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Impact of *Lactobacillus* cultures on production of B-vitamins, organic acids and biotransformation of soy isoflavones

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ABSTRACT

In the study, four potent *Lactobacillus* cultures of *L. rhamnosus* K4E, *L. plantarum* RD7, *L. fermentum* K7, and *L. fermentum* K16 were considered for the production of B-vitamins, organic acids and biotransformation of soy isoflavones. *L. plantarum* RD7 showed the highest B_2 production (0.84 μg mL^{-1}) after 36 h, while *L. fermentum* K16 exhibited maximum B_{12} production (0.084 μg mL^{-1}) after 12 h. *L. rhamnosus* K4E produced 0.24 μg mL^{-1} of folate after 12 h. Highest production of lactate (16.43 μg mL^{-1}) and acetate (5.86 μg mL^{-1}) was reported by *L. rhamnosus* K4E. *L. plantarum* RD7 showed maximum butyrate (0.253 μg mL^{-1}) production compared to the other cultures. The highest bioconversion of soy aglycones was reported by *L. rhamnosus* K4E with 55.43% for daidzein and 72.30% for genistein, during soymilk fermentation. These potent cultures have a potential to be used as functional starter cultures for the production of functional fermented soy foods.

KEYWORDS

Lactobacillus, B-vitamins, organic acids, soy isoflavones, fermented foods

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1. INTRODUCTION

Lactic acid bacteria (LAB) are auxotrophic regarding specific vitamins, and there are reports of LAB strains that exhibit efficiency in synthesising water-soluble vitamins, primarily those linked to B-group vitamins, for example B_2, B_9, B_{12}, etc. Recent reports stated malnutrition challenges and essential micronutrient deficiencies in B-vitamins in large numbers of children among the indigenous tribes (Garo, Khasi, Jaintia) residing in Meghalaya (Chyne et al., 2017). Despite the fact that there is access to both cultivable and wild-type food resources in the hills of Meghalaya, nutritional anaemia is mostly affecting these particular regions due to inadequacy of iron and vitamins A, B_2, B_6, B_9, and B_{12}. Outside the North-eastern states, the rest of the Indian population (mainly in children and adolescents) is experiencing a large numbers of vitamin B_{12} crises (Chyne et al., 2017; Hati et al., 2019).

Fermented soy-based foods may serve as ideal supplements to address the challenges with malnutrition and related adequacies in vitamins, since LAB fermentation improves the bioavailability of soy isoflavones. The absorption of isoflavone glycosides in the small intestine is very poor due to their highly polar conjugated molecules (Hati et al., 2017). The glycosides that are not absorbed pass to the colon, where probiotic microorganisms release free aglycones by removing the sugar moiety for energy conversion through β-glucosidase activity (Mishra et al., 2019). LAB are also receiving more attention due to their efficiency in production of organic acids. The food preservation efficiency of lactobacilli comes from the production of a wide variety of organic molecules that can even have antimicrobial properties. The metabolite products of lactobacilli as lactic and acetic acids are considered primary organic acids that exhibit antimicrobial characteristics (Zalán et al., 2010).

In the study, the estimation of B-vitamins, organic acids, and biotransformation of isoflavones by indigenous *Lactobacillus* strains isolated from the traditional fermented foods of Meghalaya using HPLC based methods is reported.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

The LAB selective MRS medium (De Man, Rogosa and Sharpe agar) as well as vitamin (B_2, B_9, B_{12}) free assay medium used in the study were obtained from Himedia, India. The HPLC standards for vitamins B_2, B_9, B_{12}; organic acids acetic, lactic, and butyric acid; and isoflavone aglycones daidzein and genistein were purchased from Sigma-Aldrich, India.

