in the presence of oxygen. The yogurt product with *L. bulgaricus* and *S. thermophilus* is manufactured without oxygen control. The effect of the initial level of dissolved oxygen (DO) on acid production in buffalo milk by different single lactic cultures of *Streptococcus lactis, Streptococcus diacetylactis, Streptococcus cremoris, L. bulgaricus* and *S. thermophilus* was tested by Shekar and Bhat (18). The majority of strains of lactic acid bacteria, including the four genera *Streptococcus, Leuconostoc, Pediococcus* and *Lactobacillus*, are aerotolerant to some degree (20). Sakamoto et al. (21) reported that most of the 22 strains of lactic acid bacteria including the genera *Lactobacillus, Pediococcus, Leuconostoc, Streptococcus* and *Enterococcus* grow well under aerobic conditions. Kawasaki et al. (22) investigated the effects of oxygen on the *Bifidobacterium* species using liquid shaking cultures under various oxygen

concentrations. They reported that although most of the *Bifidobacterium* species showed oxygen sensitivity, two species, *B. boum* and *B. thermophilum* showed growth stimulation in the presence of oxygen. While there have been many studies on the influence of oxygen on lactic acid bacteria, only a few studies have reported on the influence of oxygen on the symbiosis between *L. bulgaricus* and *S. thermophilus*. The yogurt starters *L. bulgaricus* and *S. thermophilus* are well-known facultative anaerobic bacteria that can grow in oxygenated environments. Starter culture LB81 composed of *L. bulgaricus* 2038 and *S. thermophilus* 1131 reduced the dissolved oxygen (DO) in the yogurt mix before acid formation. The starter began to produce acid actively after the DO concentration in the yogurt mix had been reduced to nearly 0 mg/kg. Fermentation with LB81 was suppressed by the presence of more than 1 mg/kg of DO in the yogurt mix. However, acid production by *L. bulgaricus* 2038 or *S. thermophilus* 1131 alone was neither suppressed nor advanced by the level of the DO concentration in the yogurt mix. It was suggested that DO interferes with the symbiotic relationship between *L. bulgaricus* 2038 snd *S. thermophilus* 1131(14). In this study, the author investigated the influence of oxygen (dissolved oxygen in milk) on the symbiosis between *L. bulgaricus* 2038 and *S. thermophilus* 1131, aiming at one of the growth promoting factors for formic acid.

2.2 Materials and methods

2.2.1 Culture strains used

Yogurt starter cultures of *L. bulgaricus* 2038 and *S. thermophilus* 1131 were used in this chapter. These strains were obtained from the culture collection of Research and Development Laboratories, Meiji Co., Ltd. The combination of *L. bulgaricus* 2038 and *S. thermophilus* 1131 named LB81 and these single cultures were used in this study. Starter culture LB81 has been used in the commercial production of “Meiji Bulgaria Yogurt LB81 Plain” in Japan since 1993.

2.2.2 Preparation of yogurt bulk starter of LB81

Each strain of *L. bulgaricus* 2038 or *S. thermophilus* 1131 stored at - 80°C was subcultured once at 37 C for 16 h in a skim milk and yeast extract (SMY) medium composed of 10% (wt/wt) skim milk supplemented with 0.1% (wt/wt) yeast extract (preculture, single starter) after autoclaving (121 °C, 7 min), and was cooled to 5°C. Both precultures of *L. bulgaricus* 2038 and *S. thermophilus* 1131 were inoculated (1 %; wt/wt) into a fresh, sterilized SMY medium (95 °C, 10 min), incubated at 37°C

to reach an acidity of 0.7%, and cooled immediately to 5 °C (yogurt bulk starter culture LB81). The acidity was measured by titrating a 9-g sample against 0.1 *N* sodium hydroxide using phenolphthalein as the indicator. The yogurt starter culture was then stored at 5 °C. The yogurt starter culture LB81, which was maintained at 5°C, was used until 3 d after preparation.

2.2.3 Preparation of yogurt with LB81

The method of yogurt preparation was based on a laboratory-scale manufacturing process commonly conducted at the Food Research and Development Center of Meiji limited company. The yogurt mix used in this study containing 0.1% (wt/wt) fat and 9.5% (wt/wt) SNF, was obtained by mixing skim milk powder and water. The material was supplied by the plants of the Meiji limited company. For coculture assay in the presence of formic acid or peptides, a predetermined 0.5, 1, 2, 5 mM as sodium formate (Wako Pure Chemical Industries, Ltd, Japan) or 0.1% (wt/wt) casein peptides (CE90GMM, Nippon Shinyaku., Ltd, Japan) were added into the yogurt mix. The yogurt mix was heated to 95 °C for 2 minutes and immediately cooled to 43 °C. The yogurt bulk starter LB81, *L. bulgaricus* 2038 preculture or *S. thermophilus* 1131 preculture was inoculated in the yogurt mix to a concentration of 2%. After mixing, 15 g of the mixture was placed into ten 25-ml glass test tubes. Fermentation was carried out at 43 °C (normal fermentation temperature). The yogurt fermentation end point was set at 0.7% acidity. The yogurt fermentation time was the time required for the original acidity of 0.2% to change to the end-point of 0.7%.

2.2.4 Fermentation method under reduced oxygen and fixed oxygen concentration condition

The DO reduction process was as follows. After yogurt starter culture inoculation, sterile nitrogen gas (filtered with a 0.45-μm cellulose acetate filter) was aseptically mixed and dispersed into the yogurt mix through a stainless steel pipe (approximately 3-mm bore) to reduce the DO concentration in the yogurt mix to nearly 0 mg/kg. The concentration of DO in the yogurt mix was measured with a DO meter (DO-24P, DKK-TOA Corp., Tokyo, Japan). Yogurt fermentation was carried out at 43 °C both after the DO reduction treatment and without prior treatment. Yogurt fermentation at 43 °C without prior treatment was referred to as the control fermentation. Yogurt fermentation after the reduced DO treatment at 43 °C was referred to as the reduced DO fermentation (ROF).

2.2.5 Formic acid determinations

The concentration of formic acid in the yogurt mix was measured with a formic acid enzymatic kit (R-Biopharm AG., Darmstadt, Germany).

2.3 Results

2.3.1 Fermentation under reduced DO

As shown in Figure 2.1, the acid production by LB81 at 43 °C was promoted by reducing the DO concentration in the yogurt mix to nearly 0 ppm (ROF). The time required for the acidity in the yogurt mix to reach 0.7% was approximately 30 minutes shorter than in the control fermentation at 43 °C. However, acid production by *L. bulgaricus* 2038 or *S. thermophilus* 1131 alone was neither suppressed nor advanced by ROF. Both acid production by *L. bulgaricus* 2038 alone added 1mM sodium formate and *S. thermophilus* 1131 alone added 0.1 % wt/wt casein peptides were advanced.

In a previous paper (14), the author suggested that acid production by LB81 was advanced but that acid production by single culture *L. bulgaricus* 2038 or *S. thermophilus* 1131 alone was not advanced or lowered by ROF. From these results, it could be concluded that the DO does not interfere with the growth of single cultures of *L. bulgaricus* 2038 and *S. thermophilus* 1131 but interferes with the symbiotic relationship between *L. bulgaricus* 2038 and *S. thermophilus* 1131. Then, in this study, the author investigated the influence of DO on the symbiosis between *L. bulgaricus* 2038 and *S. thermophilus* 1131, aiming at growth promoting factors for formic acid.

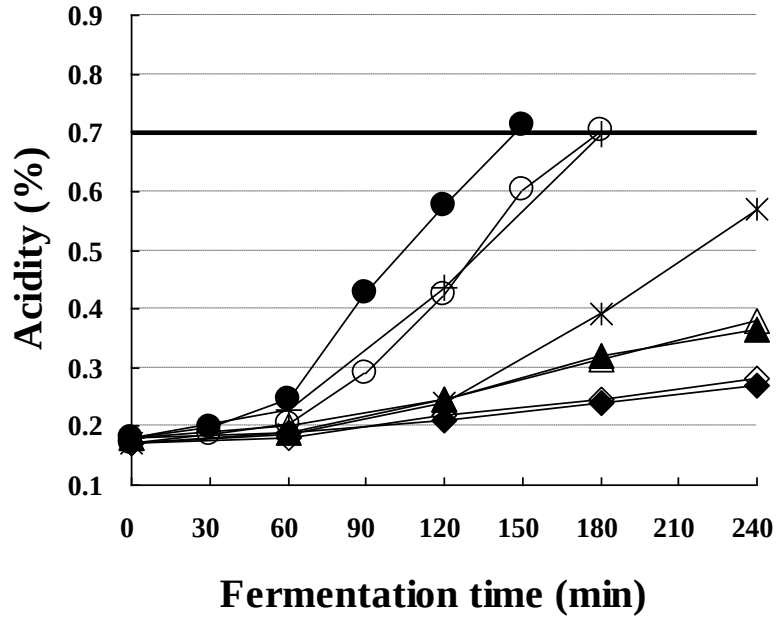


Figure 2.1

Effect of reduced dissolved oxygen (DO) fermentation (ROF) on the acid production by the yogurt culture LB81 and influence of added sodium formate, added casein peptides and ROF on acid production by the single yogurt culture *L. bulgaricus* 2038 and *S. thermophilus* 1131. Incubation was at 43 °C.

Symbols: (○) LB81 without ROF and (●) with ROF. (△) *S. thermophilus* 1131 without ROF, (▲) with ROF and (＋) added 0.1% (wt/wt) casein peptides. (◇) *L. bulgaricus* 2038 without ROF, (◆) with ROF, and (＊) added 1mM sodium formate.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2012. 95:2904-2909".

2.3.2 Influence of casein peptide or formate on the fermentation progress

The effect of formate or casein peptides on acid production velocity by LB81 or single yogurt cultures (*S. thermophilus* 1131 or *L. bulgaricus* 2038) was examined. As shown in Figure 2.2, while the addition of 1mM formate stimulated the acid production by LB81, casein peptides (0.1 % wt/wt) had almost non effect on the acidification rate of LB81. As shown in Figure 2.3A, the acid production by *L. bulgaricus* 2038 was promoted greatly with 0.5 mM or more of formate. However, the acid production by *S. thermophilus* 1131 was neither suppressed nor advanced by the addition of formate (Fig. 2.3B).

2.3.3 Formate consumption by *L. bulgaricus* 2038

The formate consumption by single yogurt culture *L. bulgaricus* 2038 was examined. As shown in Figure 2.4, the concentration of sodium formate in the yogurt mix shrank from a predetermined 0.5, 1.0, 2.0 and 5.0 mM to about 0.0, 0.5, 1.2 and 3.9 mM respectively after 240 minutes of fermentation. These results showed that the consumption of formate by *L. bulgaricus* 2038 was approximately 0.5 mM after 180 minutes of fermentation.

2.3.4 Formic acid accumulation and consumption

The formate accumulation by the single yogurt culture *S. thermophilus* 1131 or LB81 was examined.

In the case of symbiotic yogurt culture LB81, the starting point of formate accumulation in fermentation with the reduced DO (ROF) was approximately 30 minutes earlier than in the control, and the amount of formate accumulated after 180 minutes of fermentation by LB81 was approximately 0.8 mM (Figure 2.5).

It was shown that the accumulation by symbiotic yogurt cultures LB81 composed of *L. bulgaricus* 2038 and *S. thermophilus* 1131 was approximately 0.8 mM after 180 minutes of fermentation. This 0.8 mM of formic acid was not the production value of formic acid, but the accumulation value of formic acid by LB81 because *L. bulgaricus* 2038 partly used the formic acid produced by *S. thermophilus* 1131 during fermentation.

It was shown that the formate consumption by single yogurt culture *L. bulgaricus* 2038 was approximately 0.5 mM and the formate accumulation by symbiotic yogurt cultures LB81 composed with *S. thermophilus* 1131 and *L. bulgaricus* 2038 was approximately 0.8 mM. While it was not clear how much formate *S. thermophilus* 1131 consumed, from these results, it may be supposed that formate production by LB81 was approximately 1.3 mM.

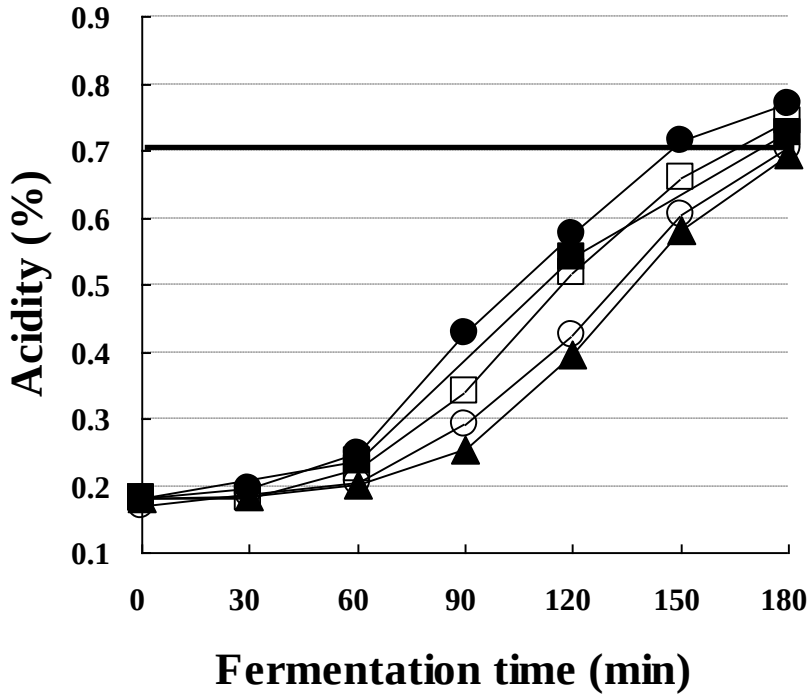


Figure 2.2

Effect of added sodium formate, added casein peptides and reduced dissolved oxygen fermentation (ROF) on acid productionby the yogurt culture LB81.

Incubation was at 43 °C.

Symbols: (●) with ROF, (□) added 1mM sodium formate, (▲) added 0.1% (wt/wt) casein peptides, (■) added 1mM sodium formate and 0.1% (wt/wt) casein peptides, and (○) control

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2012. 95:2904-2909".

