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ORIGINAL ARTICLE

Some properties of Bacillus subtilis probiotic supplements from byproducts of agricultural complex

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Previously, it was shown that B. subtilis B-8130 produce bactericides and biologically active substances while fermenting different phyto-substrates. The bacilli release somatostatin-like peptide during solid-state fermentation of beet pulp sugar, sea-buckthorn leaves and flax seeds. The growth of bacterial culture is accompanied by the formation of biofilm that encapsulates phyto-carriers. The combination of all above factors determines high biological activity of probiotics and their effects on digestion in animals. We tested the addition of 0.1% B. subtilis B-8130 fermented substrate to the pig ration. The experiments showed higher weight gain, lowered daily feed expenses and improved survival. We found substantial changes in morphology of the small intestine epithelial cells associated with intensive absorption of feed nutrients in pigs that were fed with fermented sea-buckthorn leaves. This suggests influence of probiotics on digestion mediated by hormonal system.
Key words: probiotic; bacillus; solid-state fermentation; phyto-substrate; somatostatin-like peptide; biofilm

Introduction

In modern intensive livestock industry, the probiotic products, enzyme-probiotic combinations and probiotics enriched with phyto-compounds are used to improve the digestibility and assimilation of food nutrients, to stimulate development and growth along with increasing non-specific immunity and resistance to stress (Okolelova, 2007; Anadun et al., 2006; Vanbelle et al., 1990; Ouwehand et al., 2003; Sanders et al., 2003; Simon et al., 2001; Hong et al., 2008; Szaboova et al., 2008). They are regarded as a potential alternative to antibiotics, and their use is considered essential in the production of organic foods (Simon, 2005; Shim et al., 2005). The probiotics that are balanced with phyto-compounds have highest biological activity.

There are several technologies of the food probiotic production from vegetable waste, including promising solid-state fermentation of the phyto-substrate by probiotic bacteria. Solid-state fermentation is used for production of the various biologically active substances such as antibiotics, alkaloids, growth hormones, ferments, aromatic acids and others (Perez-Guerra et al., 2003).

We developed and patented a fermentation process resembling digestion of the solid plant components in colon. It allows enrichment with valuable products of bacterial degradation and specific metabolites of probiotics. We were particularly interested in the fermentation by B. subtilis. These bacteria accumulate a wide spectrum of exogenous enzymes, secrete a variety of immunoactive and bioactive peptides, which include antibiotics and hormone-like substances, and commonly used as probiotics (Hong et al., 2008; Williams, 2007). B. subtilis are utilized for digestion of plant substrates, such as beans, and production of food supplements (Hosoi et al., 1999; Hosoi and Kiuchi, 2003). It was shown that extracts of the cells and culture medium from B. subtilis contain somatostatin-like peptides (Fries et al., 1982). Several studies demonstrated that some of the synthetic analogues of somatostatin act as antagonist to this hormone through autoimmune neutralization. Consecutively, this leads to reducing somatostatin's inhibitory effect on growth hormone, insulin, gastrin and ultimately promoting body growth

(Spencer, Hallett, 1985; Dai et al., 2008). The aim of this study was to examine the products of incomplete fermentation of the beet pulp as a phyto-substrate model by B. subtilis B-8130 strain, to investigate the secretion dynamic of somatostatin-like peptide by the bacillus during solid-state fermentation of different substrates and to evaluate their effects on digestion in piglets and their growth.

