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Development of food grade media for the preparation of *Lactobacillus plantarum* starter culture

Yuki Sawatari, Tomomi Hirano, and Atsushi Yokota*

Laboratory of Microbial Physiology, Research Faculty of Agriculture, Hokkaido University, Sapporo, Hokkaido 060–8589, Japan

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Based on MRS medium, two types of food grade (FG) culture media (FG medium I and FG medium II) for the preparation of a concentrated starter culture of *Lactobacillus plantarum* NRIC 0380 to manufacture a new type of instant Chinese noodle, the fermented instant Chinese noodle, were developed using FG materials. FG medium I, which is for normal static culture, contains table sugar (sucrose), Yeast peptone standard type F, Sunsoft Q-17S (emulsifier), sodium acetate, trisodium citrate and $MnSO_4 \cdot 4–5H_2O$. FG medium II was designed to be used for the pH-controlled jar fermentor culture conditions. Therefore, sodium acetate and trisodium citrate as a buffer to prevent acidification of medium were omitted from FG medium I. When *L. plantarum* NRIC 0380 was cultured under the pH-controlled jar fermentor culture conditions, the kinetics of growth, sugar consumption and lactic acid production in FG medium II were quite similar to those observed in the Difco Lactobacilli MRS Broth. Furthermore, growths of many lactobacilli strains isolated from various fermented foods in FG medium I were also quite similar to those observed in MRS medium. Therefore, simple and practical FG media for the culture of lactobacilli were successfully established.

Key Words——culture medium; food grade; instant Chinese noodle; lactic acid bacteria; lactobacilli; *Lactobacillus plantarum*; starter culture; Tween 80

Introduction

A new type of instant Chinese noodle, the fermented instant Chinese noodle with the application of lactic acid fermentation by *Lactobacillus plantarum* NRIC 0380 was successfully developed in our laboratory (Sawatari et al., 2005). For the industrialization of this fermented instant Chinese noodle, it is necessary to develop an appropriate medium for the preparation of the starter culture of *L. plantarum* NRIC 0380. The medium should be safe and inexpensive in terms of food manufacture. Furthermore, ease both in preparation and in cell harvest is also required. Instead of MRS medium (De Man et al., 1960), a laboratory medium for lactobacilli, several media for the preparation of starter cultures in industrial scale have been developed. In the dairy industry, semisynthetic liquid medium, skim milk and cheese whey-based media are commonly used (Gilliland, 1985). Concentrated starter cultures of lactic acid bacteria produced by general dairy methods have been commercially available for fermented vegetables (Fleming et al., 1985). A commercial culture medium for *Pediococcus cerevisiae* to ferment meat products has been described, which consisted of several natural nutrients including skim milk, dextrose and some minerals (Bacus and Brown, 1985). However, the preparation of concentrated

* Address reprint requests to: Dr. Atsushi Yokota, Kita 9 Nishi 9, Kita-ku, Sapporo, Hokkaido 060–8589, Japan.
Tel: +81–11–706–2501 Fax: +81–11–706–4961
E-mail: yokota@chem.agr.hokudai.ac.jp

starter cultures using skim milk as the culture medium is inefficient, since it is difficult to harvest the cells by centrifugation. Whey-based medium is associated with cheese factories and often requires additional nutrients for the maximum growth of starter culture bacteria (Pont and Holloway, 1968). Thus, semisynthetic liquid medium to fulfill nutritional requirements of the target starter strain seems suitable for the culture. Several liquid media containing various kinds of organic nutrients have been reported for the preparation of dairy starter cultures (Peebles et al., 1969; Piatkiewicz et al., 1977). However, it has been reported that the amino acid requirements of *L. plantarum* are much simpler than those of dairy and intestinal isolates (Morishita et al., 1981). Furthermore, recent genome analysis revealed that defects in amino acid biosynthetic capacity in *L. plantarum* (Kleerebezem et al., 2003) are less than those in *L. acidophilus* (Altermann et al., 2005) or in *L. johnsonii* (Pridmore et al., 2004). Therefore, for the culture of *L. plantarum* NRIC 0380, nutritionally simpler medium seems to be applicable. From these backgrounds, we have developed culture media suitable for the preparation of starter culture of *L. plantarum* NRIC 0380 by the modification of MRS medium. In this report, we refer this medium as "food grade (FG) medium." Furthermore, growths of other *Lactobacillus* species from different origin were tested to check the feasibility of this medium for the culture of a wide variety of lactobacilli strains.