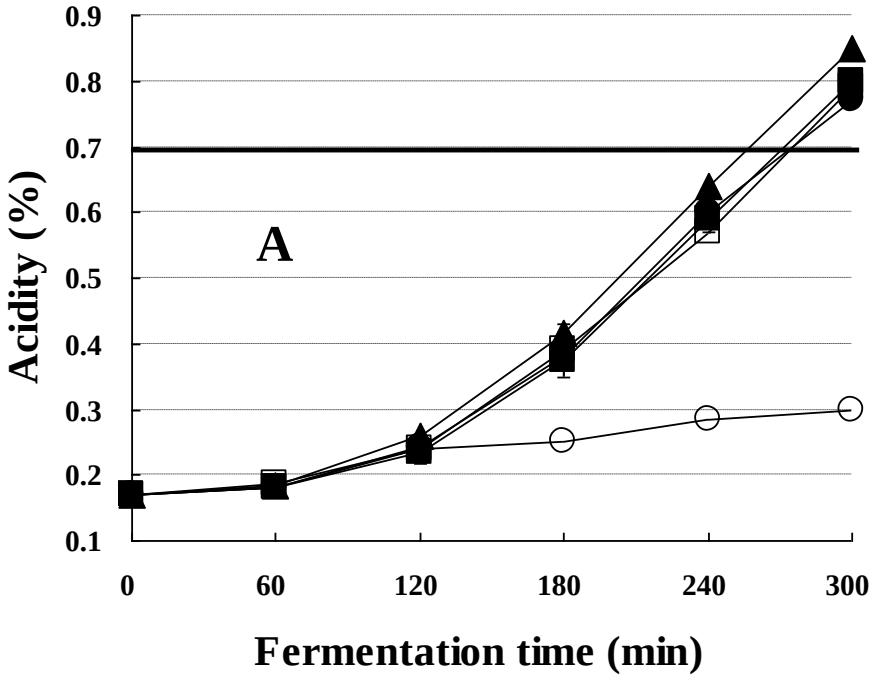


Figure 2.3A

Effect of the addition of sodium formate on the acid production by thesingle yogurt culture *Lactobacillus delbrueckii* ssp. *bulgaricus* 2038. Incubation was at 43 °C. Symbols: Sodium formate added to reach final concentrations of 5 (■), 2 (▲), 1 (□), 0.5 (●), and 0 (○) mM

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2012. 95:2904-2909".

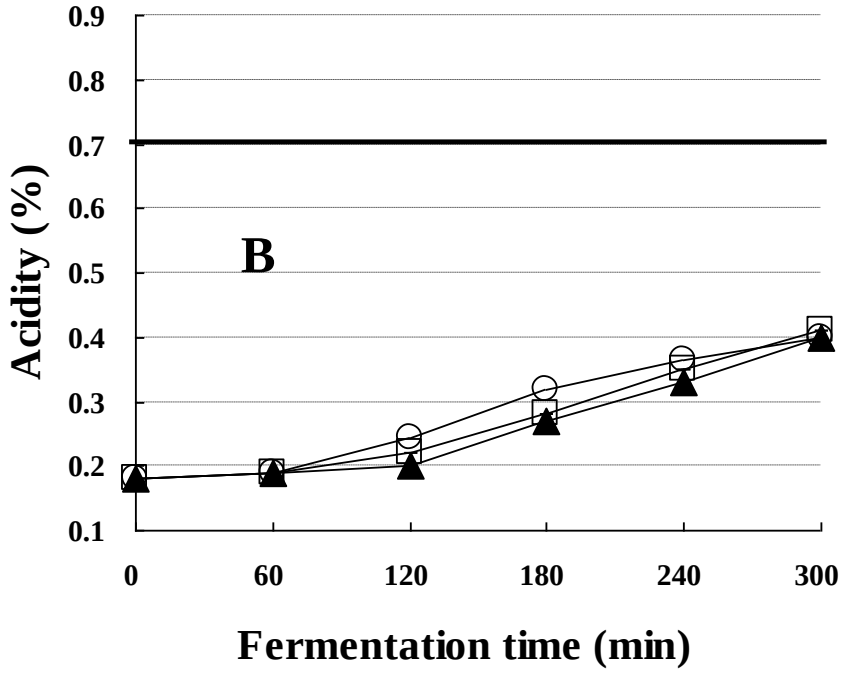


Figure 2.3B

Effect of the addition of sodium formate on acid productionby the single yogurt culture *S. thermophilus* 1131.Incubation was at 43 °C.

Symbols: Sodium formate added to reachfinal concentrations of 2(▲), 1(□) and 0(○) mM under normal air conditions.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2012. 95:2904-2909".

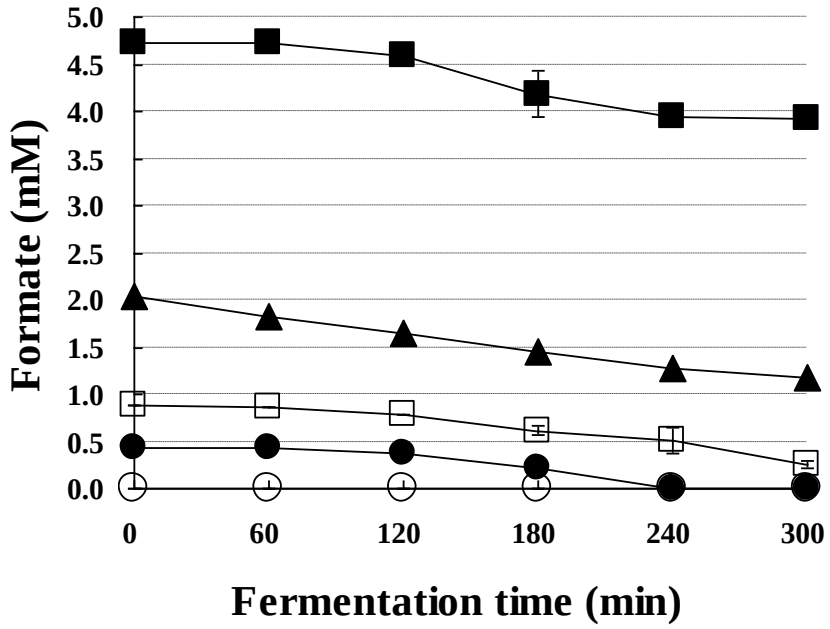


Figure 2.4

Formate consumption by the single yogurt culture *L. bulgaricus* 2038 when the sodium formate concentration in the yogurt mix was regulated to approximately 5 (■), 2 (▲), 1 (□), 0.5 (●), and 0 (○) mM beforehand under normal air conditions. Incubation was at 43 °C.

Bars represent standard deviations of averages from three experiments.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2012. 95:2904-2909".

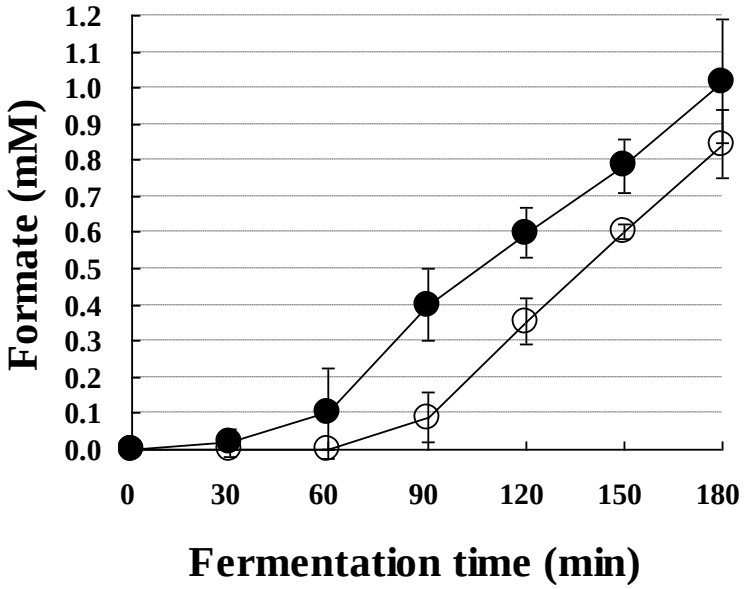


Figure 2.5

Effect of reduced dissolved oxygen fermentation (ROF) on formate production by the yogurt culture LB81when the DO concentration in the yogurt mix was regulated to approximately 0 mg/kg beforehand. incubation was at 43 °C. Symbols: (●) with ROF, and (○) without ROF.

Bars represent standard deviations of averages from three experiments.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2012. 95:2904-2909".

2.3.5 Formic acid production under constant DO concentration

The acid or formate produced by LB81 with the DO concentration in the yogurt mix fixed at 6, 4, 2, and 1 mg/kg were examined, adjusting the air or nitrogen gas bubbling into the yogurt mix.

Figure 2.6 shows that acid production by LB81 was delayed in the presence of 1 mg/kg of DO and was suppressed almost completely with more than 2 mg/kg of DO in a yogurt mix. Thus the inhibiting effect of DO on acid production during yogurt fermentation was clearly shown.

As shown in Figure 2.7A, acid production by LB81 was greatly suppressed with 6 mg/kg of DO in the yogurt mix, but, it was advanced by adding 1mM of formate.

Formate production by LB81 with the DO concentration in the yogurt mix fixed at only 1 mg/kg was completely suppressed (Figure 2.7B)

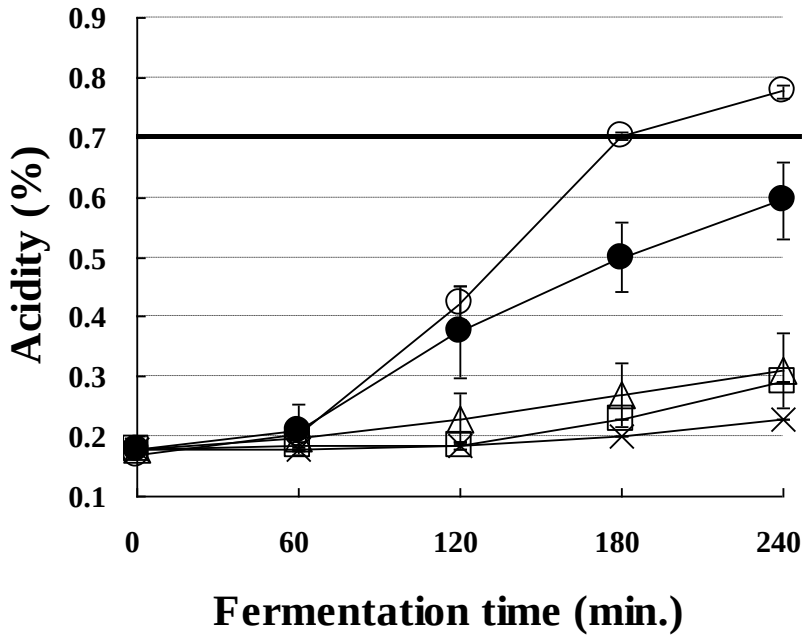


Figure 2.6

Influence of dissolved oxygen (DO) on acid production by the yogurt culture LB81. Incubation was at 43 °C.

Symbols: under DO concentration fixed at (△) 6, (×) 4, (□) 2, (●), and 1 mg/kg and (○) under normal air conditions.

Bars represent standard deviations of averages from three experiments.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2012. 95:2904-2909".

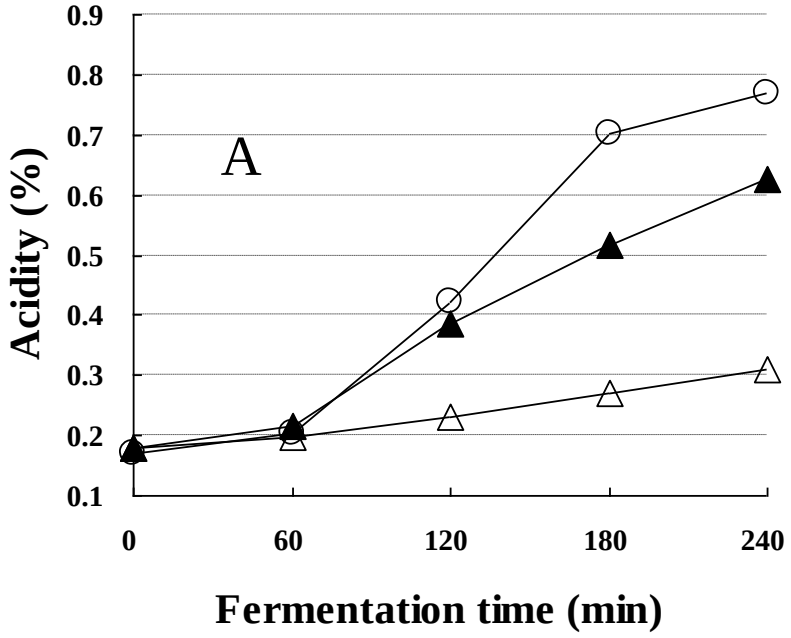


Figure 2.7A

Influence of dissolved oxygen (DO) on the acid production by the yogurt culture LB81. LB81 incubated under a DO concentration fixed at 6 mg/kg.

Incubation was at 43 °C.

Symbols: (△) under DO concentration fixed at 6 mg/kg, (▲) under DO concentration fixed at 6 mg/kg with 1mM formate, and (○) under normal air conditions.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2012. 95:2904-2909".

