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

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Abstract

Bacillus subtilis strain 87Y, isolated from the earthworm *Eisenia fetida*, decreases the growth of pathogenic *Salmonella* spp. and *Staphylococcus aureus* 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 requirements of a probiotic strain. Thanks to the production of the biosurfactant surfactin, *B. subtilis* 87Y limits the growth of the Gram-positive bacterium *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 the continuation of enriching rapeseed meal waste from solid-state fermentation with *B. subtilis* 87Y, to produce high-value animal feed supplements.

Keywords *Bacillus subtilis* · Solid-state fermentation · Surfactin · Probiotics · *Salmonella* spp. · *Staphylococcus aureus* · *Lactococcus* spp.

1 Introduction

Solid-state fermentation (SSF) exhibits numerous advantages over submerged fermentation, including lower production energy, improved cost and water equipment efficiency, and less wastewater [1]. The fermentation process improves the nutritional and functional properties of the original raw materials. *Bacillus subtilis* is widely used in the SSF processes [2–4]. For example, the traditional Japanese food “natto” is produced through fermentation of soybeans by *B. subtilis* strains [5].

B. subtilis is a Gram-positive, spore-forming bacterium. Spores of *B. subtilis* exhibit tolerance to a wide range of conditions, including acidic conditions and thermal stress [6]. Spores are thereby able to survive within the stomach, permitting live bacteria to reach the intestines [7]. Probiotic effects of *B. subtilis* have frequently been reported [8, 9], for example, improvements in the composition of *Bifidobacterium* in the faecal flora following consumption of the traditional Japanese food “natto” [8]. 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 [9].

Bacillus spp. are known for their ability to secrete extracellular enzymes, which may improve the growth of probiotic bacteria [10, 11]. For example, *Lactobacilli* cannot use starch as a carbon source, yet the metabolism of starch into sugars by α -amylase secreted by *B. subtilis* allows probiotic bacteria to utilize glucose or maltose [11]. *Bacillus* spp. are also prolific producers of lignocellulose-degrading enzymes including xylanases and cellulases. Xylooligosaccharides metabolized from hardwood and cereal xylans are reported to be utilized by *Lactobacillus brevis* and *Bifidobacterium adolescentis* [10].

The production of substances with antimicrobial activity against pathogens is another mechanism through which *Bacillus* spp. act as probiotics [12, 13]. *Bacillus* spp. produce a large number of antimicrobials, which include bacteriocins (subtilisin, nisin, subtilosin) and antibiotics (surfactin, iturin, bacitracin, bacilysin) [14]. Surfactin (SU) is a biosurfactant produced mainly by various *B. subtilis* strains. SU is a cyclic lipopeptide with high antifungal properties against *Aspergillus niger*, *Penicillium* spp., *Fusarium verticillioides* [15–17], as well as antibacterial activity against *Salmonella typhimurium*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Legionella* spp. [18–21].

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In this work, we isolate and characterize a *B. subtilis* strain, 87Y, from earthworm *Eisenia fetida*, as having probiotic properties that can be used to transform solid waste (for example, rapeseed meal) into enriched animal feed.

2 Materials and methods

2.1 Chemicals

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

2.2 Strains and growth conditions

Bacillus subtilis 87Y was isolated from heat-treated samples of *Eisenia fetida* gut microflora. The enzymatic activities of the isolate were characterized with an analytical profile index (API) 20E test. Species assignment was performed by analysing 16S rRNA sequences.

Staphylococcus aureus ATCC 6538, *Salmonella enteritidis* ATCC 13076, *Salmonella enteritidis* ATCC 49223, *Salmonella typhimurium* ATCC 14028, *Lactococcus lactis* ATCC 19435, *L. lactis* ATCC 49032, and *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.