2.2. Bacterial strains

Four well characterised indigenous *Lactobacillus* strains with NCBI GenBank accession numbers *L. rhamnosus* K4E (KX950834), *L. fermentum* K7 (KU213665), *L. fermentum* K16 (KU213667), and *L. plantarum* RD7 (MF155569.1) isolated from the traditional fermented foods and beverages of Meghalaya were used for the study based on their technological and rich probiotic properties reported previously (Das et al., 2020). The *Lactobacillus* cultures were obtained from the culture collection of the Department of RDAP, North-Eastern Hill University, Tura, Meghalaya, India.

2.3. Estimation of vitamin B_2, B_{12}, and B_9 production

The production of vitamin B_2 and B_{12} was estimated using a microbial assay adapted from Hati et al. (2019), and for B_9 production, the method of Hati et al. (2019) and Panda et al. (2018) was used. The *Lactobacillus* cultures were inoculated in vitamin B_2, B_{12}, and B_9 free assay medium (HiMedia, India) and sub-cultured in the very same medium three times. The cells were incubated at 37 °C for 12, 24, 36, and 48 h in 15 mL of the defined medium and were extracted and washed three times with phosphate buffer of 0.1 M with pH adjusted to 7.0.

For B_2 and B_{12}: The washed cells were immersed into 1 mL of extraction buffer (0.1 M Na_2HPO_4 [pH 4.5 using citric acid], 0.005% KCN) and the cells were disrupted for 30 min at 95 °C, preceded by intense vortexing for 1 min and centrifuged for 10 min at 10,000×*g*. The supernatant was transferred through a 0.45 µm syringe filter. During the HPLC study, 20 µl filtrate was injected into the HPLC (LC-10, Shimadzu, Japan) system using a micro-injector. RP18 endcapped column, LiChroCART column (250 × 4.6 mm) (Chromolith-Merck), and a Guard column (40 × 4 mm) were used with an isocratic HPLC system. The vitamins (B_2 and B_{12}) were eluted using mobile phase: 50% acetonitrile, flow rate: 0.3 mL min^{-1}, oven temperature: 40 °C, and UV–Visible detector fixed at 284 nm.

For B_9: Washed cells were resuspended in 1 mL of extraction buffer containing 20 mM sodium phosphate buffer (pH 6.2), followed by cell disruption for 30 min at 95 °C, intense vortexing for 1 min, and centrifugation for 10 min at 10,000×*g*. The supernatant obtained was let through a 0.45 µm syringe filter and was injected into the HPLC system. Five percent of acetonitrile in 20 mM sodium phosphate buffer (pH 6.2) was used as mobile phase, and the flow rate was 1 mL min^{-1} with a UV–Visible detector fixed at 280 nm.

2.4. Estimation of organic acids production

The organic acids (lactate, acetate, and butyrate) released by the *Lactobacillus* isolates were assessed using the HPLC method suggested by Leblanc et al. (2017). The bacterial strains were inoculated in 10 mL skimmed milk, and incubated at 37 °C for 24 h. Five millilitres of sample was diluted with 45 mL of water and vortexed for 5 min. The samples were let to settle for 10 min after vortexing and filtered through Whatman paper no. 42. The filtered samples were transferred through a 0.45 µm syringe filter and loaded into the HPLC system. The column was washed twice with 0.01% phosphoric acid before HPLC analysis to eliminate salts and other contaminants. The organic acids were eluted with 0.01% phosphoric acid with a flow rate of 0.5 mL min^{-1}, and the oven temperature was kept at 40 °C. The elute absorption was recorded at 210 nm.

2.5. Biotransformation of soy isoflavones

The soymilk was extracted from soybean after soaking overnight following the method of Mishra et al. (2019). Into 10 mL aliquots of soymilk, 24 h active cultures were inoculated at the rate of 2% and incubated at 37 °C for 24 h. The soy isoflavones were extracted from soymilk fermented by the *Lactobacillus* cultures adapting the method of Hati et al. (2017). The isoflavones were eluted by an isocratic flow of 30 min with mobile phase containing 0.05% TFA (trifluoroacetic acid) dissolved in 50% 100 mM ammonium acetate and 50% methanol with a flow rate of 1 mL min^{-1} and column temperature of 25 °C, and UV– Visible detector fixed at 260 nm.

