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APPLYING SPROUTS OF SELECTED LEGUMES AS CARRIERS FOR *LACTOBACILLUS RHAMNOSUS* GG – SCREENING STUDIES

S u m m a r y

Probiotics and prebiotics play an important role in human and animal nutrition. Those research studies were performed to evaluate the potential of using legume sprouts as carriers for probiotic strain of *Lactobacillus rhamnosus* GG. They determined the effect of legume species, temperature of sprouting, and inoculation methods of seeds or growing sprouts on the survival and/or growth of probiotics.

It was found that the count of bacteria in sprouts depended on the germination temperature, inoculation methods as well as on the species of legume used as a carrier. The beans examined (Adzuki and Mung) germinated effectively at a temperature between 25 ÷ 35 ºC. And the lentil sprouted most effectively at 25 ºC. In the case of soy-bean and lentil, the temperature of 35 ºC caused the germination efficiency to decrease. The growth of *Lb. rhamnosus* GG was reported only in the case of the lentil and soy-bean sprouts obtained from the seeds imbibed in an inoculum and germinated at 25 ºC. The count of probiotic bacteria was 3.1×10^6 and 7.18×10^6 CFU per grams of fresh mass, respectively. The sprouts obtained from the bean seeds analyzed did not provide any conditions for probiotic bacteria to survive and grow. The best carrier for the probiotic bacteria studied were the soy-bean sprouts; in their case, after inoculation of seeds and using a suspension of probiotic bacteria, the sprouts obtained at 25 ºC had the best quality parameters.

Key words: legumes, sprouts, probiotics, *Lb. rhamnosus*

Introduction

The consumption of probiotics is usually associated with healthy diets and an increase in consumer wellness [3]. Probiotics (or their metabolites) reduce the risk of cancer, improve heart health, enhance the immune system, reduce menopause symp-

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toms, enhance gastrointestinal health, preserve urinary tract health, decline osteoporosis, protect vision and exhibit antibacterial, anti-inflammatory and antiviral activities [4]. Many studies have confirmed those positive effects in *in vitro* studies as well as in *in vivo* animal or/and human models [4, 27].

Probiotics have been incorporated into several food products and supplements; most of them are dairy products, such as cheeses, dairy desserts, ice-cream, fermented milk products, or fermented vegetables [24]. However, in recent years, novel alternative matrices for probiotics have been developed, e.g., chocolate or meat products [3, 18]. Moreover, the need for new probiotic products has drawn attention owing to the growing interest in veganism and the higher number of consumers with diet restrictions such as lactose intolerance, allergies, and cholesterol restriction [7, 10, 19].

In modern functional food, probiotic microorganisms are usually combined with prebiotics. Prebiotic is a selective substrate for one or a limited number of probiotics strains. Prebiotics stimulate the growth and survival of probiotic strains and, consequently, they are able to alter the colonic microbiota of the host toward a healthier composition. Legumes are considered to be an excellent source of nutrients and compounds with well-documented pro-health properties, e.g., phenolics or vitamins [8, 13, 22]. Additionally, beans contain high amounts of resistant starch, which may predispose them to be effective prebiotics [1, 11, 12].

The objective of the study was to research on the possibility of using legume sprouts as carriers for *Lb. rhamnosus* GG. The study was focused on selecting the legume species, conditions of germination, and methods utilized to inoculate seeds/sprouts with a probiotic strain.

Materials and methods

Materials

All chemicals used to cultivate sprouts and microbiological media were purchased in the Sigma-Aldrich Company (Poznan, Poland) and BTL Ltd. (Łódź, Poland). Soybean, lentil, Adzuki bean, and Mung bean seeds were purchased in the PNOS S.A., Ożarów Mazowiecki, Poland.

