LEGUMES SPROUTS AS VEHICLES FOR *LACTOBACILLUS ACIDOPHILUS* – OPTIMIZATION OF SYNBIOTIC PREPARATION

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Abstract

The study evaluated legume sprouts as carriers for Lactobacillus acidophilus. Survival and/or growth of probiotics were strongly determined by germination temperature, methods of inoculation, and legume species. Compared to the initial population, an increase in the number of the probiotics was only determined in lentil and soy-bean sprouts. In lentil sprouts, growth of the probiotic microorganism was only detected in sprouts obtained from seeds imbibed in inoculums and further cultivated at 35°C (7.1× 106 CFU/ g f.m.). The number of L. acidophilus determined in soy-bean sprouts was higher - 1.07 × 107 CFU/ g f.m (25°C), 1.66 × 107 CFU/ g f.m (30°C), and 1.44 × 107 CFU/ g f.m (35°C). Soy-bean seeds were the best material for production of synbiotics (ensuring both probiotic growth and biomass accumulation). The number of probiotic microorganisms determined in the commonly consumed portions (about 10 g) classifies the obtained sprouts as probiotic functional food.

Key words: *probiotics, legumes, sprouts, L. acidophilus, synbiotics*

1. INTRODUCTION

Food containing probiotic microorganisms has been commonly consumed all over the world for many years. Diet containing such products is usually associated with good health status and an increase in consumer wellness (Saad et al. 2013). Probiotics, mainly (but not exclusively) lactobacilli and bifidobacteria, are microorganisms that are believed to provide health benefits when consumed. They are intended to colonize the large intestine and confer physiological health benefits to the host (Baena & Salinas 2015; Walsh et al. 2014). According to literature data, probiotics improve the functions of organism (e.g. alleviation of lactose intolerance, immune enhancement, decrease in fecal enzymes and mutagenicity or hypocholesterolemic effect) and reduce the risk of disease (e.g. cancer, cardio-vascular disorders, diarrhea) (Baena & Salinas 2015).

Probiotics are usually consumed as food supplements (pills, powders etc.) but are also introduced into the diet in combination with commonly consumed foods. So far, prebiotics have been incorporated with dairy products, such as cheeses, dairy desserts, ice-cream, fermented milks, or fermented vegetables (Tripathi & Giri 2014). Moreover, due to the growing interest in veganism, diet restrictions, or consumer preferences, some alternatives are being developed. Such products have to ensure high viability of probiotics during the shelf-life of foods and, in some cases, even after their expiry date. As indicated by the results of previous studies, non-dairy products such as salami, sausages, milk and dark chocolate, organic jam, and juice have been recognized as excellent vehicles to deliver probiotic health (Possemiers et al. 2010; Al-Sheraji et al. 2013; Coman et al. 2012).

There are 2 mains strategies concerning probiotics in foods. In the first approach, microorganisms are introduced in excess and their content is continuously reduced during food shelf-life. In the second scheme, microorganisms are able to grow in the food matrix and can improve its sensory properties as well as nutritional and pro-health quality (Ranadheera et al. 2010). These products can be classified to synbiotics as a compilation of pre- and probiotics.

The aim of the study was to investigate the effectiveness of legume sprouts as carriers for *L. acidophilus*. The study is focused on the optimization of synbiotic production including selection of the legume and methods of inoculation with prebiotics.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals used for cultivation of sprouts and microbiological media were purchased from Sigma–Aldrich company (Poznan, Poland) and BTL Ltd. (Łódż, Poland). Soy-bean, lentil, adzuki bean, and mung bean seeds were purchased from the PNOS S.A. in Ozarów Mazowiecki, Poland.

L. acidophilus was used in this study. The strain was isolated from a commercial probiotic preparation. The culture was tested for cell morphology; biochemical tests and 16S rDNA sequencing were also performed. Bacterial stocks used for the inoculation were stored at -20 °C in MRS broth with 20% (v/v) glycerol. Prior to inoculation, aerobic cultures on MRS agar were cultivated twice for 24 h at 37 °C. Then, the colonies were sterilely collected from Petri dishes, suspended in water, and used for inoculation of the sprouts. For preparation of the inoculum, the bacterial concentration was estimated by optical density (OD) analysis at 600 nm using a spectrophotometer Smart Spec Plus (Bio-Rad, USA). A previously determined standard curve was used to determine the number of cells in the suspension at the level of 1×10^8/ml on the basis of the OD value.

