Spore-Forming Probiotics for Poultry

Subjects: Agriculture, Dairy & Animal Science
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One of the main problems in the poultry industry is the search for a viable replacement for antibiotic growth promoters. This issue requires a “one health” approach because the uncontrolled use of antibiotics in poultry can lead to the development of antimicrobial resistance, which is a concern not only in animals, but for humans as well. One of the promising ways to overcome this challenge is found in probiotics due to their wide range of features and mechanisms of action for health promotion. Moreover, spore-forming probiotics are suitable for use in the poultry industry because of their unique ability, encapsulation, granting them protection from the harshest conditions and resulting in improved availability for hosts’ organisms.

Keywords: poultry ; spore-forming probiotics ; Bacillus ; antibiotics ; growth performance ; solid-state fermentation

1. Introduction

Spore-forming probiotics are gaining popularity in the poultry industry as natural growth promoters [1][2]. The most prevalent probiotics are lactic acid bacteria (LAB), lactobacilli, and *Bifidobacterium* spp., which are normally found in the gastrointestinal tracts (GIT) of animals and humans [3]. On the contrary, spore-forming bacteria, *Bacillus* spp. and *Clostridium* spp., due to their ability of encapsulation, can be found not only in GIT, but also in soil, water, and dust [4]. This makes the development process for spore-forming probiotics more accessible compared to LAB. Spore formation increases the survival of probiotics during the manufacturing process including fermentation, freezing, drying, thawing, and rehydration. Additionally, spores of these probiotics have a greater ability to survive passage through the gut and to proliferate and colonize the digestive tract [5][6]. This ability makes spore-forming probiotics an ideal feed additive for livestock, especially in the poultry industry.

There is an urgent need for an effective replacement for now-banned antibiotic growth promoters (AGPs). Alternatives currently under development are antibodies, prebiotics, bacteriophages, vaccines, and antimicrobial peptides [7]. However, we believe probiotics, especially spore-forming ones, are a suitable solution. Over the past several decades, they have demonstrated significant success not only in the control of pathogens, including drug-resistant strains [8][9], but also in natural growth promotion, improvement of feed conversion rates, and other zootechnical characteristics in broilers, laying hens, and other poultry species [10].

The main aim of this review is to summarize and discuss (a) the current achievements of microbiota studies in livestock birds, (b) the poultry health-promotion effects of spore-forming probiotics such as immune-modulation, (c) metabolism improvement, (d) interaction with hosts’ gene expression, and (e) the impact of spore-forming probiotics on productivity rates and egg and sperm quality. Important biotechnological aspects of spore-forming probiotics’ manufacture such as cultivation and solid-state fermentation will also be discussed.

2. Where to Start? Brief Diving into the Chicken’s Gastrointestinal Tract and Its Commensal Microbiota

Before considering the effects of probiotic bacteria on the gut microbiota, we should take a closer look at the commensal microbiota of the chicken GIT. Birds have a higher rate of passage of food through the GIT and increased activity of digestive enzymes compared to other vertebrates [11][12]. In the text below, we will study each part of the intestine separately.

2.1. Oral Cavity and Goiter

The oral cavities of birds do not contain teeth, unlike those of mammals, and therefore, food does not linger there, immediately going down the esophagus into the goiter. However, saliva production, which moistens food, occurs in the oral cavity, providing a moist and favorable environment for the development of microorganisms in the goiter [12].

A goiter is an enlargement of the esophagus where food can be stored before it enters the stomach. If the bird is hungry, food can enter the stomach, bypassing the goiter; if there is enough food, it will linger in the goiter and enter the stomach in small portions [13]. In addition to saliva from the oral cavity, a mucous secretion containing mucin is produced in the goiter. This secretion creates ideal conditions for softening food and the development of the microbiota, including microbial fermentation of food [12][14].

In chickens, the goiter microbiota is mainly represented by bacteria ingested with food. Their numbers can reach 10^9 CFU/g [15]. These are primarily *Lactobacillus* and *Clostridiaceae*, *Bifidobacterium*, *Enterobacteriaceae*, and *Enterococcus* species [16]. Han et al. (2016) confirmed these data, showing that the goiter was dominated by Firmicutes (60%) followed by Bacteroidetes (14%), Cyanobacteria (13%), and Proteobacteria (8%). Among Firmicutes, *Lactobacillus* (28% of all species), *Bacillus* (4%), and *Bacteroides* (4%) were most prevalent [17].

A review by Feye et al. (2020) also showed that *Lactobacillus*, *Bifidobacterium*, and *Enterobacter* were most often represented in the chicken goiter. However, in free-range birds, large amounts of *Bacillus* (up to 76%) can also be found in the goiter [18].

Food can stay in the goiter for up to 14 h; however, it most often does so for 1–3 h. Next, it enters the stomach, taking a part of the microbiota with it, while the rest remains on the goiter walls [12].

2.2. Glandular Stomach and Gizzard

The glandular stomach, or proventriculus processes chyme, using enzymes at acidic pH. The pH can be 2.3–4.8 [13]. The food does not linger here for very long; most often, in chickens, the time spent for food in the glandular stomach is 10–30 min [12].

In the gizzard, food is broken down by small stones or grit. It is also where the first part of the enzymatic digestion of food and the bulk of its mechanical grinding takes place [15]. The muscular intestine contents are then transported to the small intestine in small portions [12].

Due to the low pH, the number and diversity of microorganisms in the gizzard are lower than in the goiter and intestines. The number of bacteria does not exceed 10^8 CFU/g [15]. According to “Sturkie’s avian physiology,” it is possible for the contents of the small intestine to return to the gizzard [12]. Furthermore, this means the regurgitation of the microbiota of the small intestine. In general, the gizzard microbiota is represented mainly by *Lactobacillus* as well as Clostridiaceae, *Enterococcus*, and coliforms [16]. A review by Feye et al. (2020) stated that the main bacteria in the gizzard were *Lactobacillus*, *Enterobacteriaceae*, and coliform bacteria [14].

2.3. The Small Intestine

In the small intestines of birds, the duodenum, ileum, and jejunum can be distinguished; however, there are no significant functional differences between them, nor pronounced boundaries such as sphincters [13]. The pH gradually increases from 5.8 to 6.4 in the intestines [13]. In addition to the secretions of the pancreas and liver, the small intestine wall produces a secretion containing enzymes and mucin. On average, chickens have 2 to 8 h of food in the small intestine [12]. The total amount of microbiotas here increase significantly compared to other regions of the GIT, up to 10^9–10^{11} CFU/g [15].

Despite the absence of clear boundaries, the microbiota in the different sections of the intestines are distinct. Apparently, this is due to a change in the composition of the available nutrients due to the intestinal wall’s enzymatic digestion and absorption. Thus, for example, although representatives of Firmicutes (>60%) and Bacteroidetes (>10%) are the predominant species in the small intestine as a whole, the duodenum also contains a high amount of Proteobacteria (>20%). In comparison, in the rest of the sections, Proteobacteria account for less than 10% of the total microbiota. In the ileum, representatives of Actinobacteria are most widely represented [19]. In terms of individual genera, lactobacilli can be isolated, which make up more than 35% of the small intestine’s microbiota. *Enterococcus* occupies a dominant position in the ileum (up to 30%); the highest numbers of *Corynebacterium* are also found there [19].

A study by Mohd Shaufi et al. also demonstrated the predominance of Firmicutes in the ileum. According to this work, Firmicutes accounted for 85% of the microbiota, and Proteobacteria were second in number. On the other hand, the dominant orders were *Clostridiales*, bacilli (including lactobacilli), and Gammaproteobacteria, mainly Enterobacteriales [20]. According to another study, the number of lactobacilli in the ileum can be as high as 70%, with the remaining dominant genera being Clostridiaceae (11%), *Streptococcus* (6.5%), and *Enterococcus* (6.5%) [21].

2.4. Cecum

Birds have two blind guts, and in chickens, they are well developed. Food stays in the ceca for 12–20 h [15][22]. It is difficult to determine the exact time because the contents are thrown and ejected back through the same opening near the small intestine transition into the rectum. The cecum contents are constantly mixing, maintaining a stable composition of the microbiota even under the conditions of a fast digestion rate in chickens [15][22][23]. Unlike the small intestine, where the main functions are digestion and absorption of nutrients, the cecum's primary function is enzymatic activity and detoxification of harmful substances [24]. As a result of fermentation processes in the cecum, chickens receive biologically active substances such as short-chain fatty acids [25].

