The influence of *Bacillus subtilis* 87Y isolated from *Eisenia fetida* on the growth of pathogenic and probiotic microorganisms

Szmigiel I[1]., Suchodolski J[1]., Łukaszewicz M[1]., Krasowska A[1].

[1] Department of Biotransformation, Faculty of Biotechnology, University of Wroclaw, ul. F. Joliot-Curie 14a, 50-383 Wrocław, Poland

Abstract:

B. subtilis 87Y strain, isolated from *E. fetida* decreases the growth of pathogenic *Salmonella* spp. and *S. aureus* strains and promotes the growth of probiotic *Lactococcus* spp. Preserving viability in acidic conditions as well as in bile salts, *B. subtilis* 87Y meets two of the conditions of the probiotic strain. Thanks to the production of the biosurfactant surfactin, *B. subtilis* 87Y limits the growth of gram-positive bacteria *S. aureus*. In the presence of sucrose, *B. subtilis* produces levan which contributes to promoting the growth of other probiotics. Our *in vitro* studies justify their continuation with solid state fermentation using *B. subtilis* 87Y solid waste as rapeseed meal to enrich it with high-value animal feed supplements.

Keywords: *Bacillus subtilis*, solid-state fermentation, surfactin, probiotics, *Salmonella* spp., *Staphylococcus aureus*, *Lactococcus* spp.

Introduction

Solid-state fermentation (SSF) exhibits numerous advantages, including lower production energy, cost, water equipment and less waterwaste (Pandey 2003). Fermentation process improves nutritional and functional properties compared to original raw materials. *Bacillus subtilis* is widely used in SSF processes (Seo and Cho 2016; Singh and Bajaj 2016; Dai et al. 2017). Among them, traditional Japanese food “natto” is produced by fermentation of soybeans by *B. subtilis* strains (Chen et al. 2012).

B. subtilis is known Gram-positive, spore-forming bacterium. Spores of *B. subtilis* exhibit a wide range of tolerance properties like acid conditions or thermal tolerance (Setlow 2006)). Thus, the survival of spores in the stomach permits live bacteria to reach the intestines (Bernardeau et al. 2017). Probiotic effects of *B. subtilis* have been often reported (Terada et al. 2012; Yang et al. 2015), for example, the composition of *Bifidobacterium* in fecal flora was improved by consumption of traditional Japanese food “natto” (Terada et al. 2012). Co-culture of *B. subtilis* MA139 and *Lactobacillus reuteri* inhibited the growth of pathogenic *Escherichia coli* K88. Moreover, *Lactobacillus* cultured alone was less toxic towards *E. coli* than in co-culture with *B. subtilis* spores (Yang et al. 2015).

Bacillus spp. is also known for its ability to secrete extracellular enzymes, thus *Bacillus* spp. have improving effects for the growth of probiotic bacteria (Falck et al. 2013; Horie et al. 2017). *Lactobacilli* cannot use starch as a carbon source. Digesting starch into sugars by α-amylase from *B. subtilis* allows probiotic bacteria to utilize glucose or maltose (Horie et al. 2017). *Bacillus* spp. is also a great producer of lignocellulose-degrading enzymes like xylanases and cellulases. Xylooligasaccharides received from hardwood and cereal xylans were reported to be used by *Lactobacillus brevis* and *Bifidobacterium adolescentis* (Falck et al. 2013).

The production of antimicrobial substances against pathogens is another mechanism by which *Bacillus* spp. act as probiotic (Duc et al. 2003; Hong et al. 2005). *Bacillus* spp. produce a large number of antimicrobials which include bacteriocins (subtilisin, nisin, subtilosin) and antibiotics (surfactin, iturin, bacitracin, bacilysin) (Urdaci and Pinchuk 2004). Surfactin (SU) is a biosurfactant produced mainly by various *B. subtilis* strains. SU is the cyclic lipopeptide with high antifungal properties against *Aspergillus niger, Penicillium* spp., *Fusarium verticillioides* (Mohammadipour et al. 2009; Snook et al. 2009; Velmurugan et al. 2009) as well as antibacterial activity against *Salmonella typhimurium, Listeria monocytogenes, Staphylococcus aureus* or *Legionella* spp. (Gupta et al. 1990; Nitschke et al. 2009; Joe et al. 2012; Loiseau et al. 2015).

In this work, we have isolated from *Eisenia fetida* and characterized *B. subtilis* 87Y strain with probiotic properties that can be used to transform solid waste (for example rapeseed meal) into the enriched animal feed.

Materials and methods

Chemicals

Lysogeny broth (LB) mix, bacteriological agar (Lab Empire; Poland); API 20E test (Biomérieux; Poland); 16S rRNA universal primers (27F and 1492R) (Genomed; Poland); NaCl (Stanlab; Poland); Ox gall (Sigma-Aldrich; Poland); surfactin (SU) was a kind gift from InventionBio, Poland.