An *Lb. rhamnosus* GG strain was used in this study. The strain was isolated from a commercial probiotic preparation. It was identified microscopically, by biochemical tests, and by 16S rDNA sequencing. The bacterial stocks used for the inoculation were stored at -20 ºC in an MRS broth with 20 % (v/v) glycerol. Prior to inoculation, the aerobic cultures on MRS agar were cultivated twice for 24 h at 37 ºC. Then, the colonies were sterilely collected from the Petri dishes, suspended in water, and used to inoculate the sprouts. To prepare the inoculum, the bacterial concentration was estimated by an optical density (OD) analysis at 600 nm using a Smart Spec Plus spectro-

photometer (Bio-Rad, USA). A previously determined standard curve was applied to determine the number of *Lb. rhamnosus* GG cells in the suspension at a level of $1\times10^{8}\cdot ml^{-1}$ on the basis of OD value.

Sprouting conditions

The seeds were disinfected in a 1 % (v/v) sodium hypochlorite for 10 min, then drained and washed with distilled water until they reached neutral pH. Next, they were soaked in distilled water (C – control sample) or in a probiotic water suspension (1×10^{8} CFU per 1 g of seeds) (S – soaked with probiotics). The lentil, soy-bean, Mung bean, and Adzuki bean seeds were imbibed for [h]: 4, 4, 6, 8, respectively. The seeds (approximately 12 g per plate) were dark-germinated for 4 days in a growth chamber (SANYO MLR-350H) on the Petri dishes (ϕ 125 mm) lined with an absorbent paper (relative humidity of 70 %). The seedlings were sprayed every day with 5 ml of Milli-Q water (C, S) or 5 ml of probiotic water suspension (freshly prepared) on the 1st day of cultivation (1×10^{8} CFU per 1 g of seeds) (W – watered with probiotics). Sprouting was run at [ºC]: 25, 30, 35. After 4 days sprouts were manually collected and analyzed.

Assessing sprouting efficiency

The seed germination percentage rate was calculated using the following formula:

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

The sprouts biomass accumulation was expressed as a mass of 10 sprouts. For each variant of sprouting, at least 100 sprouts were weighed [23].

Counting Lb. rhamnosus GG viable cells

Once sprouting was completed, the samples were collected and the surface (SP) and total (TP) number of probiotic cells 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 made and 0.1 ml of aliquots were placed on an MRS agar in triplicate and incubated aerobically at 37 ºC for 48 h. The characteristic colonies were counted and their numbers were calculated as $CFU\cdot g^{-1}$ of fresh mass.

Efficiency factor

To sum up the final effect of the protocol as proposed for the probiotic-rich sprouts, the efficiency factor was calculated:

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

Statistical analysis

All the experimental results were expressed as a mean ± S.D. of the three parallel experiments. One-way analysis of variance (ANOVA) and Tukey's post-hoc test at a 0.05 level of significance were used to compare the groups.

Results and discussion

Currently, the development of fruits and vegetables containing probiotic strains is a highly interesting issue for consumers [2, 9, 16]. So far, legume flours or sprouted flours have been successfully used as effective prebiotics during preparation of yogurts [1], food supplements [6], or non-dairy probiotic beverages [17].

The study involved the screening of conditions for production of legume sprouts rich in *Lb. rhamnosus* GG, i.e. such conditions were selected that could simultaneously ensure the effective growth of sprouts (seed germination) and the optimal survival (even stimulation of growth) of bacteria. Therefore, the effect was determined of the temperature and inoculation method on the sprouting efficiency and sprouts biomass accumulation (Tab. 1 and 2). Generally, the two bean species (Adzuki and Mung) were effectively germinated at a temperature within the studied temperature range (Tab. 1). The Adzuki bean sprouts obtained at 30 ºC and 35 ºC were characterized by a higher biomass than that of the respective control sample; however the differences were insignificant (Tab. 2). Lentil was most effectively sprouted at 25 ºC, at 35 ºC a clearly visible decrease in the germination efficiency was reported: the seed germination rate was lower than 10 %. Similarly, in the case of the soy-bean, an undesirable effect was observed during sprouting at 35 ºC and, most importantly, it was strengthened by soaking the seeds in the probiotics (a decrease of about 40 % compared to the control sample) – Tab. 1. The introduction of *Lb. rhamnosus* into lentil seeds caused the biomass of sprouts to insignificantly increase at 25 ºC and 30 ºC compared to the respective control sample.

