Modern Status and Perspectives of *Bacillus Bacteria* as Probiotics

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Introduction

Probiotics are identified as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1]. Although different microorganisms are claimed today as probiotics, usually they represent two main genera - *Lactobacillus* and *Bifidobacterium*. Other probiotic bacteria have received limited attention of researchers. Among them are *Bacillus* bacteria - highly diverse group of microorganisms, known more than 100 years.There are strong scientific data substantiating the validity of the use of these bacteria as probiotics.

Bacillus Bacteria in Nature

Bacteria of the *Bacillus* genus are among the most widespread microorganisms in nature. *Bacillus* spp. are predominant in soil [2, 3]. These bacteria have also been isolated frequently from water[4] and air [5]. Being ubiquitous in soil, air and water they find their way easily into food products. The *Bacillus* counts in wheat, grain and wholemeal, is reported to be 10^6 CFU/g [6, 7]. Due to the high resistance of bacilli spores to heat they can survive the baking process and therefore be found in bread and bakery products [8].*Bacillus* are often present in milk microflora, remain after pasteurisation and can be the predominant microflora in pasteurised milk products [9]. *Bacillus*spp. are the major microflora of soya beans and are implicated in fermentation for preparation of soya food products and condiments [10, 11]. *Bacillus subtilis*and *B. Licheniformis* are the dominant species and their counts in fermented products are 10^8 CFU/g[12]. In Japan a pure culture of *B. Subtilis* var. *Natto* is used to produce natto – a popular food made by fermenting of cooked soybeans [13].

So, bacilli consistently enter the gastrointestinal and respiratory tracts of healthy people with food, water and air. Isolation of *Bacillus* bacteria from the gut was reported for healthy adults [14]and children [15]. The number of bacilli in the gut can reach 10^7 CFU/g [16], comparable to *Lactobacilli* count. Thus some researchers considered *Bacillus* to be one of the dominant components of the normal gut microflora [17]. Bacilli are resistant to acid and bile and keep viability in the gut [18, 19]. Under strict anaerobic conditions bacilli can use nitrate as an electron acceptor and grow anaerobically [20, 21].

Probiotic activity of *Bacillus* bacteria

Bacillus bacteria could play a significant role in the gut because of their high metabolic activity. Activity of *Bacillus* is largely determined by their ability to produce antibiotics. There were identified 795 antibiotics from *Bacillus* bacteria [22]. The most productive species is *B. subtilis*, which devotes 4-5% of genome to antibiotic synthesis and produce 66 antibiotics [23]. *Bacillus* antibiotics differ in the structure and spectrum of activity.Most of the antibiotics, produced by bacilli are peptides, but some strains produce antibiotics, belonging to other chemical classes. For example, an amini glycoside butirosin from *B. circulans*; proticin, a phosphours-containing triene from *B. licheniformis*[13]; an isocoumarin derivatives baciphelacin from *B. thiaminolyticus* [24]; amicoumacin A from *B. subtilis*[25].*Bacillus* antibiotics vary in the spectrum of antimicrobial activity. Some *Bacillus*

strains synthesize bacteriocines, which are effective only against bacteria of the same species, others produce antibiotics against Gram-negative or Gram-positive bacteria and still other strains have a wide spectrum of antibiotic activity (including antifungal and antiprotozoan). These antibiotics can be synthesized under aerobic and anaerobic conditions.

Production of lytic enzymes could also contribute to antimicrobial activity of *Bacillus* bacteria. Lytic enzyme from *B. subtilis*YU-1432 was effective against *Porphyromonas gingivalis*, which cause periodontal disease [26]. Fibrinolytic enzyme, produced by *B. vallismortis* showed high efficacyin lysis of *Streptococcus mutants*[27]. Some *Bacillus*lytic enzymes are active against Gram-negative bacteria [28], others have broad spectrum of activity, including Gram-positive and Gram-negative bacteria [29, 30]and yeasts [31].

Bacillus bacteria could support digestive function of the gut producing essential enzymes. High amylolytic activity is known for bacilli with the production of acid-resistant enzymes [32]. Pectinolytic enzymes are often isolated from *Bacillus* bacteria, especially from *B. subtilis* [33]. Some *Bacillus* strains have lipolitic and cellulolytic activity [34, 35]. Bacilli are characterized by high proteolytic activity [36]. *Bacillus* proteolytic enzymes stimulate regeneration processes [37]and enhance the fibrinolyticactivity in the plasma even after oral administration [38]. Proteolytic enzymes of *Bacillus* contribute to normal digestion by degradation of the anti nutritional factors [39] and the allergenic compounds [40]. *Bacillus* enzymes could stimulate the normal microflora of the gut. Thus subtilisin and catalase, produced by *B. subtilis* (*natto*) enhance the growth and viability of lactobacilli [41]. *Bacillus*enzymes were found to be active in live and in dead cells [42].

Bacilli produce amino-acids, including essential amino-acids[43, 44]and vitamins [45, 46]. Some *Bacillus*strains effectively degrade cholesterol *in vitro*[47]and significantly reduceplasma low-density-lipoprotein cholesterol, hepatic total cholesterol, and triglycerides after oral administration in animal studies [48].

