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ARTICLE

EFFECTS OF CO-CULTURING LAB ON GABA PRODUCTION

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Two strains of lactic acid bacteria, *Streptococcus thermophilus* IFO13957 and *Lactobacillus delbrueckii subsp. bulgaricus* IAM1120, were examined for the effects of co-culturing on acid resistance and production of GABA. When *S. thermophilus* and *L. bulgaricus* were grown separately, the medium became acidic at around pH 5 and 4, respectively. For the latter it became highly acid-sensitive. When both strains were co-cultured, they appeared to be acid resistant and grew well. The co-culturing raised GABA level in the medium, which apparently coincided with the acidification of the medium. When each of the strains was cultured in the pH 3.5 and 6 medium, only *S. thermophilus* produced a large amount of GABA at pH 3.5 and survived. The results suggest that co-culturing of the two strains raises the each other's properties of the acidification of the medium and production of GABA or acid resistance.

Key words: GABA, acid tolerance, mixed cultured, *S. thermophilus*, *L. bulgaricus*

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Abbreviations: GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; LAB, lactic acid bacteria; MSG, monosodium glutamate; OPA, *O*-phthalaldehyde; BCP, bromocresol purple

γ-Aminobutyric acid (GABA) has neurotransmission, antihypertensive, and relaxation activities that are beneficial to human health (*1*). Owing to these health benefits, food production enriched with GABA is in high demand (*2, 3*). GABA is synthesized by decarboxylation reaction of glutamate with the enzyme glutamate decarboxylase (GAD) which is widely distributed among mammals, plants and microorganisms, including lactic acid bacteria (LAB) (*4-9*). GABA production in LAB relies upon GAD and is induced when pH becomes acidic. Some of LAB are capable of surviving under strongly acidic conditions due to the presence of active GAD that utilizes intracellular proton to make LAB acid-resistance. The level of acidity during the lactate fermentation depends upon LAB strains in which the production of lactate is highly variable. GAD isolated from LAB exhibits an optimum pH at around pH 4-5. For example, the optimum pH for GAD from *i.e., L. brevis* and *L. lactis* are pH 4.2-4.5 (*9*) and pH 4.7 (*6*), respectively. The GABA production by *L. brevis* (*8, 10, 11, 12*)and by *L. Buchner* (*13*) was reported and those LAB have been used for food production; however, their detailed mechanism of GABA production has not been fully understood.

In the production process of yogurt, two strains of LAB, *L. delbrueckii subsp. bulgaricus* and *S. thermophilus*, were co-cultured in order to facilitate the growth (*14-16*) and enhance lactate fermentation (*17, 18*). *L. bulgaricus* digests casein, the milk protein, to make amino acids which feed to *S. thermophius* (*19*). *S. thermophilus* produces formate which feeds to *L. bulgaricus* (*20*). As a result, an efficient and effective lactate fermentation was accomplished.

L. bulgaricus (*10, 21*) and *S. thermophilus* (*10*) were isolated from commercial yogurt. When they were cultured separately, a minute amount of GABA, less than 0.2 mg/ml, was produced When both strains were co-cultured, a quantitative conversion of glutamate to GABA was observed (*10*). The mechanism of the co-culturing enhanced GABA production has not been fully characterized.

In this study, we examined the co-culturing and evaluated the relationship between the growth and acid-resistance.

MATERIALS AND METHODS

Culture medium - Skim milk medium contained 10% skim milk and 1% monosodium glutamate (MSG). GYP medium, a rich nutritional medium, contained 1% yeast extract, 0.5% polypeptone, 1% glucose, 15 µM sodium acetate $3H_2O$, 0.41 µM $MgSO_4$ $4H_2O$, 0.36 µM $FeSO_4$ $7H_2O$, 1.7 µM NaCl, and 1% MSG. For experiments examining the effects of acidity on the growth and GABA production, the initial pH of the medium was adjusted with HCl.

Bacteria species - LAB strains, *L. bulgaricus* IAM1120 and *S. thermophilus* IFO 13957, were from the stock in our laboratory.

Culture condition - LAB strains were pre-cultured in GYP medium for 24 h at 37 °C, then 1/1000th of the aliquot of the culture medium was cultured in the medium.

