doi: 10.17221/356/2016-CJFS

Flaxseed Varieties: Composition and Influence on the Growth of Probiotic Microorganisms in Milk

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Abstract

Smolová J., Němečková I., Klimešová M., Švandrlík Z., Bjelková M., Filip V., Kyselka J. (2017): Flaxseed varieties: composition and influence on the growth of probiotic microorganisms in milk. Czech J. Food Sci., 35: 18–23.

The influence of flaxseed variety composition on probiotics (*Lactobacillus acidophilus, Lb. helveticus, Bifidobacterium* sp.) was determined. The varieties were added into milk in four forms: whole seed, ground seed, meal, and oil. The fermentation of all samples was performed under the same conditions (37°C, 18 h) and then the count of probiotics and the concentration of formed organic acids were determined. The flaxseed meal does not seem to have any significant prebiotic effect. However, our results suggest that the flaxseed oil from varieties with a high level of ALA could improve bacterial survival in milk during storage at 4°C under acidic pH.

Keywords: flaxseed; α-linolenic acid; probiotic bacteria; flaxseed oil; fermentation profiles

Flax (*Linum usitassimum*) is an important food, oil, and fibre crop of the family *Linaceae* (Rubilar *et al*. 2010). Despite the fact that flaxseed has been consumed as a food ingredient for centuries (Oomah 2001), its nutritional benefit has not been fully appreciated yet. Flaxseed is commonly used as a whole seed (i.e. in pastry) but the substances digestible by humans occur especially inside the seeds. To get the valuable substances from flaxseed, disruption and extraction method is needed. Flaxseed contains approximately 40% lipids, 30% dietary fibre, 20% proteins, 4% ash, and 6% moisture, although chemical composition differs among varieties and environmental conditions (Zhang *et al*. 2008; Rubilar *et al*. 2010). Flaxseed is also the major source of α-linolenic acid (ALA), which may represent more than 50% of its total fatty acids depending on the variety (Bozan & Temelli 2008). ALA is one of the essential fatty acids and belongs to the family of omega-3 fatty acids. Therefore, it is not surprising that the importance of flaxseed is rising, and flax is considered to be a functional food. However, flaxseed varieties have different nutritional characteristics, especially the level of ALA, which is an important criterion for the division of flaxseeds into 3 main groups: (a) low level of ALA (less than 10% of ALA in oil); (b) medium level of ALA (30–40% of ALA in oil); and (c) high level of ALA (more than 50% of ALA in oil). Moreover, flaxseed is also a good source of phenolic compounds known as lignans, non-starch polysaccharides, and proteins of high quality (Rubilar *et al*. 2010). Non-digestible polysaccharides are known to have a prebiotic effect and additionally, flaxseed soluble fibre, often called mucilage, is proved to enhance lactic acid bacteria survival and growth

Supported by Ministry of Agriculture of the Czech Republic, Project No. QJ1510274, and by the Josef Hlávka Foundation and specific university research (Nos 20/2015 and 20-SVV/2016).

doi: 10.17221/356/2016-CJFS

in kefir (HadiNezhad *et al.* 2013). Incorporation of flaxseed and its active compounds into functional foods seems to be an attractive attempt in food development. It is profitable to combine the positive effect of lactic acid bacteria or probiotic cultures with flaxseeds in one product. However at first, the influence of flaxseed or its products on probiotic bacteria should be known. In our study, we examined the influence of added flaxseeds (whole seeds, ground seeds), flax oil, and flax fibre after oil extraction obtained from flaxseed varieties differing in the level of ALA on *Lactobacillus acidophilus*, bifidogenic culture (*Bifidobacterium* sp.), and *Lactobacillus helveticus*. We were interested in the relation between flaxseed composition and the growth of probiotic bacteria.

MATERIAL AND METHODS

Brown (Libra, Lola, Recital) and golden (Amon) cultivars of flaxseed (*Linum usitatissimum*) were used from the local producer AGRITEC, Research, Breeding & Services, Ltd. (Czech Republic). These varieties were grown at Rapotín locality in the Czech Republic, at 49°58'21.213"N altitude, 16°58'0.341"E longitude and 329 m a.s.l. The crops were conducted according to standard methods for linseed growing. Ground seeds were prepared by chopping in a Thermomix machine (Vorwerk, Germany).