There are also a high number of microorganisms in the cecum, up to 10^{11} CFU/g [15]. Due to the intestinal contents' long-term presence, the microbiota of the cecum is the most diverse among all parts of the intestine [19][20][21]. It forms a cluster that is distinct from the microbiota of the small intestine and rectum. According to a study by Xiao et al., Bacteroidetes were predominant in the cecum (>50%), while Firmicutes constituted only about 40%. The number of Lactobacillus species decreased in the cecum, while Bacteroides increased to more than 40% [19]. In another study, the dominant group was Clostridiaceae (65%) followed by *Fusobacterium* (14%), *Lactobacillus* (8%), and *Bacteroides* (5%). However, this study also highlighted a difference in the cecum and jejunum microbiomes [21].

Another genome-wide study showed the predominance of *Clostridiaceae* (>50%) and a high level of Bacteroidetes (about 20%) [20]. A study of metagenomes uploaded to public databases showed that most often, Firmicutes (78%) and Bacteroidetes (11%) prevailed in the cecum, which was consistent with the data of Shaufi et al. and Lu et al. [20][21]. Among the secondary groups, the most significant number of representatives related to Proteobacteria and Actinobacteria. Among Firmicutes, the most common were *Ruminococcus*, *Clostridium*, and *Eubacterium*; among Bacteroidetes, up to 40% were *Bacteroides*. At the phylum level, Proteobacteria, Desulfohalobium, *Escherichia*/*Shigella*, and *Neisseria* were the most abundant [26].

A study by Glendinning et al. (2020) also investigated the cecum microbiome. According to this study, Firmicutes were the dominant group, and their abundance was as high as 95%. Among Firmicutes, the majority were Clostridia (88%) followed by Lactobacillales (5%). The remaining 5% of the microbiota was distributed among Actinobacteriota, Proteobacteria (all *Escherichia coli*), Verrucomicrobiota, Bacteroidota, Campylobacterota, Cyanobacteriota, and Desulfobacterota [27]. Thus, according to various studies, the microbiota of the cecum of chickens can vary significantly.

2.5. Colon

The colon is short, and its contents stay inside for approximately one hour before entering the cloaca [13]. In general, the composition of the large intestine's microbiota is closer to that of the small intestine than that of the colon. It is also dominated by Firmicutes (>60%) and Bacteroidetes (>10%). The most widely represented genera are *Lactobacillus* and *Enterococcus* [19][28]. Due to reverse peristalsis, the cloaca contents can enter the rectum along with uric acid, negatively affecting the colon microbiota and influencing the data obtained from fecal samples [12].

2.6. Differences in the Microbiota of Chickens and Factors Affecting Them

From the data presented above, we can conclude a wide variability in the poultry microbiome. Stanley et al. (2013) conducted a study comparing the microbiomes of broiler chickens raised under the same conditions, repeated three times. To compare the results obtained, the authors used QIIME v1.3.0 open source software. Various clustering methods provided in the QIIME package showed that the resulting microbiomes formed three clusters. Non-phylogenetic beta diversity metrics grouped samples from the three trials into three fully separated groups; the Spearman metric completely separated samples from trial 3 into two separated but close groups of samples originating from trials 1 and 2. Unweighted and Weighted Unifrac also showed some, but not total, separation of the samples from each trial. The authors attributed this level of variability, even under carefully controlled conditions, to the high levels of hygiene during egg incubation that destroyed the shell microbiota. Thus, instead of the mother's shell microbiota, chicks could get bacteria from entirely different sources: egg transport boxes, staff, etc. These factors were unstable and difficult to control, and because of this, the resulting microbiota differed significantly between the repeats [29].

Oakley et al. (2014) carried out a broad comparison of various metagenomes of the ceca of birds and a comparison of their functional activity due to the genes present in the microbiome. They concluded that despite a high variability of taxa, functional variability within the chicken cecal microbiome was much lower and, to a much lesser extent, differed between samples. This meant that while the composition of the microbiota in different chicken groups could vary widely, the gut microbiota equally performed its primary functions [15].

The composition of the microbiota is highly dependent on the age of the bird. After hatching, the intestines of chicks are rapidly colonized by bacteria; however, as birds age, these alterations decrease upon reaching adulthood [21][30][31].

Diet also affects the gut microbiota of birds. Factors such as pellet size, choice of food grains, and the microbiota of those foods can cause shifts in birds' microbial communities [32][33][34][35]. Antibiotics can selectively affect the gut microbiota, which leads to changes in the ratio of different groups of gut microorganisms [36][37]. Housing conditions can also affect the microbiota. When litter is reused, the microorganisms contained in it can affect the broilers' microbiomes [38][39]. Pin Viso et al. (2021) analyzed the available metadata from MG-RAST and the NCBI Sequence Read Archive using QIIME v1.9.1 software. According to their analysis, there was a correlation between such factors as age, diet, and geographic location. The authors discussed the so-called "local microbiota" characteristics of different countries [40]. Other important factors influencing the microbiota of chickens are probiotics, prebiotics, and their compositions [41][42][43].

3. Spore-Forming Probiotics: Benefits for the Poultry Industry

As for the specific effects of spore-forming probiotics in poultry, preparations based on *B. licheniformis* have been widely used in the poultry industry for more than three decades, positively affecting feed conversion rates [44]. The range of drugs that can achieve this effect is still expanding. It has been shown that the use of preparations based on *Bacillus subtilis* equally improves the growth and productivity of broilers, and the effects are comparable to the results of the use of the antibiotics bacitracin and avilamycin. Among other things, probiotic preparation has been shown to positively affect the histomorphometry of the intestinal villi [45]. Most often, probiotics have a positive effect on the parameters of weight gain and the efficiency of food consumption in broilers [46][47][48]. According to the literature, spore-forming probiotics affect the following parameters measured in the poultry industry:

- Biochemical blood parameters showing the intensity of carbohydrate and protein metabolism (protein, glucose, urea) [49];
- Hematological blood composition (number of blood cells) as well as stimulation of the hematopoietic organs [50][51];
- Dynamics of live weight (weight gain) [52];
- Feed conversion rate (this appears to be increased by improved digestion and absorption of nutrients, leading to increased productivity) [53];
- Quantitative and qualitative composition of the microbiota [54];
- The level of oxidative stress (mRNA expression of antioxidant genes, oxidative damage index, etc.) [55];
- Meat quality (pH, cooking loss, shear, color, short-chain fatty acids, taste) [56];
- Egg production [57].
- Egg quality (yolk cholesterol, improved shell thickness, egg weight) [58];
- Sperm quality (volume of ejaculate, total number and concentration of spermatozoa in the ejaculate, number of morphologically abnormal cells in the ejaculate) [59];
- Intestinal barrier function [60][61].

All of the above-mentioned effects of spore-forming probiotics on poultry health and performance parameters are linked, as a specific and selective probiotic has not yet been identified that is associated with only one effect. The systematic action of probiotics is mediated by modulation of the GIT microbiota, resulting in a wide range of improvements to poultry performance.

It should be mentioned that there are currently no standards for probiotic poultry trials, specifically regarding definitions related to production performance parameters. Certainly, most of the in vivo probiotic research in poultry has followed the principles of blind randomization and placebo control, and has chosen suitable statistical tests for analysis. The most proven guideline for animal studies is ARRIVE (Animal Research: Reporting of In Vivo Experiments) [62]. However, it covers general items regarding study design and reporting results. In our mind, specific guidelines for probiotic poultry studies with the enumeration of essential parameters that should be evaluated in this kind of research must be created. They should include a set of minimum and possibly inexpensive parameters that must be studied to grant research reproducibility and comparability of results. As of now, there are a wide range of studies that cannot be compared because the authors did not report some production performance or quality characteristics in animals treated with spore-forming probiotics. For example, Ermakova et al. (2021) described the effects of *B. subtilis* probiotic on the yolk quality of Pharaon

quail without mentioning other egg quality parameters such as egg weight, egg shape index, eggshell color, eggshell strength, yolk weight, eggshell weight, and other factors which did not require expensive equipment or consumables to evaluate, which makes it impossible to fully compare the results of this study with others [63]. Deng et al. (2012) did not report feed conversion rates, but they did provide egg weight and feed intake rate. As for the study design, the authors only provided information about heat stress-challenged birds treated with probiotics without any data about a positive control group with probiotic treatment and without experimental conditions [64]. Many examples of studied performance parameters including egg and sperm quality characteristics are provided in **Table A1**, **Table A2** and **Table A3**. Moreover, it should be mentioned that most of the studies have not provided detailed information about spore-forming probiotics preparation, which undoubtedly affects the reproducibility of the studies.