Strains and growth conditions

Bacillus subtilis 87Y was isolated from heat-treated samples of *E. fetida* gut microflora. The enzymatic activities of the isolate were characterized by API 20E test. Species assignment was performed by the analysis of 16S rRNA sequences.

Staphylococcus aureus ATCC 6538, *Salmonella enteritidis* ATCC 13076, *S. enteritidis* ATCC 49223, *Salmonella typhimurium* ATCC 14028, *Lactococcus lactis* ATCC 19435, *L. lactis* ATCC 49032, *L. lactis* ATCC 11454 (Pol-aura; Poland) were used in the study.

Bacterial strains were routinely grown in LB agar (0.5% yeast extract, YE; 1% tryptone; 1% NaCl; 2% agar) at 37 °C.

Acid and bile salt tolerance

Acid tolerance of *B. subtilis* 87Y was determined according to Lee (2017), with modifications. 1 mL aliquot of the overnight culture of *B. subtilis* 87Y was centrifuged at 8000xg for 10 min. at room temperature. The pellets were washed in sterile PBS (100 mM, pH 7.4) and resuspended in 10 mL of sterile PBS (100 mM, pH 2.0). The bacterial suspensions were incubated at 37 ºC with agitation 180 rpm for 3 h. During the incubation, an A_{600} was measured at 1, 2 and 3 h.

Bile salt tolerance of the strain was performed similarly, an 1 mL aliquot of an overnight culture of *B. subtilis* 87Y was inoculated into 10 mL of LB broth containing 0.3% ox gall and incubated at 37 ºC for 12 h with agitation 180 rpm. During the incubation, an A_{600} was measured at 4, 8 and 12 h.

SU production

B. subtilis 87Y strain was cultivated in 20 mL of Landy's medium (Jajor et al. 2016) in 100 mL Erlenmeyer flasks. The cultures were inoculated to A_{600} = 0.1 and incubated for 24 h in 37 ºC with agitation (180 rpm). Then the cultures were centrifuged (13,500 rpm, 10 min) and the supernatants were used for Ultra High-Performance Liquid Chromatography (UHPLC, Aquity ARC Waters) analysis, equipped with a CORTEX C18 column (4.6 x 50 mm; 2.7 µm), according to (Biniarz and

Łukaszewicz 2017).

Antibacterial activity of SU

The antimicrobial activity of SU was determined against *S. aureus* ATCC 6538 by the 96-well microdilution assay, according to CLSI (2012), with modifications (Giurg et al. 2017). Here, A_{490} was measured using ASYS UVM 340 microplate reader (Biogenet; Poland). The viability was determined by normalizing A_{490} in control conditions (0 µg/mL SU) as 100%.

Co-culture on agar plates

S. aureus ATCC 6538 and *B. subtilis* 87Y inocula were prepared by resuspending freshly grown (18 h, LB agar) colonies in 0.9% NaCl solution to A_{490} = 0.125. *S. aureus* inoculum was streaked on LB agar plate, and *B. subtilis* inoculum was spotted on the agar plate. After incubation (24 h, 37 °C) the plates were photographed, using FastGene B/G GelPic imaging box (Nippon Genetics; Germany).

Co-culture insert method

The assay was performed by modifying a tissue culture protocol (Renaud and Martinoli 2016). The schematic representation of the testing conditions is given in Fig. 1. Inocula of bacteria were prepared by resuspending freshly grown (18 h, LB agar) colonies in LB to A_{490} = 0.1 (L compartment) or A_{490} = 0.4 (U compartment). After incubation (24 h, 37 °C), A_{490} was measured using Odyssey DR/2500 spectrophotometer (Hach, USA). The viability was determined by normalizing A_{490} in control conditions as 100%.

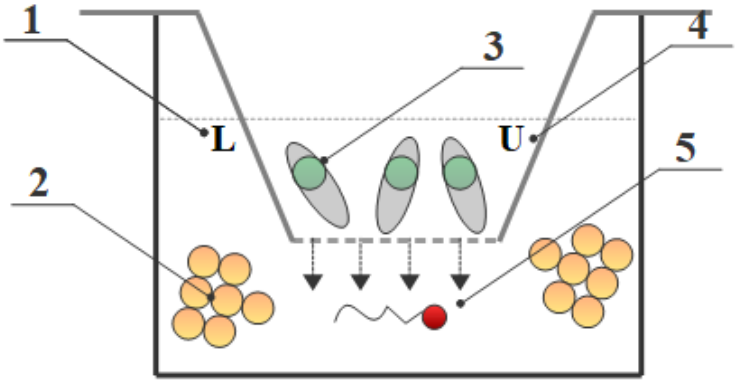

Figure 1. Schematic representation of the co-culture insert method presented on the example of *B. subtilis* 87Y influence on *S. aureus:* in the (1) lower compartment (L) (2) *S. aureus* was inhibited (A_{490} = 0.1 at t = 0) by (3) *B. subtilis* 87Y, grown (A_{490} = 0.4 at t = 0) in (4) upper compartment (U) due to (5) surfactin (SU) activity.