2.3 Acid and bile salt tolerance

Acid tolerance of *B. subtilis* 87Y was determined according to Lee et al. [22], with following modifications: 1 mL aliquot of the overnight culture of *B. subtilis* 87Y was centrifuged at 8000×g 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 °C with agitation 180 rpm for 3 h. During the incubation, A_{600} was measured at 1, 2, and 3 h.

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

2.4 SU production

B. subtilis 87Y was cultivated in 20 mL of Lysogeny broth medium and Landy's medium [23] in 100-mL Erlenmeyer

flasks. The cultures were inoculated to $A_{600} = 0.1$ and incubated for 24 h at 37 °C with agitation (180 rpm). These 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 × 50 mm; 2.7 µm), according to Biniarz and Łukaszewicz [24].

2.5 Antibacterial activity of SU

The antimicrobial activity of SU was determined against *S. aureus* ATCC 6538 with a 96-well microdilution assay, according to Clinical and Laboratory Standards Institute (CLSI) ([25]), with modifications introduced by Giurg et al. [26]. Here, the plates were inoculated to a starting $A_{490} = 0.01$. After 24-h incubation at 37 °C, A_{490} was measured using an ASYS UVM 340 microplate reader (Biogenet; Poland). The viability was determined by normalizing A_{490} to control conditions (0 µg/mL SU) as 100%.

2.6 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 onto an LB agar plate, and *B. subtilis* inoculum was spotted on an agar plate. After incubation (24 h, 37 °C), the plates were photographed using a FastGene B/G GelPic imaging box (Nippon Genetics; Germany).

2.7 Co-culture insert method

The assay was performed with a modified tissue culture protocol [27]. A 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 $A_{490} = 0.4$ (M or U compartment). Following co-culture inserts have been used, 24-well 0.4 µm (Greiner Bio-one, Austria) between U and L compartments (Fig. 1a) and between U and M compartments (Fig. 1b); 12-well 0.4 µm (Greiner Bio-one, Austria) between L and M compartment (Fig. 1b). Twenty-four or 12-well sterile plates (Greiner Bio-one, Austria) have been used for the culture (Fig. 1a, b, respectively). After incubation (24 h at 37 °C), A_{490} was measured using an Odyssey DR/2500 spectrophotometer (Hach, USA). The viability was determined by normalizing A_{490} under control conditions as 100%.

2.8 Statistical analysis

Statistical significance was determined using a binomial, unpaired Student *t* test.

Fig. 1 Schematic representation of the co-culture insert method presented using the example of **a** *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 the (4) upper compartment (U) due to (5) surfactin (SU) activity. **b** *B. subtilis* 87Y influence

3 Results and discussion

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

For the present study, we have chosen *B. subtilis* 87Y strain, which has been isolated from the earthworm *Eisenia fetida* microflora. *E. fetida* is very efficient at composting organic waste and converting it into vermicompost, which is full of nutrients and has a lower level of toxicants [28]. Thus, we expected the *B. subtilis* 87Y strain to possess enzymatic properties, which in turn may lead to production of highly additive secondary metabolites. *B. subtilis* 87Y was examined using a API 20E test to determine basic physiology and enzymatic properties [29]. *B. subtilis* 87Y was positive for beta-galactosidase; acetoin production; gelatin utilization; and fermentation of glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, and arabinose. Conversely, it was negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen disulfide production, urease production, tryptophane deaminase, and indol production.

B. subtilis is known for producing various biosurfactants [14]. Using UHPLC, we have confirmed that *B. subtilis* 87Y is a great producer of lipopeptide surfactin (Fig. 2). Depending on the culture media used, *B. subtilis* produces a wide range of surfactin analogues that vary in hydrophobic and in hydrophilic moieties [23] (Table 1). During cultivation on Landy's rich medium, containing amino acids, *B. subtilis* 87Y produced many analogues (Fig. 2b) which were also found in the surfactin standard (Fig. 2c). In turn, mainly C13–C16 analogues were identified on basic LB medium (Fig. 2a).