2.6. Statistical analysis

Analysis of variance (ANOVA) was applied and comparison was made through Tukey's test with the least significant difference of $P \leq 0.05$ using the IBM SPSS Statistical Program Ver. 20.

3. RESULTS AND DISCUSSION

3.1. Vitamin B_2 production by *Lactobacillus* cultures

The results showed highest ($P \leq 0.05$) production of B_2 by *L. plantarum* RD7 with 0.84 μg mL^{-1} after 36 h (Fig. 1). *L. fermentum* K16 presented maximum production of riboflavin (0.77 μg mL^{-1}) after 24 h, followed by declines after 36 h (0.57 μg mL^{-1}) and 48 h (0.32 μg mL^{-1}). *L. rhamnosus* K4E showed significant decrease in B_2 production from 12 h (0.83 μg mL^{-1}) to 48 h (0.35 μg mL^{-1}). *L. fermentum* K7 and *L. fermentum* K16 exhibited significant increase in riboflavin concentration after 24 h (0.54, 0.77 μg mL^{-1}, respectively), followed by gradual decrease from 36 h (0.45, 0.57 μg mL^{-1}, respectively) to 48 h (0.28, 0.32 μg mL^{-1}, respectively). Initially, 12 h culture of *L. plantarum* RD7 showed lower riboflavin production, followed by a gradual increase. However, *L. plantarum* RD7 showed B_2 production up to 36 h, *L. fermentum* K16 and *L. fermentum* K7 up to 24 h, followed by a decrease in the production afterwards (Fig. 1).

In a study by Carrizo et al. (2017), the highest total riboflavin concentrations (>250 ng mL^{-1}) were reported for *Lactococcus mesenteroides* subsp. *mesenteroides* CRL 2131 and *Enterococcus durans* CRL 2122. They isolated 29 lactic acid bacterium strains from

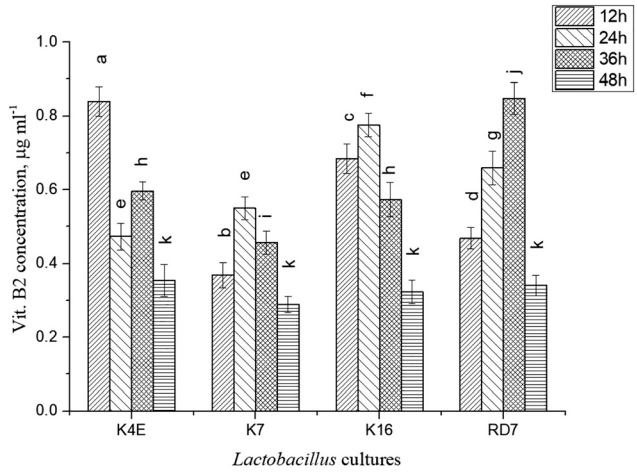

Fig. 1. Vitamin B_2 production by indigenous *Lactobacillus* cultures. Values are presented as mean ± SD with three independent determinations ($n = 3$) from each sample. Values bearing different superscripts in each bar differ significantly from each other by Tukey's test at $P \leq 0.05$

amaranth, and 79% of them were able to grow in the absence of riboflavin that agrees well with our study. The rise of riboflavin concentration in log phase and early stationary phase suggests a significant role in cellular metabolic functions, meaning that riboflavin may be used by microbes for their proliferation and growth (Kaprasob et al., 2018). Similarly to our study, Juarez Del Valle et al. (2014) studied the growth of 42 strains of lactic acid bacteria in a commercial riboflavin-free medium, and then the concentration of riboflavin produced was evaluated by HPLC analysis. From those strains, *L. plantarum* CRL 725 significantly increased the concentration of riboflavin from 309 ± 9 to 700 ± 20 ng mL^{-1} at 37 °C in soymilk after 12 h incubation.

