pISSN 2384-0269
eISSN 2508-3635

J. Milk Sci. Biotechnol. 2017;35(2):93-97
https://doi.org/10.22424/jmsb.2017.35.2.93

ARTICLE

Stimulating the Growth of Kefir-isolated Lactic Acid Bacteria using Addition of Crude Flaxseed (*Linum usitatissimum* L.) Extract

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Abstract

Linum usitatissimum L. (flaxseed) is emerging as an important functional food ingredient because of its rich contents, namely, α-linolenic acid (ALA, omega-3 fatty acid), lignans, and fiber, which are potentially beneficial for human health. Furthermore, flax or flaxseed oil has also been incorporated as a functional food ingredient into various foods such as milk, dairy products, and meat products. Flaxseed is known to possess antimicrobial activity *in vitro* and *in vivo*, but its growth-stimulating effect on lactic acid bacteria is not clear. Hence, the objective of this study was to determine whether crude flaxseed extract stimulated the growth kefir-isolated lactic acid bacteria *in vitro*. The result of this study showed that *Lactobacillus kefiranofaciens* DN1, *Lactobacillus brevis* KCTC3102, *Lactobacillus bulgaricus* KCTC3635, and *Lactobacillus plantarum* KCTC3105 treated with 100 μL of crude flaxseed extract showed significantly higher growth than the control treated with 100 μL of water (p<0.05). Based on the results of this study, crude flaxseed extract could be used as a growth stimulator for lactic acid bacteria in various food applications, including production of milk and dairy products.

Keywords

flaxseed, lactic acid bacteria, kefir, growth stimulator

Received: June 05, 2017
Revised: June 11, 2017
Accepted: June 26, 2017

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Introduction

The Latin name of the flaxseed was *Linum usitatissimum* L., which means "very useful". And flaxseed was an annual herb and originated from Egypt (Kaithwas *et al.*, 2011), and flaxseed was one of the oldest crops, having been cultivated since the beginning of civilization (Laux, 2011). Every part of the flaxseed plant was utilized commercially, either directly or after processing, while flaxseed oil and its sub-products were used in animal feed formulation (Laux, 2011; Singh *et al.*, 2011; Shim *et al.*, 2014) (Fig. 1).

There is a small difference in using the terms flaxseed (when consumed as food by humans) and linseed (when it was used in the industry and feed purpose) (Morris, 2008). Until now, flaxseed has been the focus of increased interest in the field of diet and disease research due to the potential health benefits associated with some of its biologically active components(Goyal et al., 2014; Shim *et al.*, 2014) (Table 1).

Fig. 1. *Linum usitatissimum* L. (flaxseed) was one of the oldest cultivated corps, continues to be widely grown for oil, fiber, and food (Resource: Shim *et al.*, 2014).

Flaxseeds have nutritional characteristics and are rich source of ω-3 fatty acid, α-linolenic acid (ALA), short chain polyunsaturated fatty acids (PUFA), soluble and insoluble fibers, phytoestrogenic lignans (secoisolariciresinol diglycoside-SDG), proteins and an array of antioxidants (Ivanova *et al.*, 2011; Singh *et al.*, 2011; Oomah, 2001; Alhassane and Xu, 2010).

And, according to various previous researches, flaxseed could have the health imparting benefits for reducing the cardiovascular diseases, for decreasing the risk of cancer related to the mammary and prostate gland, for alleviating the menopausal symptoms and osteoporosis, for promoting the anti-inflammatory activity and laxative effect, and so on (Goyal *et al.*, 2014). Above all, flaxseed was added to animal feed to improve animal reproductive performance and health (Heimbach, 2009; Turner *et al.*, 2014). Generally, lactic acid bacteria (LAB) like generally recognized as safe (GRAS) was an order of Gram-positive, acid-tolerant, nonsporulating, rod-(or coccus-) shaped bacteria that shared the physiological and metabolic characteristics. Usually found in decomposing plants and milk products, LAB produced lactic acid as the major metabolic end product of carbohydrate fermentation. Through the acidification's process by several LAB strains, it could be provide an additional hurdle for spoilage and pathogenic microorganisms, and hence contribute to the healthy microflora of human mucosal surfaces (Lee *et al.*, 2014; Chatterjee *et al.*, 2016). However, whether crude flaxseed extract have the growth stimulating effects of kefir-isolated lactic acid bacteria *in vitro* is not know.