Cell counts - Cell population was estimated by counting the cell numbers after diluting the culture medium including 0.25% yeast extract, 0.5% polypeptone, 0.1% glucose, 0.1% polysorbate 80, 0.83 mM cysteine, 0.1 mM bromocresol purple and 1.5% agarose and incubating for 48h at 37°.

GABA analysis in the medium - GABA was detected as a single peak on amino acids analysis. Amino acid analysis was carried out on a Shimadzu HPLC (Kyoto, Japan) that employed pre-column derivatization (*22*). After centrifugation of the culture medium, 1 ml aliquot of the supernatant was mixed with 1 ml acetonitrile and the mixture was centrifuged again. An aliquot of 30 µl supernatant was mixed with 300 µl each of *O*-phthalaldehyde (OPA) solution and mercaptoethanol solution and allowed for 120 sec for derivatization. OPA solution was prepared by dissolving 20 mg OPA in 3 ml acetonitrile and 7 ml of 0.5 M potassium borate buffer, pH

10.0. Mercaptoethanol solution was prepared by mixing 50 µl mercaptoethanol in 10 ml of 0.5 M potassium borate buffer, pH 10.0. An aliquot of 30 µl of the reaction mixture was applied onto a TSK-gel 80TM column that had been equilibrated with 4 mM sodium citrate buffer at pH 6.0 in 7% acetonitrile solution. OPA labeled amino acids were eluted by raising acetonitrile concentration from 7 to

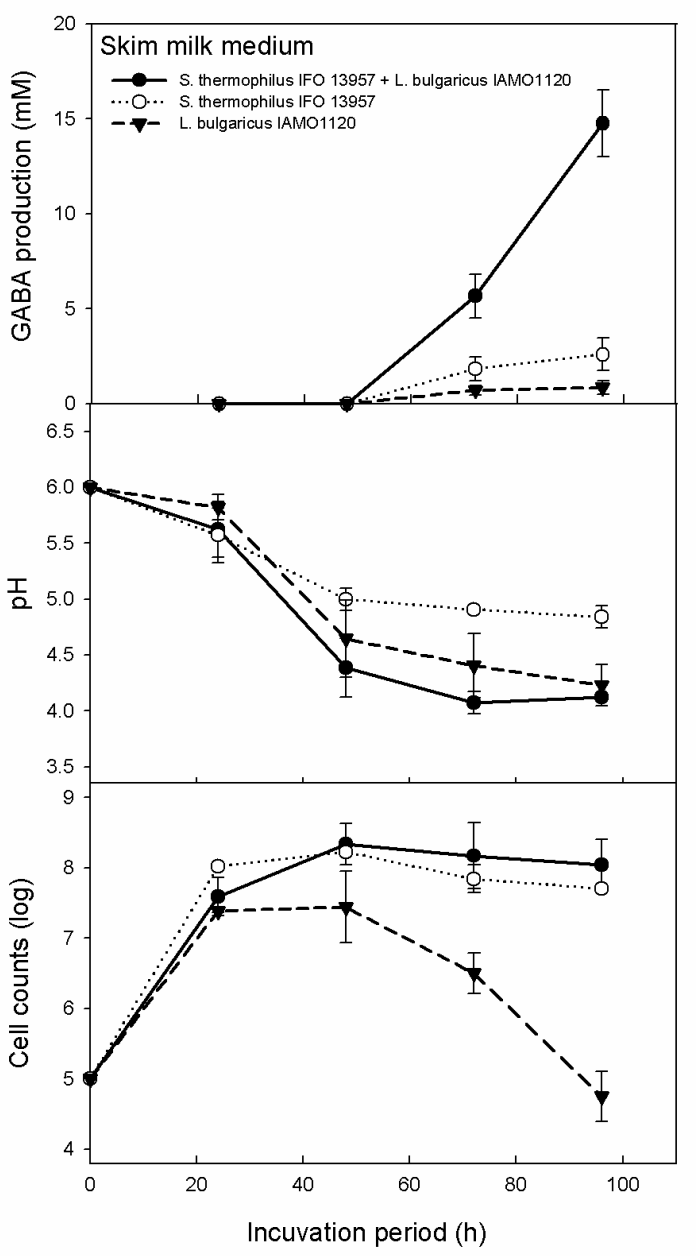

Fig. 1. The effect of the mixed culture on the growth and GABA production. *L. bulgaricus* IAM1120 and *S. thermophilus* IFO13957 were inoculated in 10% skim milk medium with 1% MSG at 37 ˚C.