Probiotic microorganisms (MO): *Lactobacillus acidophilus* – CCDM 151; *Bifidobacterium* sp. – CCDM 94; *Lactobacillus helveticus* – CCDM 92 were obtained from the Culture Collection of Dairy Microorganisms Laktoflora® (MILCOM, a.s., Czech Republic) as liquid milk starters. Skimmed UHT milk was purchased from a local market network. All chemicals were purchased from Sigma Aldrich Company (USA) and were of analytical grade.

Preparation and characterisation of flaxseed oil and flaxseed extraction meal. Brown (Libra, Lola, Recital) and golden (Amon) flaxseed cultivars were disintegrated using the mortar and pestle in our laboratory. Moisture contents were determined gravimetrically according to the ISO 665:2000 (Oilseeds – Determination of moisture and volatile matter content) method after drying (103°C). Disintegrated and conditioned samples were the input feedstock of the extraction experiments. Successive extractions were performed with *n*-hexane (Exxsol™ Hexane Hydrocarbons Fluids; Exxon Mobil, USA) in a Soxhlet extraction apparatus under inert conditions (argon). 100 g of disintegrated flaxseeds were weighed to 0.1 mg in the extraction thimble and defatted cotton was placed on top of the sample to support the distribution of the solvent and to prevent the loss of fine particles (Schneider 1980; Baümler *et al.* 2010). Afterwards, the meal was finely disintegrated in a tumbling mill and exhaustively extracted again to determine residual crude lipids extractable with *n*-hexane according to the ISO 659:2000 (Oilseeds – Determination of oil content) method. Residual content of crude lipids defined as C (g of oil/g of dry inert solid) was balanced as the oil recovered from flaxseed meal by hexane before and after milling (tumbling). Determination of nitrogen and protein content was done by the Kjeldahl method, AOCS Official Method Aa 5-91 (Nitrogen–Ammonia–Protein Modified Kjeldahl Method with titanium dioxide + copper sulphate catalyst). Fatty acid composition of flaxseed oil and residual flaxseed oil from meal extraction was determined by capillary gas-liquid chromatography (CGLC). Fatty acid composition was determined according to AOCS Official Method Ce 1f-96 (Determination of cis- and trans-fatty acids in hydrogenated and refined oils and fats by capillary GLC). Methyl heptadecanoate was used as an internal standard. The analysis was performed on a 6890N gas chromatograph (Agilent Technologies, USA) and SP™ 2560 capillary column (Supelco, USA), 0.25 mm × 100 m, film thickness 0.2 μm was used. The conditions of the analysis were as follows: hexane solution of FAME (1%) was used for the injection (1 μl), split injection (1:50) at 220°C; flow of carrier gas (He) 1 ml/min; analysis at 175°C for 120 min; FID detection at 250°C, flow of H_2 40 ml/min, air flow 450 ml/min, and make-up gas (N_2) flow 45 ml/minute. Determinations were performed at least three times.

Fermentation. In the case of whole flaxseed, ground flaxseed, or flaxseed fibre, the material was weighed and mixed into 100 ml of skimmed UHT milk. In flaxseed oil, it was necessary to emulsify the oil using soya lecithin (oil to lecithin = 10:1) in skimmed UHT milk. Emulsification was performed with a T-25 Basic Ultra-Turrax disperser (IKA) at 50°C for 5 min and the speed of 13 500/minute. Subsequently, samples were pasteurised at 90°C for 10 min and after cooling to fermentation temperature the probiotic microorganisms were added (inoculum 1% v/v). The culture of all samples was carried out at 37°C for 18 h for the detection of any positive or negative effects. All samples were performed three times.