Generally, the temperature of sprouting played a key role in the production of probiotic sprouts; it strongly affected the germination efficiency and biomass production. The results of the germination efficiency obtained in this study (sprouting at 25 ºC and 30 ºC) are comparable with the previous studies on sprouted legumes e.g. soy-bean [28], lentil [21], Adzuki beans [15], and Mung beans [26].

The number of viable *Lb. rhamnosus* GG cells in the sprouts at the beginning of cultivation showed that the inoculation method played a crucial role in the survival of bacteria after 4 days of sprouting. The seeds (sprouts) were inoculated with water infusions (1×10^8 CFU per 1 g of material). As shown in Fig. 1, the initial counts of *Lb. rhamnosus* – determined after imbibition (S) or 6 h after spraying on the 1st day of cultivation (W) – were significantly lower. This fact clearly pointed out that that step

strongly determined the quality of probiotic-rich sprouts (survival of *Lb. rhamnosus*). A significant decrease in the probiotic population in the inoculum as well as in the seeds/sprouts was probably caused by the exposure to oxygen, which had strong effects on the *Lb. rhamnosus* survival in other food matrices, e.g. in apple juice [5].

Table 1. Germination efficiency as determined by effect of sprouting temperature and method of introducing probiotics

Tabela 1. Wydajność kiełkowania determinowana wpływem temperatury kiełkowania i metody wprowadzania probiotyków

Sprouting temperature Temperatura kiełkowania [ºC]	Sample Próba	Germination efficiency / Wydajność kiełkowania [%]			
		Sprouts / Kiełki			
		Lentil Soczewica	Soy-bean Soja	Adzuki bean Fasola Adzuki	Mung bean Fasola Mung
25	C	$86.9^{bc} \pm 6.38$	$79.8^{c} \pm 3.16$	$88.8^{ab} \pm 2.22$	$94.0^{bc} \pm 1.60$
	S	$83.2^{c} \pm 0.25$	$69.7^{bc} \pm 3.04$	$89.0^{b} \pm 0.53$	$95.4^{c} \pm 0.48$
	W	$78.9^{b} \pm 0.89$	$73.8^{bc} \pm 2.48$	$92.1^{ab} \pm 5.42$	$94.0^{b} \pm 0.27$
30	C	$77.4^{bc} \pm 4.92$	$78.2^{c} \pm 3.19$	$86.3^{ab} \pm 4.09$	$94.7^{bc} \pm 1.62$
	S	$81.4^{bc} \pm 2.10$	$72.7^{bc} \pm 5.83$	$92.0^{b} \pm 2.12$	$94.9^{c} \pm 0.48$
	W	$78.1^{bc} \pm 10.66$	$75.9^{c} \pm 2.95$	$90.8^{ab} \pm 2.66$	$94.4^{c} \pm 0.97$
35	C	$9.3^{a} \pm 9.25$	$69.2^{bc} \pm 7.74$	$85.3^{a} \pm 2.15$	$89.6^{abc} \pm 6.75$
	S	$6.4^{a} \pm 0.25$	$41.9^{a} \pm 0.87$	$89.2^{b} \pm 0.27$	$91.2^{a} \pm 0.48$
	W	$6.2^{a} \pm 3.05$	$63.5^{b} \pm 6.66$	$84.3^{ab} \pm 4.75$	$93.5^{bc} \pm 1.58$

Explanatory notes / Objaśnienia:
C – control sample / próba kontrolna; S – sprouts obtained from seeds soaked in probiotic / kiełki otrzymane z nasion namaczanych w probiotyku; W – sprouts watered with probiotic / kiełki podlewane probiotykiem. Table shows mean values ± standard deviations. / W tabeli przedstawiono wartości średnie ± odchylenia standardowe; n = 3; a, b, c – mean values in columns and denoted by different letters differ statistically significantly ($p \leq 0.05$) / wartości średnie w kolumnach oznaczone różnymi literami różną się statystycznie istotnie ($p \leq 0{,}05$).