40% in a linear manner at a flow rate of 0.8 ml/min. Peaks were monitored by Shimadzu RF-4000 fluorescence detector that was equipped with 350 nm excitation filter and 455 nm emission filter. The amount of GABA was estimated by comparing the peak area with the corresponding standard GABA.

RESULTS AND DISCUSSION

The effect of co-culturing on GABA production – Both *L. bulgaricus* IAM1120 and *S. thermophilus* IFO13957 strains were originally isolated from yogurt by one of the authors (K.Y.) and distinguished from previously described strains, *L. delbrueckii subsp. bulgaricus* (*10, 21*) and *S. thermophilus* (*10*). Two strains were cultured in skim milk medium individually or together. The cell growth, pH change in the medium, and GABA concentration in the medium were analyzed (Fig. 1).

The growth of *S. thermophilus* alone and co-culturing with *L. bulgaricus* IAM1120 showed the typical growth curve with the initial log-phase growth followed by the stationary-phase up to 100 h culturing. In contrast, the growth of *L. bulgaricus* IAM1120 alone showed the initial log-phase growth followed by the short stationary-phase, and then declined. The declining of the growth appeared to coincide with the acidification of the medium, which suggests that *L. bulgaricus* IAM1120 strain seems to be acid-sensitive. As the acidity of the *L. bulgaricus* IAM1120 growth medium reached down to pH 4, lactate synthesized by this strain could be accumulated enough to kill the cells. Although *L. bulgaricus* IAM11120 is a part of LAB family that should have acid tolerance built-in, this strain may not possess full capability of acid-resistance system.

S. thermophilus IFO13957 by itself grew well, but the pH of its medium was shifted down only to 5, the pH being significantly higher than that observed for *L. bulgaricus* IAM1120. It was probably

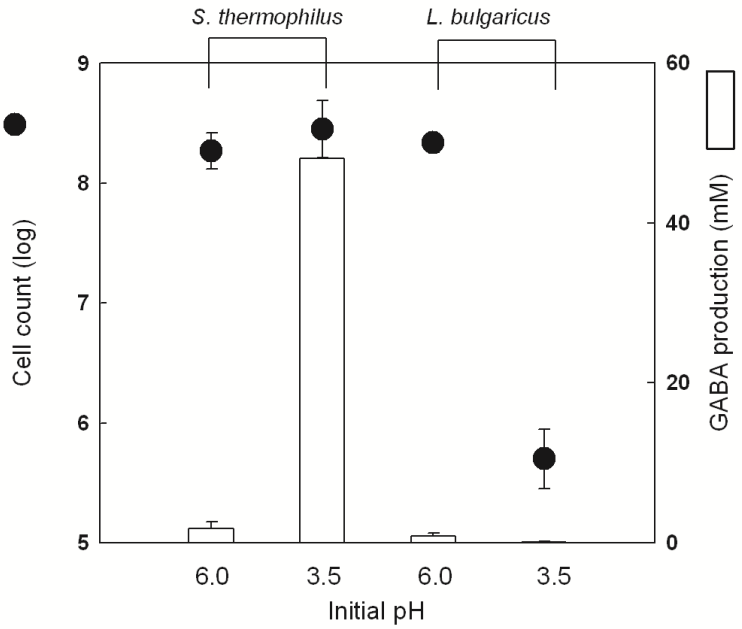

Fig. 2. The effect of the initial pH on the growth and GABA production.
L. bulgaricus IAM1120 or *S. thermophilus* IFO13957 was inoculated in GYP medium that contained 1% yeast extract, 0.5% polypeptone, 1% glucose, 15 µM sodium acetate $3H_2O$, 0.41 µM $MgSO_4$ $4H_2O$, 0.36 µM $FeSO_4$ $7H_2O$, 1.7 µM NaCl and 1% MSG at 37 °C for 48 h. Indicated pH was the initial pH of the medium.

due to the poor lactate production on this strain; although the details should wait for further study.