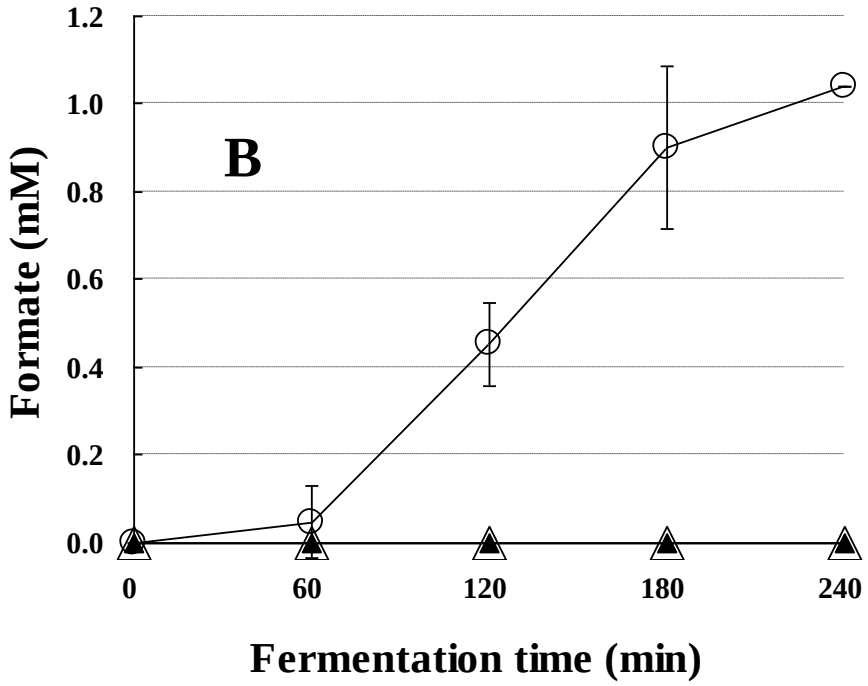


Figure 2.7B

Influence of dissolved oxygen (DO) on fomate production by the yogurt culture LB81. LB81 incubated under a DO concentration fixed at 6 mg/kg.
Incubation was at 43 °C.
Symbols: (▲) under DO concentration fixed at 6 mg/kg, (△) under DO concentration fixed at 1 mg/kg, and (○) under normal air conditions.
Bars represent standard deviations of averages from three experiments.

Created based upon data from "H. Horiuchi et al. J. Dairy Sci. 2012. 95:2904-2909".

2.4 Discussion

Veringa et al. (6) suggested that *S. thermophilus* produces a growth factor for *L. bulgaricus* under anaerobic or nearly anaerobic conditions. Galesloot et al. (8) suggested that this growth factor is equal to or can be replaced by formic acid. In author's previous paper, fermentation with LB81 was suppressed by the presence of more than 1 mg/kg of DO in the yogurt mix. In the present paper it has been proven that formate production by LB81 with the DO concentration in the yogurt mix fixed at only 1 mg/kg was completely suppressed. It is generally known that Pyruvate formate-lyase (PFL) shows oxygen-sensitivity (23) and it was reported that PFL of *Escherichia coli* or *Streptococcus mutans* are inactivated by oxygen (24, 25). The author hypothesized that PFL of *S. thermophilus* also shows oxygen-sensitivity, and then it may be concluded that this suppression of acid production was caused by the suppression of formate production.

The author investigated the influence of oxygen on the symbiosis between *L. bulgaricus* 2038 and *S. thermophilus* 1131 under ROF, normal air condition and fixed 6, 4, 2, and 1 mg/kg DO conditions. The results which ROF made nearly 30 minutes earlier formate detection than in normal fermentation (Figure 2.5) and the addition of formate stimulated acid production by LB81 (Figure 2.2) showed the effect of the fermentation time reduction by ROF mainly was the acceleration of formate production of *S. thermophilus* 1131. And the result of the fermentation progress by ROF was a little faster than by addition of formate (Figure 2.2) suggested the effect of ROF on fermentation time had factor other than formate.

2.5 Conclusion

Acid production by *L. bulgaricus* 2038 or *S. thermophilus* 1131 in single culture were neither suppressed nor advanced by the DO concentration in the yogurt mix (Figure 2.1). While acid production by starter culture LB81 composed of *L. bulgaricus* 2038 and *S. thermophilus* 1131 was greatly accelerated by reducing dissolved oxygen (DO) to nearly 0 mg/kg in the yogurt mix (fermentation method named ROF), both acid production and formate production by LB81 was suppressed by the existence of only 1 mg/kg of DO in the yogurt mix. In conclusion, these suggest that DO inhibit to the symbiotic relationship between *L. bulgaricus* 2038 and *S. thermophilus* 1131.

The author attributed the acceleration of acid production of LB81 by reduced DO mainly to the acceleration of formate production by *S. thermophilus* 1131 and the suppression of acid production of LB81 by DO mainly to the suppression of formate production by *S. thermophilus* 1131.

Chapter 3

NADH oxidase of *Streptococcus thermophilus* 1131 is required for effective yogurt fermentation with *Lactobacillus delbrueckii* subsp. *bulgaricus* 2038

3.1 Introduction

The combination *S. thermophilus* and *L. bulgaricus* is traditionally used for the manufacture of yogurt. When both bacteria gain a mutual benefit, this association is known proto-cooperation. Protocooperation (or symbiosis) exists between *S. thermophilus* and *L. bulgaricus* via an exchange of metabolites that are necessary for the growth of each bacterium in milk. For example, *L. bulgaricus* provides amino acids and peptides for *S. thermophilus*, and *S. thermophilus* provides formate and carbon dioxide for *L. bulgaricus* (5, 6, 26). Several reviews on protocooperation have been published (27-30) and the mutual benefits often result in higher acidification rates, which are important for yogurt fermentation. Pablo et al. (9) suggested that the symbiotic relationship (or protocooperation) between *S. thermophilus* and *L. bulgaricus* is very important for yogurt production because it reduces fermentation time.

Many new aspects of protocooperation in yogurt fermentation have been revealed using the current post-genomic techniques. The complete genome of five *S. thermophilus* strains (10, 31-34) and four *L. bulgaricus* strains have been characterized (11, 35-37) and post-genomic studies to analyze the proto-cooperation have recently been reported. Microarray and proteome analyses of *S. thermophilus* LMG18311 and a qRT-PCR analysis of *L. bulgaricus* ATCC 11842 have shown that coculture results in not only nutritional exchanges but also dramatic physiological changes in these two bacteria. Transcriptional changes for genes related to nitrogen, nucleotide bases and iron metabolism were observed in *S. thermophilus* LMG18311 (4). Another study (38) showed using microarray of *S. thermophilus* CNRZ1066 and *L. bulgaricus* ATCC BAA-365 that the interactions between the purine, amino acid, exopolysaccharide and long-chain fatty acid metabolisms are affected by coculture. The genome of *S. thermophilus* LMD-9 encodes eight two-component systems, and qRT-PCR analysis data indicated that the culture with *L. bulgaricus* ATCC11842 induced expression of two response regulators among them (39).

Recently, the author reported that dissolved oxygen (DO) greatly affects yogurt fermentation with an industrial starter culture composed of *L. bulgaricus* 2038 and *S.*

thermophilus 1131 (14). The starter began to produce acid actively only after the DO concentration in the yogurt mix was reduced to almost 0 mg/kg. Fermentation was suppressed in the presence of only 1 mg/kg of DO, and this suppression was compensated partially by the addition of formate to the medium (40). The author has also found that the fermentation time was shortened by 30 min if DO in the yogurt mix was removed in advance (reduced dissolved oxygen fermentation: ROF). These observations clearly demonstrated that DO in milk would greatly affect yogurt fermentation, and at the same time, they indicated that these bacteria have a DO-consuming enzyme(s) presumably required for fermentation.

Like other lactic acid bacteria, *S. thermophilus* and *L. bulgaricus* do not produce cytochrome oxidases required for energy-linked respiratory metabolism due to the inability to synthesize heme, an essential cofactor for cytochrome oxidase. Instead of heme cofactor oxidases, lactic acid bacteria consume molecular oxygen through the action of flavoprotein oxidases, including NADH oxidase, pyruvate oxidase, α-glycerophosphate oxidase, L-amino acid oxidase, and lactate oxidase. Although these oxidases have not been directly linked to energy metabolism, many functions of these oxidases in the physiology of lactic acid bacteria have been reported (41-49). Teraguchi *et al.* (50) characterized the NADH oxidase activity of *S. thermophilus* and suggested that high NADH oxidase activity might shift metabolism from homo lactic acid to mixed acid fermentation by affecting the cellular NAD^+/NADH ratio (50). NADH oxidase activity of *L. bulgaricus* has been reported to be a source of hydrogen peroxide (H_2O_2) when the bacterium was cultured under aerobic conditions (51). However, the role of these oxidases in yogurt fermentation has not been elucidated.

In the present study, the beneficial effects of ROF on yogurt fermentation were investigated from the viewpoint of protocooperation. The author also analyzed the contributions of these bacterial oxidases in yogurt fermentation, and identified the NADH oxidase of *S. thermophilus* 1131 as the major enzyme required for the DO reduction and fermentation of milk.

3.2 Materials and Methods

3.2.1 Strain and culture conditions

Yogurt starter strains of *L. bulgaricus* 2038 and *S. thermophilus* 1131 obtained from the stock cultures of Food Science Institute of Meiji Co., Ltd., which are used in the commercial production of Meiji Bulgaria Yogurt LB81 Plain in Japan since 1993. For the coculture medium, 10% (wt/wt) skim milk was prepared by heating it to 95°C

for 2 min and immediately cooling it to 43°C. For monoculture experiments of *L. bulgaricus* 2038 or *S. thermophilus* 1131, either 1 mM sodium formate (Wako Pure Chemical Industries, Ltd., Japan) or 0.1% (wt/wt) casein peptides (CE90GMM, Nippon Shinyaku Co., Ltd., Japan) was added, respectively, to the skim milk medium prepared above. Yogurt fermentation with or without deoxygenated fermentation was performed as described previously (14, 40). Since the Δ*nox* mutant (described below) did not grow well on skim milk medium, the preculture of this strain was prepared on M17L medium [M17 (Becton, Dickinson and Company) supplemented with 0.5% lactose] in monoculture. The precultures of *S. thermophilus* 1131 and the Δ*nox* mutant were grown for 16.5 hours at 37°C on M17L using an Anaero Pack system (anaerobic cultivation system; Mitsubishi Gas Chemical Company Inc.) and then inoculated to 10% skim milk supplemented with 0.1% casein peptides to give the final OD_{660} of 0.05. The growths of *S. thermophilus* 1131 and the Δ*nox* mutant were monitored at 43°C under static conditions. For coculture experiments, the preculture obtained above containing *S. thermophilus* 1131 or Δ*nox* was inoculated to 10% skim milk together with a culture of *L. bulgaricus* 2038 grown on MRS(Becton, Dickinson and Company) at 37°C. *S. thermophilus* 1131 or the Δ*nox* mutant and *L. bulgaricus* 2038 were inoculated to 10% skim milk medium at a 1:1 ratio, corresponding to a final OD_{660} of 0.025.

3.2.2 Measurements of CFU, DO, lactate and formate

In coculture experiments, a portion of skim milk medium was withdrawn at predetermined time intervals and spread on M17G agar plates and on Rogosa agar plates. The plates were incubated at 43 ˚C for 48 hours under anaerobic conditions using an Anaero Pack system. The numbers of colony-forming units (CFU) for *S. thermophilus* 1131 and *L. bulgaricus* 2038 were determined by counting colony numbers on M17G plates and on Rogosa plates, respectively.

Concentration of DO in the skim milk medium was measured using optical oxygen sensors (VisiFerm DO optical sensors, Hamilton Company, Reno, NV, USA).

l-lactate, d-lactate and formate in the skim milk medium was measured using an F-kit (Roche Diagnostics K.K.) suitable for each material as described previously (14, 40).

3.2.3 Measurement of hydrogen peroxide in skim milk medium

In order to measure small quantities of H_2O_2 in the milk medium, the following improved method was used in this study. An aliquot of culture medium (0.5 g) was harvested at predetermined time intervals and diluted with 0.5 ml of distilled water, and

30µl of Carrez I and Carrez II solutions (52) were added. After centrifugation at 12,000 rpm for 10 min at 4°C, the supernatant was diluted by three-fold with 100 mM PIPES (pH 6.5). One hundred microliters of this diluted sample received 100µl of 1mM DA64 (Wako Pure Chemical Industries Ltd., Osaka, Japan), a sensitive chromophore N-(carboxymethylaminocarbonyl)-4,4'-bis(dimethyamino)-diphenylamine sodium salt (53), and 2 µl of peroxidase (Wako Pure Chemical Industries Ltd., Osaka, Japan). After 10 min of incubation at 16°C, the absorbance at 727 nm was determined.

3.2.4 Construction of a *nox* knockout mutant of *S. thermophilus* 1131

Plasmids containing the *nox* gene, inactivated by insertion of a spectinomycin resistance cassette, were constructed as follows. A 1369-bp internal fragment of the *nox* gene was obtained by PCR using the primer pairs PYY0034 (5'-CTCGAGTCAAAAATCGTAGTTGTCGG-3') and PYY0037 (5'-CTCGAGTATTCAGCGGAGATAGCTGC-3'), genomic DNA from *S. thermophilus* 1131 and Takara ExTaq DNA polymerase (Takara Bio Inc., Otsu, Japan). The amplified fragment was cloned into pGEM-T (Promega K.K., Tokyo, Japan) to obtain pGEM-T-*nox*. The primer pairs PYY0035 (5'-GCACCAACGACTGCTACAC-3') and PYY0036 (5'-TGCGTACGATGTAGATATGG-3') were used to introduce blunt ends in the middle of the *nox* gene of pGEM-T-*nox* by PCR. pSPC1 (48) was digested with *Bam*HI obtain a DNA fragment containing a terminator-less spectinomycin resistance cassette. Then the *Bam*HI fragment was ligated with the blunt ends of the PCR product of pGEM-T-*nox* after generating blunt ends by treatment with the Klenow fragment of *E. coli* DNA polymerase I (Takara Bio Inc., Otsu, Japan). pGEM-T-*nox* carrying the spectinomycin resistance cassette in the middle of *nox* gene in the same direction was selected and designed as pGEM-T-*nox::spc*^{r}. The *nox* gene carrying the spectinomycin resistance cassette was then transferred into the *Xho*I site of pG$^+$host6 (Appligene, USA), a temperature-sensitive cloning vector for gram-positive bacteria. The resulting plasmid was designed as pG$^+$host6-*nox::spc*^{r} and introduced into *S. thermophilus* 1131 by electroporation. Double-crossover events leading to the expected gene replacements were obtained as previously described (54). Correct insertion of the spectinomycin resistance cassette into genomic DNA was confirmed by PCR analysis using primer pairs PYY0034 and PYY0037.