3.2. Vitamin B_{12} production by *Lactobacillus* cultures

The B_{12} production by the *Lactobacillus* strains ranged from 0.084 μg mL^{-1} after 12 h to 0.0022 μg mL^{-1} after 48 h ($P \leq 0.05$) (Fig. 2). *L. fermentum* K16 showed the highest B_{12} production with 0.084 μg mL^{-1} followed by *L. rhamnosus* K4E (0.071 μg mL^{-1}) and *L. fermentum* K7 (0.049 μg mL^{-1}) after 12 h. The strains achieved maximum B_{12} production after 12 h, followed by a decrease after 24 h, and further reduction after 48 h (Fig. 2).

The decrease in B_{12} concentration in the study might be related to the deficiency in nutrient concentration leading to reduced metabolic activity (Hati et al., 2019). The initial increase in vitamin B_{12} production agrees with the nature of LAB to synthesize cyanocobalamin. Torres et al. (2016) checked the production of cobalamin by *L. coryniformis* CRL 1001 (grown in a vitamin B12-free assay medium), by using *Salmonella typhimurium* AR 2680 (metE cbiB) as indicator strain, via RP-HPLC analysis. The only source of vitamin B_{12} is through bacterial synthesis. The presence of a B_{12} dependent metabolic pathway that converts

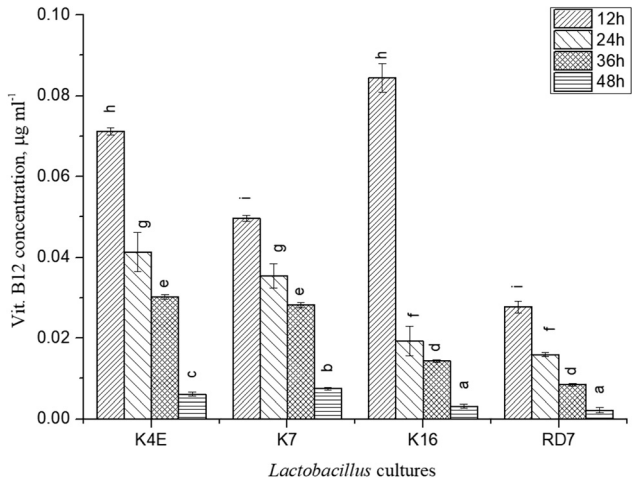

Fig. 2. Vitamin B_{12} production by indigenous *Lactobacillus* cultures. Values are presented in mean ± SD with three independent determinations ($n = 3$) from each sample. Values bearing different superscripts in each bar differ significantly from each other by Tukey's test at $P \leq 0.05$

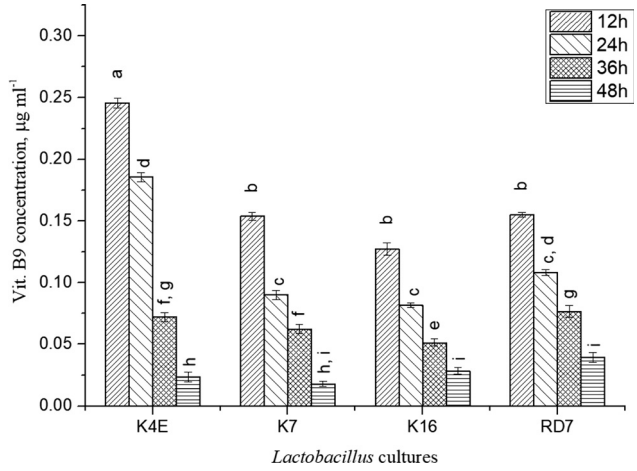

Fig. 3. Vitamin B_9 production by indigenous *Lactobacillus* cultures. Values are presented as mean ± SD with three independent determinations ($n = 3$) from each sample. Values bearing different superscripts in each bar differ significantly from each other by Tukey's test at $P \leq 0.05$

glycerol into propanediol might be the possible reason that allowed the indigenous *Lactobacillus* strains used in the study to synthesise vitamin B_{12} (Hati et al., 2019). These results are in agreement with findings of Kantachote et al. (2017), who reported a rise in vitamin B_{12} production after 24 h, which continued to increase till 48 h of fermentation in *L. plantarum* fermented coconut water.