Materials and Methods

Bacterial strains. The bacterial strains used in this study are listed in Table 2. They were obtained from the Japan Collection of Microorganisms (JCM, Wako, Japan), the Laboratory of Applied Microbiology, Research Faculty of Agriculture, Hokkaido University (AHU, Sapporo, Japan) and the Culture Collection Center, Tokyo University of Agriculture (NRIC, Tokyo, Japan).

Chemicals. Difco Lactobacilli MRS Broth (DLMB), Bacto Proteose Peptone No. 3, Bacto Beef Extract and Bacto Yeast Extract were obtained from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Tween 80 was a product from Junsei Chemical Co., Ltd., Tokyo, Japan. Yeast peptone standard type F (Deutsche Hefewerke GmbH, Hamburg, Germany), Sunsoft Q-17S (Taiyo Kagaku Co., Ltd., Yokkaichi, Japan), K_2HPO_4 (Organo Corporation, Tokyo, Japan), sodium acetate (anhydrate) (The Nippon Synthetic Chemical Industry Co., Ltd., Osaka, Japan), trisodium citrate (Tanabe Seiyaku Co., Ltd., Osaka, Japan) and $MgSO_4 \cdot 7H_2O$ (Tomita Pharmaceutical Co., Ltd., Naruto, Japan) were used as FG components for the development of FG media.

Compositions of MRS medium and FG media. MRS medium was used as the basal medium for the development of FG media for *L. plantarum* NRIC 0380. The composition of MRS medium is shown in Table 1. All the components were commercially available laboratory reagents and the pH was adjusted to 6.5 with HCl. Basal FG medium (Table 1) was designed to replace components of MRS medium with FG components described in chemicals. Glucose was replaced with table sugar (sucrose). Triammonium citrate was replaced with trisodium citrate. Yeast peptone standard type F, which is made from bakery yeast in Europe and used as flavoring, was a substitute for Bacto Proteose Peptone No. 3, Bacto Beef Extract and Bacto Yeast Extract. Tween 80 is included in MRS medium as a water-soluble oleic acid source to support the growth of oleic acid requiring lactobacilli (Briggs, 1953; Partanen et al., 2001; Williams et al., 1947). Sunsoft Q-17S, which is made from coleseed in Japan and used as an emulsifier for food processing, was added to FG medium instead of Tween 80, since oleic acid and linoleic acid are the major components of this emulsifier. An omission test yielded FG medium I suitable for normal static culture of *L. plantarum* NRIC 0380. FG medium II was the simplest medium designed to culture *L. plantarum* NRIC 0380 in a pH-controlled jar fermentor. The development of these FG media will be described in RESULTS AND DISCUSSION. The pH values of these FG media were adjusted to 6.5 with NaOH.

Omission test for the optimization of FG medium. A seed culture of *L. plantarum* NRIC 0380 was grown overnight in DLMB at 30°C. The cells were harvested by centrifugation, washed twice with sterilized saline (8.5 g/L of NaCl), and resuspended in the same solution. The cell suspension was inoculated into Basal FG medium and each omission medium shown in Fig. 1 at optical density at 660 nm (OD_{660}) of 0.03. After incubation for 24 h at 30°C, the growth was monitored by the measurement of OD_{660} after appropriate dilutions using a spectrophotometer, and the pH value of the culture supernatant was measured. All these cultures were conducted in screw-capped test tubes tightly

Table 1. Compositions of MRS medium and FG media.

Compositions	MRS medium	Basal FG medium	FG medium I	FG medium II
	g/L			
Glucose	20	—	—	—
Table sugar[a]	—	20	20	20
Bacto Proteose Peptone No. 3	10	—	—	—
Bacto Beef Extract	10	—	—	—
Bacto Yeast Extract	5	—	—	—
Yeast peptone standard type F[a]	—	25	25	25
Tween 80	1	—	—	—
Sunsoft Q-17S[a]	—	0.75	0.75	0.75
K_2HPO_4[b]	2	2	—	—
Sodium acetate[b]	5	5	5	—
Triammonium citrate	2	—	—	—
Trisodium citrate[a]	—	2.1	2.1	—
$MgSO_4 \cdot 7H_2O$[b]	0.1	0.1	—	—
$MnSO_4 \cdot 4–5H_2O$	0.05	0.05	0.05	0.05

The pH value of each media was adjusted to 6.5 using HCl or NaOH.