Material and methods

Bacterial culture

We used B. subtilis B-8130 strain culture in our study. The bacterial biomass was obtained by the aerobic fermentation in the following medium (%): glucose – 2.0, peptone – 0.5, yeast extract - 0.3 and tap water, pH 7.5; incubation at 37°C. The fermentation was completed at early stationary growth phase, before initiation of the sporulation that was controlled under the microscope. To obtain the biomass, the cells were separated from the culture liquid by centrifugation at 1500g in K-70 centrifuge (Russia) for 30 minutes. The obtained paste was divided into two portions. One part was lyophilized using device "Frost" (Russia) and the other portion of paste was resuspended in distilled water in ratio 1:5. This homogenous mass was then placed into pre-cooled to-75°C chambers for disintegration. The biomass was disintegrated using press for three minutes after complete freezing for 20 minutes. The degree of cell disruption was controlled under microscope. The cells were extracted ten times in ratio 1:5 with pre-cooled mixture of 0.2N HCl and 75% ethanol. The obtained extracts were combined and kept at 4°C overnight. The sediment was removed by centrifugation at 1500g for 30 minutes. The supernatant was evaporated using the vacuum evaporator with the pressure of 0.98 atm^{-1} at 40°C (bath) (30°C for the system). In the residual water, the pH was adjusted to 6.2 with 1N NaOH. The obtained solution was stored at – 40°C.

The lyophilized B. subtilis B-8130 biomass was resuspended in cold solution (0.2N HCl /75% ethanol) (1:5) under constant mixing for 1.5 hours. The extraction was performed in thermostat at 37.5°C for 14 hours. The extract was centrifuged at 1500 g for 30 minutes and the supernatant was evaporated at the same conditions as above. Then pH was adjusted to 5.6 with 1 N NaOH and obtained solution was stored at – 40°C.

Solid-state fermentation

To carry out the solid-state fermentation, dry beet pulp, ground sea-buckthorn leaves or flax seeds were sterilized and then mixed with B. subtilis B-8130 cultural liquid until 50% humidity of the mixture. The pH was adjusted to 8.0 with sodium bicarbonate and process was carried out in the stationary conditions at 37°C. The humid product of the fermentation was dried at 40°C.

Extraction of somatostatin-like peptide

We sampled the mixture at day one, two and three for somatostatin–like peptide. The specimens were extracted solution 0.2N HCl /75% ethanol at the ratio 1:10 at the same conditions as described above. The pH of extract was adjusted to 5.6 with 1N NaOH and samples were stored at - 40°C.

The concentration of the somatostatin-like peptide was determined by the enzyme immunoassay (EIA)(EIA-11179 kit, Peninsula Laboratories, LLC, USA). The product was measured at 450 nm wavelength (Spectramax 340, Molecular Devices, USA). We analyzed the data with software SoftmaxPro.4.1 (Molecular Devices, LLC, USA).

Chromotographic analysis

The analysis of volatile products of the fermentation was implemented by the solid-phase microextraction (SPME) in the steam phase. Each test tube with the product was placed in the covered glass chamber and the internal standard was added (10 μg of each deutero-acetone, deutero-chloroform and deutero-toluene in methanol), the sorbent fiber was injected and exposed to 50°C for one hour. This fiber was then transferred to the gas chromatography mass-spectrometry system that included Trace GC gas chromatograph and Thermo Finnigan Polaris Q GC/MS mass spectrometer (both Thermo Scientific, USA). Following test conditions were applied: SGE BPX5 GC capillary column (30 m x 0.25 mm, film thickness 0.25 μm); carrier gas helium (0.6 ml/minute flow rate); column temperature was programmed to raise from 30°C (exposure for four minutes) to 200°C with the speed 6°C/minute, then up to 280°C with the speed 8°C/minute (exposure for six minutes); injector temperature 240°C, ion-source temperature 250°C, electron ionization 70 eV; mass scan range 29-450 m/z.

The concentration of the product was determined by internal standard method of the coefficient linear dependence on the sensitivity and the chromatographic retention time (Kozel, Novak, 2002).

Electron microscopy

Electron microscopy examinations were conducted using the scanning electron microscope "CamScan" MB 2300 (Czech Republic, Brno). The samples of the fermented beet pulp were added to the drop of sucrose isotonic solution (0.3 M) and then dried on the air. After gold dusting, samples were examined under microscope.