Sample analysis. After the culture and after storage at 4–6°C for 14 and 28 days the count of probiotic bacteria

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was determined by agar plate methods (*Lactobacilli* – MRS agar, pH 6.2; *Bifidobacteria* – TOS agar with mupirocin) and pH was measured. In addition, fermentation profiles (concentration of acetic and lactic acid) were determined by isotachophoresis (VILLA Labeco analyser; Slovakia). Leading electrolyte (LE) was composed of 1.0×10^{-2} M HCl, 2.2×10^{-2} M ε-aminocaproic acid and 0.1% (w/v) methylhydroxyethylcellulose, terminating electrolyte (TE) was composed of 5.0×10^{-3} M caproic acid and 5.0×10^{-3} M tris(hydroxymethyl)aminomethane. Driving flow was 250 and 50 μA, respectively. 1 g of a sample was mixed with distilled water to the volume of 100 ml. This mixture was filtered (filtration paper KA4; Fisher Scientific, USA) and injected into the device.

RESULTS AND DISCUSSION

The oil content in used flaxseed (*Linum usitassimum*) varieties was 38.9–42.5 wt% (Table 1). Fractions rich in simple glycerolipids were removed rapidly during the Soxhlet extraction of disintegrated flaxseeds under inert conditions. The concentration of phosphoglycerolipids was negligible, when the content of phosphorus was below 15 mg/kg. Complex glycerolipids, free fatty acids, and other undesirable oil components such as pigments lowering the crude oil quality were concentrated in the extraction meal. Residual oil content of finely ground flaxseed extraction meal was 20.75–28.49 wt%. The flaxseed coat (epidermal layer, testa, and aleurone layer) had to be considered as the heterogeneous part of oilseeds because of significant differences in composition (i.e. presence of mucilage) and different sorption abilities. We studied the relationship between the growth activity of probiotic bacteria and the composition of flaxseed oil, whole flaxseed, ground flaxseed, and flaxseed meal. For better understanding of the influence of the fatty acid composition, four flaxseed varieties were chosen, two of them with a low level of ALA (Amon, Lola) and two of them with a high level of ALA (Libra, Recital). The exact level of fatty acids is shown in Table 2.

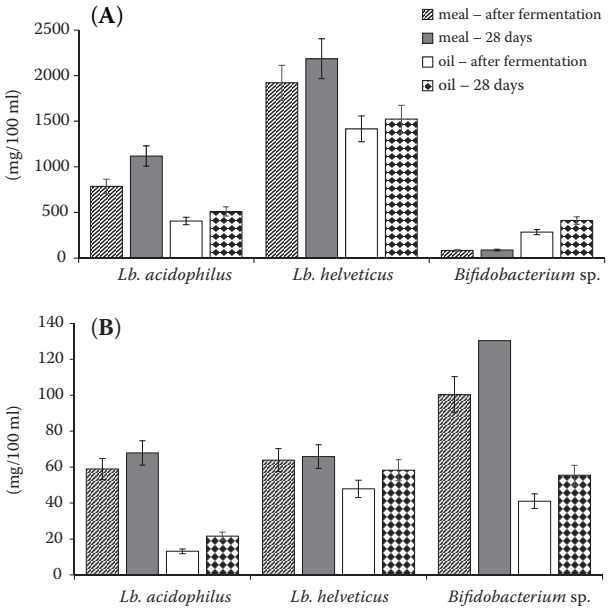

Figure 1. Lactic acid (**A**) and acetic acid (**B**) concentration (mg/100 ml) in samples of varieties with low level of α-linolenic acid

To select probiotic starters for dairy products, probiotic bacteria must be able to grow in milk rapidly and survive 4 weeks of cold storage. In our study *Lb. acidophilus* was not influenced by the presence of added flaxseed or flaxseed products and the cell count at the end of cold storage was always over 10^7 CFU/g. On the other hand, *Lb. helveticus* exhibited unstable growth in the presence of different flaxseed varieties and more repetitions of the experiment are required (Tables 3–5). *Bifidobacterium* sp. is sensitive to environmental conditions and usually requires an addition of more nutrients (e.g. MRS broth) (Jayamanne & Adams 2009). Compared to milk, *Bifidobacterium* sp. grew better in the presence of flaxseed oil with a high level of ALA (Table 5). This effect could be caused by the presence of unsaturated fatty acids such as linoleic acid and ALA. Fatty acids contained in the culture medium highly affect the composition of the cell membranes of *Bifidobacterium* sp. and these physiological modifications enable improvement of bacterial survival during storage at