In general, in the food industry, a wide range of probiotic levels (from 1×10^6 CFU·g^{-1} to 1×10^8 CFU·g^{-1}) is recommended as minimal in consumed foods for any health benefits [15]. Compared to the initial population, an increase in the total number of *Lb. rhamnosus* GG cells was detected only in the lentil and soy-bean sprouts obtained from the seeds imbibed in inoculums and further cultivated at 25 ºC. Those sprouts contained 3.1×10^6 and 7.18×10^6 CFU per g of fresh mass and, most importantly, the counts of *Lb. rhamnosus* as determined in the commonly consumed portions (about 50 g) classified the sprouts obtained as probiotic, functional food.

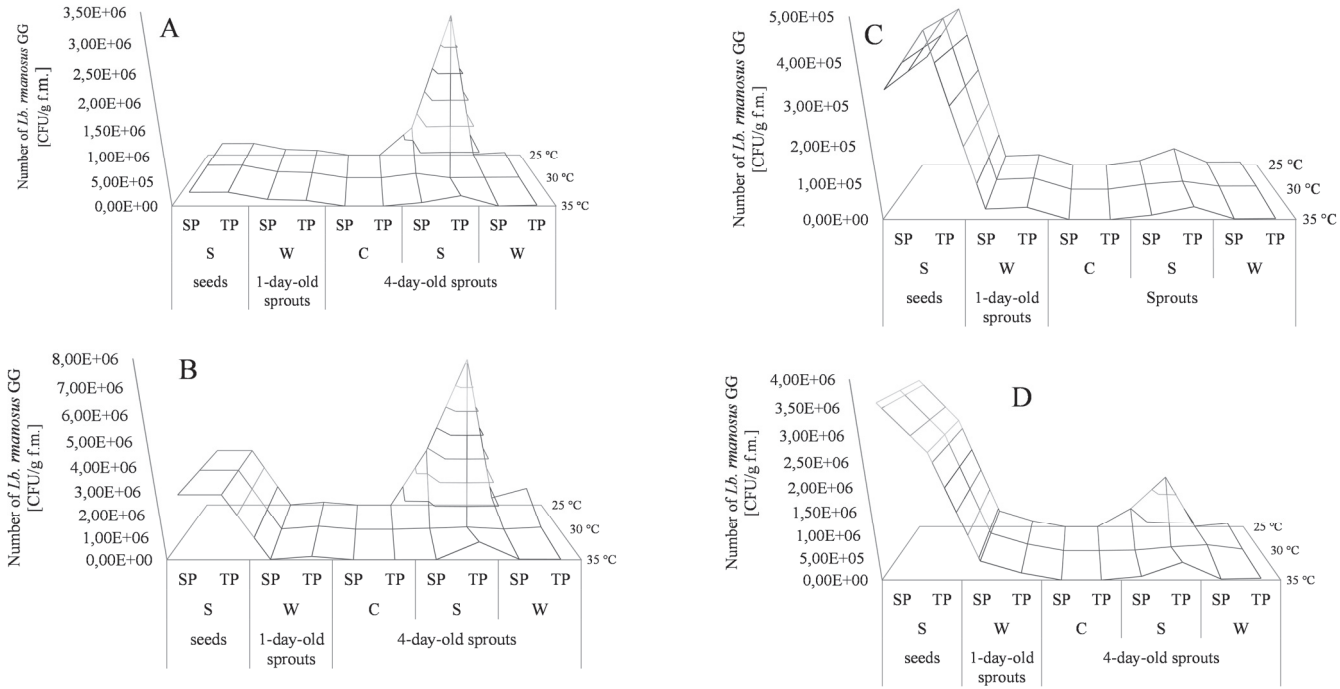
So far, no study has been conducted to evaluate sprouted foods as carriers for probiotics; however, in some studies, fruits or vegetables are involved. In the study by Alegre et al. [2] fresh-cut apples were used as carriers for the probiotic *Lb. rhamnosus*