3.3. Vitamin B_9 production by *Lactobacillus* cultures

The *Lactobacillus* strains showed a decreasing trend in B_9 production, starting with an initial value of 0.245 µg mL^{-1} after 12 h, and declining ($P \leq 0.05$) to 0.017 µg mL^{-1} after 48 h (Fig. 3). *L. rhamnosus* K4E produced 0.245 µg mL^{-1} folate after 12 h, then the concentrations decreased to 0.185 µg mL^{-1} after 24 h, and to 0.023 µg mL^{-1} after 48 h. *L. rhamnosus* K4E was found to be the best producer of folate after 12 h compared to *L. plantarum* RD7 (0.154 µg mL^{-1}), *L. fermentum* K7 (0.153 µg mL^{-1}), and *L. fermentum* K16 (0.127 µg mL^{-1}).

Microorganisms primarily biosynthesise two kinds of folate, 5-methyl tetrahydrofolate (5-MTHF) and tetrahydrofolate (THF). The *Lactobacillus* strains used in the study produced 5-MTHF according to the HPLC analysis. Similarly, to our study, Panda et al. (2018) reported LAB strains *L. rhamnosus* IFM-4 (35 ng mL^{-1}), *L. cremoris* CM22 (12.5 ng mL^{-1}), and *Lc. lactis* CM28 (14.2 ng mL^{-1}) producing 5-MTHF after 12 h. Leblanc et al. (2011) reported folate production in the range 5–291 µg L^{-1} by *Lc. lactis, Streptococcus thermophilus*, and *Leuconostoc* spp. In a study by Carrizo et al. (2016), *L. plantarum* CRL 1973 and CRL 1970, *L. rhamnosus* CRL 1972, and *L. sakei* CRL 1978 produced elevated concentrations of folate that was at par with one another. Though strain CRL 1973 showed the highest folate concentration (143 ± 6 ng mL^{-1}) in a folate free culture medium that agrees with findings of our study. Mousavi et al. (2013) reported that various studies have shown that LAB such as

Lc. lactis, Lb. bulgaricus, and *S. thermophilus* possess the ability of producing folate in fermented foods.

3.4. Determination of organic acids production by the *Lactobacillus* cultures

The highest ($P \leq 0.05$) production of lactate was shown by *L. rhamnosus* K4E (16.43 μg mL^{-1}), followed by *L. fermentum* K7 (15.78 μg mL^{-1}), *L. plantarum* RD7 (13.16 μg mL^{-1}), and *L. fermentum* K16 (11.46 μg mL^{-1}) (Table 1). *L. rhamnosus* K4E showed the highest production of acetic acid with 5.86 μg mL^{-1}, followed by *L. fermentum* K7 (5.77 μg mL^{-1}), *L. fermentum* K16 (5.18 μg mL^{-1}), and *L. plantarum* RD7 (4.07 μg mL^{-1}). *L. plantarum* RD7 showed the highest butyrate production with 0.253 μg mL^{-1}, followed by *L. fermentum* K7 (0.163 μg mL^{-1}), *L. rhamnosus* K4E (0.122 μg mL^{-1}), and *L. fermentum* K16 (0.090 μg mL^{-1}). Amongst the organic acids, lactate was secreted in the largest amounts by the *Lactobacillus* strains, followed by acetate and butyrate.

Using medium similar to ours, 10 strains of *Lactobacillus* species were studied by Zalán et al. (2010) to estimate their production of organic acids in skimmed milk, where lactic acid was produced in the concentration range 13–127 mmol L^{-1} and acetic acid in the concentration range 8–100 mmol L^{-1}. Leblanc et al. (2017) determined the in vitro potential of four probiotic bacterial strains LGG, *B. longum* SP 07/3, *B. bifidum* MF 20/5, and *L. gasseri* PA 16/8, and these strains produced 89 μM of propionate in MRS medium without butyrate or acetate production. Comparatively, the *Lactobacillus* strains in our study showed the highest production of acetic acid, lactic acid, and butyric acid after 24 h in similar medium. Under anaerobic conditions, lactic acid gets converted into acetic acid by heterofermentative LAB. Administration of LAB to the gut promotes production of organic acids, like acetic acid and lactic acid, by selectively promoting the growth of beneficial gut microbiota with antibacterial activities against a broader extent of food pathogens (Hati et al., 2019).

