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Chapter

The Use of Probiotics in Poultry Production for the Control of Bacterial Infections and Aflatoxins

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Abstract

In intensive poultry production, a large number of antimicrobials are frequently employed to prevent (prophylactic use) and treat (therapeutic use) diseases, as well as for growth promotion (subtherapeutic use), in order to increase productivity. However, it has been reported that the use of antimicrobials at subtherapeutic doses is closely related to the increase in bacterial resistance and with the treatment failure. In addition to antimicrobial resistance, another problem derived from the use of antimicrobials is the presence of residues in animal products. Therefore, these problems and the ban of antimicrobial as growth promoters have prompted the poultry industry to look for alternatives with similar benefits to antibiotics. Among these alternatives, probiotics are one of the most widely studied and interesting groups. Hence, in the present chapter, the effect of probiotics and direct-fed microbial against foodborne pathogens and mycotoxins will be summarized.

Keywords: probiotics, direct-fed microbial, foodborne pathogens, antimicrobial resistance, aflatoxins

1. Introduction

Since the discovery and application of penicillin in 1940, antibiotics have played an unprecedented role in the prevention, control, and treatment of infectious diseases in both humans and animals [1]. However, in animal production, they have also been used at subtherapeutic doses [2]. It is estimated that the global consumption of antibiotics in animal production could increase by 67% in the coming years [3] mainly because of the growing global demand for animal protein [2, 4]. Although it has been reported that in developed countries the total consumption of antibiotics has decreased by around 4%, consumption of antibiotics in the USA increased slightly [5]. Furthermore, it has been reported that the amount of antibiotics used in animal production in the USA is 100–1000 times higher than human medicine, being used ~80–90% at subtherapeutic doses, and for prophylactic purposes, while the remaining 10–20% at therapeutic doses [6, 7].

The inclusion of antibiotics at subtherapeutic doses into the feed was generalized in the early 1950s, both in the EU and the USA since they could be used to prevent diseases and positively influence the promotion of growth and feed efficiency of animals [3, 8, 9].

Nevertheless, in the last decades, these practices have changed considerably due to the concern of the increase of bacteria resistant to antibiotics, since they can be transmitted zoonotically from animals to humans, causing serious problems in public health and even death because of the failure of the antibiotic at therapeutic doses [10]. Furthermore, another problem for human health is the presence of antibiotic residues in animal-derived food, by the use of antibiotics for long periods of time, since it is associated in some cases with allergic reactions, imbalance of the intestinal microbiota, and especially, the development of antibacterial resistance [11].

Consequently, one of the measures taken in the face of the problems of bacterial resistance was the restriction of antibiotics at subtherapeutic doses in the EU in 2006 [12] and the USA in 2017 [13], and although in countries as Mexico they have not been officially banned, the Ministry of Agriculture and Rural Development (SADER), through its decentralized administrative body, the National Health Service, Food Safety and Food Quality (SENASICA), has promoted initiatives to prevent their use since 2012 [14–17]. However, as a consequence of this measure, the incidence of enteric diseases in animals has increased significantly [18], as well as the use of antibiotics, but at therapeutic doses for the purpose of controlling and preventing diseases, which could lead to a worse scenario of bacterial resistance [2, 19–21]. In this context, the European One Health Action Plan against antimicrobial resistance calls for the phasing out of routine prophylactic (Prevent) and metaphylactic (Control) antimicrobial use in animal production and investment in the research of new alternatives [22], since they could be regulated in the coming years.

Therefore, the poultry industry has been under pressure to seek and investigate new alternatives to reduce the problems of bacterial resistance, prevent and control diseases, reduce the mortality rate, and finally promote the growth of animals. Among these alternatives, the most popular are probiotics (yeasts or bacteria) since it has been reported that they can improve the performance [23, 24], as well as prevent and control enteric pathogens in poultry [25–27]. Furthermore, it has been reported that probiotics could be an interesting alternative to prevent and control the toxic effects of aflatoxins. For these reasons, the probiotic market has expanded rapidly and is expected to grow to around 7% in 2020. However, this market is led mainly by Asia and Europe given the growing demand for dietary supplements [18].