Therefore, we assessed the effects of crude flaxseed extract on the growth of Kefir-isolated lactic acid bacteria and identified the main active compounds in crude flaxseed extract responsible for the growth-stimulating effects.

Table 1. Physiological effects imparted by functional elements of flaxseeds (Resource: Goyal *et al.,* 2014; Shim *et al.,* 2014)

Increase (↑)	Decrease (↓)
1. Apoptosis	1. Angiogenesis
2. Bowel movement	2. Blood pressure
3. Fluidity of cardiac muscles	3. Blood viscosity
4. Glucose uptake	4. Cardiac arrest
5. HLD-cholesterol	5. LDL-oxidation
6. Peristalsis	6. Level of pro-inflammatory cytokines
7. Production of 'memory boosting' acetylcholine	7. Morning stiffness
8. Production of anti-inflammatory 3-series eicosanoid compound	8. Obesity
9. Production of less aggregatory 3-series eicosanoids compound	9. Prostate & colon cancer
10. Production of serotonin	10. Reduce pain in joints
11. Vasodilatory response	11. Rheumatoid arthritis
	12. Serum glucose
	13. Synthesis of VLDL
	14. Total cholesterol
	15. Triglyceride production

Materials and Methods

1. Crude flaxseed extract from flaxseed (*Linum usitatissimum* L.)

Flaxseed (*Linum usitatissimum* L.) was donated from Center for One Health, College of Veterinary Medicine, Konkuk University in Seoul, Korea. The dried flaxseed (*Linum usitatissimum* L.) was macerated in 100% sterilized distilled water for 48 hours with occasionally stirring at room temperature. And then, the soluble ingredients were concentrated were filtrated through 0.45 mm Millipore and stored at -20°C before use.

2. Preparation of 4 kefir-isolated lactic acid bacteria

As probiotic cultrure, 4 Kefir-isolated lactic acid bacteria tested in this study were originated from Kefir, and donated from Center for One Health, College of Veterinary Medicine, Konkuk University in Seoul, Korea, *Lactobacillus kefiranofaciens* DN1, *Lactobacilllus brevis* KCTC3102, *Lactobacillus bulgaricus* KCTC3635, and *Lactobacillus plantarum* KCTC3105. All 4 kefir-isolated lactic acid bacteria were grown in MRS broth and were incubated at 37℃ for 48 hrs.

3. Effect of crude flaxseed extract on the growth of various Kefir-isolated lactic acid bacteria

The effect of crude flaxseed extract on the growth of various Kefir-isolated lactic acid bacteria was determined with growth curve analysis. A total of 100 μL crude flaxseed extract was added to 1 mL of MRS broth, and also inoculated with 50 μL suspensions of each Kefir-isolated lactic acid bacteria at the level of 10^5 Log CFU. After mixing and incubating at 37°C, optical density was measured at 600 nm at 1 h intervals using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA).

4. Statistical analysis

All data were presented as mean±SD (standard deviation), and were the results of at least two independent experiments with duplicate assays. All statistical analysis were performed using one-way analysis of variance (ANOVA; GraphPad Prism 5, USA) followed by Duncan's post hoc test for mean comparison. Statistical significance was established as $p<0.05$.

Results and Discussion

In this study, the growth of 4 kefir-isolated lactic acid bacteria in MRS broth containing 10% final volumes (100 μL/1 mL) of crude flaxseed extract from flaxseed (*Linum usitatissimum* L.) was analyzed by measuring the optical density of cultures at 600 nm (Fig. 2). This results of this study showed that *Lactobacillus kefiranofaciens* DN1, *Lactobacilllus brevis* KCTC3102, *Lactobacillus bulgaricus* KCTC3635, and *Lactobacillus plantarum* KCTC3105 with the addition of 100 μL crude flaxseed extract from flaxseed (*Linum usitatissimum* L.) showed higher stimulation than control with addition of 100 μL water (Fig. 2). There was a significant difference between treated group with the addition of crude flaxseed extract from flaxseed (*Linum usitatissimum* L.) and control group ($p<0.05$).