GG strain. It was shown that the initial population of probiotic was stable during 28-day storage under cool conditions; however, only a slight growth was determined after 5 days of storage. Furthermore, some promising results were obtained by Lavermicocca et al. [14] who investigated the table olive as a vehicle for incorporating probiotic bacterial species. Bifidobacteria and one strain of *Lb. rhamnosus* (*Lactobacillus* GG) showed a good survival rate in the selected olive samples, with a recovery of about 10^6 CFU·g^{-1} after 30 days of storage. The previously cited studies demonstrated that the amount of probiotic bacteria in food products depended on the level of inoculums; their viability must be maintained throughout the product shelf life [2, 20]. In the light of those data, the growth of *Lb. rhamnosus* GG in soy-bean and lentil sprouts is very promising. In the case of the beans sprouts, it was concluded that they did not provide conditions for the survival or/and growth of probiotic *Lb. rhamnosus* strain. Generally, the population of that strain was significantly reduced during sprouting, a slight growth was observed only in the case of sprouts obtained from the seeds imbibed in the inoculum (the probiotics did not reach the initial amounts) – Fig. 1. Vesterlund et al. [25] suggested that the viability of *Lb. rhamnosus* GG was less dependent on the matrix used but it was strongly dependent on the activity of water and its content in a food matrix. During soaking the beans sprouts absorbed about 2 - 3 times lower amounts of water compared to the soy-bean and lentil seeds. Thus, the losses observed in the probiotic population at the initial stages of germination might have been caused by such factors as a lower activity of water and oxygen isolation as well as by a decreased

Table 2. Biomass accumulation as determined by sprouting temperature and probiotics added
Tabela 2. Przyrost biomasy kiełków determinowany temperaturą kiełkowania i dodatkiem probiotyków