[a] These ingredients are substitutes for components in MRS medium. Details are described in MATERIALS AND METHODS.

[b] Food additives were used for the preparation of FG media.

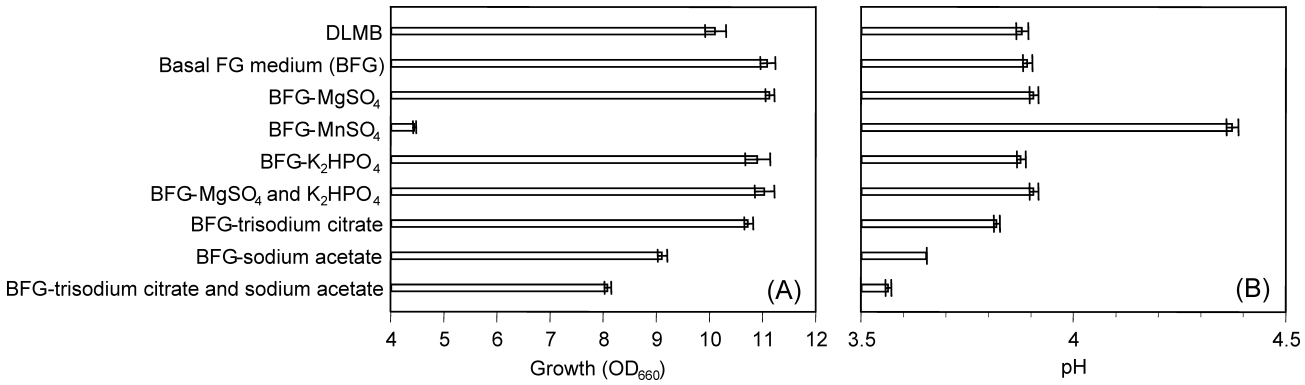

Fig. 1. Results of omission test.

The bars indicate growth (A) of *L. plantarum* NRIC 0380 and pH value (B) in each omitted FG medium after 24 h of culture. The values are the average of two independent experiments. The error bars represent the SD. DLMB, Difco Lactobacilli MRS Broth.

sealed with a rubber septum containing 3 ml of each medium. No other anaerobic treatment was applied.

Jar fermentation. To evaluate performance of FG medium II, *L. plantarum* NRIC 0380 was cultured in a jar fermentor with DLMB as a reference medium. Seed culture was conducted in 5 ml of Basal FG medium when the subsequent jar fermentor culture was carried out in FG medium II, while DLMB was used when DLMB was employed as the jar fermentor medium. Each seed culture was conducted at 30°C until the late exponential phase. Then the cells were harvested by centrifugation, washed twice and resuspended in 5 ml of sterilized saline. The jar fermentation experiments were conducted by the inoculation of 4 ml of the cell suspension into 1 L of the respective medium in a 2-L jar fermentor (ABLE Corporation, Tokyo, Japan) to give a viable cell concentration of around 1.7×10^7 colony-forming units (cfu)/ml. The jar fermentors were incubated at 30°C with stirring at 100 rpm. The pH was controlled at 6.5 with 5 M NaOH. During the culture, the

viable counts, concentrations of sugar and lactic acid in the culture broth were monitored at appropriate intervals. Viable counts of *L. plantarum* NRIC 0380 were conducted by pour-plating with half-strength DLMB containing 10 g/L of agar. The plates were incubated at 30°C for 48 h under anaerobic conditions using AnaeroPack (O_2 absorb, CO_2 generate, Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). Lactic acid in the culture supernatant was determined by HPLC as described in our preceding paper (Sawatari et al., 2005). Sucrose in FG medium II and glucose in DLMB were analyzed by the phenol-sulfuric acid method. Zero point five milliliter of 5% (w/v) phenol solution was mixed with the same volume of an appropriately diluted culture supernatant and then 2.5 ml of conc. H_2SO_4 was added to start the reaction. After 30 min of retention at room temperature, absorbance of this mixture was measured at 490 nm. Sucrose and glucose were used as the standards, respectively.