2.2. Sprouting conditions

Seeds were sterilized in 1% (v/v) sodium hypochlorite for 10 min, then drained and washed with distilled water until they reached neutral pH. Then, they were soaked in distilled water (C, control) or probiotic water suspension (1×10^8 CFU per 1 gram of seeds) (S, soaked with probiotics). Lentil, soy-bean, adzuki bean, and mung bean seeds were imbibed for 4, 4, 6, and 8 h, respectively. The seeds (approximately 12 g per plate) were dark-germinated for 4 days in a growth chamber (SANYO MLR-350H) on Petri dishes (ϕ 125 mm) lined with absorbent paper (relative humidity 70%) (Świeca and Baraniak, 2014). Seedlings were sprayed daily with 5 ml of Milli-Q water (C, S) or 5 ml of probiotic water suspension on the 1st day of cultivation (1×10^8 CFU per 1 gram of seeds)(W, watered with probiotics). Sprouting was run at 25°C, 30°C, and 35°C (25, 30, and 35, respectively). After 4 days, sprouts were manually collected and analyzed.

2.3. Assessment of sprouting efficiency

The seed germination percentage was calculated using the following formula:

$$\text{Seed germination percentage [\%]} = \frac{\text{Number of germinated seed}}{\text{Total number of seed}} \times 100$$

Biomass accumulation was expressed as the mass of 10 sprouts. For each variant of sprouting, at least 100 sprouts were weighted (Świeca & Baraniak 2014).

Counting of probiotics

Once sprouting was completed, the samples were collected and the surface (SP) and total (TP) probiotics were counted. For SP, 1 g of sprouts was washed with 10 ml of Ringer`s solution (10 min. 60 rpm). For TP, 1 g of sprouts was gently homogenized with 10 ml of Ringer`s solution and shaken for 10 min (60 rpm). Then, serial decimal dilutions of the sprout samples were done and 0.1 ml of aliquots were placed on MRS agar in triplicate and incubated aerobically at 37 °C for 48 h. The characteristic colonies were counted and their numbers were calculated as CFU/g of fresh weight.

2.4. Efficiency factor

To sum up the final effect of the protocol proposed for the synbiotic preparation, the efficiency factor was calculated:

$$\text{Efficiency factor} = \frac{\text{Seed germination percentage [\%]}}{\text{Total mass of sprouts}} \times \text{Amount of probiotics}$$

2.5. Statistical analysis

All experimental results were mean ± S.D. of three parallel experiments. One-way analysis of variance (ANOVA and Tukey`s post-hoc test) was used to compare the groups. α values < 0.05 were regarded as a significant.

3. RESULTS

The study evaluated sprouts of selected legumes as carriers for probiotic strain *Lactobacillus acidophilus.* Legumes seeds/sprouts were employed due to high content of resistant starch, which may support survival and growth of bacteria. Production of such kind of synbiotics has to be performed in condition ensuring growth of bacteria and high germination efficiency (biomass production). According to this an influence of sprouting temperature and method of probiotic inoculation on germination rate, biomass accumulation and growth of bacteria were determined.

Generally, both beans (Adzuki and mung) were effectively germinated at the studied temperature range (Table 1). Lentil was most effectively sprouted at 25°C; at 35°C a clearly visible decrease of germination efficiency was determined – seeds germination was lower than 10%. Also in the soy-bean case an undesirable effect was observed during sprouting at 35°C and most importantly it was strengthened by inoculation with probiotics (compared to the control a decrease by about 48% and 36% in the S and W sprouts, respectively) (Table 1).