When both *L. bulgaricus* IAM1120 and *S. thermophilus* IFO13957 were co-cultured, the growth pattern looked like that of *S. thermophilus* IFO13957 alone whereas the shift in medium pH was like *L. bulgaricus* IAM1120 approaching to pH 4. In the co-cultured media, we were able to observe the growth of both *L. bulgaricus* IAM1120 and *S. thermophilus* IFO13957 by using the microscope, where *S. thermophilus* IFO13957 appeared to be predominant, about 80 to 90% population over *L. bulgaricus* IAM1120. Thus, it is likely that *L. bulgaricus* IAM1120 was able to survive in the acidic environment; hence, it became acid-resistant by co-culturing. More drastic difference was observed on the production of GABA, where the extensively large amount of GABA was produced in the co-cultured medium. The GABA production appeared to correlate

with the acidification of the medium; hence, it is probable that two strains collaborated in the production of GABA as a result of acid-resistant response.

L. bulgaricus IAM1120 produced little GABA throughout the culturing period (Fig. 1). Since GABA production is a key to bacterial acid-resistance, why the poor growth observed for *L. bulgaricus* IAM1120, when the medium became acidic, could be explained because of the lack of GABA-synthesizing capability. *S. thermophilus* IFO13957 also produced small amount of GABA during the culturing condition and was suspected for its incapability of making GABA. In order to answer to this question, we designed the experiment described in the next section.

The effect of acidity on survival of LAB and GABA production - To evaluate the effect of acidity, the growth of the LAB and GABA production were examined on *L. bulgaricus* IAM1120 and *S. thermophilus* IFO13957. These strains were cultured separately in the GYP medium with pH adjusted to 3.5 and 6.0. After 48 h fermentation, the growth and GABA production were analyzed (Fig. 2). *L. bulgaricus* IAM1120 grew well at pH 6.0, but grew poorly at pH 3.5. On the other hand, *S. thermophilus* IFO13957 grew well at both pH 6.0 and 3.5.

When *S. thermophilus* IFO13957 alone was cultured for nearly 100 h in the medium in which the initial pH was adjusted to 6, pH of the medium approached to near 5 (Fig. 1). We have initially assumed that *S. thermophilus* IFO13957 was not able to lower the pH of the medium and might not be able to survive at pH below 5. However, as shown in Fig. 2, *S. thermophilus* IFO13957 was capable of growing at pH 3.5 and it produced significant amount of GABA. This may suggest that *S. thermophilus* IFO13957 was likely to be responsible for GABA production when two strains were co-cultured.

By co-culturing the two strains, it is apparent that *L. bulgaricus* IAM1120 and *S. thermophilus* IFO13957 compensate each

other's weakness. Similar effect of co-culturing was previously reported for *S. thermophilus* and *L. bulgaricus*, in which the production of amino acids and formic acid (*14-16*), respectively, enhanced the production of lactate (*17, 18*).

The action of GABA production toward acid-resistance is not unique to *S. thermophilus* IFO13957; it is also reported in *E. coli* (*23, 24*) and *Lactobacillus* (*25*), where the mechanism known as glutamate dependent acid resistance (GDAR) plays a significant role. GAD is the enzyme to make GABA from L-glutamate, and utilizes a single proton during the catalysis (*4*). This consumption of the intracellular proton is responsible for shifting the intracellular pH toward neutral; hence, it helps microbes to survive under the acidic environment. As the increase of GABA level correlates the level of the acid-resistance in *E. coli* (*23, 26, 27*) and *Lactobacillus*, the GABA level may be used for the index for the acid-resistance for the microbes. Decarboxylation of other compounds also participates in the acid-neutralizing mechanism in the microbes. For example, decarboxylation of malate decreased the acidity of the medium during the fermentation of wine (*28-30*).

Co-culturing of microbes is beneficial not only for food production, but also for human health since a large number of microbes live together in the animal intestines, in other word they are co-culturing. The microbes may collaborate to carry out important biological functions; however, very little is understood. The mechanism of the co-culturing of different microbes needs to be explored and the findings can be extended to the industrial application as well as the improvement of human health.

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