4. Spore-Forming Probiotics Manufacturing, Exploiting Their Biosynthetic Potential

4.1. Cultivation Conditions for Bacillus spp. Growth and Spore Production

The global probiotics market surpassed USD 44.2 billion in 2019 and is expected to grow at a 7.7% compound annual growth rate to hit USD 74.3 billion by 2025 [65]. The use of *Bacillus* species as probiotic formulations is also rapidly expanding, requiring them to be produced in large quantities at a low cost. A key step in the development of a bioprocess is the production of bacteria with high yield and sporulation efficiency. Therefore, to obtain new and deeper fundamental knowledge about the physiology of bacilli and the sporulation process as well as to develop industrially significant technologies for the production of probiotics, various approaches and strategies have been used including the search for new spore-forming bacteria, the use of cost-effective plant materials as growth substrates, the optimization of fermentation media and cultivation conditions, and the development of improved bioprocess technologies [66][67][68][69][70][71][72][73][74]. Nevertheless, current knowledge on the physiology of probiotic *Bacillus* spp. production is still too limited to effectively realize their biotechnological potential on an industrial scale.

In laboratory studies, chemically defined synthetic media are frequently used for growth and sporulation. Although such media provide well-reproducible and homogeneous spore preparations, they are relatively expensive and provide a relatively low spore yield (1×10^8–1×10^{10} CFU/mL). The concentration of the carbon source can play a decisive role in the process of sporogenesis by individual bacilli because it has been shown that with an increase in glucose concentration, the concentration of vegetative cells increases, but initial glucose concentrations above 5 g/L inhibit sporulation and sporulation efficiency decreases [66][68][75][76][77][78][79][80][81][82][83][84][85][86]. It has been suggested that depletion of the carbon source is the main stimulus for sporulation by *Bacillus* spp.; if that is the case, the concentration of the carbon source in the culture medium needs to be reduced to increase the sporulation efficiency and spore yield [5].

Although there is very little information in the literature on the effect of lignocellulose substrates on the formation of *Bacillus* spores, a significant number of publications are devoted to the study of solid-phase fermentation processes of spore-forming bacteria in the context of developing solid-phase processes related to the disposal of organic waste including various lignocellulose substrates such as straw, leaf-stem mass, pulp, and meal [72][73][74][76][77][78][79][80][81][82].

The use of solid-state fermentation (SSF) is an important tool in the prevailing circular bioeconomy paradigm, wherein organic solid waste is converted into value-added products. We believe that products obtained from waste by biotechnological methods differ favorably from traditional chemical sources in that the raw materials for their production are renewable raw materials of animal and plant origin, and the use of agro–industrial waste and by-products as substrates for the growth of probiotic microbes is one of the best ways to reduce production costs.

It is appropriate here to mention another area closely related to the prospects of waste recycling. SSF for plant disease biocontrol is considered to be one of the most promising alternatives to chemicals and is being commercially developed in many countries where *Bacillus* strains have a significant background [71][83][84].

However, it is necessary to identify the species and even the strain-specific lignocellulosic material to maximize the probiotic potential of spore-forming *Bacillus* spp. Thus, cornmeal and soybean meal positively influenced spore production by *B. amyloliquefaciens* BS-20, while no significant effects were found from wheat bran and molasses [70]. An optimized medium containing glucose, corn meal, soybean meal, and beef extract provided an 8.8-fold increase in spore yield compared with a control medium. In another study, a combination of tapioca with lactose in a nutrient medium for submerged cultivation of *B. amyloliquefaciens* B128 resulted in a spore yield of 5.92×10^8 spores/mL [87]. A wide range of lignocellulosic materials with different chemical compositions have been used to evaluate *B. subtilis* KATMIRA1933 spore production under submerged fermentation conditions [76]. Milled soybean and sunflower processing by-products resulted in good growth of bacilli and accumulation of vegetative cells but failed to promote mass sporulation as compared to a

control medium. On the contrary, mandarin peels followed by ethanol production residue (EPR) from corn grains provided an especially high yield of spores (5.7×10^{10} and 2.9×10^{10} spores/mL, respectively). Interestingly, the number of spores (4×10^{9} spores/mL) increased by 7 and 10 times with an increase in the concentration of mandarin peel in the medium from 10 to 30 and 40 g/L, respectively. Further increases in mandarin peel concentration did not favor spore formation. Moreover, the authors found that using cheese and curd whey instead of distilled water to prepare a culture medium containing mandarin peel, EPR, or a mixture thereof accelerated the initial growth of the bacilli and increased the spore yield to $5.8–7.4 \times 10^{10}$ spores/mL. Likewise, *B. amyloliquefaciens* B-1895 appeared to be an efficient spore-forming bacterium producing $8.2–10.8 \times 10^{9}$ spores/mL in the submerged fermentation of corn cobs, EPR from wheat grain, wheat bran, sunflower extraction cake, and mandarin peels [76]. It can be inferred then that these substrates contain all the nutrients required for both bacterial growth and effective sporulation. In addition, during the fermentation of these materials, *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 exhibited relatively low endoglucanase and xylanase activities, which hydrolyze lignocellulose polysaccharides to metabolizable sugars to provide bacterial cultures with their necessary carbon sources. Consequently, only traces of reducing sugars were detected, even at the end of submerged fermentation, when the bacterial metabolism and proliferation had significantly declined. These circumstances may lead to the prevention of sporulation inhibition caused by elevated concentrations of sugars. On the whole, comparative analysis of the data received showed that in the submerged fermentation of lignocellulosic materials, both bacilli produced higher yields of spores as compared with those in the glucose-containing medium. Thus, these results indicate that various lignocellulosic materials may be successfully exploited as growth substrates for the cultivation of spore-forming bacteria.

Typically, plant materials, being a rich carbon source, contain nitrogen in concentrations that are suboptimal for the cultivation of microorganisms, which makes it necessary to include an additional nitrogen source for their optimal growth. Several studies have shown that both the nature and concentration of nitrogen sources are crucial nutritional factors affecting bacilli growth and spore production in both synthetic and lignocellulose-based media [75][87][88][89]. In particular, mandarin peels represented an excellent growth substrate for *B. subtilis* KATMIRA1933 growth and spore production, providing an accumulation of 2×10^{10} spores/mL [13]. However, supplementation of this medium with peptone ensured a three-fold increase in the spore yield, whereas ammonium sulfate sharply inhibited the sporulation process. Moreover, the number of produced spores changed to 8.3×10^{10} spores/mL when the nitrogen concentration in the nutrient medium was increased to 40 mM. The authors attributed the positive effect of peptone to the higher production of bacterial biomass and increased sporulation efficiency. In the cultivation of *B. amyloliquefaciens* B-1895, corn cobs appeared to be an excellent growth substrate, providing an accumulation of 7.2×10^{9} spores/mL [76]. Supplementation of the medium with casein hydrolysate at a concentration of 20 mM as the nitrogen led to a three-fold increase of spore numbers. Chen et al. (2010) achieved a maximal spore yield of 1.56×10^{10} spores/mL after 40 h cultivation of *B. subtilis* WHKZ12 in a 30 L fermenter using cornstarch, wheat bran, corn flour, corn steep liquor, soybean flour, and yeast extract at optimal concentrations. Overall, the data received indicate that the determination of an optimal nitrogen source and concentration are necessary for the best growth and sporulation of *Bacillus* species, and that a consideration of the individual physiological parameters of each strain must be taken into account [76].

4.2. Fermentation Methods for the Production of Probiotics

4.2.1. Solid-State Fermentation

Probiotic yields and the cost of their production depend significantly upon the method of plant raw material fermentation. According to several studies, the preparation of probiotics using solid-state fermentation (SSF) is both cost-effective and environment-friendly [6][90][91][92][93].