Statistical analysis

Statistical significance was determined using binomial, unpaired Student's t-test.

Results and discussion

B. subtilis 87Y, isolated from *E. fetida* produces various enzymes and surfactin

Eisenia fetida is very efficient in composting organic waste and converting it into vermicompost, which is full of nutrients and with lower level of toxicants (Sharma and Garg 2018). Among isolated microflora of *E. fetida* we found 96 bacteria strains. Four of 96 isolated microorganisms were sequenced by 16S RNA and assigned as *B. subtilis* strains.

Selected strain *B. subtilis* 87Y was then examined by API 20E test for basic physiology. *B. subtilis* 87Y was beta-galactosidase-, acetoin production-, gelatin utilization- and fermentation of: glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose – positive as well as arginine dihydrolase-, lysine decarboxylase-, ornitin decarboxylase-, citrate utilization-, hydrogen disulfide production-, urease production-, tryptophane deaminase- and indol production-negative.

B. subtilis is known for producing various biosurfactants (Urdaci and Pinchuk 2004). Using Ultra-High Performance Liquid Chromatography (UHPLC), we have confirmed that strain *B. subtilis* 87Y is a great producer of lipopeptide surfactin (Fig.2). *B. subtilis* produces a wide range of surfactin analogues, that vary in hydrophobic as well as in hydrophilic moiety (Jajor et al. 2016). *B. subtilis* 87Y during cultivation on Landy's medium produced mainly analogues that differ between carbon chain length (Fig.2).

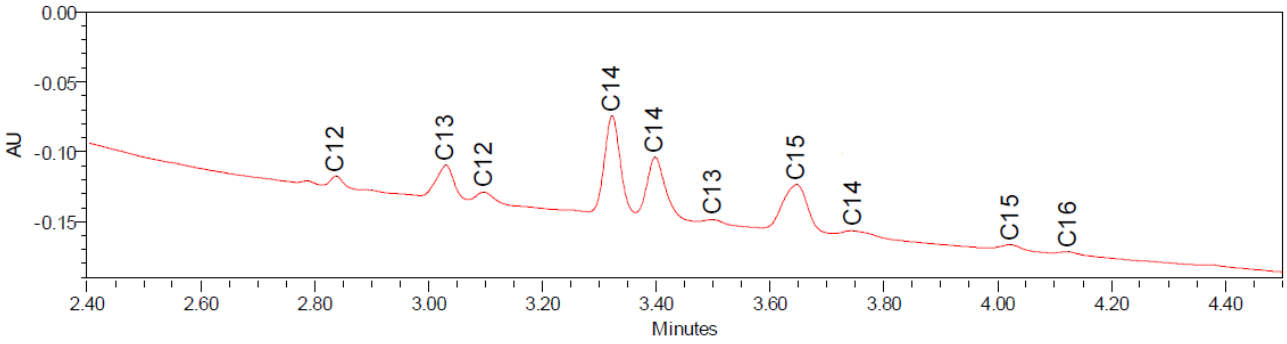

Figure 2. Production of surfactin by *B. subtilis* 87Y during cultivation on Landy's medium. C12-C16 represent analogues differing in number of carbon atoms of hydrophobic moiety.

B. subtilis 87Y is viable after acid and bile salt treatment

Spores of *Bacillus spp.* were reported to be resistant to various conditions. Among them, acid pH and bile salt tolerance were shown in the case of *B. amyloquefaciens* and *B. subtilis* strains (Spinosa et al. 2000; Lee et al. 2017). Spinosa (2000) shows the presence of *B. subtilis* spores in the intestinal tract of mice. This indicates, that spores can reach intestines after acidic conditions in the stomach.

Lee (2017) shows direct influence acidic pH and bile salts to *B. amyloliquefaciens* LN. Viability of LN strain after 3 h of acid pH, and after 12 h of bile salts treatment decrease viability of the strain about 20% and 10% respectively. We noticed similar growth of *B. subtilis* 87Y during 3 h incubation in both neutral and acidic conditions. (Fig. 3A). After 12 h of bile salts treatment, *B. subtilis* 87Y viability decreased four-fold in comparison to control conditions (LB medium), but we observed the doubling the optical density in comparison to the initial conditions (0.3% ox gall in LB, 0 h) which indicates slow cells growth (Fig. 3B).