Table 1. Effect of sprouting temperature and probiotics on germination efficiency

		Seeds germination [%]			
		Sprouts			
Sprouting		Lentil	Soy-bean	Adzuki bean	Mung bean
temperature	C	86.94±6.38bcd	79.81±3.16c	88.78±2.22bc	94.03±1.60cd
25ºC	S	92.47±0.25d	76.33±0.43c	86.61±1.92b	95.59±0.24d
	W	91.25±3.26d	72.59±3.03b	90.03±1.59bc	92.81±0.64c
	C	77.42±4.92b	78.18±3.19c	86.35±4.09abc	94.72±1.62cd
30 ºC	S	87.60±0.25c	72.18±1.30b	90.55±1.10c	93.51±0.48c
	W	85.66±1.27c	76.74±2.95bc	91.60±2.66c	96.98±0.97d
	C	9.28±9.25a	69.20±7.74bc	85.30±2.15a	89.62±6.75ab
35 ºC	S	3.02±0.25a	36.05±2.60a	82.41±2.47a	91.65±0.97b
	W	3.26±0.51a	44.35±4.64a	81.89±2.79a	86.08±3.02a

C- control; S- seeds soaked with the probiotic before germination; W- sprouts watered with the probiotic.

Means (± standard deviations) followed by different letters are significantly different (α=0.05).

The mass of sprouts in the synbiotics based on the Adzuki and mung beans was comparable with that determined for the control sprouts (Fig. 1). In the case of mung bean, a slight insignificant reduction of the biomass of sprouts inoculated with *L. acidophilus* was observed. The introduction of probiotics into lentil sprouts caused an insignificant increase in biomass at 25°C (compared to the respective control). Similarly, the soy-based synbiotics obtained after inoculation of sprouts (spraying with the inoculum on the 1st day of cultivation) were characterized by statistically significantly higher biomass (Fig. 1).

The amount of the probiotics in the sprouts at the beginning of cultivation showed that the inoculation method plays a crucial role in the survival of bacteria. Seeds (sprouts) were inoculated with water infusions to obtain 1×10^8 CFU per 1 gram of material. As shown in Fig. 2, the initial amounts of *L. acidophilus* (determined after imbibition (S) or 6 hrs after spraying on the 1st day of cultivation (W)) were significantly lower. This clearly pointed out that this step strongly determines the quality of the synbiotics (survival of probiotic bacteria). Compared to the initial population (the number of probiotics determined after soaking the seeds in probiotic or 6 hrs after spraying with the inoculum), an increase in the total number of the probiotics was found in the lentil and soy-bean sprouts. In the case of the lentil sprouts, growth of the probiotic microorganisms was only detected in sprouts obtained from seeds imbibed in the inoculums and further cultivated at 35°C (7.1E+06 CFU/ g f.m.). The number of *L. acidophilus* determined in soy-bean sprouts was higher. The growth of probiotic bacteria was determined at all the temperature values studied; however, it was most effective in sprouts obtained from seeds imbibed in the inoculum. One gram of these sprouts contained 1.07×10^7 (25°C), 1.66×10^7 (30°C), and 1.44×10^7 (35°C) and, most importantly, the amounts of *L. acidophilus* commonly consumed portions (about 10 g) classify these sprouts as probiotic functional food. As for beans sprouts, it was concluded that they did not provide conditions for survival or/and growth of the probiotic bacteria. Generally, the population of probiotics was significantly reduced during sprouting.

