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Symbiosis between Microorganisms from Kombucha and Kefir: Potential Significance to the Enhancement of Kombucha Function

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Abstract *Gluconacetobacter* sp. A4 (*G.* sp. A4), which had strong ability to produce d-saccharic acid 1, 4 lactone (DSL), was the key functional bacteria isolated from the kombucha preserved. This paper investigated the interaction between *G.* sp. A4 and ten different strains of lactic acid bacteria (LAB) obtained from kefir. The result suggested that the LAB promoted DSL production of *G.* sp. A4 to different extents, ranging from 4.86% to 86.70%. Symbiosis between *G.* sp. A4 and LAB was studied. LAB's metabolites, xylitol, and acetic acid, were utilized by *G.* sp. A4, and it promoted the growth of *G.* sp. A4 and yield of DSL. Therefore, in developing starter cultures for kombucha fermentation process, a mixed flora of LAB and *G.* sp. A4 would be the optimal combination.

Keywords Kombucha · *Gluconacetobacter* sp · Kefir · Lactic acid bacteria · Symbiosis · D-saccharic acid 1,4 lacton

Introduction

Kombucha is a traditional beverage prepared by fermenting sweetened black tea (SBT) with a mixed culture of yeast, acetic acid bacteria, and lactic acid bacteria (LAB) [1–3]. It has been claimed that kombucha can regulate cell proliferation, increase detoxification, and protect liver. Kombucha also has anti-carcinogenic effects, especially for hormone-dependent tumors [4–6]. According to the growth of the microorganisms and production of acetic acid and ethanol, the relationship of yeast and acetic acid bacteria in kombucha is symbiotic [2]. LAB only presents in certain kombucha with small amount, therefore it has not drawn much attention in the past.

D-saccharic acid 1,4 lacton (DSL) and glucuronic acid are considered to be the crucial functional components found in Kombucha [7]. DSL can inhibit glucosidase activity, and thus facilitate glucuronic acid to repel toxicants [8], including carcinogens (polycyclic

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aromatic hydrocarbons, some nitrosamines, aromatic amines, and fungal toxins), certain tumor promoters (steroid hormones) and hepatotoxins (acetaminophen) [9, 10]. In addition, they may modulate steady-state levels of estradiol and other steroid hormones and their excretion in the form of glucuronides [11]. DSL and glucuronic acid are parts of the glucuronate pathway, and the presence of DSL could indicate the existence of glucuronic acid, according to Hoffmann [12].

Kefir is a fermented milk beverage that originated in Eastern Europe. The starter is kefir grain. Kefir grain is a microbial symbiont. It has a varying and complex LAB composition, which are present as the largest portion (65–80%). Kefir can be considered a probiotic resource, because it enjoys a variety of health claims (immunomodelatory, anti-neoplastic, and pro-digestive effects) besides its nutritional status [13].

In the previous study, we found a functional strain (*G.* sp. A4) in kombucha which had the ability to readily produce more functional component: DSL (2.70~3.50 mg/mL). The aim of this paper is to investigate the interaction between microorganisms from kombucha and kefir, especially the effects of LAB on DSL production in *G.* sp. A4.

Materials and Methods

Bacterial Strains and Culture Conditions

G. sp. A4 was isolated from kombucha. It was maintained on glucose yeast extract agar (GY agar; 100 g/L glucose, 10 g/L yeast extract, 20 g/L $CaCO_3$, 15 g/L agar) and 10 mL of GY broth containing 24-h-old stock culture of *G.* sp. A4 was shaken for 24 h at 30 °C to make pure culture suspension (rotary shaker—120 rpm).

Lactobacillus sp. (LmK1, LmK2, LmK3, LmKa, LmKb), *Lactococcus lactis* subsp. (U2, U3, U4), and *Leuconostoc* sp. (Lm11, Lm2) were isolated from kefir grains as described by Han [14] and maintained on MRS (Difco Laboratories, USA). Different pure culture suspensions were obtained by adding these bacteria into 10 mL MRS broth and incubating for 48 h at 30 °C separately.

Before using in the following experiments, all the above suspensions were transferred into 50 mL sweetened black tea. Respective inoculation densities for *G.* sp. A4 and LAB strains were 4×10^7 and 2×10^7 cells/mL.