Feasibility of FG medium I for the culture of wide variety of lactobacilli strains. Growth experiments were conducted using various strains of lactobacilli shown in Table 2. Seed culture of each strain was conducted at 30 or 37°C (depending on the optimum growth temperature of each type strain, indicated in the footnote of Table 2) in 3 ml of DLMB overnight. The cells were harvested by centrifugation, washed twice with sterilized saline, and resuspended in the same solution. The cell suspension was inoculated into 3 ml of FG medium I and its modified medium in which sucrose was replaced with glucose at OD_{660} of 0.03. Incubation was carried out at the same temperature as the seed culture for 24 h, and the growth was measured by OD_{660} of the culture broth after appropriate dilutions using a spectrophotometer. The requirement for Tween 80 of each strain was also checked in MRS medium by monitoring the growth in the presence or absence of Tween 80. The same inoculum as in the feasibility test of FG medium I was transferred to 3 ml of MRS medium at OD_{660} of 0.03 and cultured as described above. All these cultures were conducted in the aforementioned screw-capped test tubes.

Results and Discussion

Design of basal FG medium

As mentioned in MATERIALS AND METHODS, Basal FG medium was designed by replacing components of MRS medium with FG materials. Among the components of Basal FG medium listed in Table 1, concentrations of Yeast peptone standard type F (substitute for Bacto Proteose Peptone No. 3, Bacto Beef Extract and Bacto Yeast Extract in MRS medium) and Sunsoft Q-17S (substitute for Tween 80 in MRS medium) were optimized for maximum growth of *L. plantarum* NRIC 0380. First, the effect of Sunsoft Q-17S concentration on the growth of *L. plantarum* NRIC 0380 was investigated under Yeast peptone standard type F concentration fixed at 25 g/L. Although *L. plantarum* NRIC 0380 showed substantial growth without addition of Sunsoft Q-17S, the growth was enhanced by the addition of this emulsifier up to 0.75 g/L (data not shown). When Yeast peptone standard type F concentration was varied at a fixed Sunsoft Q-17S concentration of 0.75 g/L, the maximum growth was obtained at a concentration of more than 25 g/L (data not shown). From these results, the composition of the Basal FG medium was established as shown in Table 1. As seen in Fig. 1, *L. plantarum* NRIC 0380 grew in this medium at the same level as that observed in DLMB. Therefore, we judged that the composition of the Basal FG medium was appropriate.

Omission test for the optimization of FG medium

To simplify the Basal FG medium, an omission test was conducted. The effects of the omission of each mineral salt and organic acid on the growth and medium pH in the culture of *L. plantarum* NRIC 0380 were investigated (Fig. 1). Omission of $MgSO_4 \cdot 7H_2O$ or K_2HPO_4 showed no adverse effect on the growth of *L. plantarum* NRIC 0380. Even when both of these components were removed, the growth and medium pH were still comparable to those observed in Basal FG medium. Probably, these mineral salts were sufficiently supplied from Yeast peptone standard type F. Thus, $MgSO_4 \cdot 7H_2O$ and K_2HPO_4 were judged to be dispensable under these conditions. On the other hand, $MnSO_4 \cdot 4–5H_2O$ was found to be indispensable for the growth of *L. plantarum* NRIC 0380. It has been reported that superoxide is removed by manganese-catalyzed scavenging activity in *L. plantarum* lacking both superoxide dismutase and catalase (Gregory and Fridovich, 1974), and that the growth of this lactic acid bacterium in the presence of oxygen requires a high concentration of Mn(II) (Archibald and Fridovich, 1981). The current result agrees well with these previous observations. Omission of sodium acetate lowered the growth of *L. plantarum* NRIC 0380 with reduction

Table 2. Growth experiments of lactobacilli strains in FG medium I and in MRS medium with or without Tween 80.