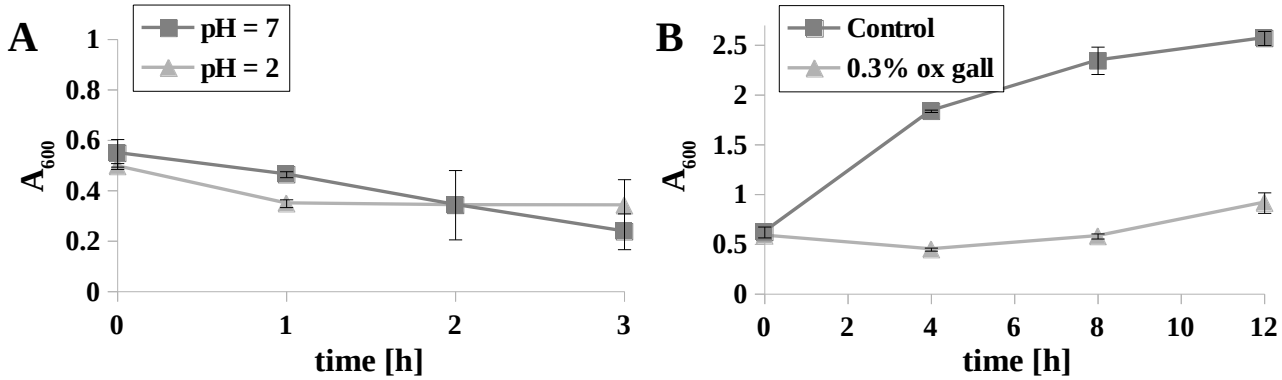

Figure 3. (**A**) The influence of acidic pH (2.0) on *B. subtilis* 87Y, expressed as A_{600} measurements in time (0 – 3 h), n = 9 ±SD; (**B**) The influence of 0.3% ox gall on *B. subtilis* 87Y, expressed as A_{600} measurements in time (0 – 12 h), n = 9 ±SD.

B. subtilis 87Y inhibits *S. aureus* growth due to SU production

Amphipathic compounds such as lipopeptides are highly toxic towards gram-positive bacteria (Silhavy et al. 2010), thus, gram-positive bacteria are more vulnerable towards SU (Jiang et al. 2016). We observed the inhibition of *S. aureus* growth, gram-positive by SU (Fig. 4A) from 20% (1 – 2 µg/mL SU) to 80% (8 – 16 µg/mL SU). Due to SU production (Fig. 2), an inhibition zone of *S. aureus* was observed in direct co-culture with *B. subtilis* 87Y on agar plates (Fig. 4B). However, we have noticed restricted SU diffusion through the agar [data not shown]. Restricted SU diffusion may result from restricted diffusion of non-polar compounds in the agar (Cleidson Valgas; et al. 2007; Jenkins and Schuetz 2012).

For further experiments we applied the direct co-culture insert method (Fig. 1), which was validated by testing the influence of SU and *B. subtilis* 87Y on the growth of *S. aureus* (Fig. 4C). 4 µg/mL SU inhibited the growth of *S. aureus* by 50% (Fig. 4C), what is in agreement with the results obtained with CLSI method (Fig. 4A). *B. subtilis* 87Y inhibited the growth of *S. aureus* by 20% (Fig. 4C) while, *S. aureus* did not influence the growth of *B. subtilis* 87Y [data not shown].