Sweetened Black Tea Preparation

SBT was prepared by the following steps: 5 g black tea (Yunnan Dianhong, China) was added into 700 mL of fresh boiling water and infused for 15 min. After filtration, 100 g glucose was added to the tea extract as well as some water to make the final volume of 1,000 mL; then the mixture was autoclaved at 121 °C for 15 min.

Growth in Single and Mixed Culture

G. sp. A4 (5 mL) and each strain of LAB (U2, U3, U4, LmK1, LmK2, LmK3, LmKa, LmKb, Lm11, Lm2) (5 mL) were inoculated into 100 mL SBT. Samples were then incubated at 30 °C for 8 days. The concentration of DSL was finally measured. In consideration of the taste and appearance of final products, LmKa was chosen for the following text.

For further investigation, fermentation progress was monitored. *G.* sp. A4 (5 mL) and LmKa (5 mL) were inoculated into 100 mL SBT. The fermentation time was 10 days at

30 °C. Sampling was performed periodically, and each jar was sampled only once in order to avoid potential contamination. Changes in *G.* sp. A4 growth, pH value, titratable acid, and DSL concentration were analyzed as described below.

The Effect of Initial pH Value on the Production of DSL

The initial pH value of autoclaved SBT was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 using 5 M HCl and 5 M NaOH. *G.* sp. A4 was inoculated into the tea broth by the proportion of 5% (*v*/*v*) and then incubated at 30 °C for 8 days. Concentration of DSL was finally measured.

The Effect of LAB Metabolites on the Production of DSL

LmKa inoculum (5 mL) was inoculated into 100 mL SBT (pH 5.0) for 8 days. After centrifugation, the supernatants were adjusted to pH 5.0, and then sterilized by passing through a 0.22 μm filter. Organic acid composition of the supernatant was measured by ion exclusion chromatography as described below. Then 5 mL of *G.* sp. A4 cultures was inoculated into the supernatant for another 8 days, and the production of DSL was measured.

For further investigation, media conditioning experiment was carried out. Supplemented SBT was prepared by adding each metabolite of LAB into tea broth. The type and concentration of components were chosen according to the LmKa metabolites analysis and were shown in table 1. The supplemented SBT was pH-adjusted to 5.0, and then 5 mL of *G.* sp. A4 was inoculated according to standard procedures. Concentration of DSL was measured after 8 days.

Analytical Methods

The pH of kombucha beverage was determined with a pH meter (Digital pH meter 110, Wheaton, USA). Total titratable acid was assessed by titrating 10 mL of kombucha beverage with 0.1 M NaOH.

Concentration of DSL was analyzed using gas chromatography. Supernatant (1 mL) was taken down to dryness using an evaporator at 90 °C for 2 h, and then dissolved in methanol. After centrifuging (1,400 g, 20 min), 1 μL of the supernatant solution was taken for GC analysis. GC was carried out on a Shimadzu GC-14C gas chromatograph (Japan), equipped with flame ionization detector (FID) and a DM-WAX capillary column (30 m×0.25 mm×0.25 μm, USA). The temperature of injector and detector was 250 °C. The samples were analyzed with the following temperature program: the initial temperature was 50 °C, the temperature increased at 12 °C/min to 200 °C and held for 1 min, then increased at 12 °C/min to 230 °C and held for 5 min. The content of DSL was calculated by reference to the peak obtained with a standard (Sigma, USA).

The concentration of *G.* sp. A4 was measured by dilution plate count. The sample was diluted appropriately in 0.85 M saline dilution blanks and plated onto GY agar. Plates were incubated at 30 °C for 24 h. Colony-forming were counted on GY agar and expressed as $\log_{10}$ cfu/mL.

Table 1 The chosen substances and their addition in 100 mL SBT.