Species	Strains	Source	Growth (OD_{660})[a]			
			FG medium I		MRS medium	
			Sucrose	Glucose	+Tween 80	−Tween 80
Lactobacillus acidophilus[b]	JCM 1132^T	Human feces	0.6±0.2	0.6±0.1	4.1±0	0.2±0
L. brevis	NRIC 0137	Fermented bamboo shoots	0.3±0.1	2.1±0.5	6.3±0	6.1±0.1
L. casei subsp. *casei*[b]	JCM 1134^T	Cheese	0.5±0	5.9±0.5	6.5±0.3	6.3±0.5
	NRIC 1917	Sugar cane wine	5.8±0.1	6.8±0.3	6.2±0.8	6.4±0.8
L. curvatus	JCM 1096^T	Milk	0.3±0	6.9±0.3	6.1±0.1	0.4±0
	AHU GD 12	"Ragi tape" (starter of sake)	0.2±0.1	5.3±0.4	5.4±0.1	0.3±0
L. delbrueckii subsp. *bulgaricus*[b]	JCM 1002^T	Bulgarian yogurt	0.3±0	0.3±0	4.7±0.6	0.2±0
L. delbrueckii subsp. *lactis*[b]	JCM 1248^T	Emmental (Swiss) cheese	0.6±0.2	0.7±0.1	3.1±0.7	0.1±0
L. fermentum[b]	JCM 1173^T	Fermented beets	4.1±0.3	6.4±0.4	6.1±0	6.1±0.3
L. gasseri[b]	JCM 1131^T	Human intestine	5.6±0.5	5.7±0.3	7.2±0.4	0.1±0
L. helveticus[b]	JCM 1120^T	Emmental (Swiss) cheese	0.1±0.1	3.6±1.9	3.4±1.1	1.4±1.2
L. mali	JCM 1116^T	Apple juice from cider press	0.3±0	5.8±0.1	5.7±0.5	5.3±0.2
L. paracasei subsp. *paracasei*	JCM 8130^T	Milk products	0.7±0	7.0±0	7.5±0.2	7.2±0.3
	NRIC 1946	Coconut juice	0.9±0	9.6±0.5	7.7±0.1	7.6±0.3
L. paracasei subsp. *tolerans*	JCM 1171^T	Pasteurized milk	0.1±0	3.2±0.2	4.9±0.3	0.3±0
	NRIC 1940	Coconut juice	1.9±0.2	8.6±0.2	8.7±0	8.1±0.4
L. pentosus	JCM 1558^T	Corn silage	10.0±0.1	11.1±0.1	9.9±0.3	9.8±0.3
	NRIC 0413	Fermented tea leaves	11.3±0.2	11.5±0.2	11.3±1.7	12.4±0.3
L. plantarum	JCM 1149^T	Pickled cabbage	11.5±0.2	12.9±0.1	12.9±0.8	12.3±0.3
	NRIC 0380	Pickles	11.2±0.2	11.6±0.1	11.8±1.1	11.5±0.3
L. sakei subsp. *sakei*	JCM 1157^T	"Moto" (starter of sake)	4.1±0.2	3.9±0.3	4.5±0.1	3.9±0.5

[a] The values indicate growth (OD_{660}) of lactobacilli strains in each media after 24 h of culture. Each value is the mean±SD of two independent experiments.

[b] Cultures were conducted at 37°C. The rest of the strains were cultured at 30°C.

of medium pH. The tendency is very obvious when trisodium citrate was omitted in combination with sodium acetate. Therefore, these components were found to work as buffers to prevent acidification of the culture medium. From these results, a simplified culture medium, designated FG medium I, was established for the culture of *L. plantarum* NRIC 0380 (Table 1).

Jar fermentation experiments

Since a pH-controlled jar fermentor will be used industrially for the culture of *L. plantarum* NRIC 0380

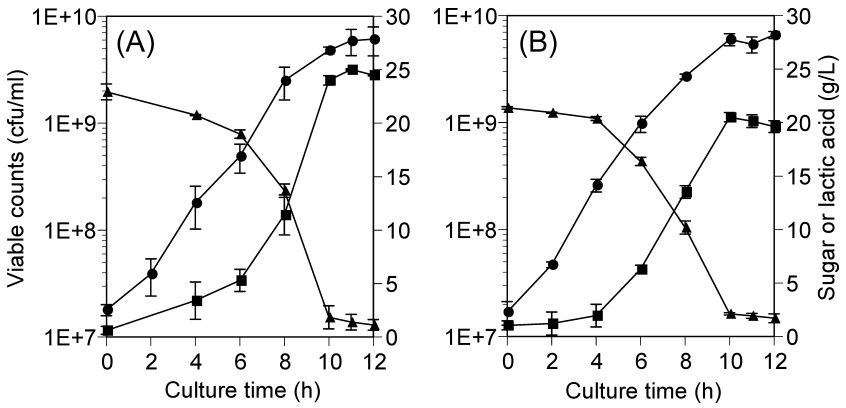

Fig. 2. Time course of the jar fermentation culture in FG medium II (A) or DLMB (B).