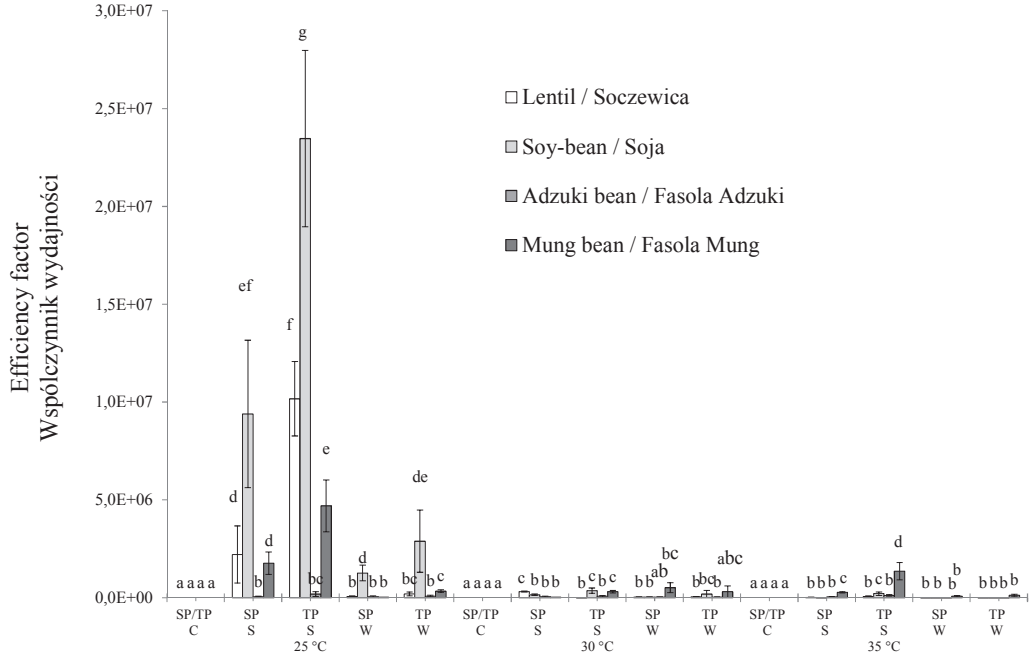
Sprouting temperature Temperatura kiełkowania [ºC]	Mass of 10 sprouts / Masa 10 kiełków [g]				
	Sprouts				
	Sample Próba	Lentil Soczewica	Soy-bean Soja	Adzuki bean Fasola Adzuki	Mung bean Fasola Mung
25	C	$1.31^{a} \pm 0.03$	$2.53^{b} \pm 0.11$	$1.47^{ab} \pm 0.09$	$1.41^{ab} \pm 0.12$
	S	$1.36^{ab} \pm 0.09$	$2.36^{abc} \pm 0.33$	$1.37^{ab} \pm 0.13$	$1.29^{a} \pm 0.10$
	W	$1.32^{ab} \pm 0.09$	$2.39^{abc} \pm 0.33$	$1.53^{abc} \pm 0.13$	$1.28^{a} \pm 0.05$
30	C	$1.43^{bc} \pm 0.09$	$2.51^{bc} \pm 0.22$	$1.57^{bc} \pm 0.07$	$1.45^{ab} \pm 0.11$
	S	$1.52^{bc} \pm 0.09$	$2.13^{a} \pm 0.04$	$1.67^{bc} \pm 0.09$	$1.51^{b} \pm 0.05$
	W	$1.47^{bc} \pm 0.15$	$2.92^{bc} \pm 0.16$	$1.60^{abc} \pm 0.16$	$1.37^{ab} \pm 0.20$
35	C	$1.29^{ab} \pm 0.10$	$2.31^{ab} \pm 0.35$	$1.46^{a} \pm 0.02$	$1.35^{a} \pm 0.10$
	S	$1.19^{a} \pm 0.09$	$2.45^{abc} \pm 0.45$	$1.58^{b} \pm 0.08$	$1.53^{ab} \pm 0.16$
	W	$1.24^{ab} \pm 0.15$	$2.52^{b} \pm 0.11$	$1.61^{abc} \pm 0.18$	$1.51^{b} \pm 0.05$

Explanatory notes as in Tab. 1. / Objaśnienia jak pod tab. 1.



Explanatory notes / Objaśnienia:
A – lentil sprouts / kiełki soczewicy; B – soy-bean sprouts / kiełki soi; C – Adzuki bean sprouts / kiełki fasoli Adzuki; D – Mung bean sprouts / kiełki fasoli Mung; C – control sample / próba kontrolna; S – sprouts obtained from seeds soaked in probiotic / nasiona otrzymane z nasion namaczanych w probiotyku; W – sprouts watered with probiotic / kiełki podlewane probiotykiem; SP – probiotics on surface of sprouts / probiotyki na powierzchni kiełków; TP – total probiotics in seeds/sprouts / całkowita ilość probiotyków w nasionach/kiełkach.

Fig. 1. Effect of sprouting conditions and inoculation methods on survival and growth of probiotics in sprouts
Rys. 1. Wpływ warunków kiełkowania i metody inokulacji na przeżywalność i wzrost probiotyków w kiełkach



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Mean values (± standard deviations) denoted by different letters differ statistically significantly (n = 3, p = 0.05 / Wartości średnie (± odchylenia standardowe) oznaczone różnymi literami różnią się statystycznie istotnie (n = 3; p = 0,05).