Xylitol	Citric acid	Succinic acid	Lactic acid	Formic acid	Acetic acid
0.5 g	0.5 g	0.5 g	40 μL	20 μL	30 μL

Organic acid composition was analyzed using high-performance liquid chromatography (HPLC) (Shimadzu, Japan) equipped with ion exclusion column (300×8 mm) and electro-conductivity detector. The mobile phase was composed of 5 mM p-Tuluensulfonic acid (p-TSA, Sigma, USA) and 0.4 M boric acid (WAKO, Japan). The post-column buffer solution was composed of 5 mM p-TSA (Sigma, USA), 20 mM Bis (2-hydroxyethyl) iminotris (hydroxymethyl) methane (Bis-Tris, Sigma, USA) and 100 μM sodium Ethylenediaminetetra-acetic (EDTA-2Na, Wako Pure Chemical Inc., Japan). The flow rate was 0.8 mL/min.

Statistical Calculations

The experiments were replicated three times in a randomized block design. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests. *P*-values<0.05 were considered to be statistical significance. Analysis was done with SPSS 13.0 (SPSS, Inc., Chicago, IL, USA).

Result and Discussion

In kombucha, positive interactions between acetic acid bacteria and yeast have long been illustrated. Yeast's production of ethanol assists bacterial production of acetic acid, which may further stimulate ethanol production. This co-existence of ethanol and acetic acid prevents competition from other microorganisms [2]. LAB is considered to be unessential in kombucha, and the role it plays in kombucha fermentation has not been fully described.

LAB Selection

In the present study, ten strains of LAB were isolated from kefir and co-cultured with *G.* sp. A4. All of them can promote the DSL production to different extents, ranging from 4.86% to 86.70% (Fig. 1). Thus, a symbiotic relationship was established between *G.* sp. A4 in kombucha and LAB in kefir. Similar observations between acetic acid bacteria and LAB were also recorded in, for example, kefir and cocoa bean fermentations [15, 16]. Addition of U2 induced the highest DSL production, however, U2 did not grow steadily with *G.* sp. A4, and the culture was fluctuant. Compared to others, the product fermented by the mix culture of A4 and Lmka was clear and stable, with a taste of moderate sour. So LmKa was finally chosen for the following experiments, which belonged to *Lactobacillus* sp.

Changes during Fermentation Progress in Single and Mixed Culture

To find out the possible underlying mechanism in which the inoculation of LmKa could improve the DSL production of *G.* sp. A4, the fermentation progress of single and mixed culture were supervised.

Growth of G. sp. A4

When *G.* sp. A4 was inoculated into SBT under anaerobic conditions, the viable cell density increased from 6.60 to 7.34 $\log_{10}$ cfu/mL within 4 days and thereafter continued to decrease. This result was different from that of Liu et al. [2] who reported the bacteria in kombucha reached about 6.74 $\log_{10}$ cfu/mL after 2 days of fermentation and remained stable from 3–6 days. Decrease of *G.* sp. A4 after 4 days of incubation in this experiment

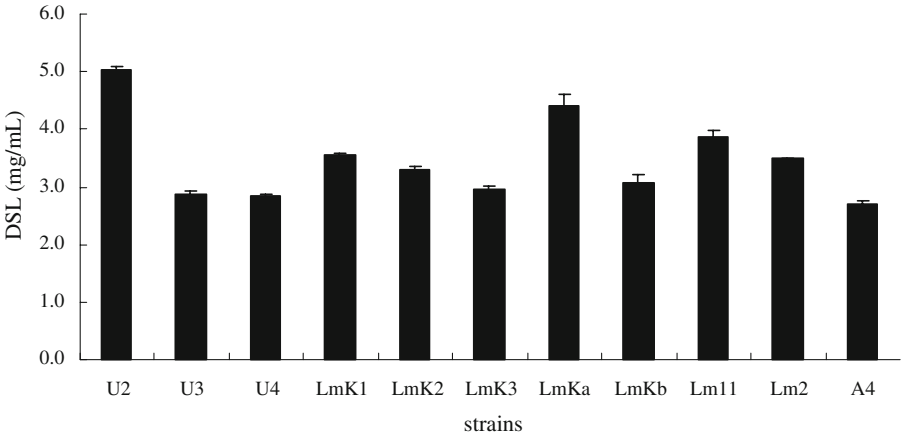

Fig. 1 DSL production of *G.* sp.A4 in co-culture with each strain of LAB

was likely caused by low pH, which influenced the multiplication of bacteria and yeast [17]. Chen and Liu [18] reported that anaerobic and starved environment created could also be the reason for the decrease of viable cell density during the fermentation period.