3.2.5 Determination of NADH oxidase and whole-cell oxygen consumption activities of *S. thermophilus*

To determine NADH oxidase activity, 1 ml of overnight culture of each strain was transferred into a 300 ml flask containing 50 ml of fresh M17G medium, and incubated at 37°C with vigorous shaking (180 cycles/min) under aerobic conditions until the mid-log phase (OD_{600} = ~1.0). Then the bacterial cells were collected by centrifugation at 6,300 g for 5 min at 4°C, and washed with 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA, and suspended in 0.8 ml of the same buffer. The cell suspensions were transferred into a screw cap tube containing 0.3 g zirconium beads and disrupted by a Mini-beadbeater (BioSpec Products,Inc. Bartlesville, OK, USA) at 4,600 rpm for 120 sec. The cell debris was removed by centrifugation at 16,000 g for 15 min at 4°C, and the resulting supernatant was used for NADH oxidase activity measurement as described previously (44). One unit of enzyme was defined as the amount that catalyzed oxidation of 1 μmol of NADH to NAD per min at 25°C. Protein concentrations were determined according to the Bradford method (55) using bovine serum albumin as a standard.

To monitor oxygen consumption activity of *S. thermophilus* cells, each strain was grown up to an early log phase (OD_{600} = ~0.5) in M17G medium with vigorous shaking (180 cycles/min) as described above. The bacterial cells were collected by centrifugation at 6,300 g for 5 min at 4°C, washed with phosphate buffered saline (PBS) and suspended in 0.8 ml of the same buffer. Then an aliquot of the suspension was injected into a rubber-capped vial filled with 3.5 ml of oxygen-saturated PBS to give an OD_{600} of 2.0. After the vial containing the bacterial suspension was incubated for 5 min at 37°C with stirring, 20% glucose solution was injected into the vial at a final concentration of 0.1%, and the rate of oxygen consumption of the bacterial suspension was monitored using an oxygen meter (Fibox3, PreSens, Regensburg, Germany) through a sensor tip fixed to the bottom of the vial.

3.3 Results

3.3.1 Growth acceleration by ROF was more prominent in *S. thermophilus* 1131 than *L. bulgaricus* 2038

It was compared that the viable cell numbers and lactate productions of *S. thermophilus* 1131 and *L. bulgaricus* 2038 in yogurt fermentation under ROF and normal fermentation conditions (14, 40). ROF in coculture of LB81 significantly increased the number of colony-forming units of *S. thermophilus* 1131 and production

of both l-lactate and formate at 90 min (Figure 3.1). However the number of colony-forming units and l-lactate concentrations of the cultures with or without ROF were almost the same at the end of fermentation. Although the number of colony-forming units of *L. bulgaricus* 2038 and d-lactate production were also slightly affected by ROF, the changes were small compared with those of *S. thermophilus* 1131. These results indicate that l-lactate, produced by *S. thermophilus* 1131, mainly contributed to the acceleration of acidification in coculture, especially between 60 and 120 min of ROF.

3.3.2 DO consumption and H_2O_2 generation in monoculture of *S. thermophilus* 1131 and *L. bulgaricus* 2038

It was examined that DO concentrations and pH changes in the skim milk medium in monoculture of each lactic acid bacterium in relation to H_2O_2 generation. The milk medium received either 0.1% of casein peptides or 1 mM sodium formate to promote growth of *S. thermophilus* 1131 or *L. bulgaricus* 2038, respectively. H_2O_2 concentrations in the culture medium were determined because *L. bulgaricus* produces H_2O_2 in the presence of oxygen (51, 56, 57) and *S. thermophilus* changes the expression of genes involved in iron metabolism during coculture with *L. bulgaricus* presumably to avoid the harmful effects of H_2O_2 (4, 38).

Although it is reportedly difficult to measure H_2O_2 concentrations in milk due to the presence of several milk components including casein (4), the author successfully determined H_2O_2 concentrations using a sensitive chromophore, DA-64. Linear standard curves of H_2O_2 in the milk were obtained repeatedly in a range between 18 and 300 μM (data not shown). This new method was used to measure H_2O_2 concentrations in the skim milk medium.

As shown in Figure 3.2, both lactic acid bacteria in monoculture reduced DO in the medium to less than 1 mg/kg within 120 min. Although *L. bulgaricus* 2038 produced up to 120 μM H_2O_2 in accordance with the decrease of DO, no H_2O_2 (less than 18 μM, the quantitative determination limit) was detected in the monoculture of *S. thermophilus* 1131. These results indicate that both lactic acid bacteria are able to consume DO in the skim milk medium and that *L. bulgaricus* 2038 does not completely reduce molecular oxygen to H_2O, resulting in production of H_2O_2.

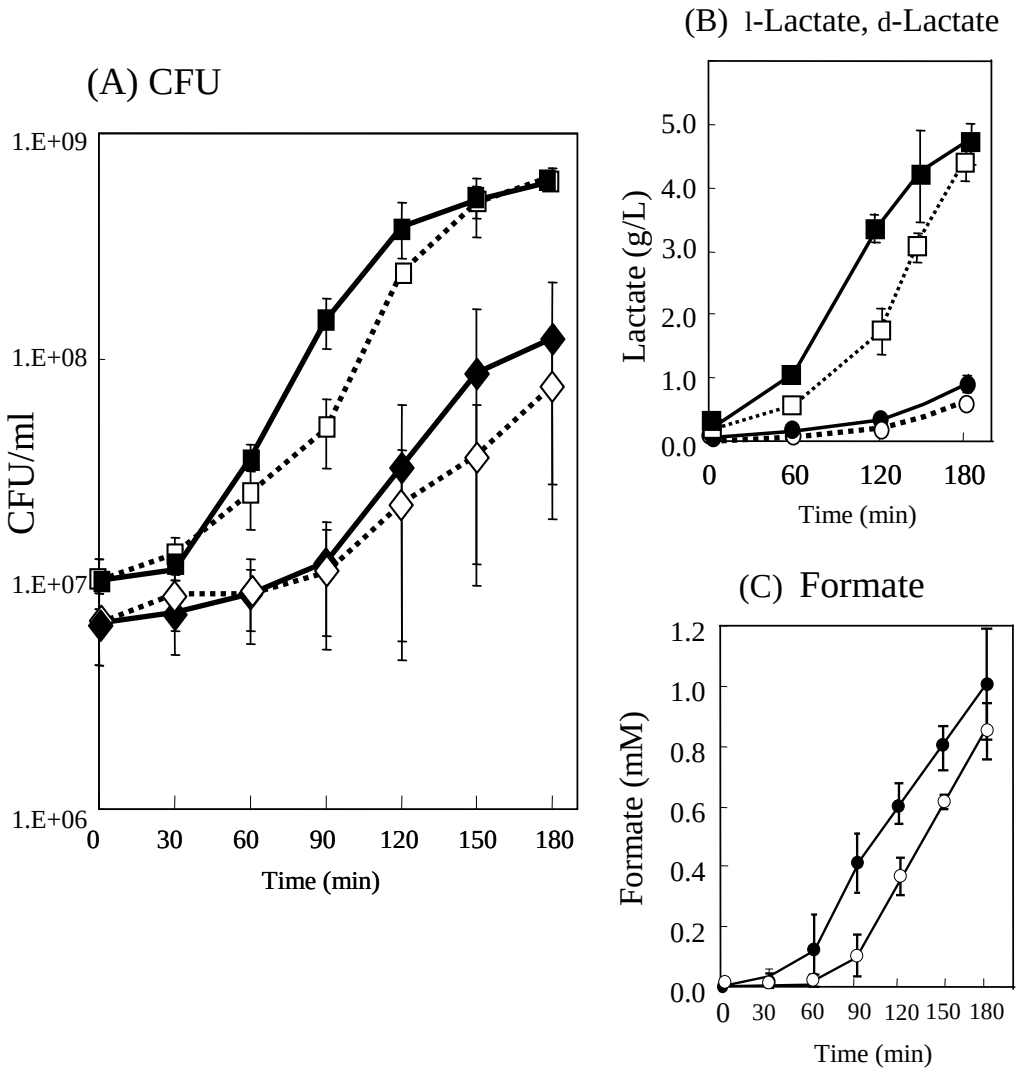


Figure 3.1.

Influence of deoxygenated fermentation on viable cell numbers and lactate and formate concentrations in cocultute of *S. thermophilus* 1131 and *L. bulgaricus* 2038. For ROF, skim milk medium was deoxygenated with nitrogen gas before fermentation. Normal fermentation (NF) was performed without this treatment. Incubation was at 43˚C. In (A), CFU of *S. thermophilus* 1131 in ROF (■) and NF (□), and CFU of *L. bulgaricus* 2038 in ROF (♦) and NF (◊) are shown. In (B), l-Lactate concentrations in ROF (■) and NF (□), and d-Lactate concentrations in ROF (●) and NF (○) are shown. In (C), formate concentrations in ROF (●) and NF (○) are shown.
The error bars represent the standard deviations of three independent experiments.

Created based upon data from "Y. Sasaki, H. Horiuchi et al. BMFH. 2014. 33:31-40".

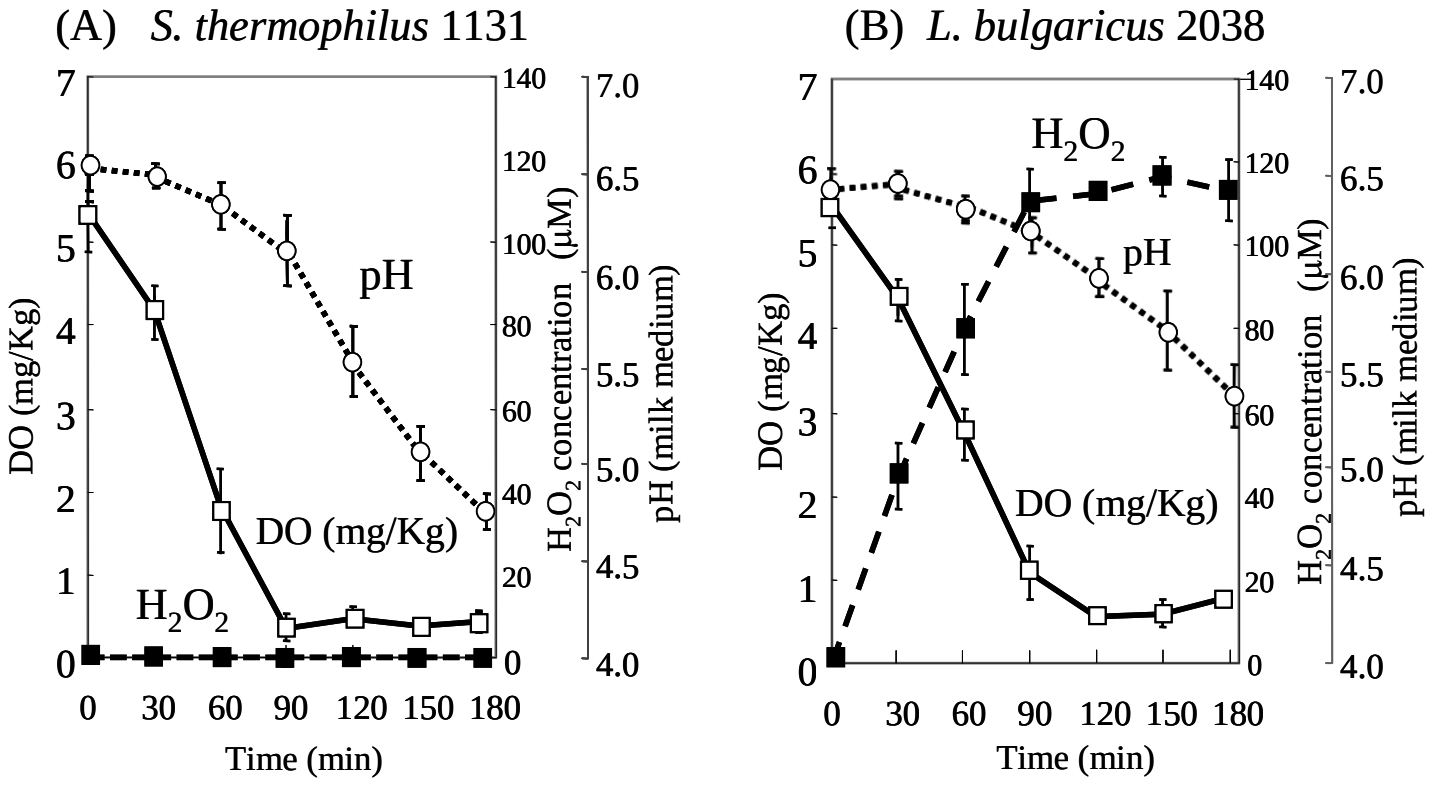


Figure 3.2.

DO consumption and H_2O_2 generation in monoculture of *S. thermophilus* 1131 and *L. bulgaricus* 2038.DO (□) and H_2O_2 (■) concentrations and pH (○) in monoculture of *S. thermophilus* 1131 (A) and *L. bulgaricus* 2038 (B) at 43˚C are shown.
The error bars represent the standard deviations of three independent experiments.

Created based upon data from "Y. Sasaki, H. Horiuchi et al. BMFH. 2014. 33:31-40".

3.3.3 DO consumption and H_2O_2 generation in coculture of LB81

It was measured that the concentrations of DO and H_2O_2 in the culture medium together with the pH values in coculture of *S. thermophilus* 1131 and *L. bulgaricus* 2038. As shown in Fig. 3.3, DO in the medium decreased to less than 1 mg/kg within 90 min, and H_2O_2 was not detected until 60 min of fermentation. The maximum concentration of H_2O_2 was around 40 μM, which was approximately 3 times lower than that of the *L. bulgaricus* 2038 monoculture (Figure 3.2B).

The result showing that H_2O_2 was not detected in the first 60 min of fermentation, when the rapid decrease of DO occurred, strongly suggests that the DO decrease in coculture can be attributed to *S. thermophilus* 1131. This result together with the increased cell number and production of L-lactate in yogurt fermentation (Figure 3.1A and 3.1B) indicates that the metabolic activity of *S. thermophilus* 1131 is the main contributor to DO reduction during yogurt fermentation.

H_2O_2 was transitionally detected at 90 and 120 min of fermentation. This suggested that *L. bulgaricus* 2038 might also contribute to DO reduction and either *S. thermophilus* 1131 or the skim milk medium may scavenge H_2O_2. To assess the fate of H_2O_2 during the coculture, the author measured the whole cell H_2O_2-scavenging activity of *S. thermophilus* 1131. H_2O_2 degradation was not promoted by the addition of *S. thermohpilus* 1131 cells to the PBS or skim milk medium to a final concentration of 10^9 CFU/ml (data not shown). Instead, this experiment indicated that the medium itself has an ability to decrease H_2O_2, i.e., 200 μM H_2O_2 added to the skim milk medium at 43 ˚C decreased to about 90 μM in one hour.