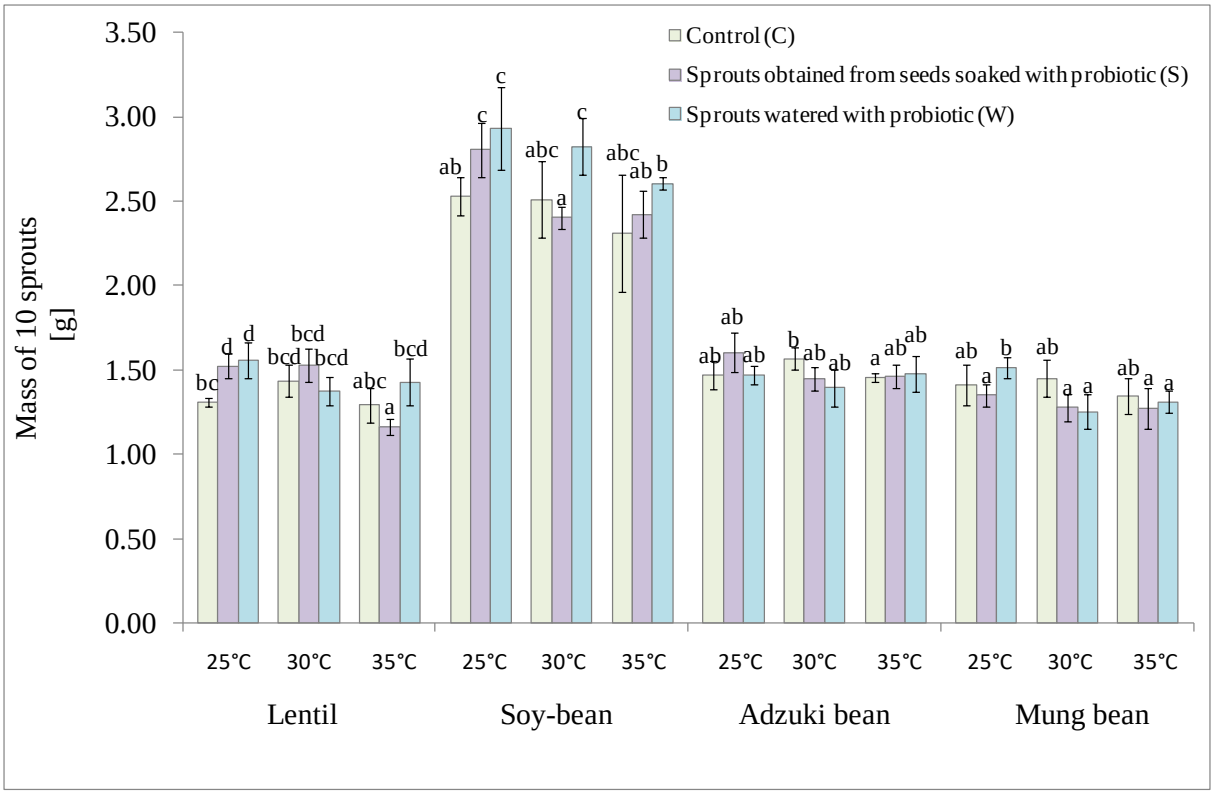


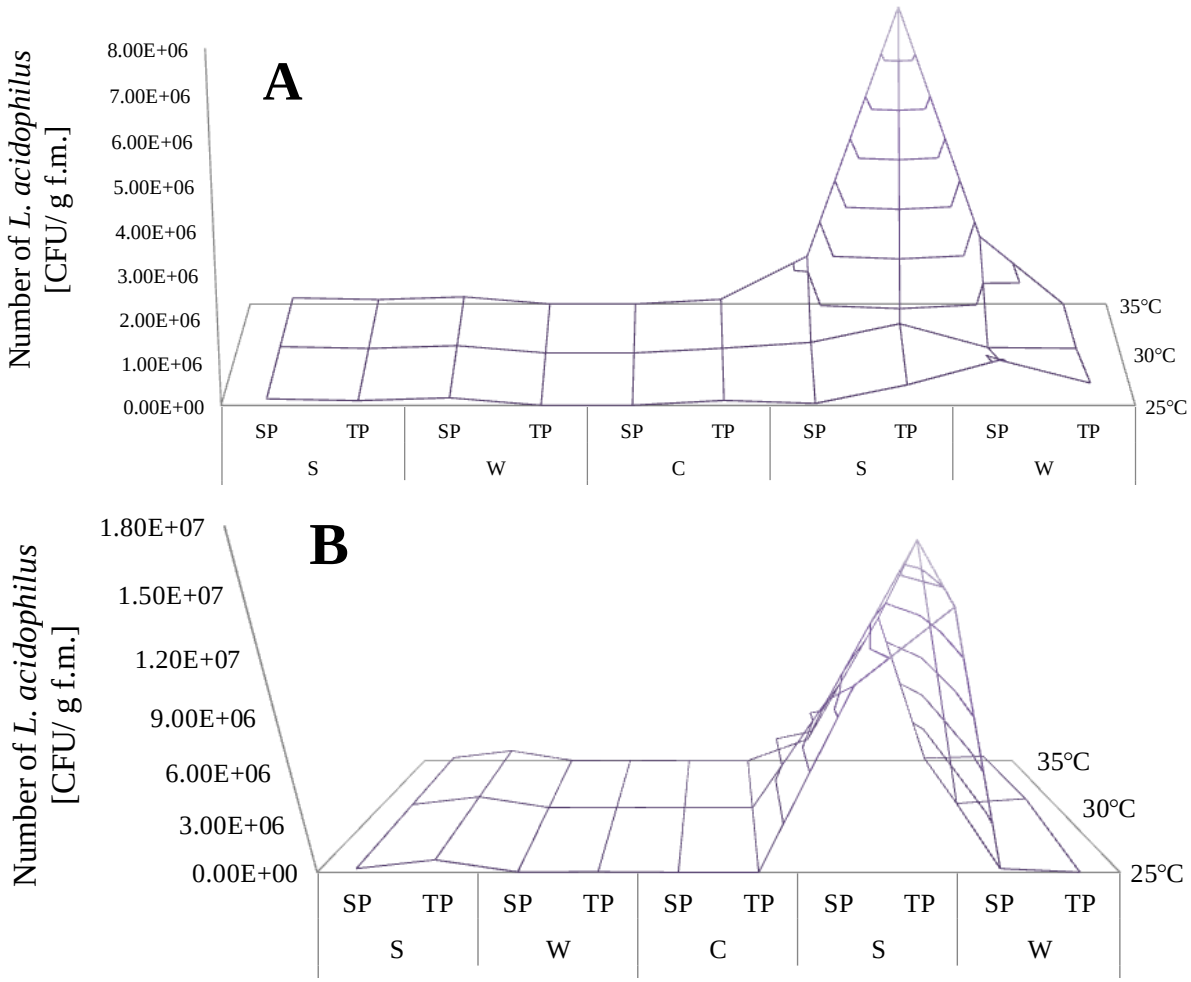
Fig. 1. Effect of sprouting temperature and probiotics on biomass accumulation

Means (± standard deviations) followed by different letters for selected legume species are significantly different (α=0.05).

Conditions for production of legume-based synbiotic ought to ensure growth (survival) of probiotic microorganisms as well as high sprouting efficiency and biomass accumulation. To combine all these issues, an efficacy factor was proposed (Table 2). The values of the efficiency factors determined in the study clearly pointed out that soy-bean sprouts were the best carriers for *L. acidophilus*. At temperature 35°C, lentil sprouts contained a satisfactory population of the probiotic; however, the sprouting efficiency and biomass accumulation were extremely low, which disqualifies these conditions

4. DISCUSSION

Synbiotics designed by combination of low processed fruits/vegetables and probiotic organisms are rare. The main factors limiting probiotic survival and/or growth are insufficient amounts of nutrients and physicochemical conditions (osmotic pressure of the product, a high oxygen level). In the most cases, probiotics are introduced in excess, and their level decreases during the shelf-life of synbiotic products. This strategy has already been successfully employed during development of new functional foods based on table olives (Lavermicocca et al. 2005), an apple-based fruit juice (Champagne et al. 2008), or fresh cut apple (Alegre et al. 2011).