However, when LmKa was simultaneously inoculated into the same culture, growth of *G.* sp. A4 was slightly promoted and the maximum viable cell density increased to 7.87 $\log_{10}$ cfu/mL. This experiment suggested LmKa can positive influence the growth of *G.* sp. A4, which was an effective way to increase the DSL production. Similar observations were reported between LAB and yeasts, that some LAB species release galactose which may favor the growth of lactose-negative yeasts [19]. This positive interaction influenced the growth and metabolism of either LAB or yeasts and may modify ripening time and/or the production of essential odors [20].

DSL Concentration

Concentration of DSL increased considerably during the first 8 days and reached its maximum value on the eighth day, which is the inflexion point of the curve. Then, there was a remarkable decrease until the end of fermentation (Fig. 2b). Differences in DSL production between single and mixed culture were observed at fourth day after starting the fermentation, when mixed culture exhibited a rapider increase. *G.* sp. A4 together with LmKa produced much more DSL than *G.* sp. A4 alone, which were 3.97 mg/mL compared with 2.78 mg/mL at eighth day.

pH and Total Titratable Acid Concentration

pH decreased continuously and reached a final value between 2.5 and 3.0 (Fig. 2c). The pH of SBT fermented by mixed culture decreased from 5.10 to 2.60, while that by G. sp. A4 decreased to 3.06.

Concentration of total titratable acid was elevated considerably during the first 6 days and thereafter remained relative constant, showing only a slight decrease at the end of fermentation process (Fig. 2d). These observations were largely in agreement with the results of other studies [17, 18, 21, 22]. Large differences in the production of total titratable acid were observed in single and mixed culture. At the eighth day of incubation,

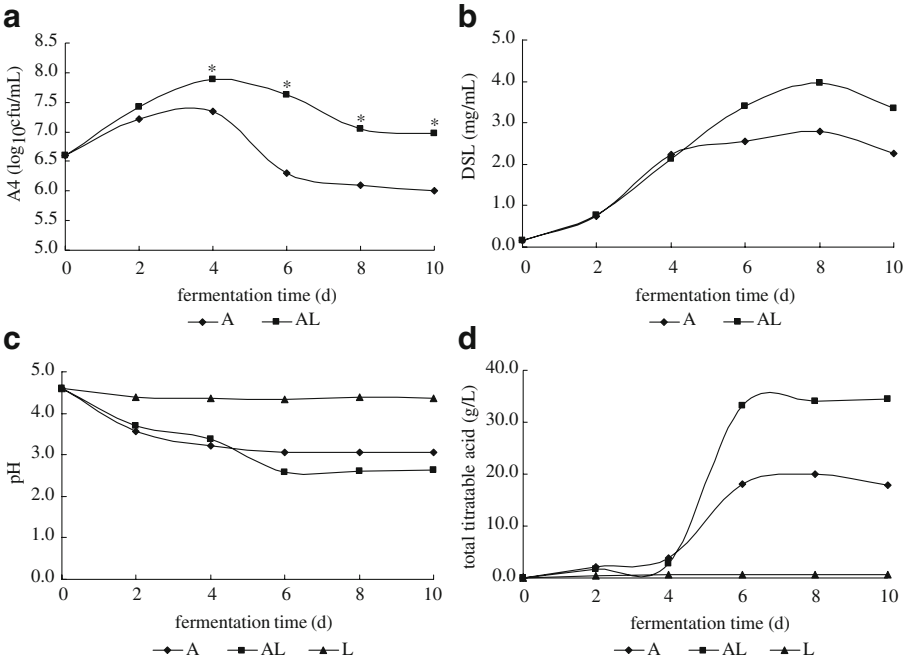

Fig. 2 Symbiosis between G. sp. A4 and LmKa: **a** growth curve of *G.* sp.A4, **b** change in DSL, **c** change in pH and **d** change in total titratable acid in SBT fermented by: *G.* sp. A4 (*A*), *G.* sp. A4 and LAB (*AL*) and LmKa (*L*). *means statistically promoted the growth of *G.* sp. A4 at $P<0.05$

the production of total titratable acid by the mixed culture was almost two times more than that by G. sp. A4 or LmKa alone (34.06 g/L, 19.02 g/L, and 0.67 g/L, respectively). The main organic acids were gluconic acid and 2-keto gluconic acid. There was little or no lactic acid and acetic acid present.