3.3.4 Construction of an NADH oxidase knockout mutant of *S. thermophilus* 1131

It was strongly suggested the importance of the DO reduction activity of *S. thermophilus* 1131 during yogurt fermentation by the results obtained above. To identify the oxidase(s) that contributes to DO reduction during yogurt fermentation, the author focused on an H_2O-forming NADH oxidase (Nox) homologue of *S. thermophilus* 1131.

An *S. thermophilus nox*-inactivated strain was constracted by insertion into *nox* of a terminator-less sepectinomycin resistance gene as described in Materials and Methods. insersion of the sepectinomycin resistance gene into the chromosomal *nox* gene was confirmed by PCR analysis (data not shown). Log phase cultures of the resulting Δ*nox* mutant and *S. thermophilus* 1131 were prepared under aerobic conditions and used for NADH oxidase and rate of whole-cell oxygen consumption determinations. As shown in Fig. 3.4, NADH oxidase activity was diminished in the Δ*nox* mutant to less than 4% of that in wild-type strain. Inactivation of the *nox* gene also reduced the rate of

whole-cell oxygen consumption by 65%. These results indicate that NADH oxidase encoded by the *nox* gene is the major oxygen-consuming enzyme in *S. thermophilus* 1131.

3.3.5 Comparison of growth and DO consumption in monoculture of *S. thermophilus* 1131 and the Δ*nox* mutant

It was compared that growth properties of *S. thermophiles* 1131 and the Δ*nox* mutant in a monoculture on skim milk medium supplemented with 0.1% casein peptides. As shown in Figure 3.5, *S. thermophilus* 1131 effectively reduced the DO concentrations of the medium, and fermentation seemed to be accelerated after the DO concentrations were below 1 mg/kg. By contrast, the Δ*nox* mutant did not rapidly reduce the DO concentrations, and the pH value of the medium was kept high even after 4 hours of fermentation. The author also compared the growth of both strains on M17L medium under the same static conditions, and found no significant differences between the two strains (data not shown). These results demonstrated that NADH oxidase of *S. thermophilus* 1131 was required for the effective fermentation in the skim milk medium but not in M17L medium.

Formate concentrations of the skim milk medium were determined because NADH oxidase has a possible role in activating formate production in *S. thermophilus*. Under anaerobic conditions, formate is generally produced by pyruvate formate lyase (Pfl), an oxygen-sensitive enzyme whose activity is easily inactivated by molecular oxygen (24). NADH oxidase may be able to activate Pfl by removing DO in the medium. As shown in Figure 3.5B, formate was detected in the culture medium of *S. thermophilus* 1131 after 2 hours of fermentation, but not in that of the Δ*nox* mutant, supporting this hypothesis.

3.3.6 Growth and DO removal of *S. thermophilus* 1131 and the Δ*nox* mutant in coculture with *L. bulgaricus* 2038

It was examined that the behavior of the *S. thermophilus* 1131 Δ*nox* mutant in coculture with *L. bulgaricus* 2038. The coculture conditions were essentially the same as standard coculture conditions except that M17L was used as a preculture of *S. thermophilus* 1131 and the Δ*nox* mutant. The consumption of DO and decrease in pH caused by coculture of the Δ*nox* mutant were by far slower than those caused by coculture of the wild type (WT) strain, resulting in a strong retardation of yogurt fermentation (Figure 3.5). The H_2O_2 concentrations in the coculture medium of the Δ*nox* mutant were in the range of 40 to 70 μM, a little higher than the concentration

observed in Figure 3.3, indicating that NADH oxidase activity might contribute to the reduction of H_2O_2 generation during coculture.

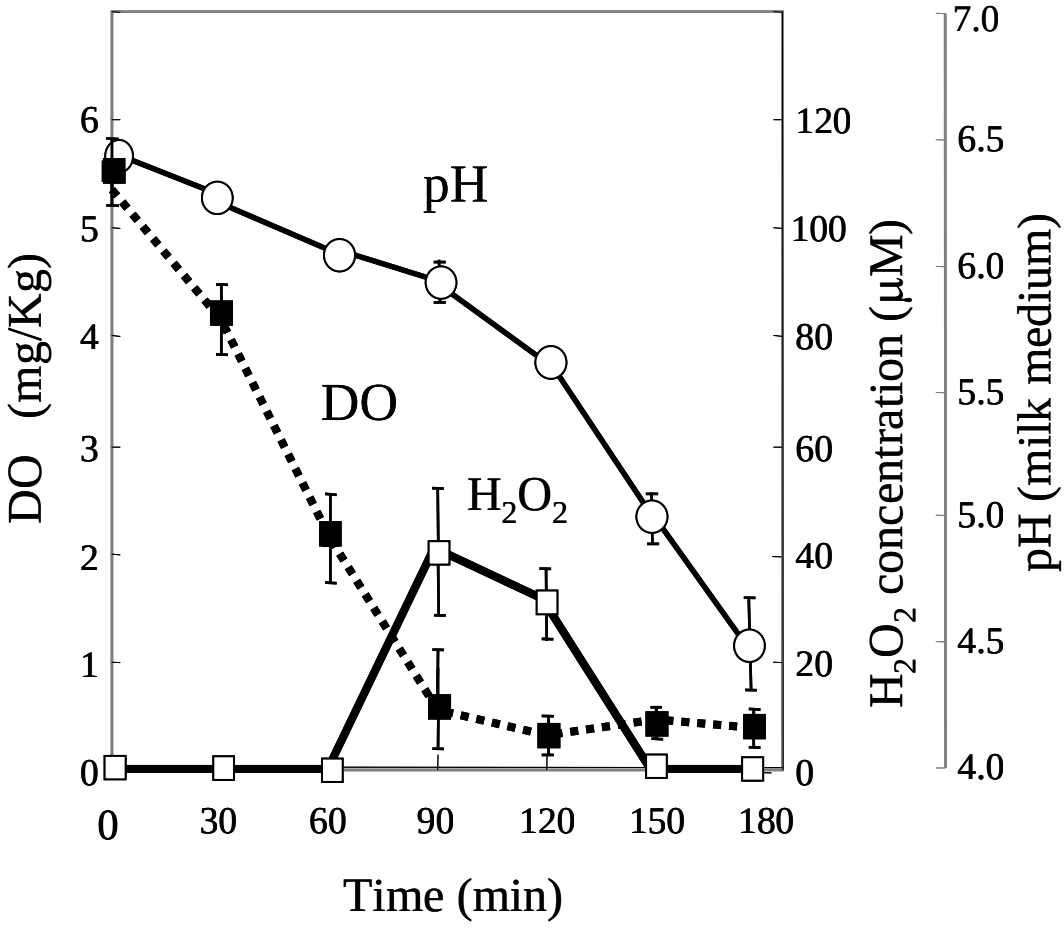


Figure 3.3. DO consumption and H_2O_2 generation in coculture.
DO (□) and H_2O_2 (■) concentrations and pH (○) in coculture of *S. thermophilus* 1131 and *L. bulgaricus* 2038 at 43˚C are shown.
The error bars represent the standard deviations of three independent experiments.

Created based upon data from "Y. Sasaki, H. Horiuchi et al. BMFH. 2014. 33:31-40".

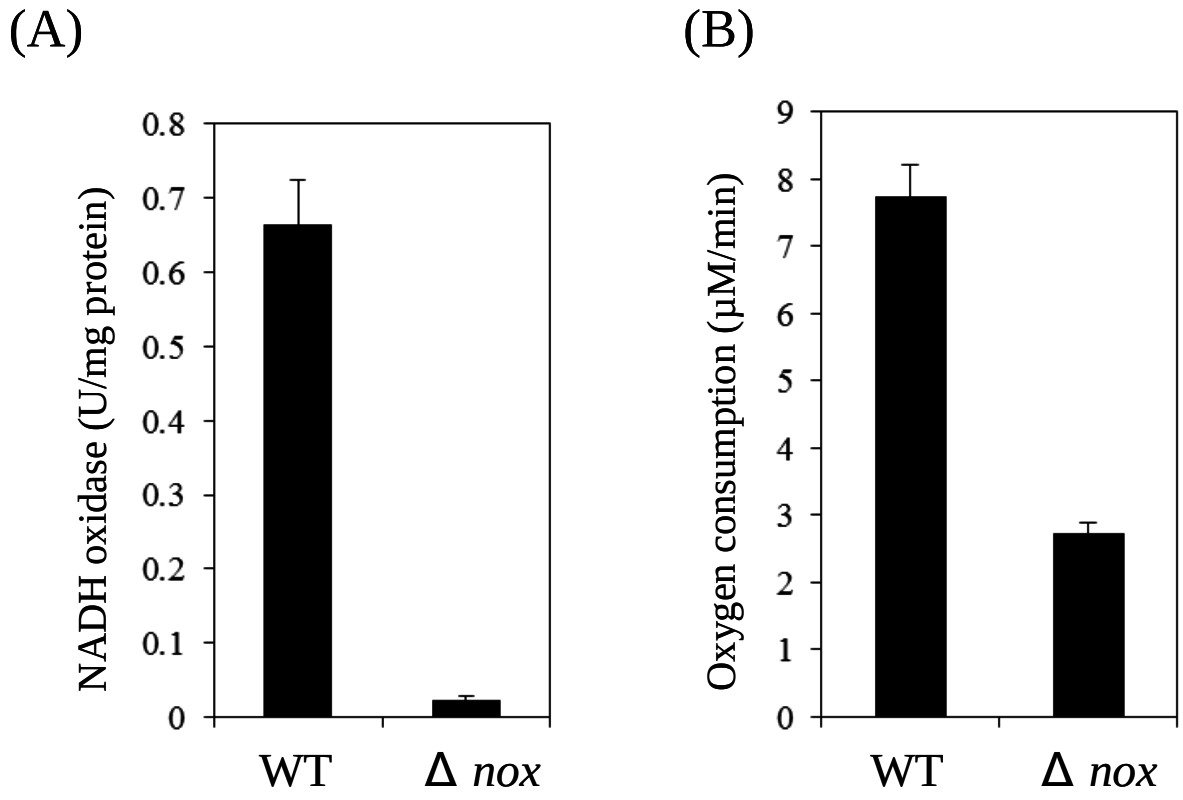


Figure 3.4. NADH oxidase and whole-cell oxygen consumption activities of *S. thermophilus* 1131 and Δ*nox* mutant. Log phase cultures of *S. thermophilus* 1131 and Δ*nox* were used for NADH oxidase activity (A) and whole-cell oxygen consumption determinations.
The error bars represent the standard deviations of three independent experiments.

Created based upon data from "Y. Sasaki, H. Horiuchi et al. BMFH. 2014. 33:31-40".

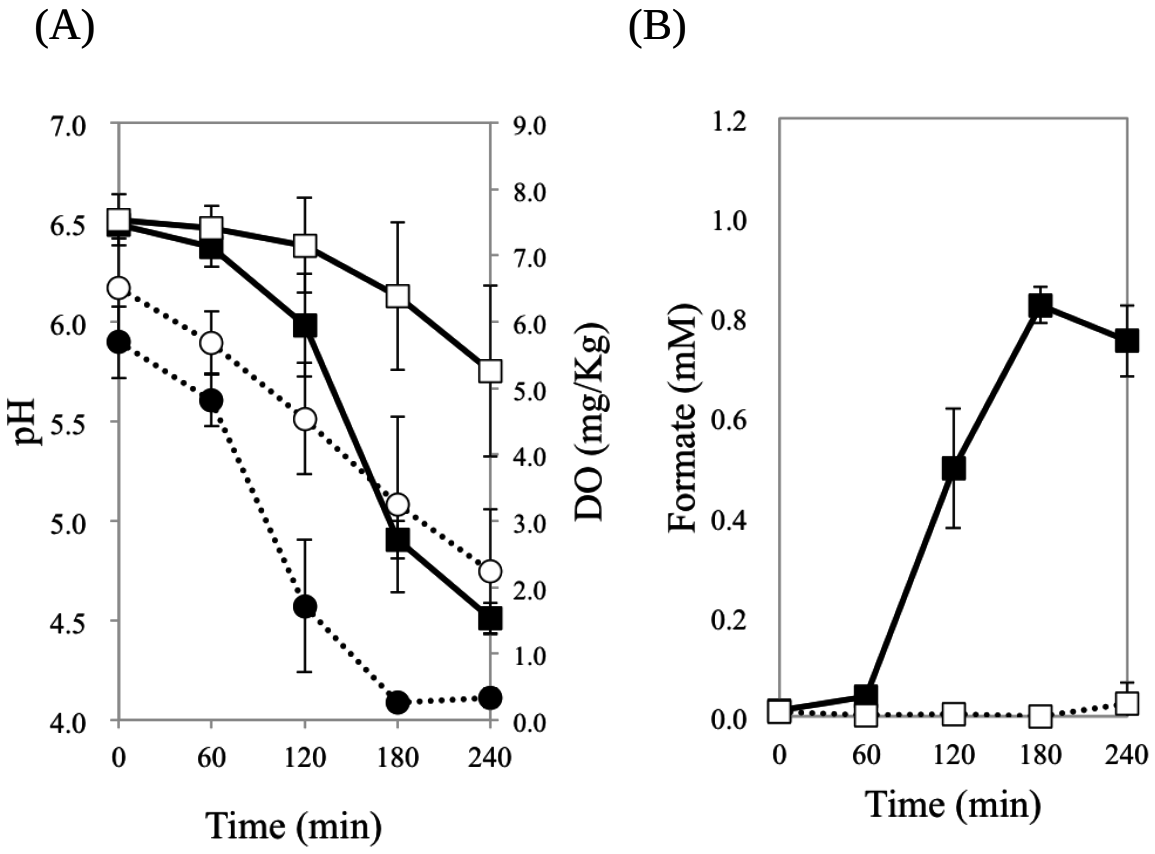


Figure 3.5.

Growth, DO concentration, and formate production in monoculture of *S. thermophilus* 1131 and Δ*nox* mutant.Growth of *S. thermophilus* 1131 and Δ*nox* mutant were monitored at 43˚C in skim milk medium supplemented with 0.1 % casein peptides. In (A), pH (■) and DO (●) of *S. thermophilus* 1131, and pH (□) and DO (○) of Δ*nox* mutant are shown. In (B), formate concentrations in monoculture of *S. thermophilus* 1131 (■) and Δ*nox* mutant (□) are shown.