2. Probiotics

Probiotics are defined as "live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [28]. The most common microorganisms used as probiotics in livestock production are lactic acid bacteria (LAB) from the genus *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, and *Leuconostoc*. Nevertheless, only the genera *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, and *Weissella* are the most frequently used in poultry production [29]. Although the efficacy of probiotics reducing enteric pathogens is evident, one of the disadvantages is that they require refrigeration or lyophilization to survive for long storage periods or can be encapsulated to increase their stability/viability when included in the feed, which would increase the cost of production at the industrial level, making it unprofitable [30]. Unlike LAB, direct-fed microbials (DFM) as *Bacillus* spores, other types of probiotics, have several potential applications since they can be included as feed additives in poultry diets, due to their remarkable heat stability and long shelf life [31, 32]. Bacteria of the genus *Bacillus* are Gram-positive, frequently found in the soil. However, several studies have shown that *Bacillus* spores can also be present, germinate, and survive

in the gastrointestinal tract (GIT) of different animal species [25]. The survival rate and persistence of some *Bacillus* strains in the GIT could be related to their capacity to synthesize biofilms, thus protecting themselves against the different conditions present in the gut [33]. Furthermore, another advantage of *Bacillus* strains is that they are frequently used by biotechnology companies for the production of enzymes and antibiotics. Therefore, these multifunctional microorganisms have different applications, since they are useful inside or outside a host [34, 35].

2.1 Mechanisms of action probiotics

2.1.1 Pathogenic bacteria

Although a large number of studies have shown the possible mechanisms by which probiotics have a beneficial action in inhibiting of pathogens, more studies are needed to elucidate them.

The possible modes of action of probiotics for the inhibition of pathogens include two basic mechanisms [29, 36, 37]: competitive exclusion and modulation of the host immune system (**Figure 1**). Competitive exclusion involves mechanisms such as (1) production of inhibitory compounds, that is, hydrogen peroxide, bacteriocins, and defensins [38, 39], (2) prevention of the pathogen adhesion [38], (3) competition for nutrients [40], and (4) reduction of toxin bioavailability [36]. Meanwhile, in the modulation of the host immune system, both innate and adaptive immune responses are involved [29]. The adaptive immune response depends on B and T lymphocytes to induce an antigen-specific response and produce antibodies [29, 41]. In contrast, physical and chemical barriers (innate immunity), such as intestinal epithelial cells (IEC), are the first line of defense to prevent the spread of pathogens and subsequent infections. Furthermore, IEC are the target cells for probiotics, which can improve the function of the intestinal barrier by stimulating the production of mucus and antimicrobial peptides such as defensins [42, 43].