Compared with the single culture at the fourth day of incubation, some significant differences were observed in the mixed culture: the production of DSL increased significantly; the change of pH value and total titratable acid content became obvious; the viable cell density of *G.* sp. A4 achieved its maximum. Based on the above observations, positive influence on the growth of *G.* sp. A4 was proposed to be one reason for the promotion of DSL production. pH and metabolites produced by LmKa may be also responsible for the commensal relationship demonstrated between *G.* sp. A4 and *Lactobacillus* sp. LmK. It was partly in accordance with the results reported by Pybus. They proposed that increased availability of amino acids by *Prevotella bivia* was the mechanism to support the observed commensal symbiosis between *P. bivia* and *Peptostreptococcus anaerobius* [23]. The following experiments were carried out to investigate the effect of pH and metabolites by LAB on the production of DSL.

The Effect of Initial pH Value on the Production of DSL

To further elucidate the influence of pH value, we consequently adjusted the pH of SBT to various values from 2.0 to 7.0. The optimal initial pH for *G.* sp. A4 to produce DSL was 5.0, which was similar to the natural pH of SBT. Then the DSL production decreased with changes in the original pH value (Fig. 3). But from Fig. 2b and c, we can see that when the

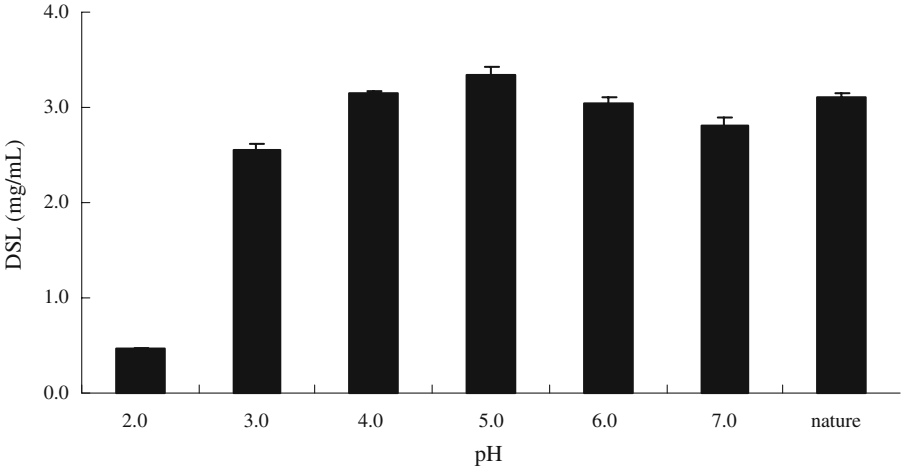

Fig. 3 Influence of initial pH value on DSL production of *G.* sp. A4

highest DSL production was obtained at the eighth day, the pH value of the SBT was around 2.5. Apparently, pH shift characteristic of LmKa was possibly not responsible for the promotion of DSL production, and low pH value would inhibit the growth of acetic acid bacteria [17, 24]. It agreed in part with the results reported by Soh [25], that the initial pH value affected the acidity during kombucha fermentation.

The Effect of LAB Metabolites on the Production of DSL

LAB metabolites experiment was designed to test the metabolites influence. When *G.* sp. A4 was grown in SBT previously fermented by LmKa, the production of DSL increased significantly to the control (3.82 mg/mL and 3.10 mg/mL, respectively, Fig. 4). Hence, metabolites by LmKa in SBT appeared to have a positive effect on the DSL production of *G.* sp. A4. To determine a biochemical basis for the apparent promoting effect, SBT previously fermented by LmKa was analyzed using HPLC (Fig. 5). Xylitol, citic acid, succinic acid, lactic acid, formic acid and acetic acid were finally chosen for media conditioning experiments.