The exploitation of biofilm growth is the key feature determining SSF's advantages because biofilms are perhaps the most natural form of microbial communities' existence [94][95]. Bacteria assembled in a consortium are considerably persistent, just as an organized community is much stronger than a group of separated individuals in higher-level organisms. As is known, the high persistence of pathogenic biofilms creates a number of problems in the treatment of infections. However, this phenomenon has a second side. Probiotic bacteria combined in biofilms grow better, are more resistant to drying, and can colonize the gastrointestinal tract of the host more efficiently [96].

SSF of plant raw materials is attractive compared to the submerged fermentation process because its implementation requires relatively low investment and less sophisticated equipment. It is easy to handle and has higher productivity and concentration of the final product, which can be dried directly without centrifugation, as well as a low wastewater output. Therefore, the cost-effectiveness of SSF is not as dramatically dependent on scaling as in the case of liquid-state fermentation. Moreover, we believe that the indisputable advantage of solid-phase fermentation is the possibility of its organization precisely within the framework of a small-scale technology focused on local raw materials.

However, the cultivation of microbes using SSF depends on several technological issues such as oxygen supply for aerobic metabolism; the removal of heat, CO_2, and volatile components produced from metabolic processes; and the maintenance of suitable moisture content for optimal growth [87][88].

SSF is widely applied in the cultivation of filamentous fungi. The attention of researchers in East and Southeast Asia studying the technological and dietological experience of obtaining traditional food products through the fermentation of soybeans using *Aspergillus* and *Rhizopus* mushrooms has significantly enriched biotechnology worldwide [89]. It was found that in the process of solid-phase fermentation, not only did the protein concentration and the nutritional value increase, the content of anti-nutritional factors of the substrate decreased [80][97][98][99]. The biosynthesis of active substances is also more efficient [100], including antibiotics [101][102], phytohormones, food pigments, and alkaloids. [103][104].

Bacteria and fungi are the two main types of microorganisms used in Asian fermented foods, with most of them, primarily Japanese natto, being fermented with *Bacillus* spp. During fermentation, *Bacillus subtilis* produces various metabolites including peptones, peptides, amino acids, sugars, organic acids, and the enzyme nattokinase [105] that are capable of modulating human and animal health. There is evidence that SSF with several species of the genus *Aspergillus* such as *A. niger* and *A. oryzae* is inferior in its efficiency in increasing the availability of nutrients in soybean substrate and reducing the pool of anti-nutritional factors as compared to bacterial fermentation using *B. subtilis* [106]. Since the discovery of the health benefits of fermented foods [107], the number of publications devoted to this topic are progressively increasing, and new aspects are opening up [105][108][109][110][111].

Bacterial cultures can also be successfully used for the SSF of plant raw materials by microorganisms adapted for the fermentation of lignocellulosic substrates, or, that is to say, capable of secreting lignocellulose-degrading enzymes. Until now, only a few studies have exploited the SSF method for *Bacillus* probiotic production, and there is a lack of comparative information on the production of *Bacillus* spp. probiotics under submerged and SSF conditions. In particular, Zhao et al. (2008) achieved the highest yield of spores (1×10^{11} spores/g) when a mixture of 15 g wheat bran and 5 g rice straw powder was used as a growth substrate for SSF by *B. licheniformis* B36. Supplementation of this medium with an additional carbon source, either glucose or sucrose, increased spore production by 35% and 25%, respectively, while additional nitrogen sources, peptone and yeast extract, increased the spore yield by 16% and 24%, respectively [97]. In our studies, *B. amyloliquefaciens* B-1895 [6][76] and *B. subtilis* KATMIRA1933 [13] showed a capability to utilize various inexpensive lignocellulosic wastes/by-products as growth substrates for high-yield spore production. The summarized data [5] showed that in most media, SSF was a suitable method for bacilli cultivation, favoring a significant increase in the number of spores compared to those produced during the same time using submerged fermentation. SSF of wheat bran followed by mandarin peels provided especially high yields of B. subtilis KATMIRA1933 spores (5.7 and 4.9 × 10^{11} spores/g, respectively), whereas EPR from wheat grains and wheat straw promoted spore formation in the SSF by *B. amyloliquefaciens* B-1895 (3.8 and 3.1 × 10^{11} spores/g, respectively). Interestingly, depending on the cultivation method, both bacteria showed different preferences for growth substrates. For example, wheat straw appeared to be the worst growth substrate for sporulation by *B. amyloliquefaciens* B-1895 in submerged fermentation, but it was a preferable source of nutrients in SSF conditions. In the submerged fermentation, mandarin peels appeared to be the superior growth substrate for B. subtilis KATMIRA1933 spore production, whereas *B. amyloliquefaciens* B-1895 was capable of efficiently sporulating following fermentation of the majority of tested materials. Overall, these findings suggest that both bacilli possess sufficiently potent enzymatic systems to deconstruct plant raw materials and provide all necessary nutrients required for abundant bacterial growth, whereas the chemical composition, particle structure, and adhesive properties of these materials favors biofilm formation and efficient sporulation.

4.2.2. Perspectives on Scaling up Fermentation Processes

Scaling up fermentation is the last step in the development of the production process, and several research groups have demonstrated the technical feasibility of scaled-up production of *Bacillus* spp. spores. Sen and Babu (2005) developed a two-stage strategy for *B. coagulans* RK-02 cultivation and sporulation in a 20 L fermenter. During the first stage, cultivation conditions were created that were favorable for the production of biomass, while for the second stage, in the stationary phase, conditions optimal for sporulation were maintained to obtain a maximum spore yield of 9 × 10^{11} spores/g [111]. Monteiro et al. (2014) cultivated *B. subtilis* in a 2 L bioreactor, using an optimized, chemically defined medium, and during the exponential growth phase, the authors increased the agitation rate from 100 to 1200 rpm to compensate for the oxygen consumption rate. The maximum vegetative cell concentration (1.3×10^{10} cells/mL) was obtained at the end of the exponential growth phase. Thereafter, cell lysis was observed, but only 48% of vegetative cells produced heat-resistant spores, with a final concentration of 6.3×10^{9} spores/mL [75].

Cultivation of *B. subtilis* KATMIRA1933 was performed in a 7 L fermenter filled with an optimized medium containing mandarin peels as a growth substrate [76]. At a fermenter stirring speed of 300 rpm and aeration rate of 1.0 L/L/min, bacilli multiplication proceeded rapidly, and after 24 h of fermentation, the number of vegetative cells increased from 3×10^6 CFU/mL to 2.4×10^{10} CFU/mL, with a spore concentration of 3×10^8 spores/mL. During the second day, the vegetative cells and spore numbers increased to 8.1×10^{10}/mL and 9.3×10^9 spores/mL, respectively. In subsequent cultivation, the *B. subtilis* KATMIRA1933 cell number increased to 1.04×10^{11} CFU/mL after 96 h fermentation, with a maximum yield of 6.5 × 1010 spores/mL.

Undoubtedly, a promising strategy for the mass production of probiotics is the use of fed-batch cultures when the concentration of the limiting substrate (usually, carbon source) can be kept very low, thus avoiding the repressive effects of high concentrations of the substrate. In this case, all other nutrients are present in sufficient quantities so that the growth of the microorganism is controlled solely by the concentration of the carbon source present [69][112]. Thus, a fed-batch cultivation process in a 2 L bioreactor was developed for *B. subtilis* spore production with a high yield. Initially, the culture was grown for 5 h in batch mode in a medium containing 3.5 g glucose/L. Before the complete depletion of glucose in the middle of the exponential growth phase, a nutrient feed was started to extend the exponential growth phase, prevent sporulation, and accumulate a maximum concentration of vegetative cells (3.6×10^{10} cells/mL). At the end of the fed-batch phase, glucose was completely depleted from the medium, causing a spike in dissolved oxygen concentrations and indicating the onset of the sporulation process. This fed-batch process of *B. subtilis* cultivation resulted in an increase in spore production, with the highest yield of 7.4×10^9 spores/mL. To obtain a high yield of the probiotic *Bacillus coagulans*, Pandey and Vakil (2016) first achieved a high cell density in batch culture followed by fed-batch fermentation in which glucose was added intermittently in portions. The maximum biomass yield reached was 30 g/L, which corresponded to 3.8×10^{11} cells/mL, with a high spore titer of 1.9×10^{11}/mL and a sporulation efficiency of about 81%. High biomass production was achieved by maintaining the dissolved oxygen (DO) concentration above a critical level (20% DO) to meet the organism's maximum specific oxygen demand [69].