Table 1. Fat and fatty acid content (%) in the used flaxseed varieties

Variety	Humidity	Fat	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Amon	6.36	41.45	7.4	2.3	18.3	68.7	3.3
Libra	5.80	42.50	6.3	2.5	15.7	16.1	59.3
Lola	6.28	38.88	6.9	2.1	15.3	64.4	11.3
Recital	5.76	41.47	6.0	3.6	21.5	15.2	53.6

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Table 2. Fatty acid composition of oil and extraction meal (g/100 g) from different flaxseed varieties

Fatty acid	Amon		Libra		Lola		Recital	
	oil	meal	oil	meal	oil	meal	oil	meal
Myriistic	0.1	0.1	0.1	0.0	0.1	0.1	0.0	0.0
Palmitic	6.2	6.9	5.6	6.1	6.3	6.9	5.6	6.1
Palmitolejic	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Stearic	3.8	3.9	4.0	3.7	3.2	3.0	4.3	4.0
Elaidic	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0
Oleic	16.0	16.7	14.6	16.6	14.7	15.2	19.4	20.0
C18:2 *trans*	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1
Linoleic	70.6	69.8	15.8	15.4	63.0	63.6	16.1	15.8
C18:3 *trans*	0.2	0.2	0.5	0.3	0.1	0.1	0.2	0.2
Linolenic	2.5	1.8	58.8	57.4	12.1	10.5	53.5	53.0
Saturated fatty acid	10.4	11.2	10.0	10.1	9.9	10.3	10.4	10.6
Cis-MUFA	16.2	16.9	14.8	16.8	14.9	15.4	19.7	20.2
Cis-PUFA	73.1	71.6	74.6	72.8	75.1	74.1	69.6	68.9
Trans-unsaturated fatty acid	0.3	0.3	0.6	0.4	0.1	0.2	0.3	0.3

4°C under acidic pH (Florence *et al*. 2016). However, unsaturated fatty acids are susceptible to fast oxidation due to unsaturated bonds in their molecules. ALA most frequently undergoes autooxidation caused by atmospheric oxygen and oxidation caused by hydrogen peroxide which is produced naturally in foodstuffs (Velíšek 2009). Oxidation products are usually aldehydes that are known to cause unpleasant sensory properties in fermented products.

The pH decrease in all samples correlates with the cell count very well, however, in case of lactobacilli, the pH drops below 4.0, which is a sign of over-fermentation. Apparently, 18 h fermentation is a too long time for the starters *Lb. helveticus* and *Lb. acidophilus*.

By comparing the impact of added flaxseed meal and oil, we find out that the meal seems to be a richer source of polysaccharides and proteins than oil, which