3.5. Biotransformation of soy isoflavones by *Lactobacillus* cultures

A significant increase ($P \leq 0.05$) in the concentration of daidzein and genistein was observed during the soymilk fermentation by the four *Lactobacillus* cultures studied. Before fermentation, daidzin and genistin accounted for 25.11% and 22.55% of the glycosides, respectively, in soymilk (Fig. 4). *L. rhamnosus* K4E showed the highest bioconversion of soy aglycones with values of

Table 1. Organic acids production by the indigenous *Lactobacillus* isolates

Lactobacillus strains	Organic acids production (μg mL^{-1})		
	Acetate	Lactate	Butyrate
K4E	$(5.86 \pm 0.04)^{c}$	$(16.43 \pm 0.70)^{d}$	$(0.122 \pm 0.002)^{g,h}$
K7	$(5.77 \pm 0.05)^{c}$	$(15.78 \pm 0.37)^{d}$	$(0.163 \pm 0.002)^{h}$
K16	$(5.18 \pm 0.02)^{b}$	$(11.46 \pm 0.04)^{e}$	$(0.090 \pm 0.001)^{g}$
RD7	$(4.07 \pm 0.03)^{a}$	$(13.16 \pm 0.29)^{f}$	$(0.253 \pm 0.002)^{i}$

Values are presented as mean ± SD with three independent determinations ($n = 3$) from each sample. Values bearing different superscripts in each cell differ significantly from each other by Tukey's test at $P \leq 0.05$.