The upscaling of the SSF process to a pilot level for probiotic production was carried out for the first time by Berikashvili et al. (2018) after optimizing the composition of the culture medium for *B. amyloliquefaciens* B-1895. In these experiments, bacilli cultivation in 1 kg of milled corncobs soaked by an optimized cheese whey-containing medium and placed in polypropylene gas-permeable bags resulted in the accumulation of 1.0×10^{12} spores per gram of dry biomass [6]. Recently, the feasibility of the developed medium and SSF strategy was proven for *B. subtilis* KATMIRA1933 probiotic production, when bacilli were cultivated in polypropylene bags or trays filled with 2 kg of wheat bran or milled corn cobs and formed 4.9×10^{11} spores/g and 4.3×10^{11} spores/g, respectively (unpublished results). These findings show that the SSF of plant raw materials by spore-forming bacteria has great potential for the efficient production of cheap probiotics.

In conclusion, the analysis of literature data shows that only a few *Bacillus* spp. have been extensively studied so far, and current knowledge on their physiology is still too limited to effectively realize their biotechnological potential on an industrial scale. Especially little is known about the physiological peculiarities of bacilli growth and spore production during lignocellulose fermentation, under solid-state conditions in particular. Moreover, information on hydrolytic enzyme production by probiotic bacilli during lignocellulose fermentation is limited, although polysaccharides are typically the main resource for bacterial growth, and cellulases play a decisive role in steadily supplying a carbon and energy source to the bacteria. It is necessary to elucidate the physiological mechanisms that regulate (enhance or suppress) the growth and sporulation of individual bacilli as well as understand the optimal nutrient requirements for both processes. Finally, to develop effective technology for the production of spore-forming bacteria, a reasonable strategy for increasing the production of probiotics is to create conditions at the beginning of cultivation that ensure high cell density as well as conditions that allow sporulation to occur.

References

1. Ruiz Sella, S.R.B.; Bueno, T.; de Oliveira, A.A.B.; Karp, S.G.; Soccol, C.R. Bacillus subtilis natto as a potential probiotic in animal nutrition. Crit. Rev. Biotechnol. 2021, 41, 355–369.
2. Angelakis, E. Weight gain by gut microbiota manipulation in productive animals. Microb. Pathog. 2017, 106, 162–170.
3. Zommiti, M.; Chikindas, M.L.; Ferchichi, M. Probiotics-Live Biotherapeutics: A Story of Success, Limitations, and Future Prospects-Not Only for Humans. Probiotics Antimicrob. Proteins 2020, 12, 1266–1289.
4. Mingmongkolchai, S.; Panbangred, W. Bacillus probiotics: An alternative to antibiotics for livestock production. J. Appl. Microbiol. 2018, 124, 1334–1346.

5. Elisashvili, V.; Kachlishvili, E.; Chikindas, M.L. Recent Advances in the Physiology of Spore Formation for Bacillus Probiotic Production. Probiotics Antimicrob. Proteins 2019, 11, 731–747.
6. Berikashvili, V.; Sokhadze, K.; Kachlishvili, E.; Elisashvili, V.; Chikindas, M.L. Bacillus amyloliquefaciens Spore Production Under Solid-State Fermentation of Lignocellulosic Residues. Probiotics Antimicrob. Proteins 2018, 10, 755–761.
7. McAllister, T.A.; Wang, Y.; Diarra, M.S.; Alexander, T.; Stanford, K. Challenges of a one-health approach to the development of alternatives to antibiotics. Anim. Front. 2018, 8, 10–20.
8. Demin, K.A.; Refeld, A.G.; Bogdanova, A.A.; Prazdnova, E.V.; Popov, I.V.; Kutsevalova, O.Y.; Ermakov, A.M.; Bren, A.B.; Rudoy, D.V.; Chistyakov, V.A.; et al. Mechanisms of Candida Resistance to Antimycotics and Promising Ways to Overcome It: The Role of Probiotics. Probiotics Antimicrob. Proteins 2021.
9. Grant, A.; Gay, C.G.; Lillehoj, H.S. Bacillus spp. as direct-fed microbial antibiotic alternatives to enhance growth, immunity, and gut health in poultry. Avian Pathol. 2018, 47, 339–351.
10. Neijat, M.; Shirley, R.B.; Barton, J.; Thiery, P.; Welsher, A.; Kiarie, E. Effect of dietary supplementation of Bacillus subtilis DSM29784 on hen performance, egg quality indices, and apparent retention of dietary components in laying hens from 19 to 48 weeks of age. Poult. Sci. 2019, 98, 5622–5635.
11. Rougière, N.; Carré, B. Comparison of gastrointestinal transit times between chickens from D+ and D− genetic lines selected for divergent digestion efficiency. Animal 2010, 4, 1861–1872.
12. Scanes, C.G. Sturkie's Avian Physiology, 6th ed.; Elsevier: Amsterdam, The Netherlands, 2014; p. 1056.
13. Grist, A. Poultry Inspection: Anatomy, Physiology, and Disease Conditions, 2nd ed.; Nottingham University Press: Nottingham, UK, 2006; p. 262.
14. Feye, K.M.; Baxter, M.F.A.; Tellez-Isaias, G.; Kogut, M.H.; Ricke, S.C. Influential factors on the composition of the conventionally raised broiler gastrointestinal microbiomes. Poult. Sci. 2020, 99, 653–659.
15. Oakley, B.B.; Lillehoj, H.S.; Kogut, M.H.; Kim, W.K.; Maurer, J.J.; Pedroso, A.; Lee, M.D.; Collett, S.R.; Johnson, T.J.; Cox, N.A. The chicken gastrointestinal microbiome. FEMS Microbiol. Lett. 2014, 360, 100–112.
16. Stanley, D.; Hughes, R.J.; Moore, R.J. Microbiota of the chicken gastrointestinal tract: Influence on health, productivity and disease. Appl. Microbiol. Biotechnol. 2014, 98, 4301–4310.
17. Han, G.G.; Kim, E.B.; Lee, J.; Lee, J.Y.; Jin, G.; Park, J.; Huh, C.S.; Kwon, I.K.; Kil, D.Y.; Choi, Y.J.; et al. Relationship between the microbiota in different sections of the gastrointestinal tract, and the body weight of broiler chickens. Springerplus 2016, 5, 911.
18. Saxena, S.; Saxena, V.K.; Tomar, S.; Sapcota, D.; Gonmei, G. Characterisation of caecum and crop microbiota of Indian indigenous chicken targeting multiple hypervariable regions within 16S rRNA gene. Br. Poult. Sci. 2016, 57, 381–389.
19. Xiao, Y.; Xiang, Y.; Zhou, W.; Chen, J.; Li, K.; Yang, H. Microbial community mapping in intestinal tract of broiler chicken. Poult. Sci. 2017, 96, 1387–1393.
20. Mohd Shaufi, M.A.; Sieo, C.C.; Chong, C.W.; Gan, H.M.; Ho, Y.W. Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. Gut Pathog. 2015, 7, 4.
21. Lu, J.; Idris, U.; Harmon, B.; Hofacre, C.; Maurer, J.J.; Lee, M.D. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Appl. Environ. Microbiol. 2003, 69, 6816–6824.
22. Clavijo, V.; Flórez, M.J.V. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. Poult. Sci. 2018, 97, 1006–1021.
23. Apajalahti, J.; Kettunen, A. Microbes of the chicken gastrointestinal tract. In Avian Gut Function in Health and Disease; Graham, C.P., Ed.; CABI: Wallingford, UK, 2006; Volume 28, pp. 124–137.
24. Gong, J.; Forster, R.J.; Yu, H.; Chambers, J.R.; Wheatcroft, R.; Sabour, P.M.; Chen, S. Molecular analysis of bacterial populations in the ileum of broiler chickens and comparison with bacteria in the cecum. FEMS Microbiol. Ecol. 2002, 41, 171–179.
25. Józefiak, D.; Rutkowski, A.; Martin, S.A. Carbohydrate fermentation in the avian ceca: A review. Anim. Feed Sci. Technol. 2004, 113, 1–15.
26. Wei, S.; Morrison, M.; Yu, Z. Bacterial census of poultry intestinal microbiome. Poult. Sci. 2013, 92, 671–683.
27. Glendinning, L.; Stewart, R.D.; Pallen, M.J.; Watson, K.A.; Watson, M. Assembly of hundreds of novel bacterial genomes from the chicken caecum. Genome Biol. 2020, 21, 34.
28. Waite, D.W.; Taylor, M.W. Exploring the avian gut microbiota: Current trends and future directions. Front Microbiol. 2015, 6, 673.