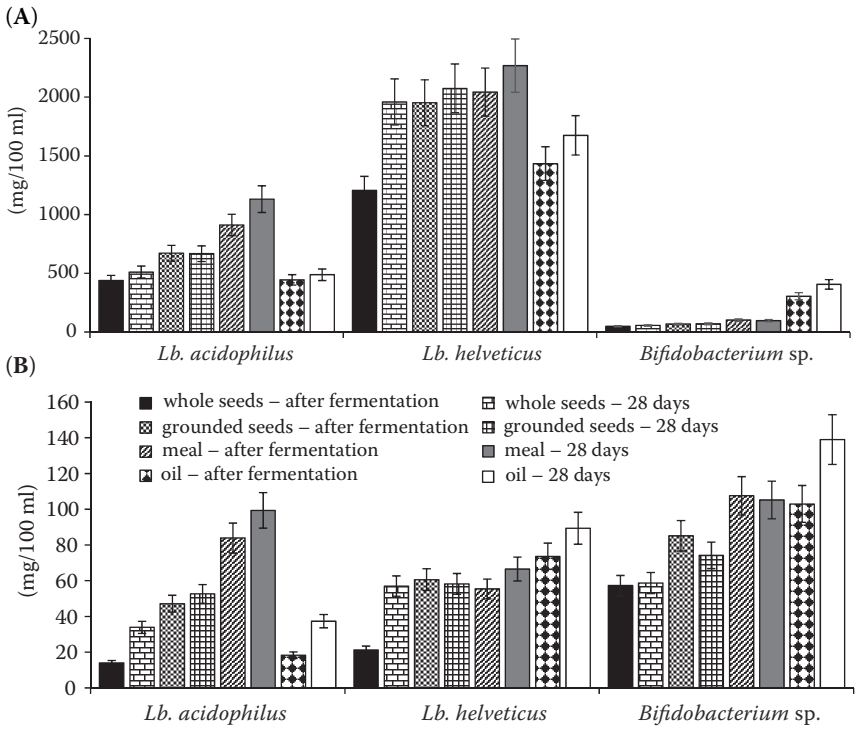

Figure 2. Lactic acid (**A**) and acetic acid (**B**) concentration (mg/100 ml) in samples of varieties with high level of α-linolenic acid

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Table 3. The influence of low-level α-linolenic acid extraction meal and oil on the growth and survival of probiotics

Variety	MO	Meal						Oil					
		after fermentation		14 days of cool storage		28 days of cool storage		after fermentation		14 days of cool storage		28 days of cool storage	
		CFU/g	pH	CFU/g	pH	CFU/g	pH	CFU/g	pH	CFU/g	pH	CFU/g	pH
Amon	151	5.4×10^8	4.09	5.6×10^8	3.93	2.6×10^8	3.89	1.7×10^8	4.36	5.9×10^8	4.24	7.5×10^7	4.15
	92	1.8×10^7	3.44	9.4×10^6	3.40	7.9×10^6	3.39	6.0×10^6	3.47	5.0×10^6	3.40	$< 10^4$	3.41
	94	1.3×10^8	5.63	2.0×10^8	5.77	1.1×10^8	5.90	1.4×10^8	4.42	1.1×10^8	4.02	5.3×10^7	4.18
Lola	151	1.2×10^9	4.46	5.8×10^8	4.26	2.5×10^8	4.12	6.1×10^7	5.55	6.5×10^7	5.42	4.9×10^7	5.31
	92	1.6×10^9	3.55	1.0×10^9	3.50	9.7×10^8	3.55	1.0×10^9	3.60	4.3×10^8	3.65	5.9×10^7	3.74
	94	2.3×10^8	5.26	1.9×10^8	5.32	1.3×10^8	5.40	1.6×10^7	5.77	1.8×10^7	5.65	1.7×10^7	5.59

MO – microorganism signification; CFU – colony forming unit; 151 – *Lbc. acidophilus* CCDM 151; 92 – *Lbc. helveticus* CCDM 92; 94 – *Bifidobacterium* sp. CCDM 94

Table 4. The influence of high-level α-linolenic acid flaxseed and ground flaxseed on the growth and survival of probiotics

Variety	MO	Whole flaxseed						Grounded flaxseed					
		after fermentation		14 days of cool storage		28 days of cool storage		after fermentation		14 days of cool storage		28 days of cool storage	
		CFU/g	pH	CFU/g	pH	CFU/g	pH	CFU/g	pH	CFU/g	pH	CFU/g	pH
Libra	151	2.0×10^8	4.87	2.8×10^8	4.79	3.1×10^8	4.48	3.3×10^9	4.14	4.3×10^8	4.02	2.0×10^8	4.31
	92	ND	3.41	1.9×10^7	3.40	1.0×10^6	3.40	6.0×10^7	3.40	3.0×10^6	3.39	1.5×10^5	3.38
	94	1.4×10^5	5.85	6.0×10^8	5.60	1.5×10^8	5.92	4.2×10^8	5.66	3.5×10^8	5.62	3.4×10^8	5.62
Recital	151	3.4×10^8	4.97	2.6×10^8	5.02	1.8×10^8	5.04	2.6×10^8	4.80	1.1×10^8	4.65	6.5×10^7	4.57
	92	1.1×10^8	3.52	3.1×10^8	3.48	4.4×10^8	3.54	1.5×10^8	3.47	1.9×10^8	3.50	5.8×10^8	3.52
	94	4.0×10^8	6.08	2.8×10^8	5.95	3.6×10^8	6.00	3.1×10^8	6.00	7.6×10^7	6.02	3.3×10^7	6.09