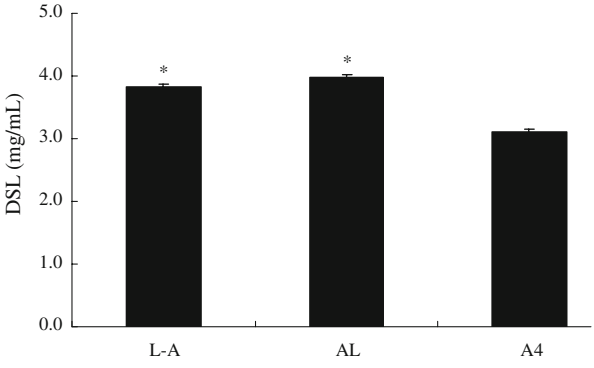

Fig. 4 DSL production of *G.* sp. A4 in SBT *L-A*: fermented by *G.* sp. A4 in LAB supernatant; *AL*: fermented by *G.* sp. A4 and LAB together in SBT; *A4*: fermented by *G.* sp. A4 in SBT. *means statistically increased the DSL production of *G.* sp. A4 at $P<0.05$

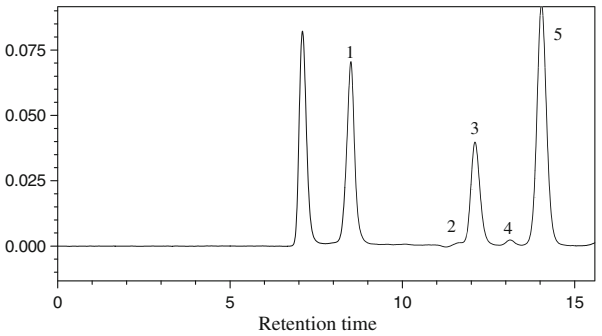

Fig. 5 Chemical composition of LmKa culture. *1* citric acid, *2* succinic acid, *3* lactic acid, *4* formic acid; *5* acetic acid

In reciprocal media conditioning experiments, it was observed that acetic acid and xylitol could increase the DSL production by 24.31% and 40.60% respectively. Lactic acid, citric acid had no positive effect, while succinic acid and formic acid even showed a slight adverse effect (Fig. 6). We proposed the production of xylitol and acetic acid by LAB and their subsequent utilization by *G.* sp. A4 as one specific biochemical pathway contributing to the commensal relationship demonstrated between these two organisms.

In our previous study, we found that the optimal medium for *G.* sp. A4 fermentation were glucose (5%, *w*/*v*) and black tea (0.5%, *w*/*v*) [26]. DSL was synthesized from glucose through the glucuronate pathway [12]. Glucuronate reductase and UDP-glucose reductase are key enzymes of the glucuronate pathway [27, 28]. Possible reasons for the ability of xylitol to enhance the production of DSL may be that the formation of DSL from xylitol avoids speed limiting processes in glucuronate pathway—xylitol can elevate the metabolic flux in glucuronate pathway. That was partly in accordance with the results reported by Ishihara et al. [29] and Granstrom et al. [30]. Ishihara reported that D-xylose/D-xylulose was the best mixed medium for fermentation of *Acetobacter xylinus* IFO 15606. Granstrom