29. Stanley, D.; Geier, M.S.; Hughes, R.J.; Denman, S.E.; Moore, R.J. Highly variable microbiota development in the chick en gastrointestinal tract. PLoS ONE 2013, 8, e84290.

30. Ballou, A.L.; Ali, R.A.; Mendoza, M.A.; Ellis, J.C.; Hassan, H.M.; Croom, W.J.; Koci, M.D. Development of the Chick Mic robiome: How Early Exposure Influences Future Microbial Diversity. Front Vet. Sci. 2016, 3, 2.

31. Awad, W.A.; Mann, E.; Dzieciol, M.; Hess, C.; Schmitz-Esser, S.; Wagner, M.; Hess, M. Age-Related Differences in the Luminal and Mucosa-Associated Gut Microbiome of Broiler Chickens and Shifts Associated with Campylobacter jejuni I nfection. Front Cell Infect. Microbiol. 2016, 6, 154.

32. Apajalahti, J.H.; Kettunen, A.; Bedford, M.R.; Holben, W.E. Percent G+C profiling accurately reveals diet-related differe nces in the gastrointestinal microbial community of broiler chickens. Appl. Environ. Microbiol. 2001, 67, 5656–5667.

33. Engberg, R.M.; Hedemann, M.S.; Jensen, B.B. The influence of grinding and pelleting of feed on the microbial composi tion and activity in the digestive tract of broiler chickens. Br. Poult. Sci. 2002, 43, 569–579.

34. Tellez, G.; Latorre, J.D.; Kuttappan, V.A.; Kogut, M.H.; Wolfenden, A.; Hernandez-Velasco, X.; Hargis, B.M.; Bottje, W. G.; Bielke, L.R.; Faulkner, O.B. Utilization of rye as energy source affects bacterial translocation, intestinal viscosity, mi crobiota composition, and bone mineralization in broiler chickens. Front. Genet. 2014, 5, 339.

35. Haberecht, S.; Bajagai, Y.S.; Moore, R.J.; Van, T.T.H.; Stanley, D. Poultry feeds carry diverse microbial communities tha t influence chicken intestinal microbiota colonisation and maturation. AMB Express 2020, 10, 143.

36. Xing, Z.; Li, H.; Li, M.; Gao, R.; Guo, C.; Mi, S. Disequilibrium in chicken gut microflora with avian colibacillosis is relate d to microenvironment damaged by antibiotics. Sci. Total Environ. 2021, 762, 143058.

37. Zhu, C.; Huang, K.; Bai, Y.; Feng, X.; Gong, L.; Wei, C.; Huang, H.; Zhang, H. Dietary supplementation with berberine i mproves growth performance and modulates the composition and function of cecal microbiota in yellow-feathered broil ers. Poult. Sci. 2021, 100, 1034–1048.

38. De Cesare, A.; Caselli, E.; Lucchi, A.; Sala, C.; Parisi, A.; Manfreda, G.; Mazzacane, S. Impact of a probiotic-based cle aning product on the microbiological profile of broiler litters and chicken caeca microbiota. Poult. Sci. 2019, 98, 3602–3 610.

39. De Toledo, T.D.S.; Roll, A.A.P.; Rutz, F.; Dallmann, H.M.; Dai Prá, M.A.; Leite, F.P.L.; Roll, V.F.B. An assessment of the i mpacts of litter treatments on the litter quality and broiler performance: A systematic review and meta-analysis. PLoS O NE 2020, 15, e0232853.

40. Pin Viso, N.; Redondo, E.; Díaz Carrasco, J.M.; Redondo, L.; Sabio, Y.; Garcia, J.; Fernández Miyakawa, M.; Farber, M.D. Geography as non-genetic modulation factor of chicken cecal microbiota. PLoS ONE 2021, 16, e0244724.

41. Khan, S.; Moore, R.J.; Stanley, D.; Chousalkar, K.K. The Gut Microbiota of Laying Hens and Its Manipulation with Prebi otics and Probiotics To Enhance Gut Health and Food Safety. Appl. Environ. Microbiol. 2020, 86, e00600-20.

42. Ricke, S.C.; Lee, S.I.; Kim, S.A.; Park, S.H.; Shi, Z. Prebiotics and the poultry gastrointestinal tract microbiome. Poult. Sci. 2020, 99, 670–677.

43. Azad, M.A.K.; Sarker, M.; Li, T.; Yin, J. Probiotic Species in the Modulation of Gut Microbiota: An Overview. Biomed. Re s. Int. 2018, 2018, 9478630.

44. Sögaard, H.; Suhr-Jessen, T. Microbials for feed: Beyond lactic acid bacteria. Feed Int. Ernational. 1990, 11, 32–38.

45. Jayaraman, S.; Das, P.P.; Saini, P.C.; Roy, B.; Chatterjee, P.N. Use of Bacillus Subtilis PB6 as a potential antibiotic gro wth promoter replacement in improving performance of broiler birds. Poult. Sci. 2017, 96, 2614–2622.

46. Li, Y.B.; Xu, Q.Q.; Yang, C.J.; Yang, X.; Lv, L.; Yin, C.H.; Liu, X.L.; Yan, H. Effects of probiotics on the growth performan ce and intestinal micro flora of broiler chickens. Pak. J. Pharm. Sci. 2014, 27, 713–717.

47. Timmerman, H.M.; Veldman, A.; van den Elsen, E.; Rombouts, F.M.; Beynen, A.C. Mortality and growth performance of broilers given drinking water supplemented with chicken-specific probiotics. Poult. Sci. 2006, 85, 1383–1388.

48. Liu, X.; Yan, H.; Lv, L.; Xu, Q.; Yin, C.; Zhang, K.; Wang, P.; Hu, J. Growth Performance and Meat Quality of Broiler Chi ckens Supplemented with Bacillus licheniformis in Drinking Water. Asian-Australas. J. Anim. Sci. 2012, 25, 682–689.

49. Bodinga, B.M.; Hayat, K.; Liu, X.; Zhou, J.; Yang, X.; Ismaila, A.; Soomro, R.N.; Ren, Z.; Zhang, W.; Yang, X. Effects of Bacillus Subtilis DSM 32315 on Immunity, Nutrient Transporters and Functional Diversity of Cecal Microbiome of Broiler Chickens in Necrotic Enteritis Challenge. J. World Poult. Res. 2020, 10, 527–544.

50. Cetin, N.; Güçlü, B.K.; Cetin, E. The effects of probiotic and mannanoligosaccharide on some haematological and imm unological parameters in turkeys. J. Vet. Med. A Physiol. Pathol. Clin. Med. 2005, 52, 263–267.

51. Rahman, M.; Mustari, A.; Salauddin, M.; Rahman, M. Effects of probiotics and enzymes on growth performance and ha ematobiochemical parameters in broilers. J. Bangladesh Agric. Univ. 2014, 11, 111–118.

52. Keerqin, C.; Rhayat, L.; Zhang, Z.H.; Gharib-Naseri, K.; Kheravii, S.K.; Devillard, E.; Crowley, T.M.; Wu, S.B. Probiotic Bacillus subtilis 29,784 improved weight gain and enhanced gut health status of broilers under necrotic enteritis conditi on. Poult. Sci. 2021, 100, 100981.

53. Zhang, S.; Zhong, G.; Shao, D.; Wang, Q.; Hu, Y.; Wu, T.; Ji, C.; Shi, S. Dietary supplementation with Bacillus subtilis pr omotes growth performance of broilers by altering the dominant microbial community. Poult. Sci. 2021, 100, 100935.