MO – microorganism signification; CFU – colony forming unit; ND – unsuccessful determination; 151 – *Lbc. acidophilus* CCDM 151; 92 – *Lbc. helveticus* CCDM 92; 94 – *Bifidobacterium* sp. CCDM 94

Table 5. The influence of high-level α-linolenic acid extraction meal and oil on the growth and survival of probiotics

Variety	MO	Meal						Oil					
		after fermentation		14 days of cool storage		28 days of cool storage		after fermentation		14 days of cool storage		28 days of cool storage	
		CFU/g	pH	CFU/g	pH	CFU/g	pH	CFU/g	pH	CFU/g	pH	CFU/g	pH
Libra	151	6.3×10^8	4.04	4.2×10^8	3.97	3.9×10^8	3.87	4.5×10^8	4.82	4.3×10^8	4.57	2.5×10^8	4.62
	92	1.7×10^7	3.42	1.2×10^7	3.41	6.5×10^5	3.40	8.0×10^6	3.56	$< 10^6$	3.48	6.5×10^7	3.35
	94	7.3×10^8	5.22	2.0×10^9	5.48	2.7×10^8	5.42	3.3×10^{10}	4.97	6.9×10^8	4.68	5.4×10^8	4.65
Recital	151	2.4×10^8	4.28	1.6×10^8	4.15	9.5×10^7	4.11						
	92	6.9×10^7	3.47	5.5×10^7	3.45	4.2×10^8	3.49						
	94	7.9×10^7	5.56	6.3×10^7	5.60	5.0×10^7	5.72						

MO – microorganism signification; CFU – colony forming unit; 151 – *Lbc. acidophilus* CCDM 151; 92 – *Lbc. helveticus* CCDM 92; 94 – *Bifidobacterium* sp. CCDM 94

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is evident from the increased CFU/g (Table 3). In the general balance of ATP, polysaccharides and proteins are preferred energy sources in comparison with β-oxidation of fatty acids and utilisation of glycerols.

The results of the lactic and acetic acid concentrations in samples of varieties with both low level of ALA and high level of ALA (Figures 1 and 2) show that the composition of fermentation medium influences the fermentation profiles. These profiles in milk with flaxseed components differ from generally known profiles in milk according to which *Bifidobacterium* sp. produces lactic acid and acetic acid at the 3:2 ratio (Salminen & von Wright 2004) while *Lb. acidophilus* and *Lb. helveticus* use homofermentative pathways to form lactic acid only. Fermentation profiles can be influenced by the fermentation conditions (inoculum, temperature, time) as well (Chramostová *et al.* 2014). For lactobacilli, the meal of low-level ALA varieties stimulated acid production more than oil while the influence of high-level ALA varieties was not so pronounced. For bifidobacteria, both the low-level and high-level ALA meal stimulated the formation of acids and increased the ratio of acetic acid. The increased ratio of acetic acid negatively influenced the sensory evaluation of the samples.

CONCLUSION

To the best of our knowledge, we are the first to study the influence of flaxseed and its products on probiotic bacteria. Our results indicate that flaxseed oil with high level of ALA has a positive effect on the growth of *Bifidobacterium* sp. and the oil of both high-level ALA and low-level ALA varieties stimulates them to produce organic acids. These results contribute to the development of novel food products with health benefits, however, it should be explored further.

Acknowledgements. The author is indebted to Erik Pesek for the determination of chemical and physical characteristics of fats and oils.

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Received: 2016–09–13
Accepted after corrections: 2016–12–14