Bacilli could significantly influence the immunological status of the host. Oral treatment with *B. subtilis*spores increased expression of activation markers on lymphocytes in dose-dependent manner [49]. The effect of lymphocyte activation by *B. subilis*spores was both quantitatively and qualitatively similar to that induced by the mitogens phytohaemagglutinin (PHA) and Concanavalin A (ConA) [50]. *B. subtilis*spores stimulated cytokine production *in vitro*and after oral

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administration in mice [51, 52]. Adjuvant properties of *Bacillus* bacteria were confirmed in many studies. *B. subtilis* spores induced systemic antibody response to tetanus toxoid fragment Cand ovalbumin in mice [53]. Killed *B. subtilis* spores enhanced the levels of systemic IgG and mucosal sIgA specific to the influenza when intra-nasally were administered to mice [54].

The quorum-sensing pentapeptide, competence and sporulation factor (CSF) of *B. subtilis* activate key survival pathways in intestinal epithelial cells of the host [55]. It was shown, that CSF induces heat shock proteins, which protect intestinal epithelial cells against injury and loss of barrier function. Thus *Bacillus* bacteria may provide the host with the ability to maintain intestinal homeostasis.

Presented data show the possibility to isolate highly effective probiotic strains among *Bacillus* bacteria. However, probiotic activity is strain-specific. For example, analyze of various *Bacillus* probiotic strains for their antimicrobial activity against clinical multi-resistant strains of pathogens, showed that only one *B. subtilis* 3 strain was effective *in vitro* studies and in animals testing [56, 57]. Thus every selected *Bacillus* bacterium should be studied individually to confirm probiotic activity.

Safety of *Bacillus* bacteria

Humans have been in contact with these bacteria during their entire existence as a species. Evidence of this is the presence of bacilli in samples of permafrost soil, as old as 10^5 years [58], isolation of *Bacillus* spp. from Dominican amber, 25-40 million years old [59, 60], discovery of spores in salt crystal, 250 million years old [61]. Some scientists consider the *Bacillus* spores to be the longest-lived cellular structures known [62, 63].

Bacillus bacteria have a long history of safe use in foods. Over a period of many centuries these bacteria have been used for preparation of alkaline-fermented foods where they are dominated microflora [64]. Humans are constantly exposed to the *Bacillus* species from the environment with no evidence of infections outbreak due to these bacteria (except *B. anthracis* and *B. cereus*) or apparent ill effects. Some cases of infection associated with "nonpathogenic" *Bacillus* species are described [65]. But frequency of such cases is low and comparable with the frequency of infections known for other bacteria of normal microbiota, such as *Lactobacillus* [66] and *Bifido bacteria* [67]. It is well known that bacterial pathogenicity is strain-specific, so every bacterial strain, promising as probiotic, should be tested individually. Pathogenic potential of some *Bacillus* strains is known, so the European Scientific Committee on Animal Nutrition proposed a scheme for the testing of toxin production in *Bacillus* bacteria intended for use as feed additives [68]. Several *Bacillus* strains - *B. subtilis* and *B. licheniformis* [69], *B. subtilis* and *B. indicus* [70], were tested according to this scheme and showed no evidence of toxicity. Additional testing in animals, including acute and chronic toxicity studies, also indicated safety of these strains. *B. subtilis* RO179 was safe *in vitro* toxicity study and in chronic oral toxicity challenges, performed in rats [71]. Safety of *B. coagulans* was demonstrated in acute and sub-chronic oral toxicity [72] and in chronic one-year oral toxicity [73]. Results of these studies indicated that treatment of animals with *Bacillus* bacteria even in the high doses caused no signs of toxicity or any other adverse effects, related to tested cultures. Toxicity data, obtained for *Bacillus* strains [69] were in accordance with the safety records for *Lactobacillus* and *Bifidobacteria* [74, 75].

Current Status of *Bacillus* Probiotics

First clinical data about effective treatment of gastrointestinal infections with *Bacillus* bacteria were published in France [76]. Additionally, spores of *Bacillus* sp. IP 5832 used during therapy with antibiotics, prevented dysbiosis in patients [77]. *B. subtilis* therapy was highly effective in treatment of various infectious pathologies in patients [78]. Mazza [79] summarized the main results about clinical efficacy of *B. subtilis* as an antidiarrheal agent, used in different counties. Author concluded that *B. subtilis* is one of the most important microorganisms for the therapy and prophylaxis of intestinal disorders in humans. *B. subtilis* bacteria were more effective in treatment of acute diarrhea, than lactobacilli [80].

Clinical efficacy of *B. coagulans* has been shown in treatment of diarrhea, dysbiosis and as adjunct therapy for rheumatoid arthritis [81]. Anti-diarrheal activity of *B. clausii* was proved in clinical trials, resulted in significant decrease of diarrhea and pain duration in patients [82].

Resistance of *Bacillus* bacteria to environmental conditions makes them attractive for use as food supplements [83,84].