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

Spores of *Bacillus* spp. have been reported to be resistant to a broad range of environmental conditions. Among

on *L. lactis* and *Salmonella* spp.: in the (1) L compartment (2), *Salmonella* spp. was inhibited ($A_{490} = 0.1$ at $t = 0$) by the (3) middle compartment (M) grown (4) *L. lactis* ($A_{490} = 0.4$ at $t = 0$), which on the other hand was promoted by (5) U-grown (6) *B. subtilis* 87Y ($A_{490} = 0.4$ at $t = 0$)

them, acid pH and bile salt tolerance were shown in the case of *B. amyloquefaciens* and *B. subtilis* [22, 30]. Spinosa et al. [30] showed the presence of *B. subtilis* spores in the intestinal tract of mice. This indicates that spores can reach the intestine in a viable state after withstanding acidic conditions in the stomach. Lee et al. [22] showed a direct influence of acidic pH and bile salts on *B. amyloquefaciens* LN growth. Viability of the LN strain after 3 h of acid pH, and after 12 h of bile salt treatment, decreased by approximately 20% and 10%, respectively. We noticed similar growth of *B. subtilis* 87Y during 3-h incubations in both neutral and acidic conditions (Fig. 3a). After 12 h of bile salts treatment, *B. subtilis* 87Y viability decreased fourfold in comparison with control conditions (LB medium), but we observed the doubling of optical density in comparison with the initial conditions (0.3% ox gall in LB, 0 h) which indicates slow cell growth (Fig. 3b).

3.3 *B. subtilis* 87Y inhibits *S. aureus* growth through SU production

Amphipathic compounds such as lipopeptides are highly toxic towards Gram-positive bacteria [31]; thus, Gram-positive bacteria are more vulnerable to SU [32]. We observed inhibition of *S. aureus* growth by SU (Fig. 4a), ranging from 20% (1–2 $\mu\text{g/mL}$ SU) to 80% (8–16 $\mu\text{g/mL}$ SU). Due to SU production (Fig. 2), an inhibition zone of *S. aureus* was observed on co-culture with *B. subtilis* 87Y on agar plates (Fig. 4b). However, we 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 [33, 34].

In subsequent experiments, we applied the direct co-culture insert method (Fig. 1a), which was validated by testing the influence of SU and *B. subtilis* 87Y on the growth of *S. aureus* (Fig. 4c). At 4 $\mu\text{g/mL}$, SU inhibited the growth of *S. aureus* by 50% (Fig. 4c), which is in agreement with

Fig. 2 Production of surfactin by *B. subtilis* 87Y after 24-h cultivation on LB (a) and Landy's medium (b), surfactin standard (c). C12–C17 represent analogues differing in hydrophobic and hydrophilic moieties

results obtained with the modified 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).

Table 1 Surfactin analogues identified in the SU standard and in the cultivation broths (Fig. 2). (+)—peak found, (ND)—peak not detected

Analogues	Pseudo-molecular mass [H^+] and/or [Na^+] adducts (m/z)	SU standard	<i>B. subtilis</i> 87Y LB medium	<i>B. subtilis</i> 87Y Landy's medium
C12	994, 1016	+	ND	+
C13	1008, 1030	+	+	+
C14	1022, 1044	+	+	+
C15	1036, 1058	+	+	+
C16	1050, 1072	+	+	+
C17	1064	+	ND	+

Fig. 3 **a** The influence of acidic pH (2.0) on growth of *B. subtilis* 87Y, expressed as A_{600} measurements in time (0–3 h), $n = 9 \pm \text{SD}$. **b** The influence of 0.3% ox gall on growth of *B. subtilis* 87Y, expressed as A_{600} measurements in time (0–12 h), $n = 9 \pm \text{SD}$

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

The fermentation process provides improved nutritional and functional properties compared with the original raw materials. *B. subtilis* is widely used in SSF processes [3, 4, 35].