As shown in Fig. 2, *Lactobacillus kefiranofaciens* DN1 with the addition of 100 μL crude flaxseed extract from flaxseed (*Linum usitatissimum* L.) showed the significant growth-stimulation from 14 hours to 24 hours ($p<0.05$), *Lactobacilllus brevis* KCTC3102 and *Lactobacillus plantarum* KCTC3105 with the addition of 100 μL crude flaxseed extract from flaxseed (*Linum usitatissimum* L.) showed the significant growth-stimulation from 17 hours to 24 hours and from 18 hours to 24 hours, respectively ($p<0.05$). And *Lactobacillus bulgaricus* KCTC3635 with the addition of 100 μL crude flaxseed extract from flaxseed (*Linum usitatissimum* L.) showed the significant growth-stimulation from 14 hours to 21 hours. However, *Lactobacillus bulgaricus* KCTC3635 without the addition of 100 μL water showed the significant stimulation from 23 hours to 24 hours ($p<0.05$), maybe it was thought to be due to pH.

Until now, several bifidogenic (or lactic acid bacteria) growth stimulators have been reported such as 2-amino-3-carboxy-1,4-naphthoquinone, 1,4-dihydroxy-2-naphthoic acid, O-α-d-Glucopyranosyl-(1→6)-O-α-d-glucopyranosyl-(1→4)-α-d-glucose, and so on (Isawa *et al.*, 2002; Makelainen *et al.*, 2009).

Lee *et al.* (2014) reported that fermented rice extract (0.2%) stimulated the growth of the lactic acid bacteria such as *Lactobacillus acidophilus, Streptococcus thermophilus*, and *Bifidobacterium lactis*. Specifically, the number of bacteria increased significantly to 11~53 fold after 12-18 hours incubation, compared with negative control significant ($p<0.05$). Namely, fermented rice extract with known or unknown substances strongly stimulated the growth of both bifidobacteria and lactic acid bacteria.

Also, Chatterjee *et al.* (2016) studies the growth of lactic acid bacteria using fruit pectin. Pectin could be classified as a prebiotic on food ingredient. Generally, however, it is assumed that a prebiotic should increase the number or activity of bifidobacteria and lactic acid bacteria (Gibson