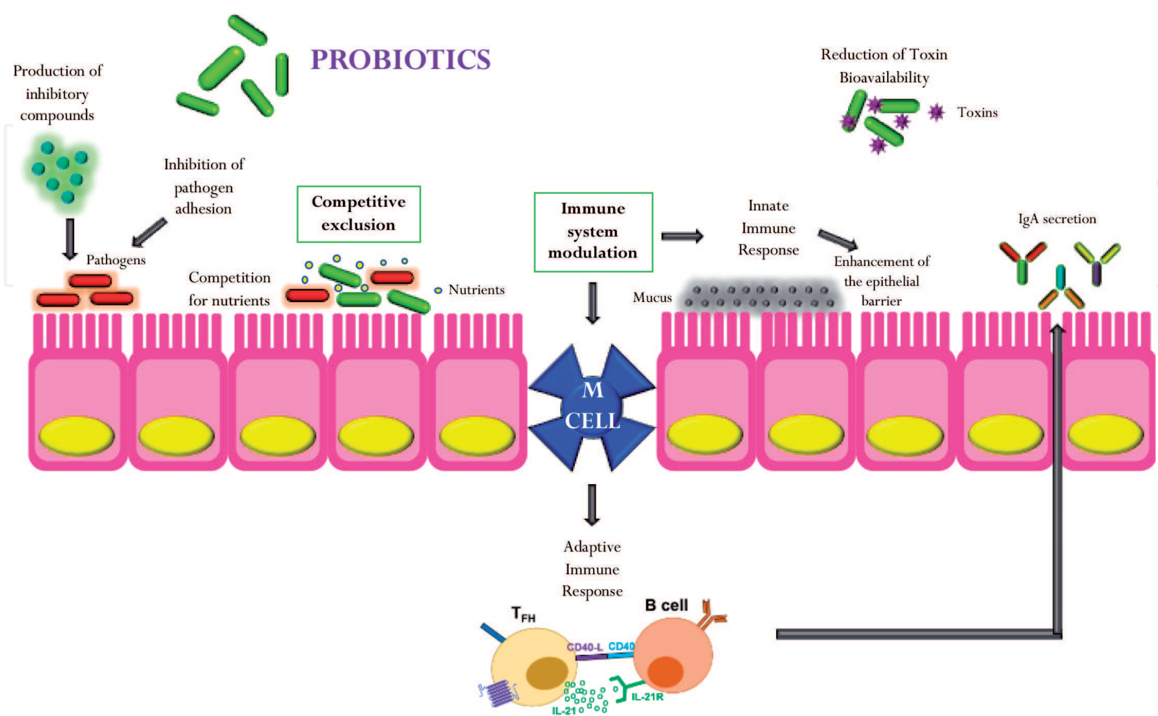

Figure 1.
Mechanism of action of probiotics.

2.1.2 Aflatoxins

Similar as for pathogenic bacteria, probiotics can (1) compete for space and nutrients with aflatoxigenic mold strains, (2) degrade aflatoxins by the production of enzymes, or (3) avoid the intestinal absorption of AFB1 by its binding to the cell walls of probiotic strains [44].

3. Probiotic application in poultry industry

Although probiotics are considered potential alternatives to antibiotic use in poultry because they leave no residues in the meats and eggs given their modes of action, the variety of microorganisms in terms of species and even between strains of the same species, as well as their variation in metabolic activity, could affect their effectiveness. Furthermore, other factors that influence the effectiveness of probiotics in poultry are the species of origin, the probiotic preparation method, the survival of colonizing microorganisms in the gastrointestinal tract conditions, the environment where the birds are raised, the application time and administration route of probiotics, the immunologic state, the lineage of poultry, as well as age and concomitant use of antibiotics [45, 46]. Below are some of the applications of probiotics in poultry.

3.1 Effects of lactic acid bacteria against *pathogens* of importance in poultry

Several articles published by our laboratory have shown that the use of probiotics as a replacement of antibiotics in poultry production has had positive effects by reducing the growth of pathogens in *in vitro* models that simulate or not the three main compartments in birds (crop, proventriculus, and intestine) [47, 48], as well as the colonization of pathogens through the gastrointestinal tract in both turkeys and broiler chickens [26, 27, 49–51]. Although the results obtained have been promising, it is a fact that the isolated probiotics were characterized biochemically and by 16S rRNA sequence analyses (Microbial ID Inc., Newark, DE 19713, USA), subsequently, they were evaluated using *in vitro* models to determine their activity against pathogens, and, finally, the candidates were tested in *in vivo* models with the purpose of obtaining a well-characterized functional product.

Extensive research conducted by our laboratory determined the antimicrobial capability of several lactic acid bacteria (LAB) isolates mainly against *Salmonella* in *in vitro* models. However, only 11 were selected to produce a product called FloraMax®-B11 given their effect against *Salmonella*. Subsequently, these LAB were characterized by 16S rRNA sequence analyses (**Table 1**) [52].

However, since these LAB were grown together in a culture, the only LAB that remained viable were *Lactobacillus salivarius* and *Pediococcus parvulus*, two strains of poultry gastrointestinal origin. Despite this, *in vitro* studies showed that FloraMax®-B11 presented antimicrobial activity against *Salmonella* enteritidis, *Escherichia coli* (O157:H7), and *Campylobacter jejuni* [47] (**Table 2**). The antimicrobial activity of this probiotic culture could be due to the accumulation of primary metabolites such as lactic acid, ethanol, and carbon dioxide and to the production of other antimicrobial compounds such as bacteriocins [53]. Furthermore, the probiotic culture was capable of maintaining its viability under acidic conditions (pH = 3) for 4 h, which agrees with other studies where *Lactobacillus* spp. isolates were resistant to low pH, with high survival rates at

LAB identification	16S rRNA sequence analyses (Microbial ID Inc.)
18	*Pediococcus parvulus*
24	*Weissella confusa*
27	*Weissella confusa*
29	*Pediococcus parvulus*
36	*Lactobacillus salivarius*
37B	*Weissella confusa*
40	*Weissella confusa*
44	*Weissella paramesenteroides*
46	*Lactobacillus salivarius*
48	*Lactobacillus salivarius*
42	*Pediococcus parvulus*

Table 1.
Identifications of FloraMax®-B11 (FM-B11) lactic acid bacteria (LAB).

	Salmonella enteritidis	*Escherichia coli* (O157:H7)	*Campylobacter jejuni*
Lactobacillus salivarius	+	+	+
Pediococcus parvulus	+	+	+
Symbols: +, inhibition.			