Several *Bacillus* probiotic strains have been approved for human use. *B. subtilis* 3 and *B. licheniformis* 31 (Biosporin) received a drug status by the national authorities of Russia and Ukraine and are used in these countries for prophylaxis and treatment of acute intestinal infections [80,85]. *B. clausii* (Enterogermina) and *B. cereus* (Bactisubtil) were approved for medical use in Europe. Recently, *B. coagulans* (GanedenBC30) obtained self-affirmed GRAS status in USA. *B. subtilis* (*natto*) OUV23481 was approved in Japan as FOSHU (food for specified health use) [86].

Perspectives of *Bacillus* Probiotics

Probiotic therapy gains more attention of researchers as alternative approach to conventional antibiotic therapy, especially because of emerging of new multi-resistant pathogens. Efficacy of probiotics against pathogenic bacteria was well documented in many scientific reports. But the variety of pathogens and the limited spectrum of specific activity of existing probiotic bacteria raise the question about improvement of probiotics. The recent advances in the genetic engineering can be used to modify probiotic cultures both to strengthen their existing activity and to create new strains with the desired properties. So it is possible to influence the mechanism of probiotics' action.

Bacillus bacteria are promising system for developing of new recombinant probiotics. They are genetically well studied and used as a model for cloning of different pro- and eukaryotic genes. Recombinant *Bacillus* strains are known as commercial producers of biologically active compounds (enzymes, antibiotics, etc.) [87]. *Bacilli* do not colonize mucous membranes permanently, so the amount of these bacteria and recombinant protein can be controlled by the different doses and schemes of administration. Additionally, this reduces the possibility of gene transfer or any adverse effects dealing with such transfer.

It is known that bacilli are able to secrete large amount of proteins into the culture medium, thus it opens the opportunity to develop *Bacillus* strains with extracellular production of recombinant proteins. This approach has been used for construction of *B. Subtilis* strain with antiviral activity. *Bacillus subtilis* probiotic strain was transformed with plasmid DNA containing the chemically synthesized gene of human alfa-2 interferon. This plasmid was stable in the bacterial cell

even after sporulation and repeated germination. The recombinant bacteria produced interferon extracellularly in an amount of $10^4 – 10^5$ IU/mL [88]. Antiviral activity of *B. subtilis* recombinant strain against influenza virus, herpes virus and equine encephalomyelitis virus was shown *in vitro* and in experimental animal infections [89]. Efficacy of animal protection against 10 LD_{50} dose of influenza virus by oral administration of recombinant strain was 70%, against 100 LD_{50} dose – 50% [90]. Oral treatment of mice with *B.subtilis* recombinant strain significantly increased cytotoxic activity of macrophages and inhibited the growth of Lewis lung carcinoma [91]. Safety of *B. subtilis* recombinant strain (Subalin) was demonstrated *in vitro*, in animals [92] and in healthy volunteers [93]. Subalin was effective in combined treatment of meningoencephalitis [94] and hepatitis A and B [95]. Recombinant *B. subtilis* strain (Subalin) is approved as a drug by the national authority of Ukraine.

B. brevis strain was transformed with the plasmid, carrying a synthetic gene of a single chain human insulin precursor [96]. Authors demonstrated that recombinant product, obtained from the culture supernatant, had the biological activity, similar to human insulin. Proinsulin (PI) was efficiently overproduced and secreted into the culture medium by recombinant *B. subtilis* strain [97]. The maximum concentration of PI in supernatant has reached 1 mg/mL.

Plasmid DNA, coding for the intracellular production of the human IL-1 receptor antagonist (IL-Ra) was used to transform *B. subtilis* strain [98]. After treatment of rats and rabbits with recombinant bacteria, human IL-1Ra protein was found intact and biologically active in the intestinal lavage and in the serum of animals.

Bacillus spores can be engineered to express heterologous antigens. *B. subtilis* is one of the most studied spore surface display system [99]. Structural coat proteins CotB, CotC and CotG were used to anchor the heterologous passenger proteins on the spore surface. The spore display approach was applied to construction of *B. subtilis*-based tetanus vaccine [100, 101], rotavirus vaccine [102], anthrax vaccine [103], vaccine against trermatode *Clonorchissinensis* [104]. Other proteins were successfully exposed on the spore surface by using this method: green fluorescent protein GFP_{UV} [105]; proinsulin [106]; human serum albumin [107].

Conclusion

Bacillus bacteria are attracting increasing attention of the researchers as promising probiotics.

More scientific data indicate the valuable role of these bacteria in the environment and in the host organism. *Bacillus* bacteria are ubiquitous in nature; they are normal microflora of foods. Bacilli consistently enter the gastrointestinal and respiratory tract of humans and animals with food, water and air and they are component of the normal gut microflora. These bacteria are known as highly effective producers of biotechnologically important products – antibiotics, enzymes, vitamins, amino acids. Strains with unique activity can be isolated among *Bacillus* bacteria. Bacilli have a great potential for developing of new recombinant probiotics with desired properties. Currently several approaches have been applied to construct recombinant bacilli, producing heterologous proteins for treatment of pathological conditions or for vaccine delivery. Further studies of the mechanisms of *Bacillus* probiotics' action will lead to elaboration of new probiotics, based on new selected strains or genetically modified strains with predetermined properties.

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