The unwashed biopsy samples of porcine caecum (2 x 2 cm) were spread with its chime up and submerged into 0.3M isotonic sucrose solution followed by fixation in 9% formalin solution. Then, they were dehydrated through a series of increasing alcohol

concentrations (50°, 70°, 80°, 96°, 100°), followed by mixture of alcohol and acetone (1:1) and pure acetone (Соколов et al., 1988). Prior to microscopy samples were removed from the acetone, air-dried, secured with glue on the sample table and dusted with a gold in vacuum.

Animal experiments

The research involving animals was complied with all relevant federal guidelines from the Federal Service for Veterinary and Phyto-Sanitary Surveillance and institutional policies. We used Large White breed (LW) and F-1 generation of Large White x Landrace (LW x L) piglets (~40kg weight). The animals were randomly divided into three experimental groups (see details in Table 3) and controls (same number for each group). The groups were fed with pig fodder designed for growing piglets based on nutrient and energy requirements. Experimental animals received 0.1 % probiotic produced by solid-state fermentation of sea-buckthorn leaves, pulp beet and flax seeds by B. subtilis B-8130. All pigs were weighed at the beginning and end of experiment, and actual feed consumption was evaluated daily. Animal survival rate was estimated in percent alive piglets by the end of experiment.

Histological evaluation

The tissue samples from thin (duodenum) and colon (cecum) intestine were collected from piglets fed with sea buckthorn leaves and controls at the end of experiment for histological evaluation. The samples were fixed in 10 % neutralized formalin and processed according to the following procedure: after 2 days, 5x5 mm tissue pieces were washed in running water, dehydrated in isoprene (BioVitrum, Russia) and mixture of absolute isopropanol and Triton X-15 (rising concentrations), then embedded in paraffin Histomix (BioVitrum, Russia). The 5um-thick samples were cut on a rotary microtome (Shandon Finesse 325, Thermo Electron Corporation, UK) and stained with hematoxylin-eosin. Microscopy evaluation was performed using brightfield microscope («Opton», Germany) (16x and 40x). Histomorphometry was performed using ImageScope software (LLC "System for Microscopy and Analysis"). The value of nuclear-cytoplasmic ratio (NCR) was determined by following formula: NCR = S(n)/S(c), where S(n) is the area of cell nucleus; S(c) is cytoplasmic area.

Statistics

Student's two-tailed t-test was used to compare groups. All data are expressed as mean± standard deviation (sd). Statistical significance was set at $P<0.05$.

Results

In vitro studies

The highest concentration of a somatostatin-like peptide was recorded around 48 hours, except for flax seeds (Table 1). According to our measurements, the extract of disintegrated B.subtilis B-8130 cells contained 0.06 ng of peptide per gram of the raw biomass whereas the lyophilized intact cells had 0.032 ng.

Table 1. The concentration of somatostatin-like peptide in fermented substrate

Substrate	Incubation, hours	Concentration, ng/g
Beet pulp	0	0.002
	24	0.003
	48	0.185
	72	0.094
Hippophae leaves	0	0.006
	24	0.007
	48	0.028
	72	0.027
Fax seeds	0	0.003
	24	0.004
	48	0.018
	72	0.088

The final fermentation product contained substances that possess bactericidal activity including phenol compounds and their products. The addition of bicarbonate to our fermentation process resulted in accumulation of the restoration products such as alcohols (Table 2). Specifically, benzyl alcohol and monoterpenoids content increased up to 20% and 30%, respectively.

Table 2. Components of the fermented B.subtilis B-8130 beet pulp

Components	Content, μg
Acetic acid	0.025
3-Methylbutanoate	0.003
Phenol	0.004
Toluene	0.019
Ethylbenzol	0.001
m-and p-xyloles	0.002
o-xylol	0.001
p-guaiacol	0.015
Styrene	0.030
Ethylhexanoate	0.058
Benzolbutane acid	0.063
Benzyl alcohol	0.154
Sum of monoterpenoids	0.225
Naphthalene	0.005
Methilnaphthalene	0.003
Dimethilnaphthalene	0.005

In current conditions, B.subtilis cells generated micro-particles of bacterial biofilm on the surface of phyto-carrier (Fig. 1).