Table 2.
In vitro assessment of antimicrobial activity of Lactobacillus salivarius and Pediococcus parvulus present in FloraMax®-B11 against enteropathogenic bacteria.

pH 3.0 for 1 h [54]. Although probiotic bacteria need to survive passage through the stomach (pH 1.5–2.0) [55], and maintain their viability for 4 h or more [56] before reaching the intestine, the feed passage rate for birds is faster; therefore, bacterial acid tolerance is not as critical in chickens as it is in other animals [57]. Additionally, this probiotic culture grew at low and high temperatures for 4 h of incubation. However, the ability to grow at high temperatures is an important advantage since the production of lactic acid increases, and, therefore, the bacterial load decreases [58]. The probiotic culture was also able to tolerate high osmotic concentrations of NaCl, but it is extremely important since it has been reported that a high salt concentration could affect the physiology of probiotics, as well as their enzymatic activity, water activity, and metabolism [58]. Finally, this probiotic culture has its ability to tolerate bile salt concentrations of 0.4, 0.5, and 0.6% for 2, 4, and 24 h of incubation. Bile resistance of probiotics is related to their enzyme activity of bile salt hydrolase that helps to hydrolyze conjugated bile, reducing its toxic effect [59, 60].

Furthermore, the effect of this commercial product (FloraMax®-B11) has been evaluated in different models of infection both in broiler chickens and turkeys. In neonatal broilers, the administration of 1×10^6 cfu/bird FloraMax®-B11 by oral gavage 1 h after the chicks were challenged with *Salmonella* enteritidis (SE) and *Salmonella* typhimurium (ST) (1×10^4 cfu/bird) reduced the incidence of SE and ST, as well as the SE counts by >2.9 log, 24 h post-LAB administration [61] (**Table 3**). In contrast, there were no significant differences at 6- and 12-h post-LAB administration, but a slight reduction was observed at 12-h post-LAB

Rep.	Treatment	ST cecal tonsil +/– (%)	SE cecal tonsil +/– (%)	Log SE cecal recovery (all samples)	Log SE cecal recovery (only positive samples)
1	Control	20/25 (80)	22/25 (88)	3.81 ± 0.32	4.33 ± 0.17
	LAB	2/25 (8)*	8/25 (32)*	0.62 ± 0.19*	1.95 ± 0.09*
2	Control	18/25 (72)	25/25 (100)	3.59 ± 0.23	3.59 ± 0.23
	LAB	2/25 (8)*	7/25 (28)*	0.42 ± 0.18*	1.91 ± 0.29*
3	Control	20/25 (80)	25/25 (100)	3.91 ± 0.19	3.91 ± 0.19
	LAB	1/25 (4)*	11/25 (40)*	1.00 ± 0.25*	2.22 ± 0.24*

**A significant ($p \leq 0.05$) difference was observed between control and treated within a single experiment in each column.*

Table 3.
Effect of lactic acid bacteria (LAB) on Salmonella typhimurium (ST) or Salmonella enteritidis (SE) recovered from cecal tonsils or ceca of broiler chicks 24-h post-LAB administration.

administration. These data suggest that the mechanism to reduce *Salmonella* was initiated within the first 12 h after treatment. Probably the reduction of *Salmonella* is due to the set of mechanisms of action of probiotics: bacterial interactions (competitive exclusion) or stimulation of a host innate immune response. The competitive exclusion could have included competition for receptor sites, production of volatile fatty acids that are inhibitors of certain enteric pathogens, production of bacteriocins, or competition with pathogens and native flora for limiting nutrients [62]. Furthermore, since the *Salmonella* recovery was performed in the early stages of infection, the innate immune response could be responsible for the reduction of *Salmonella*.

In our other studies, the administration of FloraMax®-B11 in drinking water (10^6 cfu/mL) for 3 days post-SE challenge (10^4 cfu/bird) using two presentations, liquid and lyophilized significantly reduced the incidence of *Salmonella* [63], which agrees with other studies [64]. Furthermore, the administration of FloraMax®-B11 at the same concentration as the previous study after 1-h post-*Salmonella* Heidelberg (SH) challenge practically eliminated the concentration of SH, as well as its incidence, since only one sample was positive. However, in turkey poults under the same experimental conditions (**Table 4**), although similar significant results were observed at day 3 post-FloraMax®-B11 administration, it is clear that poults were more susceptible to SH colonization than chicks [51].