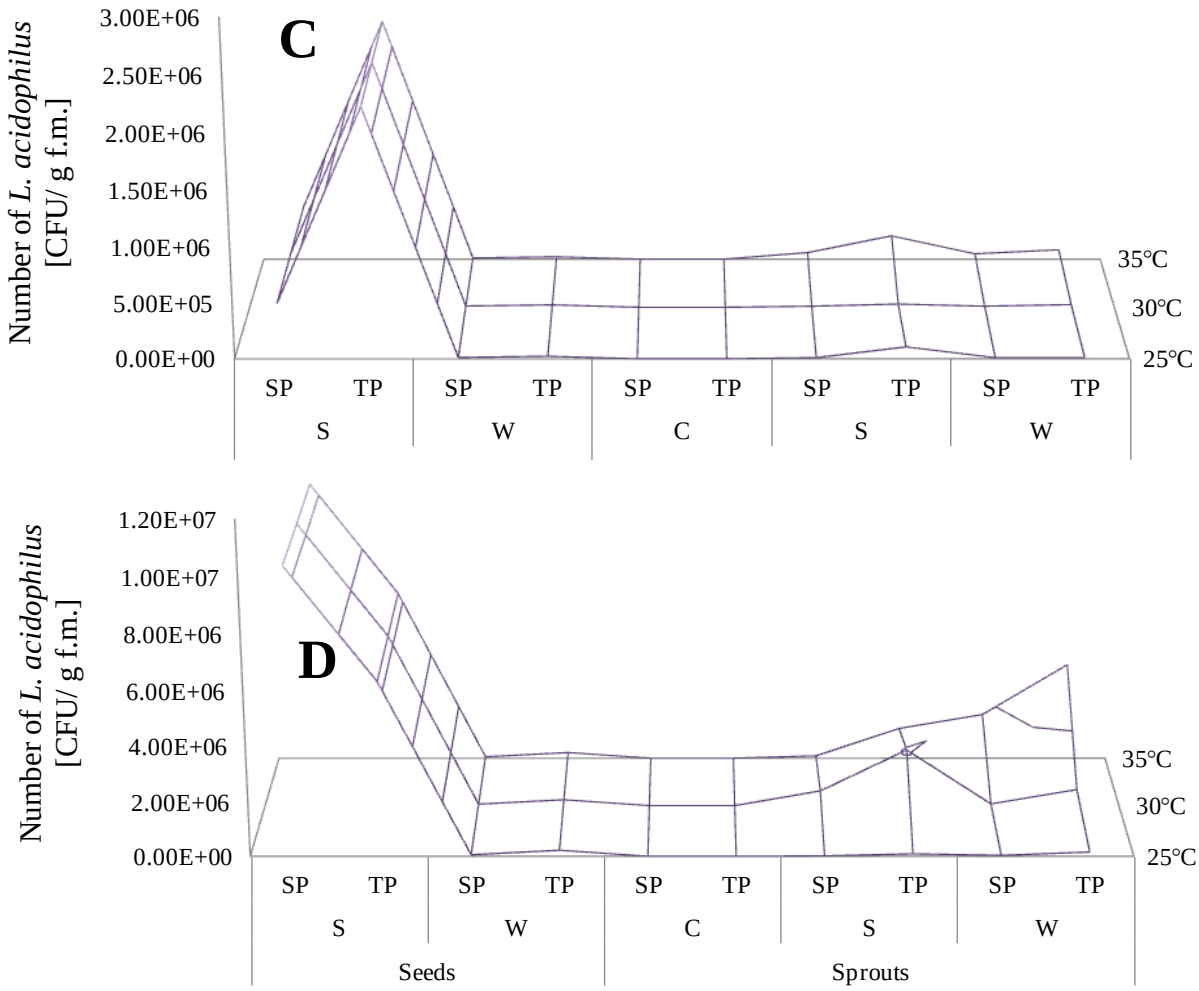


Fig. 2. Effect of sprouting conditions and inoculation methods on survival of probiotics in sprouts

A-lentil sprouts; B- soy-bean sprouts; C- adzuki bean sprouts; D- mung bean sprouts.

C- control; S- seeds soaked with the probiotic before germination; W- sprouts watered with the probiotic.