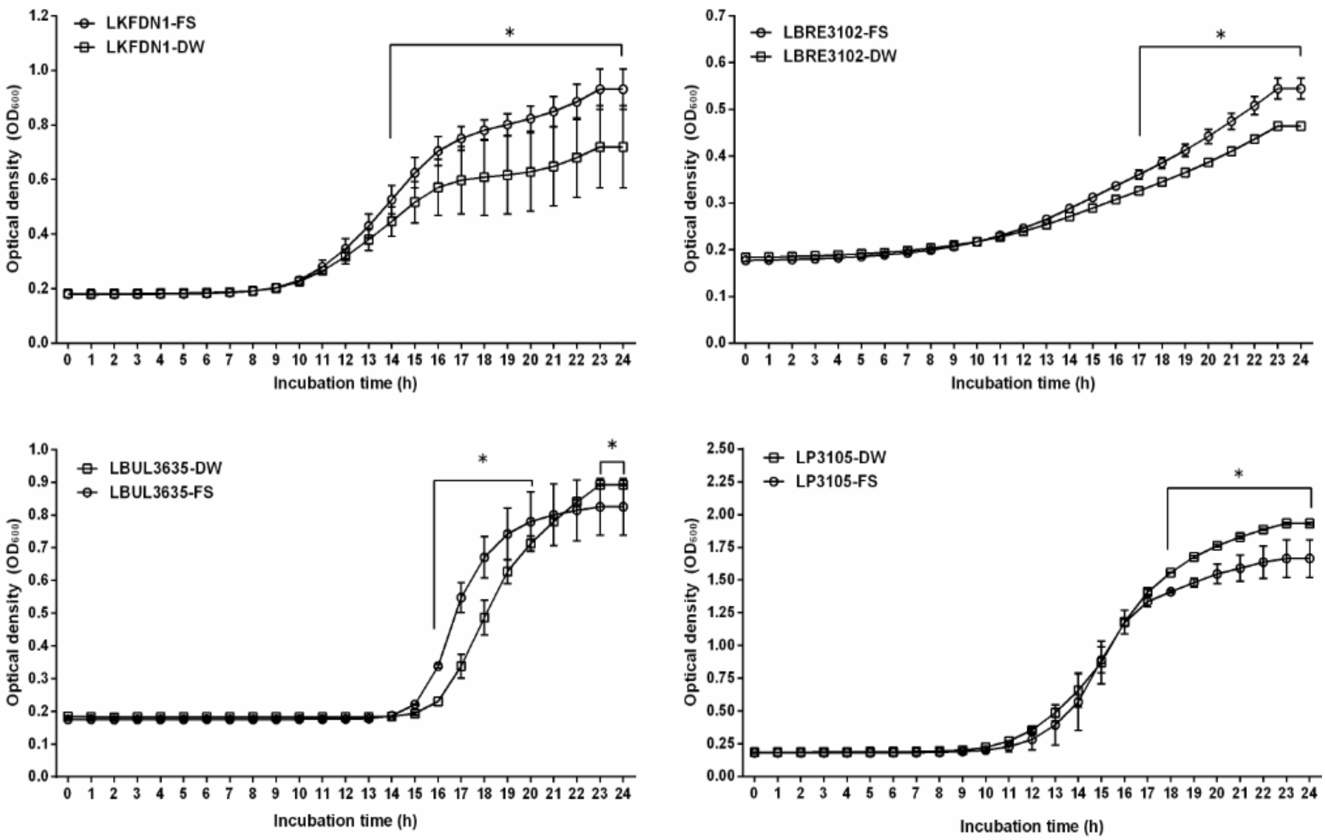

Fig. 2. Growth curves of *Lactobacillus kefiranofaciens* DN1, *Lactobacilllus brevis* KCTC3102, *Lactobacillus bulgaricus* KCTC3635, and *Lactobacillus plantarum* KCTC3105 as kefir-isolated lactic acid bacteria grown in MRS broth mixed with crude flaxseed extract from flaxseed (*Linum usitatissimum* L.) at 10 % final concentration at 37°C for 24 h. Growth was analyzed by measuring optical density at 600 nm.

and Collins, 1999). According to research results of Chatterjee *et al.* (2016), the optical density was measured at 660 nm, for *Lactobacillus acidophilus* culture it was found to be 0.8 at 48 hrs of incubation and 0.77 by 60 hrs of incubation at 37˚C. Whereas *Bifidobacterium bifidum* O.D. value was 0.6 at 48 hrs and 0.56 at 60 hrs of incubation. There was no significant change in the growth of lactic acid bacteria on incubation for 60 hrs, *Lactobacillus casei* the OD was 1.004 at 48 hrs and 0.985 at 60 hrs. There was a reduction in the absorbance when the cultures were incubated more than 48 hrs. Especially, the pH at which the fermentation was carried out had a direct effect on the substrate metabolism due to the change in enzyme activity at different pH (Palframan *et al.*, 2002). According to the results of this study, crude flaxseed extract from flaxseed (*Linum usitatissimum* L.) could be a potential prebiotic so as to stimulate the bacteria to grow on colon and then alter the colonic microflora toward a more health composition. Further understanding of the protective mechanisms conferred by crude flaxseed extract from flaxseed (*Linum usitatissimum* L.) will further enhance the numerous beneficial effects of prebiotics. The results of the current study suggest that crude flaxseed extract from flaxseed (*Linum usitatissimum* L.) could be used as the growth-stimulating agents for Kefir-isolated lactic acid bacteria.

Disclaimer

The views expressed herein do not necessarily reflect those of the US Food and Drug Administration or the US Department of Health and Human Services.

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