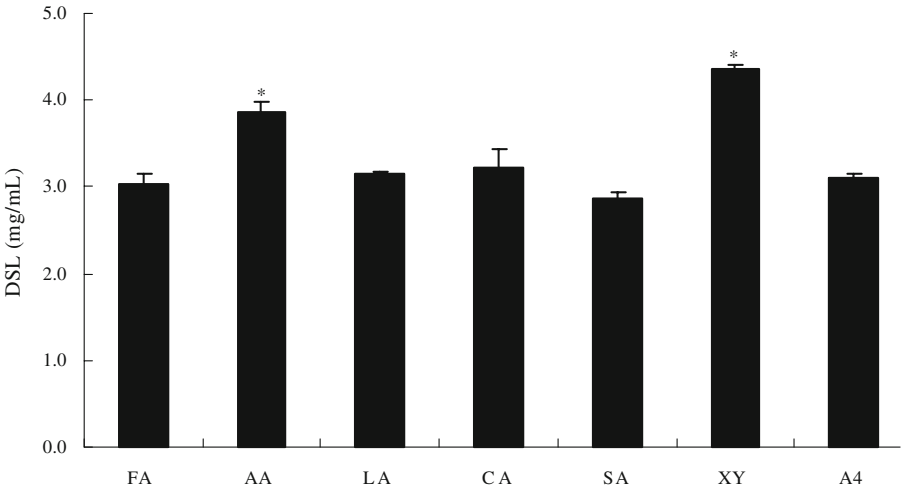

Fig. 6 Influence of carbohydrate metabolites on DSL production of G. sp. A4. *means statistically increased the DSL production of *G.* sp. A4 at $P<0.05$. *FA* formic acid, *AA* acetic acid, *LA* lactic acid, *CA* citric acid, *SA* succinic acid, *XY* xylitol, *A4* control. *DSL* d-saccharic acid 1,4 lactone; *LAB* lactic acid bacteria from kefir; *G. sp. A4 Gluconacetobacter* sp. A4, *SBT* sweetened black tea

thought that D-xylitol can induce those enzyme activities required for L-xylulose production from xylitol, which may be further transformed into DSL.

Acetic acid is the final metabolite of glycolysis. According to feedback inhibition theory, its presence could inhibit the glycolysis pathway of *G.* sp. A4 and therefore enhances metabolic flux in the glucuronate pathway.

Lactate was found to stimulate the cell growth of bacteria during the early stages of batch culture by linking with the respiratory chain and generating energy for growth [31]. However, that was not responsible for the increase of DSL in our experiment, due to the rather small lactate produced by Lmka observed in SBT (less than 0.47 g/L). According to Takaaki, to increase the bacterial cellulose production rates and cell concentration at a steady state, lactate concentrations in the feed medium should be from 4.5~12.5 g/L [32].

Conclusion

There is a symbiosis between microorganisms from kombucha and kefir. LAB appeared to play important roles in the survival and activities of *G.* sp. A4. LAB was also involved in the metabolism of glucose to xylose, acetic acid, and other metabolites, which influenced DSL production of *G.* sp. A4. Therefore, in developing starter cultures for kombucha fermentation process, mixed flora of LAB and *G.* sp. A4 should be the optimal combination. The possible underlying mechanisms for this finding, however, need further investigations.

References

1. Greenwalt, C. J., Steinkraus, K. H., & Ledford, R. A. (2000). *Journal of Food Protection*, *63*(7), 976–981.
2. Liu, C. H., Hsu, S. H., & Lee, F. L. (1996). *Food Microbiology*, *13*(6), 407–415. doi:10.1006/fmic.1996.0047.
3. Kurtzman, C. P., Robnett, C. J., & Basehoar-Power, E. (2001). *FEMS Yeast Res*, *1*(2), 133–138. doi:10.1111/j.1567-1364.2001.tb00024.x.
4. Yoram, A. (1998) Results of Study of Magic Kombucha ® and kombuchion® in patients With HIV. Available from: persweb.direct.ca/chaugen/kombucha-research.html. Accessed February 1, 2003.
5. Walaszek, Z. (1990). *Cancer Letters*, *54*(1–2), 1–8. doi:10.1016/0304-3835(90)90083-A.
6. Valentine, T. (1993) Kombucha, a fermented beverage with real “Zing”in it. Available from: www.kombu.de/val-gwf.htm. Accessed Jun 1, 1997.
7. Günther, W.F. (1996) The Kombucha Journal. Avaible from: www.kombu.de/index.htm. Accessed May 1, 2003.
8. Roussin, M. R. (1996) Analyses of Kombucha Ferments: Report on Growers. Available from: persweb.direct.ca/chaugen/kombucha-research-mroussin2toc.html. Accessed April 2, 2001.
9. Dutton, G. J. (1980), in Baca Raton: Glucuronidation of Drugs and Other Compounds, Florida, pp. 1–96.
10. Levvy, G. A., & Conchie, J. (1965), In Dutton G. J. (Ed.), Glucuronic acid: Free and combined: β-Glucuronidase and its hydrohysis of glucuronides. New York, pp, 301–364.
11. Walaszer, Z. (1993). *Cancer Bulletin*, *45*(5), 453–457.
12. Hoffmann, N. (2000) The Ubiquitous Co-Enzyme UDPGlucuronic Acid Detoxifying Agent in Kombucha Tea. Available from: www.bluemarble.de/Norbert/kombucha/Glucuron/body_glucuron.htm. Accessed July 1, 2002.
13. Moreno, A., Matar, C., Farnworth, E., & Perdigon, G. (2006). *Cytokine*, *34*, 1–8. doi:10.1016/j.cyto.2006.03.008.
14. Han, H. (2007) Study on optimization of kefir fermentation and freeze dry technics. Beijing, pp.12–13.
15. Judith, A. P., Mariano, L. C. and Analía, G. A. (2007) Food Hydrocolloids doi:10.1016/j.foodhyd.2007.10.005.
16. Ardhana, M. M., & Graham, H. F. (2003). *International Journal of Food Microbiology*, *86*, 87–99. doi:10.1016/S0168-1605(03)00081-3.