Finally, trying to find FloraMax®-B11 applications in poultry, we opted for spray application since it could be more efficient and has lower cost than its application in

Treatment	24 h		72 h	
	Cecal tonsils[1]	SH[2] ($\log_{10}$ cfu/g of ceca content)	Cecal tonsils[1]	SH[2] ($\log_{10}$ cfu/g of ceca content)
Control SH	20/20 (100)	7.04 ± 0.19^a	20/20 (100)	6.05 ± 0.28^a
FloraMax®-B11	13/20 (65)*	4.36 ± 0.74^b	9/20 (45)*	2.15 ± 0.75^b

^{a,b}Different superscripts within columns indicate significant differences ($p < 0.05$).
[1]Data expressed as positive/total poults (%).
[2]Data expressed as mean ± SE.
**$p < 0.001$.*

Table 4.
In vivo evaluation of FloraMax-B11 against Salmonella Heidelberg (SH) at 24 and 72 h in poults.

drinking water since it is important to take into account water quality and medicator/proportioner function [65]. The results obtained were promising since when the probiotic was applied by spray and in drinking water, there was a reduction in the recovery of SE (55 and 50%, respectively; controls 85%) when chicks were held for 8 h prior to SE challenge and placement. In the same way, when probiotic was applied by spray or in drinking water and SE challenge occurred simultaneously, with placement 8 h after treatment, a marked and significant reduction of SE recovery was noted after 5d (10 and 40%, respectively; controls 55%). Furthermore, when the probiotic was sprayed and chickens were SE challenged simultaneously, with placement 8 h after treatment, a significant reduction of SE recovery was again noted in both the spray and DW application (80% controls, 15% spray, 15% drinking water) (**Table 5**). These results suggest that the spray application of this probiotic can be effective in protecting chicks against *Salmonella* infection. Furthermore, hatchery administration could prove to be a more effective way to administer probiotics because the chicks will be receiving the beneficial bacteria at the earliest possible time, in the absence of *in ovo* administration.

In this regard, an *in ovo* study was performed to know the effectiveness of FloraMax®-B11 [66]. For this, 18-day-old embryos were candled and inoculated with either saline or 10^4 cfu FloraMax®-B11 via *in ovo* injection into the amnion. On day 21, chicks were pulled from hatchers to measure hatchability. Subsequently, all chickens were then orally gavaged with SE on the day of hatch (~10^4 cfu/chick) and maintained for 7 days. *Salmonella* recovery was done 24-h post-SE challenge. Body weight (BW) was determined at days 1, 3, and 7. In this experiment, a significant increase in BW was observed. Furthermore, chickens that received the probiotic culture showed a significant reduction in the incidence and counts of SE in cecal tonsils when compared with saline control chickens (**Table 6**).

These results agree with another study where the *in ovo* colonization with a probiotic could become an important method to reduce *Salmonella* and other intestinal bacterial infections in poultry [67]. Regarding the increase of BW in the group treated with the probiotic, this could be due to the significant morphometric changes in the duodenum and ileum observed at day 1 of age.

Treatment regimen	Group	Cecal tonsils	
		Exp. 1	Exp. 2
Treat-challenge-place immediately	Control	95% (19/20)	95% (19/20)
	Probiotic (drinking water)	75% (15/20)	25% (5/25)**
	Probiotic spray	90% (18/20)	80% (16/20)
Treat-hold 8 h-challenge-place	Control	85% (17/20)	70% (14/20)
	Probiotic (drinking water)	50% (10/20)*	70% (14/20)
	Probiotic spray	55% (11/20)*	80% (16/20)
Treat-challenge-hold 8 h-place	Control	55% (11/20)	80% (16/20)
	Probiotic (drinking water)	44% (7/20)*	15% (2/20)*
	Probiotic spray	20% (2/20)**	15% (2/20)*

Indicates significant ($p < 0.05$) differences were observed between control and treated within a single experiment and treatment regime in each column.
**Significantly ($p < 0.01$) different than all groups within a single experiment and treatment regime in each column.*

Table 5.
Salmonella enteritidis recovery from cecal tonsils of broiler chicks 5-day post-challenge.