Supplementing poultry feed with *B. subtilis* leads to an increase in the number of lactic acid bacteria (LAB) in the gastrointestinal tract [36]. *L. lactis* stimulates the growth of broilers [37, 38] and is one of the dominant LAB species isolated from the faeces of broilers [39]. In this study, in LB medium, *B. subtilis* 87Y did not stimulate a significant increase in *L. lactis* growth (Fig. 5a). However, *B. subtilis* 87Y growth was promoted by the presence of *L. lactis* (Fig. 5c).

Al-Hijazeen and Al-Rabadi [40] reported that a sucrose-rich diet positively affected the quality of broilers' meat. Here, the addition of sucrose resulted in ~10% growth promotion in case of *B. subtilis* 87Y and ~14, 26, or 52% promotion of *L. lactis*, ATCC 11454, ATCC 19435, and ATCC 49032, respectively. In co-cultures, the significant promotion of the growth of two *L. lactis* strains by *B. subtilis* 87Y was also observed (Fig. 5b). Most likely, this effect occurred since sucrose is a substrate for the synthesis of levan, a prebiotic polymer produced by *B. subtilis* [41]. The growth of *B. subtilis* 87Y increased by ~10% in the presence of

L. lactis (Fig. 5c). The lower level promotion of *B. subtilis* 87Y growth on LB + 5% sucrose by *L. lactis*, in comparison with growth on LB might result from stronger *L. lactis* growth under those conditions (Fig. 5b).

3.5 *B. subtilis* 87Y and *L. lactis* inhibit *Salmonella* spp.

B. subtilis has been reported to inhibit the growth of pathogenic Gram-negative bacteria in poultry feeding [42, 43]. In this study, both *B. subtilis* 87Y or *L. lactis* strains inhibited *S. enteritidis* and *S. typhimurium* (Fig. 6a). The effect of probiotic bacteria was similar, regardless of the *Salmonella* strain or species. *B. subtilis* 87Y displayed the greatest inhibitory activity (~40% growth inhibition); whereas *L. lactis* ATCC 49032 and ATCC 11454 showed lower inhibitory activity (~20% growth inhibition).

The addition of sucrose resulted in significant inhibition of the growth of *S. enteritidis* ATCC 13076 by both *B. subtilis* 87Y and *L. lactis* (Fig. 6b). *S. enteritidis* ATCC 49223 and *S. typhimurium* ATCC 14028 were inhibited to a lesser extent than when cultured without sucrose (Fig. 6a). The reduction in percentage growth of both *Salmonella* spp. strains were still significant in the case of co-culture with *B. subtilis* 87Y or *L. lactis* ATCC 19435 (Fig. 6b).

Yang et al. [9] reported that the combination of *Lactobacillus* spp. and *B. subtilis* had a higher inhibitory effect towards *E. coli* than *Lactobacillus* spp. alone. Here, we

Fig. 4 **a** Percentage growth of *S. aureus* ATCC 6538 in the presence of surfactin (SU) in the range 0–16 $\mu\text{g/mL}$, $n = 6 \pm \text{SD}$. Statistical analysis at each concentration was performed in relation to a reference value of 0 $\mu\text{g/mL}$ 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, $n = 2$). **c**

The % of growth of *S. aureus* (L compartment) untreated (control), in the presence of *B. subtilis* 87Y (U compartment, here: + 87Y) or with 4 $\mu\text{g/mL}$ SU (U compartment, here: + SU), $n = 6 \pm \text{SD}$. Statistical analysis was performed comparing control conditions and + 87Y and + SU conditions (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

Fig. 5 Percentage growth of *L. lactis* strains ATCC 19435, ATCC 49032, and ATCC 11454 alone (control) or in the presence of *B. subtilis* 87Y, in **a** LB ($n=6$) or **b** LB + 5% sucrose ($n=6$). **c** Percentage growth of *B. subtilis* 87Y alone (control) or in the presence of *L. lactis* in LB or

LB + 5% sucrose ($n=6$). *B. subtilis* 87Y was grown in compartment U, and *L. lactis* in compartment L. Statistical analysis was performed considering control conditions as reference values (* $P<0.05$; ** $P<0.01$; *** $P<0.001$)

applied a triple co-culture insert method (Fig. 1b) to check the effect of the presence of *B. subtilis* 87Y and *L. lactis* on *Salmonella* growth (Fig. 7).