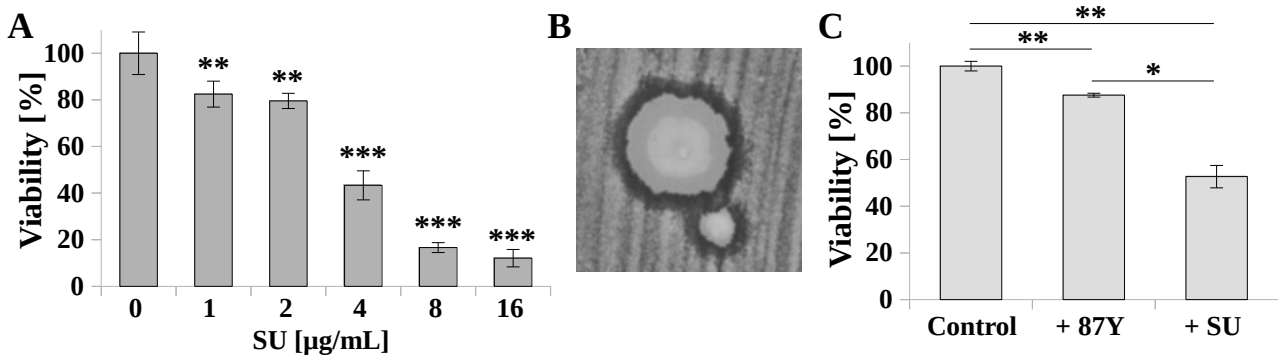

Figure 4. (**A**) The % of the growth of *S. aureus* ATCC 6538 in the presence of surfactin (SU) in a range 0 – 16 µg/mL, n = 6 ±SD. Statistical analysis at each concentration was performed in accordance to 0 µg/mL SU (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$); (**B**) The growth inhibition zone of *S. aureus* (external), induced by *B. subtilis* 87Y (center), representative out of n = 2; (**C**) The % of growth of *S. aureus* (L compartment) untreated (Control), in the presence of *B. subtilis* 87Y (U compartment, here: + 87Y) or 4 µg/mL SU (U compartment, here: + SU), n = 6 ±SD. Statistical analysis was performed in accordance to control conditions or between + 87Y and + SU conditions (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

***B. subtilis* 87Y and *L. lactis* promote their mutual growth in the presence of sucrose**

Fermentation process improves nutritional and functional properties compared to original raw materials. *B. subtilis* is widely used in SSF processes (Seo and Cho 2016; Singh and Bajaj 2016; Dai et al. 2017).

Supplementing poultry feeding with *B. subtilis* leads to an increase in the number of lactic acid bacteria (LAB) in the gastrointestinal tract (Teo and Tan 2007). *L. lactis* stimulates the growth of broilers (Fajardo et al. 2012; Brzóska et al. 2013) and is one of the dominant LAB species isolated from faeces of broilers (Shazali et al. 2014). Here, in LB medium *B. subtilis* 87Y did not lead to a significant increase in *L. lactis* growth (Fig. 5A). However, *B. subtilis* 87Y was promoted by the presence of *L. lactis* (Fig. 5C).

Al-Hijazeen and Al-Rabadi (2017) reported that a sucrose-rich diet positively affected the quality of broilers meat. Here, the addition of sucrose resulted in the significant promotion of the growth of two *L. lactis* strains by *B. subtilis* 87Y (Fig. 5B). Most likely, the effect occurred since sucrose is a substrate for the synthesis of levan, a prebiotic polymer produced by *B. subtilis* (Domżał-Kędzia et al. 2019). The growth of *B. subtilis* 87Y was promoted by ~10% in the presence of *L. lactis* (Fig. 5C). The lower degree of promoting *B. subtilis* 87Y on LB + 5% sucrose by *L. lactis*, than on LB might result from strong *L. lactis* growth in those conditions (Fig. 5B).