The error bars represent the standard deviations of three independent experiments.

Created based upon data from "Y. Sasaki, H. Horiuchi et al. BMFH. 2014. 33:31-40".

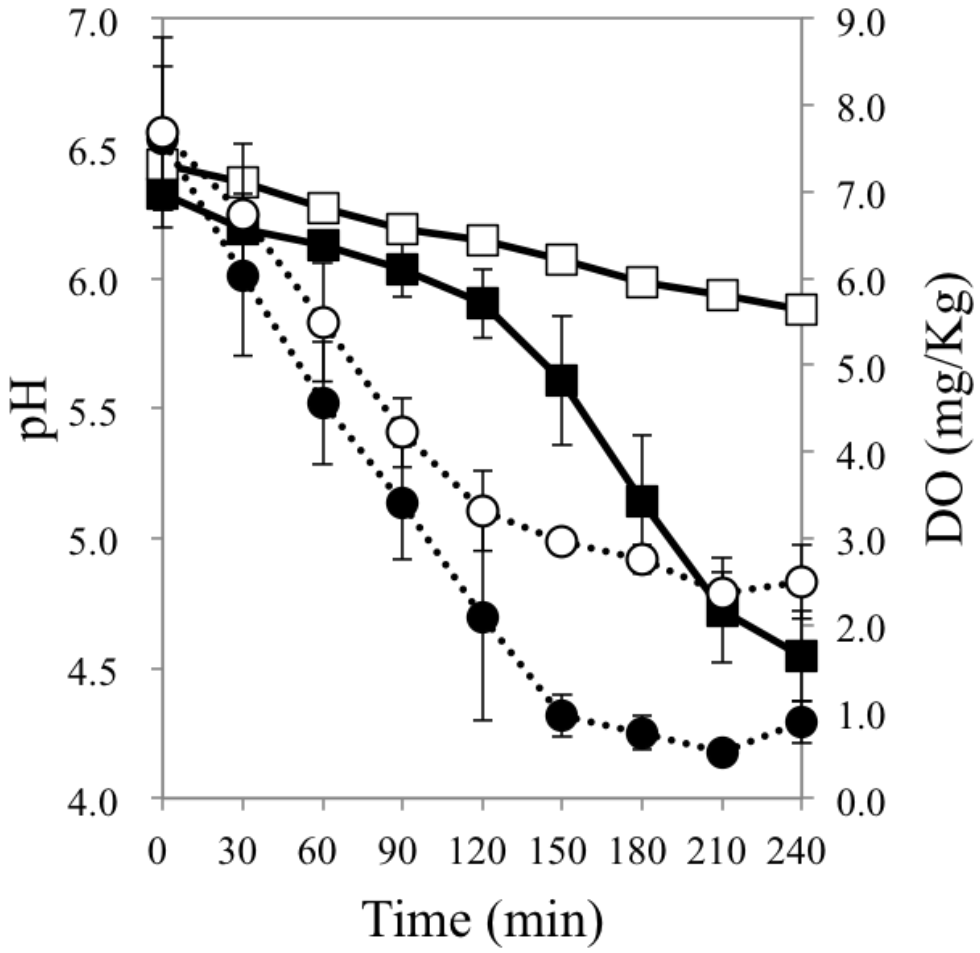


Figure 3.6.

Growth and DO concentration in coculture with *L. bulgaricus* 2038.

pH (■) and DO (●) in coculture of S. thermophilus 1131 and *L. bulgaricus* 2038, and pH (□) and DO (○) in coculture of *S. thermophilus* Δ*nox* mutant and *L. bulgaricus* 2038 at 43˚C are shown.

The error bars represent the standard deviations of three independent experiments.

Created based upon data from "Y. Sasaki, H. Horiuchi et al. BMFH. 2014. 33:31-40".

3.4 Discussion

The author have previously reported that ROF (reduced dissolved oxygen fermentation with LB81 composed with *S. thermophilus* 1131 and *L. bulgaricus* 2038) shortened the yogurt fermentation time by 30 min, and the details of this preferable effect were investigated in the present study. Monitoring the growth of *S. thermophilus* 1131 and *L. bulgaricus* 2038 in skim milk demonstrated that DO removal mainly accelerates the growth of *S. thermophilus* 1131. Formate production was also increased by pre-fermentation removal of DO from the medium. Accumulation of formate would favor to the growth of *L. bulgaricus* 2038, because formate is a well-known growth factor for *L. bulgaricus* in milk (7). However, DO removal stimulated the growth of *S. thermophilus* 1131. This result implies that formate accumulation may also facilitate the growth of *S. thermophilus* in milk. Using proteome analysis, Derzelle *et al.* identified Pfl of *S. thermophilus* lMG18311 as a protein strongly induced during growth in skim milk medium (58). They also demonstrated that the addition of formate or purine bases diminishes the overexpression of Pfl and increases the growth yield of *S. thermophilus* in skim milk medium (58). Their observations suggest that formate produced by Pfl may be utilized in an essential biosynthetic pathway such as purine biosynthesis of *S. thermophilus* in milk. It would therefore not be surprising if the deoxygenated milk activated the Pfl, thereby promoting the growth of *S. thermophilus* 1131 by the addition of formate.

In this study, the author identified Nox of *S. thermophilus* 1131 as the major oxygen-consuming enzyme that promotes the fermentation of milk. The *S. thermophilus* 1131 Δ*nox* mutant could not effectively reduce the DO concentrations and pH values of the skim milk mediums in either monoculture or coculture. Nox is flavoration conserved in most Streptococcaceae and has been reported to be a major part of the oxidase machinery in several lactic acid bacteria. Although most flavoration oxidases of lactic acid bacteria generate H_2O_2 as an end product of the reaction, Nox is able to reduce molecular oxygen to H_2O without producing H_2O_2.

In this study, the author demonstrated the H_2O_2-producing activity of *L. bulgaricus* 2038 in milk, while *S. thermophilus* 1131 did not produce detectable H_2O_2 under the same conditions (Fig3.2). During coculture of these bacteria in milk, H_2O_2 was transitionally detected at 90 and 120 min of fermentation (Fig.3.3). This suggested that *L. bulgaricus* 2038 might also contribute to DO reduction and that either *S. thermophilus* 1131 or skim milk medium may scavenge H_2O_2. To assess the fate of H_2O_2 during coculture, the author measured the whole-cell H_2O_2-scavenging activity of *S. thermophilus* 1131. H_2O_2 degradation was not promoted by the addition of *S.*

thermophilus 1131 cells to the PBS or skim milk medium to a final concentration of 10^9 CFU/ml (data not shown). Instead, this experiment indicated that the medium itself has the ability to decrease H_2O_2, i.e., 200 µM H_2O_2 added to the skim milk medium at 43˚C decreased to about 90 µM in one hour. The presence of H_2O_2 during milk fermentation has been suggested in several studies (4, 29). The author believes that further research will clarify the effects of H_2O_2 on yogurt fermentation.

Chapter 4
Development of superior fat free set yogurt with LT-ROF

4.1 Introduction

Yogurt is beneficial to human health when it is consumed regularly. However, some consumers do not eat yogurt daily because they are concerned about gaining weight. Younger people are often on a diet and many people over forty are very careful about eating too much fat due to concerns about diseases related to metabolic syndrome in Japan. Therefore, the author hypothesized that there should be great demand for fat free yogurt.

Modern industrial yogurt products are classified into stirred, drinking yogurt, and set yogurt (1, 28). Even though composition of yogurt product has fat free, both drinking yogurt and fat free stirred yogurt were able to have good taste, but then there are shortcomings that low/no-fat composition is reduced taste on set yogurt. While there is great demand for set type yogurt in Japan, taste of fat free set yogurt has notably below. Therefore, the author tried to develop a superior fat free set yogurt and wanted to develop a manufacturing method for excellent fat free set yogurt without using thickeners or stabilizers. The shortcomings of fat free set yogurt are reduced taste and remarkably lower quality. Fat free results in a thin taste and becomes the yogurt with a coarse structure and increases whey syneresis. Then, the author focused on the effect of reduced dissolved oxygen low-temperature fermentation (LT-ROF) (14). LT-ROF is a favorable method for fat free set yogurt that possesses a smooth texture, strong curd structure, rich taste and in reduces whey syneresis (14).

4.2 Materials and methods

4.2.1 Culture strains used

The culture LB81, which contains *L. bulgaricus* 2038 and *S. thermophilus* 1131, was the most frequently used in this chapter. These strains were obtained from the culture collection of Research and Development Laboratories, Meiji Co., Ltd. This starter culture has been used in the commercial production of Meiji Bulgaria Yogurt LB81 Plain in Japan since 1993.

4.2.2 Preparation of yogurt bulk starter of LB81

Each strain of *L. bulgaricus* 2038 or *S. thermophilus* 1131 stored at - 80°C was

subcultured once at 37°C for 16 h in skim milk and yeast extract (SMY) medium, composed of 10% (wt/wt) skim milk supplemented with 0.1% (wt/wt) yeast extract (preculture) after autoclaving (121°C, 7 min), and was cooled to 5°C. Both precultures of *L. bulgaricus* 2038 and *S. thermophilus* 1131 were inoculated (1 %; wt/wt) into fresh, sterilized SMY medium (95°C, 10 min), incubated at 37°C to reach an acidity of 0.7%, and cooled immediately to 5°C (yogurt bulk starter culture). The acidity was measured by titrating a 9-g sample against 0.1 *N* sodium hydroxide using phenolphthalein as the indicator. The yogurt starter culture was then stored at 5°C. The yogurt starter culture LB81, which was maintained at 5°C, was used until 3 d after preparation.

4.2.3 Preparation of fat free set yogurt with LT-ROF

The fat free set yogurt mixture used in this chapter containing 0.2% (wt/wt) fat and 12.0% (wt/wt) SNF, was obtained by mixing skim milk powder, whey protein concentrated (WPC), and water. These materials were supplied by the plants of Meiji limited company. The yogurt mix was heated to 95°C for 2 min and immediately cooled to 37°C. The yogurt bulk starter LB81 was inoculated in the yogurt mix to a concentration of 2% (wt/wt). After yogurt starter culture inoculation, sterile nitrogen gas (filtered with a 0.45 μm cellulose acetate filter) was aseptically mixed and dispersed into the yogurt mix through a stainless steel pipe (about 3-mm bore) to reduce the DO concentration in the yogurt mix to nearly 0 mg/kg. Concentration of DO in the yogurt mix was measured with a DO meter (DO-24P, DKK-TOA Corp., Tokyo, Japan). Then, 90 g of the mixture was placed into ten 100-ml polystyrene cups that were oxygen permeable. Fermentation was carried out at 37°C. The yogurt fermentation end point was set at 0.8% acidity.

The normal fat yogurt mix as control used in this study containing 3.0% (wt/wt) milk fat and 9.5% (wt/wt) SNF, was obtained by mixing raw milk [3.6% (wt/wt) fat], skim milk powder, butter [80.0% (wt/wt) milk fat], whey protein concentrated (WPC) and water. These materials were supplied by the plants of Meiji Co., Ltd. After being homogenized at 15 MPa, the yogurt mix was heated to 95°C for 2 min and immediately cooled to 43°C. The yogurt bulk starter was inoculated in the yogurt mix to a concentration of 2%. After mixing, 90 g of the mixture was placed into ten 100-ml polystyrene cups that were oxygen permeable. Fermentation was carried out at 43°C (control fermentation). The yogurt fermentation end point was set at 0.7% acidity.

Both yogurt samples were stored at 5°C for 1 d before the analysis.

4.2.4 Physical characterization of fat free set yogurt

The properties of yogurt samples were measured after 24 h storage at 5°C. The pH was measured with a pH meter (HM-50V, DKK-TOA Corp.).

The physical properties of smoothness and hardness of yogurt were measured, using a curd meter (ME-302; Iio denki Tokyo, Japan) as shown chapter 1. However, there is no optimum method for evaluating the smoothness of set yogurt. The ME-302 curd meter is specially designed to evaluate the hardness of set yogurt and can also be used for evaluating smoothness. Specifically, the surface angle formed by pressure of a yogurt knife with a weight of 100 g was measured. Here, the weight at which the elastic surface curve was broken and there is penetration occurred was defined as hardness (g), whereas the angle of that curve (the penetration angle) was used as an indicator of smoothness (with the angle having a value from 0 to 90°, and with smaller values representing a smoother tissue). Three yogurt samples were analyzed at each trial and average readings were taken.

The degree of whey syneresis of yogurt was defined as the ratio (%) of the volume of the supernatant after centrifugation (2,150 × *g*, 10 min, 5°C) to yogurt sample. This method helped us to estimate rapidly and in advance the actual whey syneresis of yogurt after storage.

4.2.5 Microbiological analysis

To count the viable cells of yogurt bacteria, aliquots of the yogurt sample after 1 d of storage at 5°C were poured onto plates and mixed with 15 ml of bromocresol purple plate count agar (Eiken Chemical Co., Ltd, Tokyo Japan) that had been autoclaved and kept at 50°C. The plates were incubated at 37°C for 72 h. The colonies of *L. bulgaricus* 2038 and *S. thermophilus* 1131 were identified by their rough shape and smooth shape, respectively, in the agar plates.

4.2.6 Sensory Evaluation

Two yogurt samples in 100 ml cups of fat free set yogurt made by the LT-ROF method and normal fat set yogurt made by the control fermentation at 43°C were stored at 5°C for 1 d before evaluation.

The sensory evaluation was carried out using 17 trained panelists of Research and Development Laboratories, Meiji Co., Ltd. Each panelist evaluated only one kind of yogurt at a session. Yogurt samples were coded using 3-digid random number and served to the panelists in individually partitioned booths (19). Panelists evaluated both a normal set yogurt and a no fat set yogurt made by the LT-ROF method for smoothness,

sourness and mildness at 2 sessions. Sessions were held at least 1 h apart. The absolute evaluation data (scored 1 through 5 for either sample).

4.3 Results and Discussions

The author compared the characteristics of the fat free set yogurt prepared by LT-ROF and the normal fat set yogurt containing 3.0% (wt/wt) milk fat prepared by control fermentation at 43°C with sensory evaluation, microbiological analysis and physical characterization.