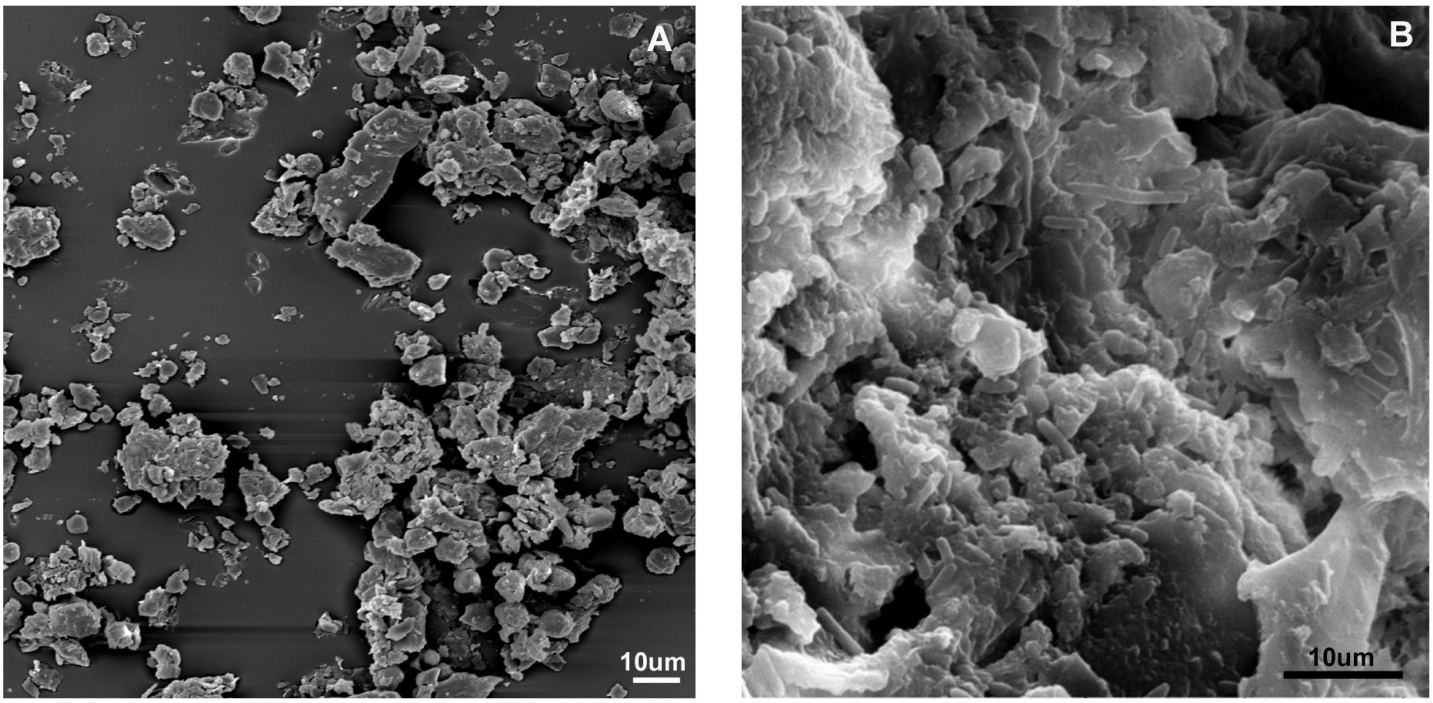

Figure 1. The electron micrograph of (A) microparticles of the sorbent (fermented beet pulp) and (B) *B.subtilis* **B-8130 in the fermented beet pulp.**

Animal experiments

The probiotic supplements were evaluated on pure and crossbred piglets. We examined the growth and development parameters, digestion and morphology of intestinal epithelial cells. The addition of probiotic produced by B. subtilis B-8130 fermentation of sea buckthorn leaves resulted in 16.3% higher weight gain while reducing feed costs per unit of production by 14.1%. Similar results were obtained when piglets were fed fermented sugar beet and flax seeds (Table 3).