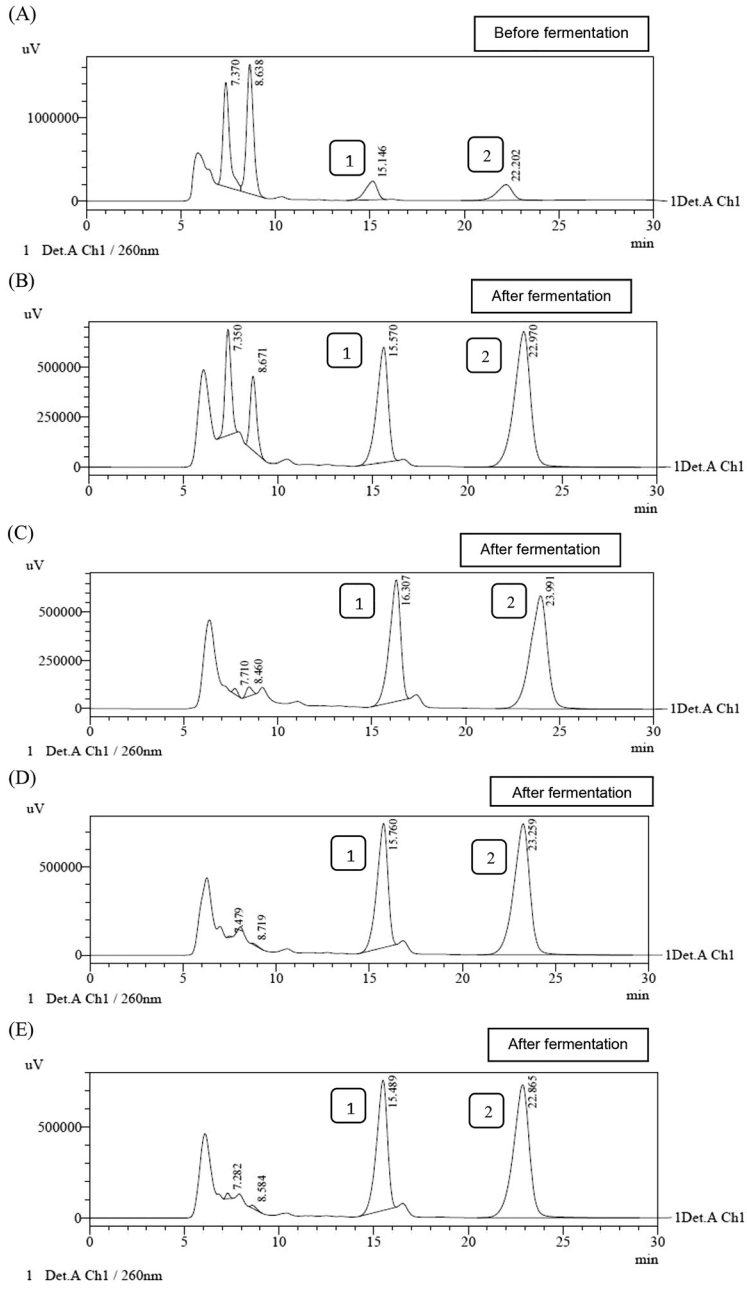

Fig. 4. Chromatograms for biotransformation of isoflavones in: A) unfermented soy milk and soymilk fermented by: B) *L. rhamnosus* K4E, C) *L. fermentum* K7, D) *L. fermentum* K16, and E) *L. plantarum* RD7. 1 = daidzein, 2 = genistein

55.43% for daidzein and 72.30% for genistein. *L. fermentum* K7 biotransformed aglycones to 52.14% daidzein and 68.16% genistein. Similarly, *L. fermentum* K16 and *L. plantarum* RD7 produced effective amounts of aglycones, 47.36% and 44.56% daidzein and 65.77% and 50.32% genistein, respectively. Genistein was the major bioactive isoflavone aglycone in each soymilk fermented by the four *Lactobacillus* strains used in the study.

Similarly, to our study, Hati et al. (2017) reported the production of 49.42% genistein and 23.49% daidzein by *L. bulgaricus* NCDC (09), 44.81% genistein and 25.14% daidzein by *L. rhamnosus* MTCC 5945 (NS4), and 47.47% genistein and 22.83% daidzein by *L. helveticus* MTCC 5463 (V3) in fermented soymilk. According to Rekha and Vijayalakshmi (2010), after 24 and 48 h of soymilk fermentation, bioactive aglycones of genistein and daidzein varied from 97.4 to 98.5% and 62.7 to 92.3%, respectively, with various lactic acid bacteria combinations. Chen et al. (2011) reported similar findings for production of aglycones from soymilk by employing the strains *Lactobacillus paracasei* and *B. longum,* since aglycones production increased with 52 and 60% compared to unfermented soymilk, after incubation of 48 h. Compared to unfermented soy milk, the lactobacilli strains used in the study showed 9–10 times higher concentrations of soy aglycones (Chen et al., 2011).

4. CONCLUSIONS

Lactobacillus strains producing vitamins, organic acids, and soy aglycones are considered viable, cost-effective alternatives to develop vitamin fortified fermented foods. The highest B_2, B_{12}, and B_9 production was shown by *L. plantarum* RD7 (0.84 μg mL^{-1}), *L. fermentum* K16 (0.084 μg mL^{-1}), and *L. rhamnosus* K4E (0.24 μg mL^{-1}). The highest lactate (16.43 μg mL^{-1}) and acetate (5.86 μg mL^{-1}) production was obtained for *L. rhamnosus* K4E, and the highest butyrate production (0.253 μg mL^{-1}) was measured for *L. plantarum* RD7. *L. rhamnosus* K4E successfully biotransformed soy aglycones during soymilk fermentation (daidzein: 55.43%, genistein: 72.30%). These four *Lactobacillus* cultures could be used as promising strains for developing functional fermented dairy foods and beverages with extra health benefits.

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