4.3.1 Sensory evaluation of no fat set yogurt prepared by LT-ROF

The author compared the characteristics of the fat free set yogurt prepared by LT-ROF and the normal fat set yogurt containing 3.0% (wt/wt) prepared by control fermentation at 43°C. The results of the sensory test with 17 trained panelists showed that the fat free set yogurt prepared by LT-ROF had almost the same score with respect to “fatty taste”, “smooth texture”, “sweet taste” and “sour taste” compared with the yogurt made by the control fermentation at 43°C (Table 4.1). This indicated that the fat free set yogurt prepared by LT-ROF had almost the same good taste as the normal fat set yogurt containing 3.0% (wt/wt) milk fat.

Table 4.1 A sensory test with 17 trained panelists was carried out on the fat free set yogurt made by low-temperature reduced dissolved oxygen fermentation (LT-ROF; A) and the normal fat set yogurt made by control fermentation method at 43°C (B), served in 100-ml cups

	Scores[1]	
Term	A	B
Fattiness	2.64	2.87
Smoothness	2.67	2.79
Sweetness	2.17	1.92
Sourness	3.32	3.53

[1]The absolute evaluation data were scored 1 thorough 5 for both of the control and test yogurt for their Fattiness (1 = not at all, 5 = very fatty), smoothness (1 = not at all, 5 = very smooth), sweetness (1 = weak, 5 = strong), and sourness (1 = weak, 5 = strong).

4.3.2 Microbiological analysis and physical characterization of fat free set yogurt prepared by LT-ROF

The viable cell counts of *L. bulgaricus* 2038 and *S. thermophilus* 1131 of the fat free set yogurt prepared by LT-ROF were 2.5×10^8 and 12.5×10^8 cfu/g, which were nearly the same as those of the normal fat set yogurt.

The fat free set yogurt prepared by LT-ROF had nearly the same smooth texture as the normal fat set yogurt containing 3.0% (wt/wt) milk fat prepared by control fermentation at 43°C. To express these physical properties with numerical values, 2 methods were then used. First, the degree of whey syneresis of yogurt (%) was measured; this was defined as the ratio of the supernatants obtained by centrifugation (2,150 × *g*, 10 min, 5°C) of the yogurt sample. The degree of whey syneresis of the fat free set yogurt prepared by LT-ROF was approximately 22%, while the normal fat set yogurt containing 3.0% (wt/wt) milk fat prepared by control fermentation at 43°C was approximately 20%. This result indicates that the no fat set yogurt made by LT-ROF had almost the same close structure as the normal fat set yogurt made by the control fermentation at 43°C (14). Second, the hardness and the penetration angle of the yogurt curd were determined with a 100-g yogurt knife and the ME-302 curd meter. In our experience, a hardness of 40 g and greater is sufficient to stand up to the impact of shaking during transport, whereas 30 g or less is not. The penetration angle could be a value up to 90°, with a smaller value indicating a smoother texture. As shown in Table4.2, the hardness and smoothness of the fat free set yogurt prepared by LT-ROF were 50 g and 61°, respectively, whereas those of the normal fat set yogurt prepared by control fermentation at 43°C were 50 g and 57°. This indicated that the fat free set yogurt prepared by LT-ROF had almost the same hardness and smoothness as the normal fat set yogurt (14).

Table 4.2 Mean values (± SD) of the physical properties of the fat free set yogurt prepared by low-temperature reduced dissolved oxygen fermentation (LT-ROF) and the normal fat set yogurt containing 3.0% (wt/wt) prepared by control fermentation at 43°C

	Physical properties of the yogurt[1]	
Yogurt production	Hardness (g)	Penetration angle (°)
Fat free set yogurt made by LT-ROF	50 ± 1	61 ± 5
Normal fat set yogurt	50 ± 4	57 ± 3

[1]Physical properties of the yogurt are defined in the Materials and Methods section.

4.4 Conclusion

The low-temperature reduced dissolved oxygen fermentation (LT-ROF) method has made it possible to manufacture excellent fat free set yogurt without using sugar, stabilizers and thickeners. The fat free set yogurt product prepared by LT-ROF had almost the same fatty taste, smooth texture, and sufficient hardness to stand up to the impact of shaking during transport as the normal fat set yogurt product containing 3.0% (wt/wt) milk fat prepared by control fermentation at 43°C. This fat free set yogurt product prepared by LT-ROF had been sold as commercial production of Meiji Bulgaria Yogurt LB81 Zero Fat Plain in Japan since 2009. This yogurt product has a big seller now (2014).

General conclusion

Yogurt starter culture LB81, composed of *L. bulgaricus* 2038 and *S. thermophilus* 1131, reduced the DO in the yogurt mix before acid formation. The starter began to produce acid actively after the DO concentration in the yogurt mix had been reduced to nearly 0 mg/kg. Fermentation with LB81 was suppressed by the presence of more than 1 mg/kg of DO in the yogurt mix. The author concluded that DO interferes with the symbiotic relationship between *L. bulgaricus* and *S. thermophilus*. The author found that reducing DO concentration in the yogurt mix with LB81 to nearly 0 mg/kg reduced the fermentation time at 43°C compared with the control fermentation at 43°C (Chapter 1).

Acid production resulting from either *L. bulgaricus* 2038 or *S. thermophilus* 1131 in single culture was neither suppressed or enhanced by the DO concentration in the yogurt mix. While acid production by starter culture LB81 composed of *L. bulgaricus* 2038 and *S. thermophilus* 1131 was greatly accelerated by reducing dissolved oxygen (DO) to nearly 0 mg/kg in the yogurt mix (ROF), both acid production and formate production by LB81 were suppressed by the existence of more than 1 mg/kg of DO in the yogurt mix. In conclusion, these results suggest that DO inhibits the symbiotic relationship between *L. bulgaricus* 2038 and *S. thermophilus* 1131. The author attributes the acceleration of acid production of LB81 by reduced DO mainly to the acceleration of formate production and the suppression of acid production of LB81 by DO mainly to the suppression of formate production (Chapter 2).

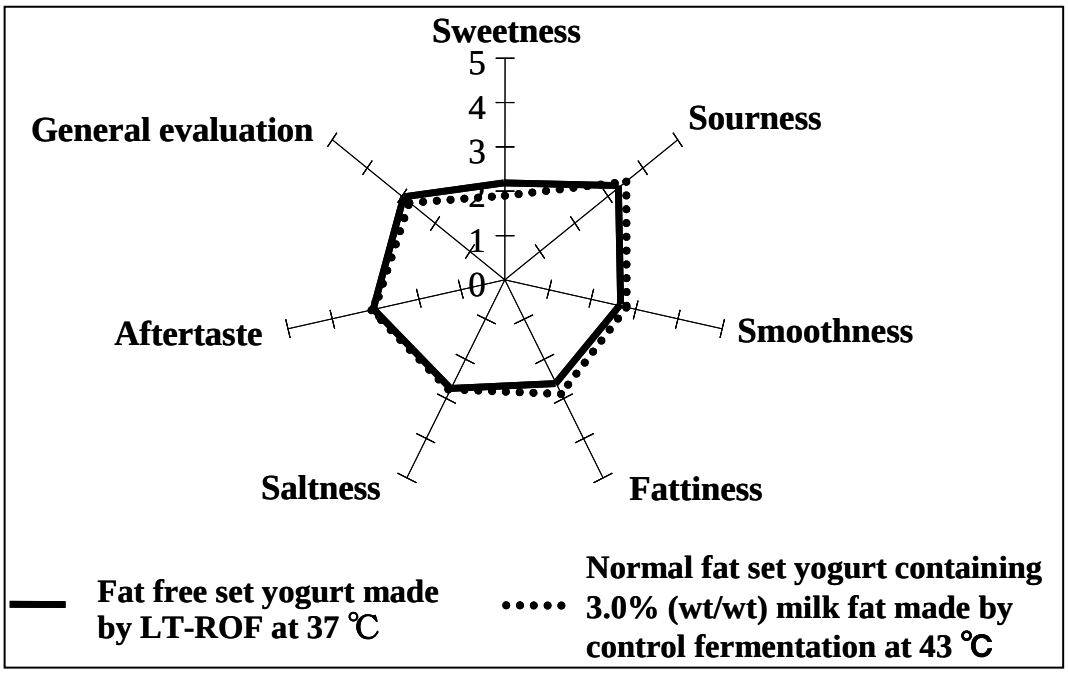
In this paper, the author shows that ROF brought about faster cell growth of *S. thermophilus* 1131 and earlier l-lactate and formate accumulation in milk medium, which is favorable to industrial yogurt production. *S. thermophilus* 1131 was found to work mainly to remove DO in yogurt fermentation. The results using H_2O-forming NADH oxidase (*nox-2*) defective mutant (Δ*nox*) of *S. thermophilus* 1131 revealed that Nox-2 played an important role for DO reduction during yogurt fermentation. Yogurt fermentation by a starter composed of Δ*nox* and *L. bulgaricus* 2038 was significantly slow, presumably because this starter could not reduce DO concentration to less than 2 mg/kg. These observations suggest that Nox-2 of *S. thermophilus* 1131 contributes greatly to yogurt fermentation. One of the remaining problems to be solved in relation of proto-cooperation in yogurt fermentation is the fact that depending on the specific combination of *L. bulgaricus* and *S. thermophilus*, strains, the efficiency of yogurt fermentation differs significantly. A favorable combination of two strains, *L. bulgaricus*

2038 and *S. thermophilus* 1131, was selected and used in this study. Further experiments are necessary to know whether the results obtained in this study are applicable to other combinations of two lactic acid bacteria (Chapter 3).

The set yogurt made by controlled fermentation at 37°C (low-temperature fermentation) had a smooth texture but had insufficient hardness to stand up to the impact of shaking during transport. Moreover, this low-temperature fermentation took a much longer time, which reduced the yogurt manufacturing efficiency. Combining ROF with low-temperature fermentation (LT-ROF) reduced the time required for low-temperature fermentation, and the yogurt made by the LT-ROF method also had sufficient hardness. Meiji Co., Ltd. has marketed a yogurt product made by LT-ROF which was named Meiji Bulgaria Yogurt LB81 Domashno Plain (2004 to 2008). “Domashno” means home-made in Bulgarian. This yogurt was a commercial product which effectively reproduced the Bulgarian traditional home-made yogurt prepared in unglazed pots.

Meiji Bulgaria Yogurt LB81 Domashno Plain

The author believes that LT-ROF is a favorable method for industrial-scale manufacture of set yogurt that possesses a smooth texture and strong curd structure (Chapter 1). LT-ROF method has made it possible to manufacture excellent fat free set yogurt without using sugar, stabilizers and thickeners. The fat free set yogurt product prepared by LT-ROF had almost the same fatty taste, smooth texture, and sufficient hardness to stand up to the impact of shaking during transport as the Japanese normal fat set yogurt product containing 3.0% (wt/wt) milk fat prepared by control fermentation at 43°C (Chapter 4).



Result of a sensory test carried out on the fat free set yogurt made by LT-ROF and the normal fat set yogurt made by control fermentation.

Meiji Co., Ltd. has marketed a fat free set yogurt product made by LT-ROF called Meiji Bulgaria Yogurt LB81 Zero Fat Plain since 2009. This yogurt product has become a big seller. In early 2014, the main product in the Meiji Bulgaria series also started to employ this LT-ROF technology (Meiji Bulgaria Yogurt LB81 Plain).

Therefore, even though Meiji Bulgaria Yogurt LB81 Domashno Plain is no longer sold, the LT-ROF fermentation technology developed for that product is still being used.

Meiji Bulgaria Yogurt LB81 Zero Fat Plain

Meiji Bulgaria Yogurt LB81 Plain

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3. NADH oxidase of *Streptococcus thermophilus* 1131 is required for effective yogurt fermentation with *Lactobacillus delbrueckii* ssp. *bulgaricus* 2038
Y. Sasaki[a], H. Horiuchi[a], H. Kawashima, T. Mukai, and Y. Yamamoto
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[a] These authours contributed equally to this work.

Patents

1. Method for producing fermented milk and fermented milk
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WO2004/049812, JP3644505B, US08236361B, etc.

2. Method for producing fermented milk and fermented milk
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3. Delicious Fermented Milk with Low Fat Content and Process for Producing the Same
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7. Cultured milk with low lactose content and method for manufacturing same
H. Horiuchi
WO2010/098086, SG173789B, CA2753348B, etc.