The histological evaluation of the intestine specimens showed similar morphology between controls and animals fed with the probiotics (Fig. 2). Despite smaller size, crypt cells in the small intestine of experimental animals had larger villi and greater nuclear-cytoplasmic ratio ($P<0.001$) (Table 4). The morphometric parameters for large intestine epithelial cells were not significantly different among groups (the data are not shown).

Table 3. The analysis of growth performance in piglets fed with supplemented rations and evaluation of their economic efficiency

		Substrate					
		Flax seeds		Beet pulp		Sea-buckthorn	
Index		mean	sd	mean	sd	mean	sd
Age of piglets, days		65-95		36-75		60-120	
Breed		LW		F-1: LWxL		LW	
Number per group		3		75		50	
Weight gain per day, g	control	446.7	129.2	428.2	56.7	578.0	83.3
	experiment	498.3	126.8	464.1	67.5	672.0	80.0
Weight gain, %	control	100.0		100.0		100.0	
	experiment	111.4		108.4		116.3	
Feed spent per unit of weight gain, kg	control	3.10		1.63		2.96	
	experiment	2.75		1.55		2.55	
Exchange energy spent per unit of weight gain, kJ	control	38.2		22.7		36.2	
	experiment	33.9		21.5		31.1	
Rate of survival, %	control	100.0		94.7		93.5	
	experiment	100.0		96.0		95.3	

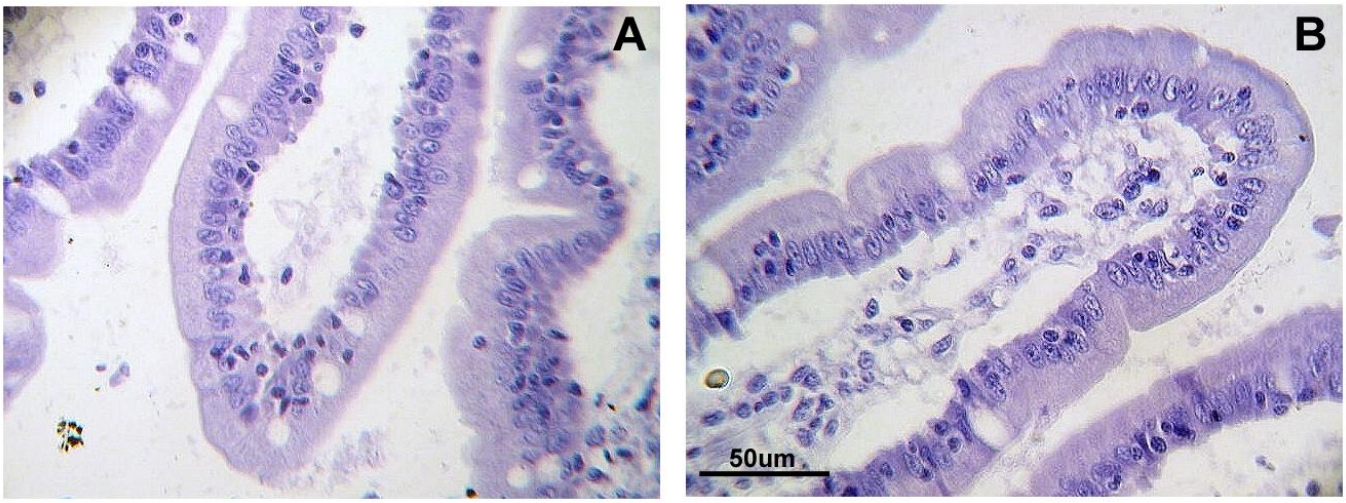

Figure 2. Histological evaluation of the small intestine (40x). Villi: (A) control group, (B) experimental group.