17. Jayabalan, R., Marimuthu, S., & Swaminathan, K. (2007). *Food Chemistry*, *102*(1), 392–398. doi:10.1016/j.foodchem.2006.05.032.
18. Chen, C., & Liu, B. Y. (2000). *Journal of Applied Microbiology*, *89*, 834–839. doi:10.1046/j.1365-2672.2000.01188.x.
19. Marshall, V. M. E., & Tamine, A. Y. (1997). In B. A. Law (Ed.), *Microbiology and chemistry of cheese and fermented milks: Physiology and biochemistry of fermented milk* pp. 153–192. London: Blackie Academic and Professional.
20. Gadaga, T. H., Mtukumira, A. N., & Narvhus, J. A. (2001). *International Journal of Food Microbiology*, *68*, 21–32. doi:10.1016/S0168-1605(01)00466-4.
21. Reiss, J. (1994). *Lebensmittel-Untersuchung und-Forschung*, *198*, 258–261.
22. Sreeramulu, G., Zhu, Y., & Knol, W. (2000). *Journal of Agricultural and Food Chemistry*, *48*, 2589–2594. doi:10.1021/jf991333m.
23. Pybus, V., & Onderdonk, A. B. (1998). *FEMS Immunology and Medical Microbiology*, *22*, 317–327. doi:10.1111/j.1574-695X.1998.tb01221.x.
24. Hwang, J. W., Young, K. Y., Jae, K. H., Yu, R. P., & Yu, S. K. (1999). *Journal of Bioscience and Bioengineering*, *88*(2), 183–188. doi:10.1016/S1389-1723(99)80199-6.
25. Soh, H. S., & Lee, S. P. (2002). *International Journal of Food Sciences and Nutrition*, *7*(1), 37–42.
26. Tian, W. L., Ji, B. P., Li, B., & Zhang, H. (2004). *Food Science China*, *25*(2), 161–163.
27. Goode, D., Lewis, M. E., & Crabbe, J. C. (1996). *FEBS Letters*, *395*, 174–178. doi:10.1016/0014-5793(96)01012-5.
28. Henrik, J., Fredrik, S., & Bahram, A. (2002). *BBA*, *1576*, 53–58.
29. Ishihara, M., Matsunaga, M., Hayashi, N., & Tišler, V. (2002). *Enzyme and Microbial Technology*, *31*(7), 986–991. doi:10.1016/S0141-0229(02)00215-6.
30. Granström, T. B., Takata, G., Morimoto, K., Leisola, M., & Izumori, K. (2005). *Enzyme and Microbial Technology*, *36*(7), 976–981. doi:10.1016/j.enzmictec.2005.01.027.
31. Matsuoka, M., Tsnchida, T., Matsushima, K., Adachi, O., & Yoshinaga, F. (1996). *Bioscience, Biotechnology, and Biochemistry*, *60*, 575–579.
32. Naritomi, T., Kouda, T., Yano, H., & Yoshinaga, F. (1998). *Journal of Fermentation and Bioengineering*, *85*(1), 89–95. doi:10.1016/S0922-338X(97)80360-1.