54. Zhang, G.; Wang, H.; Zhang, J.; Tang, X.; Raheem, A.; Wang, M.; Lin, W.; Liang, L.; Qi, Y.; Zhu, Y.; et al. Modulatory Eff ects of Bacillus subtilis on the Performance, Morphology, Cecal Microbiota and Gut Barrier Function of Laying Hens. An imals 2021, 11, 1523.

55. Neveling, D.P.; Dicks, L.M.T. Probiotics: An Antibiotic Replacement Strategy for Healthy Broilers and Productive Rearin g. Probiotics Antimicrob. Proteins 2021, 13, 1–11.

56. Duskaev, G.; Rakhmatullin, S.; Kvan, O. Effects of Bacillus cereus and coumarin on growth performance, blood bioche mical parameters, and meat quality in broilers. Vet. World 2020, 13, 2484–2492.

57. Lei, K.; Li, Y.L.; Yu, D.Y.; Rajput, I.R.; Li, W.F. Influence of dietary inclusion of Bacillus licheniformis on laying performan ce, egg quality, antioxidant enzyme activities, and intestinal barrier function of laying hens. Poult. Sci. 2013, 92, 2389–2 395.

58. Abdelqader, A.; Irshaid, R.; Al-Fataftah, A.R. Effects of dietary probiotic inclusion on performance, eggshell quality, ceca l microflora composition, and tibia traits of laying hens in the late phase of production. Trop. Anim. Health Prod. 2013, 4 5, 1017–1024.

59. Mazanko, M.S.; Gorlov, I.F.; Prazdnova, E.V.; Makarenko, M.S.; Usatov, A.V.; Bren, A.B.; Chistyakov, V.A.; Tutelyan, A. V.; Komarova, Z.B.; Mosolova, N.I.; et al. Bacillus Probiotic Supplementations Improve Laying Performance, Egg Qualit y, Hatching of Laying Hens, and Sperm Quality of Roosters. Probiotics Antimicrob. Proteins 2018, 10, 367–373.

60. Chaucheyras-Durand, F.; Durand, H. Probiotics in animal nutrition and health. Benef. Microbes 2010, 1, 3–9.

61. Bai, W.K.; Zhang, F.J.; He, T.J.; Su, P.W.; Ying, X.Z.; Zhang, L.L.; Wang, T. Dietary Probiotic Bacillus subtilis Strain fmbj Increases Antioxidant Capacity and Oxidative Stability of Chicken Breast Meat during Storage. PLoS ONE 2016, 11, e0 167339.

62. Percie Du Sert, N.; Hurst, V.; Ahluwalia, A.; Alam, S.; Avey, M.T.; Baker, M.; Browne, W.J.; Clark, A.; Cuthill, I.C.; Dirnag l, U.; et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol. 2020, 18, e30004 10.

63. Ermakova, L.P.; Nozdrin, G.A.; Tishkov, S.N.; Novik, Y.V.; Gotovchikov, N.A.; Mensh, I.K. Effects of a probiotic containin g Bacillus subtilis on the gut microflora, yolk quality and blood lipid concentrations of laying Pharaon quails. Vet. Stanic a 2021, 52, 297–306.

64. Deng, W.; Dong, X.F.; Tong, J.M.; Zhang, Q. The probiotic Bacillus licheniformis ameliorates heat stress-induced impair ment of egg production, gut morphology, and intestinal mucosal immunity in laying hens. Poult. Sci. 2012, 91, 575–582.

65. Global Probiotics Market Size 2019, by Application (Functional Food & Beverages [Dairy Products, Non-dairy Beverage s, Infant Formula, Cereals], Dietary Supplements, Feed), Ingredient (Bacteria, Yeast), End-User (Human, Animal), Regi on and Forecast to 2025. Available online: (accessed on 9 May 2021).

66. Monteiro, S.M.; Clemente, J.J.; Henriques, A.O.; Gomes, R.J.; Carrondo, M.J.; Cunha, A.E. A procedure for high-yield s pore production by Bacillus subtilis. Biotechnol. Prog. 2005, 21, 1026–1031.

67. Cutting, S.M. Bacillus probiotics. Food Microbiol. 2011, 28, 214–220.

68. Posada-Uribe, L.F.; Romero-Tabarez, M.; Villegas-Escobar, V. Effect of medium components and culture conditions in Bacillus subtilis EA-CB0575 spore production. Bioprocess. Biosyst. Eng. 2015, 38, 1879–1888.

69. Pandey, K.R.; Vakil, B.V. Development of bioprocess for high density cultivation yield of the probiotic Bacillus coagulans and its spores. J. BioSci. Biotechnol. 2016, 5, 173–181.

70. Ren, H.; Su, Y.T.; Guo, X.H. Rapid optimization of spore production from Bacillus amyloliquefaciens in submerged cultu res based on dipicolinic acid fluorimetry assay. AMB Express 2018, 8, 21.

71. Ballardo, C.; Barrena, R.; Artola, A.; Sánchez, A. A novel strategy for producing compost with enhanced biopesticide pr operties through solid-state fermentation of biowaste and inoculation with Bacillus thuringiensis. Waste Manag. 2017, 7 0, 53–58.

72. Martelli, F.; Favari, C.; Mena, P.; Guazzetti, S.; Ricci, A.; Del Rio, D.; Lazzi, C.; Neviani, E.; Bernini, V. Antimicrobial and Fermentation Potential of Himanthalia elongata in Food Applications. Microorganisms 2020, 8, 248.

73. Aslam, F.; Ansari, A.; Aman, A.; Baloch, G.; Nisar, G.; Baloch, A.H.; Rehman, H.U. Production of commercially importan t enzymes from Bacillus licheniformis KIBGE-IB3 using date fruit wastes as substrate. J. Genet. Eng. Biotechnol. 2020,

18, 46.