8. Fermented milk having little lactose and method for manufacturing same
H. Horiuchi
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脱酸素低温発酵法による新規なセットタイプヨーグルトの製造

堀内　啓史

論文内容の要旨

ヨーグルトを始めとする発酵乳の歴史は古く、約 5000 年前に遡るといわれている。その発祥地については中央アジアからブルガリアを中心としたバルカン半島にかけての一帯、トルコ周辺が知られている。発酵乳は人間が意識的に生み出したものではなく、乳が自然環境の中で偶然に発酵して出来上がったものである。やがて、乳を利用・発酵させる技術が発展し、それが伝播・継承されて、世界各地にその気候・風土に適した伝統的な発酵乳製品が生み出されていったといわれている。

一般に、ヨーグルトは 2 種の乳酸菌 *Lactobacillus delbrueckii* subsp. *bulgaricus*（*L. bulgaricus*）と *Streptococcus thermophilus*（*S. thermophilus*）の共生作用で乳を発酵させて生産する。これらの乳酸菌は、通性嫌気性菌であり、酸素の存在下でも活発に生育する。そのため、酸素がヨーグルトの発酵に及ぼす影響について着目した研究は殆ど無かったが、ブルガリア国伝統の素焼きの壷で作るヨーグルトの研究がきっかけとなり、ヨーグルトスターターである LB81 スターター（*L. bulgaricus* 2038, *S. thermophilus* 1131）を用いて、酸素がヨーグルトの発酵に及ぼす影響についての研究を行った。

1.「脱酸素発酵法」および「脱酸素低温発酵法」の開発

1-1.　ブルガリア国伝統のヨーグルト

ヨーグルトの本場ブルガリアには、素焼きの壷で作る昔ながらの伝統的なヨーグルトがある。絞りたての牛乳を煮立てて人肌くらいに冷ましてから、素焼きの壷に入れ、前日に作っておいたヨーグルトを種菌（スターター）として加える。その壷を布で包んで保温して放置すると、発酵してヨーグルトになっていく。発酵中に素焼きの壷が牛乳から水分を吸収し、牛乳が濃縮され、更に、壷の表面からその水分が蒸発する際に気化熱を奪うため「低温発酵」となる。このようにして作られたヨーグルトは、なめらかでコクがあり、非常においしい。そこで、この伝統的なヨーグルト（セットタイプのプレーンヨーグルト）を工業的に再現しようと試みた。

1-2.　ブルガリア国伝統のヨーグルトの工業的再現

セットタイプのプレーンヨーグルト（以降、ヨーグルトと略す）の工業生産は、まず、乳（生乳）および乳製品（脱脂粉乳、生クリーム、ホエイ濃縮物、等）を用いて、ヨーグルト原料（ヨーグルトベース）を調合する。これを 95 ℃ で殺菌、43 ℃ まで冷却し、ヨーグルトスターター（*L. bulgaricus* と *S. thermophilus*）を接種した後、容器に充填し、発酵室（43℃培養庫）にて静置発酵を行う。発酵時間の経過と共に乳酸菌が乳酸を生成するため、ヨーグルトベースは酸凝固により固まっていく（カードの形成）。発酵は、pH が約 4.7（無脂乳固形分が 10 % 程度の場合、酸度約 0.7 % に相当）になるまで行い、冷却によって発酵を止める。その結果、プリン状のヨーグルトとなる。

伝統的なヨーグルトの工業的再現のため、まず、43 ℃ に加温した生乳を素焼きの壺に注ぎ、ヨーグルトスターター（LB81 スターター：*L. bulgaricus* 2038, *S. thermophilus* 1131）を加え、43 ℃ の培養庫にて発酵させた。結果、時間と共に牛乳の温度は低下し、37℃程度で安定化した。また、発酵終了後、生乳は約 1.2 倍に濃縮されていた。従って、この伝統的なヨーグルトは、“濃縮した生乳”を 37 ℃ 程度の「低温発酵」させることで工業的に再現出来ると考えられた。“濃縮した生乳”は、生乳に脱脂粉乳、バター等を加え、無脂乳固形分および乳脂肪分を高めることで容易に工業的な再現ができた。

1-3.　「低温発酵」による発酵遅延

次に、「低温発酵」について検討した。「低温発酵」は、非常になめらかな組織のヨーグルトを作り出すことが出来るメリットがある反面、発酵に長時間を要するデメリットがある。通常ヨーグルトの発酵は、乳酸菌の乳酸生成が最も活発な 43 ℃ で行う（通常発酵）が、37 ℃ 程度で「低温発酵」を行うと、発酵の進行は著しくは遅延する。LB81 スターターを用いた「低温発酵」では、「通常発酵」に比べて発酵時間が約 40 分間長くなった。これは、工業的大量生産を行う場合の生産性を大きく低下させる。よって、「低温発酵」を工業化するためには、発酵時間を短縮させる新たな発酵法の確立が不可欠であった。

1-4.「脱酸素発酵法」による発酵時間の短縮

発酵時間を短縮する方法を検討する中で、酸素がヨーグルトの発酵に及ぼす影響についての研究を行った。ヨーグルトに用いる *L. bulgaricus* と *S. thermophilus* は通性嫌気性菌であり、酸素の存在下においても活発に生育する。そのため、その発酵において酸素（乳中の溶存酸素）は殆ど着目されてこなかった（元々ヨーグルトは羊や牛などの乳に偶然入り込んだ乳酸菌の作用で自然に誕生した）。しかし、LB81 スターターを用いて、酸素が発酵に及ぼす影響についての研究を行った結果、発酵開始時は 6〜7 ppm(mg/kg)程度ある溶存酸素濃度が、発酵の進行に伴って減り、その濃度が 0 ppm 程度まで下がってから乳酸酸度が上昇することが分かった。そこで、酸素がヨーグルトの発酵を阻害していると推察し、あ

らかじめ酸素を減らし、嫌気状態としてから発酵する方法を検討した。具体的には、LB81スターターを乳に接種後、窒素ガス置換処理によって乳中の酸素を 0 ppm に減らしてから発酵することで発酵時間が短縮されることを見出した。本発酵法を「脱酸素発酵法 (reduced dissolved oxygen fermentation: ROF)」と命名した。

1-5.「脱酸素低温発酵法」の開発

「脱酸素発酵法」を 37 ℃ の「低温発酵」と組み合わせたところ、発酵時間を 40 分間短縮することができた。これにより「低温発酵」の問題点を解決でき、ブルガリア国伝統の素焼きの壺で作るヨーグルトの工業的再現に成功した。「低温発酵」と「脱酸素発酵法」を組み合わせた発酵法を「脱酸素低温発酵法 (low temperature reduced dissolved oxygen fermentation: LT-ROF)」と命名した。

1-6.「脱酸素低温発酵法」で作られるヨーグルトの物性

「脱酸素低温発酵法」で作られるヨーグルトはなめらかな物性は、官能評価および物性測定装置によって示される。その物性は主に「低温発酵」に起因する。「低温発酵」で作られるヨーグルトがなめらかな物性になることは一般的に知られていることであるが、それは、「低温発酵」が発酵に長時間を要し、ヨーグルトのカードが時間をかけてゆっくり形成されることが要因の 1 つとされている。しかし、「脱酸素低温発酵法」で作られたヨーグルトは短時間の発酵にも拘わらずなめらかな物性となるため、その理由について考察した。一般に、ヨーグルトは、酸度 0.4 %程度からカード形成が始まる。そして、酸度 0.7 % を発酵終了点と定めているため、酸度 0.4 % から 0.7 % に到達するのに要する発酵時間が「カード形成時間」となる。このカード形成時間が長いほど、なめらかな物性のヨーグルトになると考えられる。43 ℃ の「通常発酵」では、発酵時間 180 分間に対してカード形成時間が 50 分間であった。一方、「脱酸素低温発酵法」では、発酵時間全体は「通常発酵」と同じ 180 分間であるが、カード形成時間は、90 分間であった。「脱酸素低温発酵法」は、全体の発酵時間は短いが、カード形成時間が長いため、なめらかな物性のヨーグルトができると推察した。

2. 酸素が LB81 スターターの共生発酵に及ぼす影響（発酵時間短縮のメカニズム）

2-1. LB81 スターターの共生発酵

予め酸素を低減してから LB81 スターター（*L. bulgaricus* 2038 と *S. thermophilus* 1131）で発酵する「脱酸素発酵法」および「脱酸素低温発酵法」により発酵時間が短縮されるメカニズムの解明に取り組んだ。

乳を *L. bulgaricus* と *S. thermophilus* 単菌で培養した場合に比べて、混合で培養した場合、乳酸の生成が活発になる場合がある。これを *L. bulgaricus* と *S. thermophilus* の共生発酵というが、*L. bulgaricus* は乳中のカゼインを分解してペプチド等を生成し、*S.*

thermophilus は蟻酸等を生成し、これらの物質がお互いの生育を促進することが知られている。*L. bulgaricus* 2038 および *S. thermophilus* 1131 においても共生発酵が認められ、カゼインペプチドと蟻酸による生育促進効果も認められた。

2-2. 「脱酸素発酵法」による LB81 スターターの共生発酵促進

L. bulgaricus 2038 および *S. thermophilus* 1131 の脱脂粉乳培地（以降、培地と略す）における混合培養（共生発酵）では「脱酸素発酵法」による発酵促進が認められたが、それぞれの単菌培養時には「脱酸素発酵法」による発酵時間短縮効果は認められなかった。このことから、酸素は乳酸菌の生育を阻害するのではなく、共生発酵を阻害すると考えられた。

そこで、*L. bulgaricus* の生育促進物質の 1 つである蟻酸に着目し、酸素が共生発酵におよぼす影響について検討した。次にヨーグルト発酵中に培地に蓄積される蟻酸量を測定した（発酵中、*L. bulgaricus* 2038 は蟻酸を消費すると考えられるので、培地の蟻酸濃度は *S. thermophilus* 1131 が生成した蟻酸と *L. bulgaricus* が消費した蟻酸の差であると考えられる)。「脱酸素発酵法」と「通常発酵」で比較した結果、培地中に蟻酸が検出され始める時間が脱酸素発酵法では、約 30 分早まった。すわわち、「脱酸素発酵法」による発酵時間の短縮は、*S. thermophilus* 1131 による蟻酸生成が早まり、結果として LB81 スターター（*L. bulgaricus* 2038 と *S. thermophilus* 1131 ）の共生発酵が促進されたためであると推察された。

2-3. 酸素による LB81 スターターの共生発酵抑制

次に、LB81 スターターの発酵中の培地への蟻酸蓄積量に及ぼす溶存酸素濃度の影響について調べた。培地中の溶存酸素濃度を 1, 2, 4, 6 ppm の一定濃度に固定した場合、いずれも、発酵が遅延し、特に 2 ppm, 4ppm, 6 ppm では発酵が殆ど進まなかった。溶存酸素を 1ppm, 6ppm に固定した時の蟻酸濃度を測定した結果、いずれの条件においても培地への蟻酸の蓄積は全く認められなかった。すなわち、僅か 1 ppm の溶存酸素が蟻酸の生成を抑制し、共生発酵を抑制したと考えられた。

なお、*L. bulgaricus* 2038 および *S. thermophilus* 1131 それぞれの単菌発酵では、酸素の有無は発酵速度に影響しなかった。

また、溶存酸素濃度を固定したヨーグルト発酵の系に蟻酸を添加した場合について検討した。蟻酸 Na を 1 mM 濃度で添加し、溶存酸素濃度を 0 ppm, 6 ppm に固定して発酵した。結果、元々発酵が促進されている 0 ppm に固定した発酵においては、蟻酸添加により更に発酵が促進されることはなかったが、発酵が著しく阻害される 6 ppm に固定した場合においては、蟻酸を添加することで発酵の遅延が大幅に回復した。

3. ヨーグルトの発酵における *S. thermophilus* 1131 の NADH オキシダーゼの重要性

L. bulgaricus 2038 および *S. thermophilus* 1131 は、両菌ともに、単菌発酵時においても、混合発酵時と同様に溶存酸素濃度が減少してから発酵が活性化するが、*L. bulgaricus* 2038 では、溶存酸素が過酸化水素に還元されるのに対して、*S. thermophilus* 1131 では過酸化水素は検出されなかった。*S. thermophilus* 1131 は添加した過酸化水素を分解できないことから、過酸化水素を生じないで溶存酸素を処理することが推定され、*S. thermophilus* 1131 の水生成型 NADH オキシダーゼ遺伝子のノックアウト株を作成した。ノックアウト株と *L. bulgaricus* 2038 との共生発酵では、*S. thermophilus* 1131 の野生株と *L. bulgaricus* 2038 との共生発酵に比べて、溶存酸素の低下が遅くなり、生育も遅くなり、過酸化水素の蓄積量も増えた。このことから、*S. thermophilus* 1131 の NADH オキシダーゼは、ヨーグルトの発酵において溶存酸素を減らすための重要な働きをしていることが示唆された。

4.「脱酸素低温発酵法」による無脂肪ヨーグルトの開発

「脱酸素低温発酵法」は、コクのあるセットタイプヨーグルトの製造を可能とした。まろやかさとコクは、官能的な脂肪感の向上に繋がるが、「脱酸素低温発酵法」で作られた脂肪分の無い（無脂肪）ヨーグルトは、脂肪分は 3%の通常のヨーグルトと同等の脂肪感になることを官能検査により確認した。すなわち、「脱酸素低温発酵法」により、従来には無い“おいしい無脂肪ヨーグルト”が実現できた。無脂肪ヨーグルトの欠点である“コクの無さ”や“水っぽさ”を、寒天やゼラチン等の添加物を加えることなく（原料コストをかけることなく）、“発酵技術”によって補うことが出来た。

5. 総括

L. bulgaricus 2038 、*S. thermophilus* 1131 を用いたヨーグルトの発酵において、発酵開始時は 6～7 ppm 程度ある溶存酸素濃度が、発酵の進行に伴って減り、その濃度が 0 ppm 程度まで下がってから乳酸酸度が上昇することが分かった。乳中の溶存酸素を低減してから *L. bulgaricus* 2038 と *S. thermophilus* 1131 で発酵する（脱酸素発酵法: ROF）ことで、ヨーグルトの発酵時間 が短縮された。そして、「脱酸素発酵法」と低温発酵を組み合わせる（脱酸素低温発酵法: LT-ROF）ことで、短時間の発酵でなめらかなセットタイプヨーグルトを製造することが可能となり、従来に無い“おいしい無脂肪ヨーグルト”の商品開発に成功した。「脱酸素発酵法」の発酵時間短縮のメカニズムを検討した結果、酸素の有無が *L. bulgaricus* 2038 と *S. thermophilus* 1131 それぞれ単菌の発酵に影響しなかったことから、酸素は両菌の共生発酵に影響すると推察した。共生発酵では、*L. bulgaricus* は乳中のカゼインを分解してペプチド等を生成し *S. thermophilus* は蟻酸等を生成し、これらの物質がお互いの生育を促進することが知られている。「脱酸素発酵法」において、*L. bulgaricus* の生育促進物質の 1 つである蟻酸に着目した結果、*S.*

thermophilus 1131 の蟻酸生成が通常の発酵に比べて早まることが分かった。これにより、*L. bulgaricus* 2038 と *S. thermophilus* 1131 の共生発酵の発現が早まり、発酵時間が短縮されると推察した。また、*S. thermophilus* 1131 の水生成型 NADH オキシダーゼ遺伝子のノックアウト株と *L. bulgaricus* 2038 との共生発酵では、溶存酸素の低下が遅くなり、生育も遅くなったことから、*S. thermophilus* 1131 の NADH オキシダーゼは、ヨーグルトの発酵において溶存酸素を減らすための重要な働きをしていることが示唆された。

ヨーグルトはその歴史が非常に古く、その発酵については、分かっているようで、解明しなければならないことが数多くあり、*L. bulgaricus* と *S. thermophilus* の共生発酵はその代表例である。今回、「脱酸素発酵法」に関する研究を進めていく中で、酸素が共生発酵に影響していることを突き止めたことは、共生発酵のメカニズム解明の大きな手がかりになると考える。