Table 4. The morphological characteristics of small intestine villi in piglets

Group	Number of cells	Total area, mean	Total area, sd	S(n), μm² mean	S(n), μm² sd	S(c), μm² mean	S(c), μm² sd	NCR
control	102	165.30	24.52	43.64	5.40	121.66	21.36	0.36
experiment	100	189.03	13.05	50.09	7.65	138.94	18.71	0.36

S(n), μm^2 – area of cell nucleus, S(c), μm^2- area of cytoplasm, NCR – nuclear-cytoplasmic ratio

Discussion

Our study has shown that solid-state fermentation by B. subtilis B-8130 with addition of bicarbonate converted different substrates into biomass enriched with variety of immunoactive and bioactive peptides including hormone-like substances and specific metabolites of probiotics. We focused our study on production of somatostatin-like peptide due to its importance as a regulator of digestive function and its effects on hypothalamo-hypophysial system that regulates the rate of growth hormone secretion. In vitro data demonstrated that B. subtilis cells synthesize this immunoactive peptide when bacterial culture starts its transition to the stationary growth phase since we controlled the formation of spores in our bacterial cultures. It is well established that gram-positive bacteria including B. subtilis interact with each other using oligopeptide signal molecules

(Gruzina, 2003; Shpakov, 2009; Pottathil et al., 2008; Van Dijl et al., 2013). Also, some bacteria undergo cell death at this fermentation stage, thus increasing intercellular proteins' content in substrate.

The fermentation product was also enriched with cellulose fiber particles - microsorbents as well as with low molecular weight metabolites. Some of these substances have profound effect on multiple functions in organism. For example, benzyl alcohol, which was the major fermentation product of the beet pulp, is well-known natural preservative and antiseptic. Other components such as terpenoids attracted significant attention as alternative to antibiotics. They have high antioxidant activity and can change permeability of mitochondrial internal membrane; they interact with important metabolic enzymes, affect respiratory enzymes, regulate calcium homeostasis and have neuro-protective effect (Melentyeva et al., 1985, Ovchinnikov, 1987). Terpenoids represented more than 30% of identified substances in the fermented beet pulp. Another component was guaiacol that is widely used for suppressing putrefactive bacteria in an intestine (Fiege et al., 2000).

Our solid-state fermentation process resulted in immobilization of probiotics on phyto-carriers followed by biofilm formation, что повышает выживаемость бактерий в неблагоприятных условиях внешней среды, включая высушивание, грануляцию, а также кислые условия желудка. It has been demonstrated that probiotic supplements with spore-forming bacteria significantly improve food efficiency and weight gain in animals. It has been postulated that improvements in animal health are due to optimization of microbiocenosis, activization of fermental and hormonal systems which lead to increase in metabolism and thus, in growth rate (Meng et al., 2010; Chu et al., 2011).

The presence of biologically active substances as well as immobilized probiotics in the fermented substrates undoubtedly contributed to the improvements in weight gain in experimental group in animal experiment. Our results reassure the view that probiotics reinforce bioactive properties of phyto-substrate. The increase in digestibility of nutrients in experimental group can be linked primarily to the fiber digestibility. B.subtillis bacteria are involved in the initial stages of cellulose digestion through excretion of cellulolytic enzyme endoglucanase (Ushakova et al., 2003). This consecutively improves access digestive tract enzymes to the other nutrients. Additionally, the sorption properties of biotransformed phyto-substrate microparticles can also increase the intestinal movements and excretion of the toxins. The histological examination showed larger small intestine villi and epithelial cells in experimental pigs as compared to controls (Fig. 2). This indicates better absorption and a more active digestion in experimental animals ($P < 0.001$) and thus, suggests a beneficial effect of fermented substrate on the development of the gastrointestinal tract in young pigs.

Conclusion

The presence of bactericidal, bioactive substances such as somatostatin-like peptide as well as bacterial biofilm encapsulating phyto-carrier determine the effect of probiotics on animals. This was confirmed by experiments using all types of phyto-substrate: beet pulp, sea buckthorn leaves and flax seeds. The efficiency of digestion, growth rate and survival rate were increased with addition of fermented substrates to the pig diet. Regardless of the type phyto-substrate, we recommend to use 0.5-1.0 kg of fermented substrate per ton of pig fodder.

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