74. Shi, H.; Su, B.; Chen, X.; Pian, R. Solid state fermentation of Moringa oleifera leaf meal by mixed strains for the protein enrichment and the improvement of nutritional value. PeerJ 2020, 8, e10358.
75. Monteiro, S.M.S.; Clemente, J.J.; Carrondo, M.J.T.; Cunha, A.E. Enhanced spore production of Bacillus subtilis grown i n a chemically defined medium. Adv. Microbiol. 2014, 4, 444–454.
76. Khardziani, T.; Kachlishvili, E.; Sokhadze, K.; Elisashvili, V.; Weeks, R.; Chikindas, M.L.; Chistyakov, V. Elucidation of B acillus subtilis KATMIRA 1933 Potential for Spore Production in Submerged Fermentation of Plant Raw Materials. Probi otics Antimicrob. Proteins 2017, 9, 435–443.
77. Zhu, Z.; Zhang, F.; Wei, Z.; Ran, W.; Shen, Q. The usage of rice straw as a major substrate for the production of surfact in by Bacillus amyloliquefaciens XZ-173 in solid-state fermentation. J. Environ. Manag. 2013, 127, 96–102.
78. Gowdhaman, D.; Manaswini, V.S.; Jayanthi, V.; Dhanasri, M.; Jeyalakshmi, G.; Gunasekar, V.; Sugumaran, K.R.; Ponn usami, V. Xylanase production from Bacillus aerophilus KGJ2 and its application in xylooligosaccharides preparation. In t. J. Biol. Macromol. 2014, 64, 90–98.
79. Viayaraghavan, P.; Jeba Kumar, S.; Valan Arasu, M.; Al-Dhabi, N.A. Simultaneous production of commercial enzymes u sing agro industrial residues by statistical approach. J. Sci. Food Agric. 2019, 99, 2685–2696.
80. Chen, L.; Zhao, Z.; Yu, W.; Zheng, L.; Li, L.; Gu, W.; Xu, H.; Wei, B.; Yan, X. Nutritional quality improvement of soybean meal by Bacillus velezensis and Lactobacillus plantarum during two-stage solid- state fermentation. AMB Express 202 1, 11, 23.
81. Cheng, Y.H.; Horng, Y.B.; Dybus, A.; Yu, Y.H. Bacillus licheniformis-Fermented Products Improve Growth Performance and Intestinal Gut Morphology in Broilers under Clostridium perfringens Challenge. J. Poult. Sci. 2021, 58, 30–39.
82. Feng, Y.; Wang, L.; Khan, A.; Zhao, R.; Wei, S.; Jing, X. Fermented wheat bran by xylanase-producing Bacillus cereus boosts the intestinal microflora of broiler chickens. Poult. Sci. 2020, 99, 263–271.
83. Kim, S.Y.; Lee, S.Y.; Weon, H.-Y.; Sang, M.K.; Song, J. Complete genome sequence of Bacillus velezensis M75, a bioc ontrol agent against fungal plant pathogens, isolated from cotton waste. J. Biotechnol. 2017, 241, 112–115.
84. Ongena, M.; Jacques, P. Bacillus lipopeptides: Versatile weapons for plant disease biocontrol. Trends Microbiol. 2008, 16, 115–125.
85. Rao, Y.K.; Tsay, K.J.; Wu, W.S.; Tzeng, Y.M. Medium optimization of carbon and nitrogen sources for the production of spores from Bacillus amyloliquefaciens B128 using response surface methodology. Process. Biochem. 2007, 42, 535–541.
86. Chen, Z.M.; Li, Q.; Liu, H.M.; Yu, N.; Xie, T.J.; Yang, M.Y.; Shen, P.; Chen, X.D. Greater enhancement of Bacillus subtili s spore yields in submerged cultures by optimization of medium composition through statistical experimental designs. A ppl. Microbiol. Biotechnol. 2010, 85, 1353–1360.
87. Gangadharan, D.; Sivaramakrishnan, S.; Nampoothiri, K.M.; Pandey, A. Solid culturing of Bacillus amyloliquefaciens for alpha amylase production. Food Technol. Biotechnol. 2006, 44, 269–274.
88. Hölker, U.; Höfer, M.; Lenz, J. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Appl. Microbiol. Biotechnol. 2004, 64, 175–186.
89. Schultze, M.O. Nutritional value of plant materials; growth of rats on purified rations containing soybean protein. J. Nutr. 1950, 41, 103–113.
90. Zhao, S.; Hu, N.; Huang, J.; Liang, Y.; Zhao, B. High-yield spore production from Bacillus licheniformis by solid state fer mentation. Biotechnol. Lett. 2008, 30, 295–297.
91. Shim, Y.H.; Shinde, P.L.; Choi, J.Y.; Kim, J.S.; Seo, D.K.; Pak, J.I.; Chae, B.J.; Kwon, I.K. Evaluation of multi-microbial probiotics produced by submerged liquid and solid substrate fermentation methods in broilers. Asian-Australas. J. Ani m. Sci. 2010, 23, 521–529.
92. Su, Y.T.; Liu, C.; Long, Z.; Ren, H.; Guo, X.H. Improved Production of Spores and Bioactive Metabolites from Bacillus a myloliquefaciens in Solid-state Fermentation by a Rapid Optimization Process. Probiotics Antimicrob. Proteins 2019, 1 1, 921–930.
93. Mahoney, R.; Weeks, R.; Zheng, T.; Huang, Q.; Dai, W.; Cao, Y.; Liu, G.; Guo, Y.; Chistyakov, V.; Chikindas, M.L. Evalu ation of an Industrial Soybean Byproduct for the Potential Development of a Probiotic Animal Feed Additive with Bacillu s Species. Probiotics Antimicrob. Proteins 2020, 12, 1173–1178.
94. Kragh, K.N.; Hutchison, J.B.; Melaugh, G.; Rodesney, C.; Roberts, A.E.; Irie, Y.; Jensen, P.Ø.; Diggle, S.P.; Allen, R.J.; Gordon, V.; et al. Role of Multicellular Aggregates in Biofilm Formation. mBio 2016, 7, e00237.

95. Rossi, E.; Cimdins, A.; Lüthje, P.; Brauner, A.; Sjöling, Å.; Landini, P.; Römling, U. “It’s a gut feeling”—Escherichia coli biofilm formation in the gastrointestinal tract environment. Crit. Rev. Microbiol. 2018, 44, 1–30.
96. Ushakova, N.A.; Abramov, V.M.; Khlebnikov, V.S.; Semenov, A.M.; Kuznetsov, B.B.; Kozlova, A.A.; Nifatov, A.V.; Sakulin, V.K.; Kosarev, I.V.; Vasilenko, R.N.; et al. Properties of the Probiotic Strain Lactobacillus plantarum 8-RA-3 Grown in a Biofilm by Solid Substrate Cultivation Method. Probiotics Antimicrob. Proteins 2012, 4, 180–186.
97. Hesseltine, C.W. Biotechnology report. Solid state fermentations. Biotechnol. Bioeng. 1972, 14, 517–532.
98. Anderson, R.L.; Wolf, W.J. Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. J. Nutr. 1995, 125, 581S–588S.
99. Li, W.; Wang, T. Effect of solid-state fermentation with Bacillus subtilis lwo on the proteolysis and the antioxidative properties of chickpeas. Int. J. Food Microbiol. 2021, 338, 108988.
100. McGovern, E.P.; Bentley, R. Biosynthesis of flaviolin and 5,8-dihydroxy-2,7-dimethoxy-1,4-naphthoquinone. Biochemistry 1975, 14, 3138–3143.
101. Wang, H.H.; Chiou, J.Y.; Wang, J.Y.; Hong, C.Y.; Tsen, W.C. Cephalosporin C production by solid state fermentation with rice grains. Zhonghua Min. Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi 1984, 17, 55–69.
102. Yang, S.S.; Ling, M.Y. Tetracycline production with sweet potato residue by solid state fermentation. Biotechnol. Bioeng. 1989, 33, 1021–1028.
103. Barrios-González, J.; Mejía, A. Production of secondary metabolites by solid-state fermentation. Biotechnol. Annu. Rev. 1996, 2, 85–121.
104. Kumar, V.; Ahluwalia, V.; Saran, S.; Kumar, J.; Patel, A.K.; Singhania, R.R. Recent developments on solid-state fermentation for production of microbial secondary metabolites: Challenges and solutions. Bioresour. Technol. 2021, 323, 124566.
105. Jang, C.H.; Oh, J.; Lim, J.S.; Kim, H.J.; Kim, J.S. Fermented Soy Products: Beneficial Potential in Neurodegenerative Diseases. Foods 2021, 10, 636.
106. Mukherjee, R.; Chakraborty, R.; Dutta, A. Role of Fermentation in Improving Nutritional Quality of Soybean Meal—A Review. Asian-Australas. J. Anim. Sci. 2016, 29, 1523–1529.
107. Hayashi, U.; Nagao, K.; Tosa, Y.; Yoshioka, Y. Relationship between food containing “Natto” (fermented soybeans) and the blood pressure of SHR. JPN Heart J. 1976, 17, 343–344.
108. Chou, H.Y.; Liu, L.H.; Chen, C.Y.; Lin, I.F.; Ali, D.; Yueh-Luen Lee, A.; David Wang, H.M. Bifunctional mechanisms of autophagy and apoptosis regulations in melanoma from Bacillus subtilis natto fermentation extract. Food Chem. Toxicol. 2021, 150, 112020.
109. Iwasaki, M.; Sato, M.; Yoshihara, A.; Saito, T.; Kitamura, K.; Ansai, T.; Nakamura, K. A 5-year longitudinal association between dietary fermented soya bean (natto) intake and tooth loss through bone mineral density in postmenopausal women: The Yokogoshi cohort study. Gerodontology 2021. Epub ahead of print.
110. Jiang, X.; Ding, H.; Liu, Q.; Wei, Y.; Zhang, Y.; Wang, Y.; Lu, Y.; Ma, A.; Li, Z.; Hu, Y. Effects of peanut meal extracts fermented by Bacillus natto on the growth performance, learning and memory skills and gut microbiota modulation in mice. Br. J. Nutr. 2020, 123, 383–393.
111. Sen, R.; Babu, K.S. Modelling and optimization of the process conditions for biomass production and sporulation of a probiotic culture. Process. Biochem. 2005, 40, 2531–2538.
112. Riesenberg, D.; Guthke, R. High-cell-density cultivation of microorganisms. Appl. Microbiol. Biotechnol. 1999, 51, 422–430.