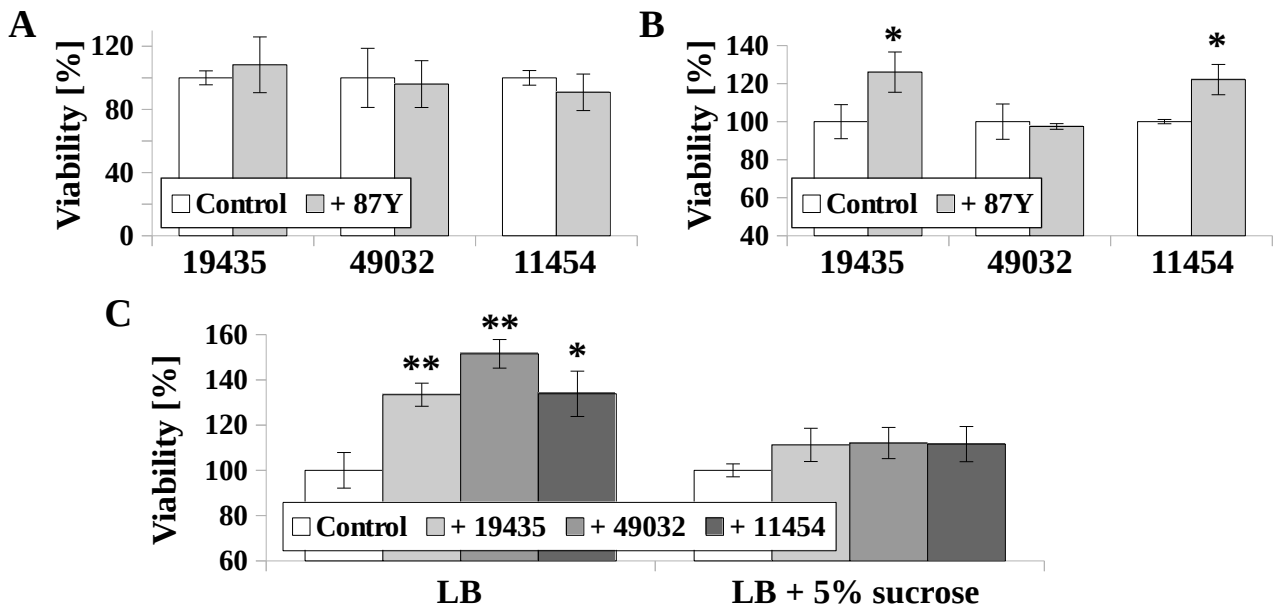

Figure 5. The % of growth of *L. lactis* ATCC 19435, ATCC 49032 and ATCC 11454 alone (Control) or in the presence of *B. subtilis* 87Y, in (**A**) LB, n = 6 ±SD or (**B**) LB + 5% sucrose, n = 6 ±SD; (**C**) The % of growth of *B. subtilis* 87 alone (Control) or in the presence of *L. lactis* in LB or LB + 5% sucrose, n = 6 ±SD. *B. subtilis* 87 Y was grown in U compartment; *L. lactis* in D compartment. Statistical analysis was performed in accordance to control conditions (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

***B. subtilis* 87Y or *L. lactis* inhibits *Salmonella* spp.**

B. subtilis was reported to inhibit the growth of pathogenic gram-negative bacteria in poultry feeding (La Ragione et al. 2001; Gao et al. 2017). Here, both *B. subtilis* 87Y or *L. lactis* strains inhibited *S. enteritidis* and *S. typhimurium* (Fig. 6). The effect of probiotic bacteria was similar, regardless of the *Salmonella* strain or species. *B. subtilis* 87Y displayed the best inhibitory activity (~40% growth inhibition); whereas *L. lactis* ATCC 49032 and ATCC 11454 the lower inhibitory activity (~20% growth inhibition).

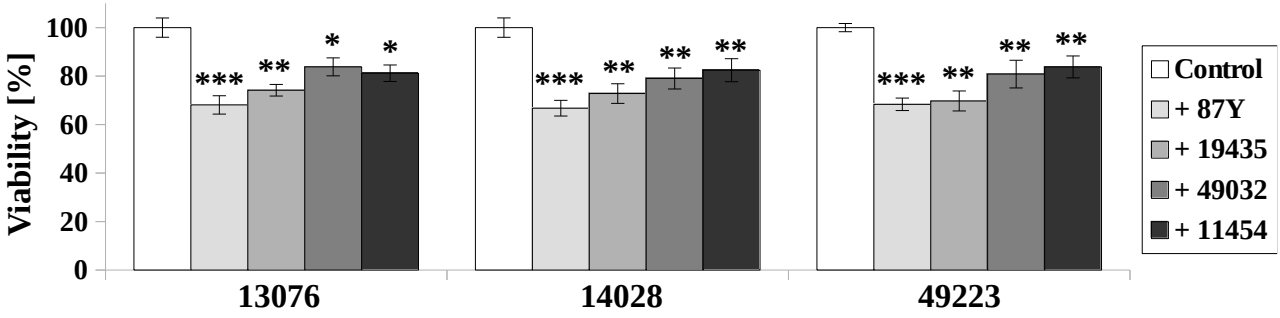

Figure 6. The % of the growth of *S. enteritidis* ATCC 13076, *S. enteritidis* ATCC 49223 and *S. typhimurium* ATCC 14028 untreated (Control), in the presence of *B. subtilis* 87Y, or *L. lactis* ATCC 19435, ATCC 49032 and ATCC 11454 in LB, n = 6 ±SD. *B. subtilis* 87 Y and *L. lactis* were grown in U compartment; *Salmonella* spp. in L compartment. Statistical analysis was performed in accordance to control conditions (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Conclusions

B. subtilis 87Y strain isolated from *E. fetida* compost worm might be promising strain for production of fermented animal food. Following probiotic characteristics were noticed in our *in vitro* studies:

1. is viable in bile salts and low pH
2. inhibits *S. aureus* growth due to SU production
3. promotes the growth of *L. lactis* in the presence of sucrose
4. is promoted by the presence of *L. lactis*
5. inhibits the growth of *Salmonella* spp.

Acknowledgments

This work was partially supported by POIR grant 01.02.00-00-0064/17 and Wrocław Center for Biotechnology (WCB) the Leading National Research Centre (KNOW).

References

Bernardeau M, Lehtinen MJ, Forssten SD, Nurminen P (2017) Importance of the gastrointestinal life cycle of Bacillus for probiotic functionality. J Food Sci Technol 54:2570–2584. doi: 10.1007/s13197-017-2688-3

Biniarz P, Łukaszewicz M (2017) Direct quantification of lipopeptide biosurfactants in biological samples

via HPLC and UPLC-MS requires sample modification with an organic solvent. Appl Microbiol Biotechnol doi: 10.1007/s00253-017-8272-y

Brzóska F, Śliwiński B, Stecka K (2013) Effect of Lactococcus lactis vs. Lactobacillus spp. bacteria on chicken body weight, mortality, feed conversion and carcass quality. Ann Anim Sci 12:549–559. doi: 10.2478/v10220-012-0046-y