Fig. 2. Effect of sprouting conditions and inoculation methods on production of probiotic-rich sprouts
Rys. 2. Wpływ warunków kiełkowania i metody inokulacji na produkcję kiełków bogatych w probiotyki

availability of nutrients (loose seed structure and inadequate activity of enzymes responsible for mobilization of storage materials). Since the conditions optimal for the growth of probiotics (survival) and sprout development are not always the same, an efficiency factor is suggested in the study. It combines the results of the germination rate, biomass accumulation, and the number of probiotic bacteria cells (Fig. 2). The values of the efficiency factors determined in the study clearly pointed out that the soybean sprouts were the best carriers for *Lb. rhamnosus* GG. Based on the efficiency factor, it was verified that the lentil sprouts (that had comparable amounts of probiotic bacteria – Fig. 1) were not predisposed for to be carriers for *Lb. rhamnosus* GG: they were characterized by a lower germination efficiency and biomass accumulation.

The study provides some promising results; however, a detailed evaluation of probiotic preparation based on legume sprouts and *Lb. rhamnosus* requires additional studies on the survival of probiotic strain during storage and digestion as well as changes in the nutritional and nutraceutical quality of sprouts.

Conclusions

1. Legume sprouts may be used as carriers for *Lb. rhamnosus* GG.
2. A number of probiotic bacteria in sprouts are strongly determined by the germination temperature, inoculation methods, and legume species.
3. Lentil and soy-bean sprouts obtained at 25 ºC were the most effective carrier for the probiotic strain studied.
4. Due to the fact that soy-bean seeds ensure both the growth and the biomass accumulations of sprouts and probiotic bacteria, they are the best material for the production of *Lb. rhamnosus*-rich sprouts.

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ZASTOSOWNIE KIEŁKÓW WYBRANYCH ROŚLIN STRĄCZKOWYCH JAKO NOŚNIKA DLA *LACTOBACILLUS RHAMNOSUS* GG – BADANIA PRZESIEWOWE

S t r e s z c z e n i e

Probiotyki i prebiotyki odgrywają ważna rolę w żywieniu zwierząt i człowieka. W badaniach oceniono możliwość zastosowania kiełków roślin strączkowych jako nośników probiotycznych bakterii *Lac-*

tobacillus rhamnosus GG. W pracy określono wpływ gatunku rośliny, temperatury kiełkowania, metody inokulacji nasion lub wzrastających już kiełków na przeżywalność i/lub wzrost probiotyków w kiełkach.

Stwierdzono, że liczba bakterii w kiełkach zależała od temperatury prowadzenia hodowli, metody inokulacji, jak też gatunku rośliny zastosowanej jako nośnik. Badane fasole (Adzuki oraz Mung) efektywnie kiełkowały w zakresie temp. 25 ÷ 35 ºC. Soczewica natomiast najwydajniej kiełkowała w temp. 25 ºC. W przypadku kiełków soczewicy i soi temperatura 35 ºC wpłynęła na zmniejszenie wydajności kiełkowania. Wzrost *Lb. rhamnosus* GG stwierdzono tylko w przypadku kiełków soczewicy i soi otrzymanych z nasion namoczonych w inokulum i kiełkowanych w temp. 25 ºC. Ich liczba w kiełkach wynosiła odpowiednio 3.1×10^6 i 7.18×10^6 jkt/g świeżej masy. Kiełki uzyskane z nasion analizowanych fasoli nie zapewniały warunków do przeżywalności i wzrostu bakterii probiotycznych. Najlepszym nośnikiem badanych bakterii probiotycznych były nasiona soi, w przypadku których w temp. 25 ºC otrzymano kiełki o najlepszych parametrach jakościowych po zastosowaniu inokulacji nasion zawiesiną bakterii probiotycznych.

Słowa kluczowe: rośliny strączkowe, kiełki, probiotyki, *Lb. rhamnosus*