SP- surface probiotics; TP- total probiotics

Table 2. Effect of sprouting conditions and inoculation methods on synbiotic efficiency

			Efficiency factor			
Sprouting temperature		Sprouts:	Lentil	Soy-bean	Adzuki Bean	Mung Bean
25°C	C	SP/TP	0a	0a	0a	0a
	S	SP	2.2E+06±1.5E+06^{c}	9.4E+06±3.8E+06^{g}	5.1E+04±2.6E+04^{c}	1.8E+06±5.7E+05^{bc}
		TP	1.0E+07±1.9E+06cd	2.3E+07±4.5E+06g	1.9E+05±1.2E+05cd	4.7E+06±1.3E+06e
	W	SP	6.6E+04±3.3E+04bcde	1.3E+06±4.1E+05cd	5.7E+04±3.8E+04bc	3.8E+04±1.5E+03e
		TP	2.0E+05±9.7E+04d	2.9E+06±1.6E+06cd	9.0E+04±4.7E+04bc	3.4E+05±6.7E+04ef
30°C	C	SP/TP	0	0	0	0
	S	SP	3.2E+05±2.1E+04bcd	1.5E+05±3.6E+04f	5.3E+04±3.4E+04b	3.4E+04±1.6E+03de
		TP	3.3E+03±3.1E+02de	3.6E+05±1.4E+05g	8.2E+04±4.3E+04bc	3.1E+05±6.4E+04e

	W	SP	3.0E+04±1.6E+03c	3.3E+04±5.3E+03d	3.3E+04±2.6E+03b	5.2E+05±2.6E+05ab
		TP	4.5E+04±1.7E+04bc	1.9E+05±1.9E+05d	3.3E+04±2.7E+03b	3.1E+05±2.9E+05abc
35°C	C	SP/TP	0	0	0	0
	S	SP	1.9E+04±4.3E+03^{b}	8.4E+02±8.8E+02^{abcd}	4.3E+04±2.1E+04^{b}	2.8E+05±1.2E+04^{cd}
		TP	5.7E+04±4.2E+04d	2.2E+05±4.0E+04bc	1.2E+05±3.8E+04c	1.4E+06±4.4E+05bc
	W	SP	4.5E+02±3.4E+02b	5.9E+03±3.5E+03b	6.5E+03±2.9E+03b	7.7E+04±3.4E+04b
		TP	5.0E+03±2.6E+03b	5.1E+03±3.3E+03b	9.3E+03±4.9E+03b	1.3E+05±1.9E+04bc

C- control; S- sprouts obtained from seeds soaked with the probiotic; W- sprouts watered with the probiotic. SP- surface probiotics; TP- total probiotics

Means (± standard deviations) followed by different letters are significantly different (α=0.05).

In our study, *L. acodophilus* was introduced into legume sprouts, which are an excellent source of starch (potential prebiotic) (Świeca & Gawlik-Dziki 2015). The study focused on selection of an optimal protocol for preparation of synbiotics allowed us to find conditions that would ensure growth of both components, i.e. the sprouts and the microorganisms. Screening of the conditions proposed for lentil and soy-bean sprouting confirmed that those legumes prefer lower temperatures for germination. A decrease in the sprouting efficiency was comparable with that observed previously by Egli et al. (2005) Unfortunately, 35°C (the highest temperature studied in the screening) was optimal for bacterial growth. Most importantly, a parallel growth of sprouts and *L. acidophilus* was determined for the soy-bean sprouts at 25-30°C. In the available literature, there is no data concerning application of bean flour as probiotic. So far, increased growth of probiotics has been observed after addition of lentil flour to yogurts (Agil et al. 2013). The results of the cited study demonstrated that green lentils selectively enhanced the number of probiotic bacteria in yogurt in the initial stages of storage and maintained overall microbial counts (starter cultures and probiotics) over a 28-day storage period. It has already been proved that the viability of *L. acidophilus* is less dependent on the matrix used, but strongly dependent on water activity and content in the food matrix. Therefore, it can be concluded that the losses observed in the probiotic population in the initial stages of germination may have been caused by lower activity of water (the bean seeds absorbed about 2-3-fold lower amounts of water compared to the soy-bean and lentil seeds). Since *L. acidophilus* growth was detected only in sprouts obtained from seeds soaked in the inoculum, it may be suggested that an important role is also played by oxygen isolation.

The study provides some promising results; however, a detailed evaluation of synbiotics based on legume sprouts and *L. acidophilus* needs additional studies of the survival of probiotics during storage and digestion as well as changes in the nutritional and nutraceutical quality of sprouts.

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