Chen KI, Erh MH, Su NW, Liu WH, Chou CC, Cheng KC (2012) Soyfoods and soybean products: From traditional use to modern applications. Appl Microbiol Biotechnol 96:9–22. doi: 10.1007/s00253-012-4330-7

Valgas C, Souza SMD, Smânia EF, Smânia Jr A (2007) Screening methods to determine antibacterial activity of natural products. Brazilian J Microbiol 38:369–380

Dai C, Ma H, He R, Huang L, Zhu S, Ding Q, Luo L (2017) Improvement of nutritional value and bioactivity of soybean meal by solid-state fermentation with Bacillus subtilis. LWT - Food Sci Technol 86:1–7. doi: 10.1016/j.lwt.2017.07.041

Domżał-Kędzia M, Lewińska A, Jaromin A, Weselski M, Pluskota R, Łukaszewicz M (2019) Fermentation parameters and conditions affecting levan production and its potential applications in cosmetics. Bioorg Chem 1–8. doi: 10.1016/j.bioorg.2019.02.012

Duc LH, Hong HA, Cutting SM (2003) Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen delivery. Vaccine 21:4215–4224

Fajardo P, Pastrana L, Méndez J, Rodríguez I, Fuciños C, Guerra NP (2012) Effects of feeding of two potentially probiotic preparations from lactic acid bacteria on the performance and faecal microflora of broiler chickens. Sci World J 2012:14–16. doi: 10.1100/2012/562635

Falck P, Precha-Atsawanan S, Grey C, Immerzeel P, Stålbrand H, Adlercreutz P, Nordberg Karlsson E (2013) Xylooligosaccharides from hardwood and cereal xylans produced by a thermostable xylanase as carbon sources for lactobacillus brevis and bifidobacterium adolescentis. J Agric Food Chem 61:7333–7340. doi: 10.1021/jf401249g

Gao Z, Wu H, Shi L, Zhang X, Sheng R, Yin F, Gooneratne R (2017) Study of Bacillus subtilis on growth performance, nutrition metabolism and intestinal microflora of 1 to 42 d broiler chickens. Anim Nutr 3:109–113. doi: 10.1016/j.aninu.2017.02.002

Giurg M, Gołąb A, Suchodolski J, Kaleta R, Krasowska A, Piasecki E, Piętka-Ottlik M (2017) Reaction of bis[(2-chlorocarbonyl)phenyl] diselenide with phenols, aminophenols, and other amines towards diphenyl diselenides with antimicrobial and antiviral properties. Molecules 22: 974-982. doi: 10.3390/molecules22060974

Gupta SK, Das P, Singh SK, Akhtar MS, Meena DK, Mandal SC (2011) Microbial levan , an ideal prebiotic and immunonutrient in aquaculture. World Aquac 61–64. doi: 10.1142/9789814360807

Hong HA, Le HD, Cutting SM (2005) The use of bacterial spore formers as probiotics. FEMS Microbiol Rev 29:813–835. doi: 10.1016/j.femsre.2004.12.001

Horie M, Koike T, Sugino S, Umeno A, Yoshida Y (2017) Evaluation of probiotic and prebiotic-like effects of *Bacillus subtilis* BN on growth of lactobacilli. J Gen Appl Microbiol 64:26–33. doi:

10.2323/jgam.2017.03.002

Jajor P, Piłakowska-Pietras D, Krasowska A, Łukaszewicz M (2016) Surfactin analogues produced by Bacillus subtilis strains grown on rapeseed cake. J Mol Struct 1126:141–146. doi: 10.1016/j.molstruc.2016.02.014

Jenkins SG, Schuetz AN (2012) Current concepts in laboratory testing to guide antimicrobial therapy. Mayo Clin Proc 87:290–308. doi: 10.1016/j.mayocp.2012.01.007

Jiang J, Gao L, Bie X, et al (2016) Identification of novel surfactin derivatives from NRPS modification of Bacillus subtilis and its antifungal activity against Fusarium moniliforme. BMC Microbiol 16:1–14. doi: 10.1186/s12866-016-0645-3

Joe MM, Bradeeba K, Parthasarathi R, et al (2012) Development of surfactin based nanoemulsion formulation from selected cooking oils: Evaluation for antimicrobial activity against selected food associated microorganisms. J Taiwan Inst Chem Eng 43:172–180. doi: 10.1016/j.jtice.2011.08.008

La Ragione RM, Casula G, Cutting SM, Woodward MJ (2001) Bacillus subtilis spores competitively exclude Escherichia coli O78:K80 in poultry. Vet Microbiol 79:133–142. doi: 10.1016/S0378-1135(00)00350-3