Changes in viable counts (●), concentrations of sugar (▲) and lactic acid (■). The values are the average of two independent experiments. The error bars represent the SD.

and also for most of the other cases, there is a possibility that the buffer components, sodium acetate and trisodium citrate, can be further removed from FG medium I. Therefore, we have investigated this possibility by culturing *L. plantarum* NRIC 0380 in FG medium I without these organic acids (termed FG medium II, Table 1) using a 2-L jar fermentor with pH controlled at 6.5. DLMB served as a reference medium. As shown in Fig. 2, kinetics of growth, sugar consumption and lactic acid production in FG medium II (Fig. 2A) were quite similar to those observed in the DLMB (Fig. 2B). Fermentable sugar(s) in both media were consumed completely after 10 h. The similar growth rates, 0.81 h^{-1} in FG medium II and 0.84 h^{-1} in the DLMB, were obtained with the final cell concentrations of 6.1×10^9 cfu/ml in FG medium II and 6.6×10^9 cfu/ml in the DLMB after the culture for 12 h. *L. plantarum* is a facultatively heterofermentative lactic acid bacterium that can convert 1 mol of sucrose to 4 mol of lactic aicd and 1 mol of glucose to 2 mol of lactic acid. In this experiment, 63.7 mM sucrose (21.8 g/L) was converted to 265.3 mM lactic acid (23.9 g/L) in FG medium II, and 109.3 mM glucose (19.7 g/L) was converted 206.4 mM (18.6 g/L) lactic acid in the DLMB. Therefore, the conversion rates of lactic acid fermentation were almost theoretical in these cultures. From these results, the composition of FG medium II was found to be sufficient for the culture of *L. plantarum* NRIC 0380 in a pH-controlled jar fermentor. Almost the same fermentation profile was also obtained in this medium when *L. plantarum* NRIC 0380 was cultured in 1,200 L FG medium II prepared in a pH-controlled jar fermentor (data not shown), thereby confirming the feasibility of FG medium II in industrial operation. Furthermore, the starter culture thus prepared was successfully applied for the manufacture of fermented instant Chinese noodles (data not shown). Therefore, it was concluded that FG medium II was the simplest and most cost-saving practical medium for the preparation of concentrated starter culture of *L. plantarum* NRIC 0380 in the food industry.

Feasibility of FG medium I for the culture of a wide variety of lactobacilli strains

To evaluate the feasibility of FG medium I for the culture of 15 species (including subspecies) of lactobacilli, growth experiments were conducted. Since several strains used in this experiment did not ferment sucrose, a modified FG medium I in which sucrose was replaced with glucose was also used (FG-Glu medium I). At the same time, the Tween 80 requirement of the each strain was checked in MRS mdium. Table 2 shows the results of those experiments. *L. casei* subsp. *casei*, *L. curvatus*, *L. fermentum*, *L. gasseri*, *L. helveticus*, *L. mali*, *L. paracasei* subsp. *paracasei*, *L. paracasei* subsp. *tolerans*, *L. pentosus*, *L. plantarum* and *L. sakei* subsp. *sakei* grew in both FG medium I and FG-Glu medium I or at least in FG-Glu medium I at the comparable level as that observed in MRS medium. These strains were isolated from a variety of fermented foods. Among these strains, *L. curvatus* JCM 1096^T and AHU GD 12, *L. gasseri* JCM 1131^T, *L. helveticus* JCM 1120^T and *L. paracasei* subsp. *tolerans* JCM 1171^T showed a requirement for Tween 80 in MRS medium for maximum growth. Thus, it was demonstrated that Sunsoft Q-17S functioned as

a source of oleic acid instead of Tween 80. Therefore, the feasibility of culture of lactobacilli strains isolated from various kinds of fermented foods in FG medium I was demonstrated. On the other hand, *L. acidophilus*, *L. delbrueckii* subsp. *blugaricus* and *L. delbrueckii* subsp. *lactis* grew neither in FG medium I nor in FG-Glu medium I. These strains originated from human feces and dairy products. It is not clear why these strains did not grow in FG medium I or in FG-Glu medium I. According to the investigation of the amino acid requirements of the several species of lactobacilli in chemically defined media (Grobben et al., 1998; Hébert et al., 2004; Morishita et al., 1981; Møretrø et al., 1998), it has been demonstrated that *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* exhibit wider requirements than *L. plantarum*, *L. pentosus* and *L. curvatus*. Thus, it was assumed that the requirements for amino acids for growth in these species of lactobacilli were not fulfilled in FG medium I. These results indicated that the established FG media, FG medium I and FG medium II, can be employed for the culture of not all but a wide variety of lactobacilli strains for food manufacture. The contribution of these media in the food industry will be expected.

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