For the experiment, we used *L. lactis* ATCC 19435 as the strain demonstrating the greatest inhibitory effect on *Salmonella* spp. (Fig. 6) among other *L. lactis* strains, as well being promoted at the highest rate by *B. subtilis* 87Y (Fig. 5). *S. typhimurium* ATCC 14028 was chosen due to its wide animal host range, including poultry, cattle, and pigs [44]. *S. enteritidis* is associated mostly with poultry [44].

We observed a ~20% growth inhibition of *S. typhimurium* when co-cultured with *B. subtilis* 87Y and *L. lactis*, regardless of sucrose concentration (Fig. 7a), which was similar across strains (Fig. 6). However,

the promotion rate of *L. lactis* (Fig. 7b) in triple inserts was much higher than in the case of co-culture with *B. subtilis* 87Y alone (Fig. 5). This effect most likely occurred due to the presence of *S. typhimurium*, which is in agreement with data reported by Denkova et al. [45]. During co-culture with *Lactobacillus acidophilus* and *Salmonella* spp., an increase in viable *L. acidophilus* cells was observed [45].

The external *B. subtilis* 87Y supplementation should not lead to *B. subtilis* overgrowth in animal guts. Thus, we examined the viability of *B. subtilis* 87Y in co-culture with *L. lactis* and *S. typhimurium*, which decreased by ~30 or 10% on LB, and LB + 5% sucrose, respectively (Fig. 7c). The reason for this may have been interspecies competition for nutrients.

Fig. 6 Percentage 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, $n=6 \pm SD$ in **a** LB or **b** LB + 5% sucrose.

B. subtilis 87Y and *L. lactis* were grown in compartment U and *Salmonella* spp. in compartment L. Statistical analysis was performed using control conditions as reference values (* $P<0.05$; ** $P<0.01$; *** $P<0.001$)

Fig. 7 **a** Percentage growth of *S. typhimurium* ATCC 14028 untreated (control) or in the presence of *B. subtilis* 87Y and *L. lactis* ATCC 19435 ($n = 3 \pm \text{SD}$). **b** *L. lactis* ATCC 19435 untreated (control) or in the presence of *B. subtilis* 87Y and *S. typhimurium* ATCC 14028. **c** *B. subtilis* 87Y untreated (control) or in the presence of *L. lactis* ATCC 19435 and

S. typhimurium ATCC 14028. All experiments were performed on LB or LB + 5% sucrose. *B. subtilis* 87Y was grown in compartment U, *L. lactis* in compartment M, and *Salmonella* spp. in compartment L. Statistical analysis was performed considering control conditions as reference values (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

4 Conclusions

B. subtilis strain 87Y, which can be isolated from *E. fetida* (earthworm), may be a promising strain for use in the production of fermented animal food. The following probiotic characteristics were noticed in our in vitro studies:

(1) *B. subtilis* 87Y produces surfactin and a variety of enzymes, which may contribute to improving gastrointestinal digestion and nutrient assimilation.

(2) *B. subtilis* 87Y would be able to survive gastrointestinal conditions due to its viability under low pH and bile salts.

(3) *B. subtilis* 87Y promotes the growth of *L. lactis* in the presence of sucrose and is promoted by the presence of *L. lactis*.

(4) *B. subtilis* 87Y inhibits the growth of *Salmonella* spp. directly and indirectly through the promotion of *L. lactis*.

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