Lee A, Cheng KC, Liu JR (2017) Isolation and characterization of a Bacillus amyloliquefaciens strain with zearalenone removal ability and its probiotic potential. PLoS One. doi: 10.1371/journal.pone.0182220

Loiseau C, Schlusselhuber M, Bigot R, Bertaux J, Berjeaud JM, Verdon J (2015) Surfactin from Bacillus subtilis displays an unexpected anti-Legionella activity. Appl Microbiol Biotechnol 99:5083–5093. doi: 10.1007/s00253-014-6317-z

Mohammadipour M, Mousivand M, Salehi Jouzani G, Abbasalizadeh S (2009) Molecular and biochemical characterization of Iranian surfactin-producing Bacillus subtilis isolates and evaluation of their biocontrol potential against Aspergillus flavus and Colletotrichum gloeosporioides . Can J Microbiol 55:395–404. doi: 10.1139/w08-141

Nitschke M, Araújo L V., Costa SG, et al (2009) Surfactin reduces the adhesion of food-borne pathogenic bacteria to solid surfaces. Lett Appl Microbiol 49:241–247. doi: 10.1111/j.1472-765X.2009.02646.x

Pandey A (2003) Solid-state fermentation. Biochem Eng J 13:81–84

Renaud J, Martinoli MG (2016) Development of an Insert Co-culture System of Two Cellular Types in the Absence of Cell-Cell Contact. J Vis Exp 113:. doi: 10.3791/54356

Seo SH, Cho SJ (2016) Changes in allergenic and antinutritional protein profiles of soybean meal during solid-state fermentation with Bacillus subtilis. LWT - Food Sci Technol 70:208–212. doi: 10.1016/j.lwt.2016.02.035

Setlow P (2006) Spores of Bacillus subtilis: Their resistance to and killing by radiation, heat and chemicals. J Appl Microbiol 101:514–525. doi: 10.1111/j.1365-2672.2005.02736.x

Sharma K, Garg VK (2018) Comparative analysis of vermicompost quality produced from rice straw and paper waste employing earthworm Eisenia fetida (Sav.). Bioresour Technol 250:708–715. doi: 10.1016/j.biortech.2017.11.101

Shazali N, Foo HL, Loh TC, Choe DW, Rahim RA (2014) Prevalence of antibiotic resistance in lactic acid bacteria isolated from the faeces of broiler chicken in Malaysia. Gut Pathog 6:1–7. doi: 10.1186/1757-

4749-6-1

Silhavy TJ, Kahne D, Walker S (2010) The Bacterial Cell Envelope1, Cold Spring Harbor perspectives in biology. 2.5: a000414. doi: 10.1101/cshperspect.a000414

Singh S, Bajaj BK (2016) Bioprocess optimization for production of thermoalkali-stable protease from Bacillus subtilis K-1 under solid-state fermentation. Prep Biochem Biotechnol 46:717–724. doi: 10.1080/10826068.2015.1135455

Snook ME, Mitchell T, Hinton DM, Bacon CW (2009) Isolation and characterization of Leu 7-surfactin from the endophytic bacterium Bacillus mojavensis RRC 101, a biocontrol agent for fusarium Verticillioides. J Agric Food Chem 57:4287–4292. doi: 10.1021/jf900164h

Spinosa MR, Braccini T, Ricca E, De Felice M, Morelli L, Pozzi G, Oggioni MR (2000) On the fate of ingested Bacillus spores. Res Microbiol 151:361–368. doi: 10.1016/S0923-2508(00)00159-5

Teo AY, Tan HM (2007) Evaluation of the performance and intestinal gut microflora of broilers fed on corn-soy diets supplemented with Bacillus subtilis PB6 (CloSTAT). J Appl Poult Res 16:296–303. doi: 10.1093/japr/16.3.296

Terada A, Yamamoto M, Yoshimura E (2012) Effect of the Fermented Soybean Product “Natto” on the Composition and Metabolic Activity of the Human Fecal Flora. Japanese J Food Microbiol 16:221–230. doi: 10.5803/jsfm.16.221

Urdaci MC, Bressollier P, Pinchuk I (2004) Bacillus clausii probiotic strains: antimicrobial and immunomodulatory activities. Journal of Clinical Gastroenterology 38: S86-S90.

Velmurugan N, Choi MS, Han SS, Lee YS (2009) Evaluation of antagonistic activities of Bacillus subtilis and Bacillus licheniformis against wood-staining fungi: In vitro and in vivo experiments. J Microbiol 47:385–392. doi: 10.1007/s12275-009-0018-9

Yang JJ, Niu CC, Guo XH (2015) Mixed culture models for predicting intestinal microbial interactions between escherichia coli and Lactobacillus in the presence of probiotic Bacillus subtilis. Benef Microbes 6:871–877. doi: 10.3920/BM2015.0033