Treatment	Day 1 BW (g)	Day 3 BW (g)	Day 7 BW (g)	SE incidence cecal tonsils 24 h PI	Log SE/g of ceca content 24 h PI
Saline	49.13 ± 0.30^{a}	62.53 ± 0.81^{b}	132.89 ± 3.06^{b}	20/20 (100%)	7.13 ± 1.01^{a}
FloraMax®-B11	49.72 ± 0.36^{a}	65.42 ± 0.77^{a}	144.98 ± 3.02^{a}	9/20 (45%)*	5.45 ± 1.25^{b}

^{a,b}*Superscripts within columns indicate significant differences $p < 0.05$, n = 12/group.*
**Indicates significant differences $p < 0.001$, n = 20/group.*

Table 6.
Evaluation of in ovo administration of FloraMax®-B11 on body weight and Salmonella enteritidis (SE) recovery in broiler chickens.

3.2 The use of direct-fed microbials (DFM) for the control of pathogens in poultry

Although the use of LAB has been promising for the control of pathogens such as *Salmonella* spp., as described above, it is important to mention that one limitation is their sensitivity to pelletizing processes for feed production (heating) [30, 68, 69], environmental factors [70], and the low pH of the stomach and the presence of bile salts in the small intestine [71, 72]. For this reason, some strategies to increase the viability of these bacteria include their microencapsulation in polymer matrices [73, 74], as well as their freezing or lyophilization [75, 76]. However, production costs increase, so it becomes nonviable in animal production. Although LAB are better probiotics than *Bacillus*, the latter is more stable due to their ability to form spores, which are more resistant to severe environmental conditions, feed pelleting process with extreme temperatures, as well as tolerance to extremes of pH, dehydration, high pressures, and chemicals, and therefore, stability to long period storage conditions, making them suitable for commercialization [77, 78] since they could be used as direct-fed microbials (DFM) [68].

Previously in our laboratory, we have screened and identified *Bacillus* spp. isolates as DFM. Some of these demonstrated to be effective as potential DFM candidates by reducing *Salmonella* colonization and having a positive effect on the increase in body weight gained in both chickens and turkeys, as well as tolerance to acidic condition (pH = 2), high osmotic pressure (NaCl at 6.5%), and 0.037% bile salts after 24 h of incubation [79–81].

Several studies have reported that some *Bacillus* species are capable of producing different exogenous enzymes such as protease, lipase, cellulase, xylanase, phytase, and keratinase [82–86], which agrees with one of our studies already published [25]. These enzymes could improve the digestion of nutrients, making them more bioavailable, and also, they help to reduce intestinal viscosity in non-starch polysaccharide diets and decrease the substrates available for the growth of pathogenic bacteria. Considering this information, we performed a study in order to evaluate the effect of three *Bacillus*-DFM candidates with excellent to good relative enzyme activity values (cellulase and xylanase) on digesta viscosity and *Clostridium perfringens* (CP) proliferation in different poultry diets using an *in vitro* digestive model [87]. One of the three *Bacillus* strains was identified as *Bacillus subtilis* and the other two isolates as *Bacillus amyloliquefaciens* by 16S rRNA sequence analysis. Subsequently, *Bacillus* candidate strains were sporulated and mixed in equal amounts during the *Bacillus*-DFM preparation process [88] and incorporated into the experimental diets (10^{8} spores/g). The results of this study demonstrated that *Bacillus* candidate significantly reduced the viscosity of non-corn-based diets.

This could be due to the capability of these *Bacillus* strains to produce cellulase and xylanase, which could help improve the digestibility of cereals with high-soluble non-starch polysaccharides [89]. Furthermore, *Bacillus*-DFM candidate demonstrated effective antimicrobial properties against CP (**Table 7**), given their capability to produce antimicrobial-like compounds and/or compete for nutrients. Likewise, it was shown that the persistence of *Bacillus*-DFM candidate spores changes in each compartment of the *in vitro* digestive model mainly due to the conditions of pH and suggests that their full life cycle is developed in the gastrointestinal tract.

Based on the previous results, the effect of *Bacillus*-DFM candidate spores formed by an isolate of *Bacillus subtilis* and two of *Bacillus amyloliquefaciens* on growth performance, intestinal integrity, necrotic enteritis (NE) lesions, and ileal microbiota in broiler chickens using a previously established NE-challenged model [90] was evaluated [24]. This study consisted of three experimental groups: negative control (NC), positive control (PC), and *Bacillus*-DFM group (DFM). The last two groups were challenged with *Salmonella* typhimurium (ST, day 1), *Eimeria maxima* (EM, day 13), and *Clostridium perfringens* (CP, day 18–19). The overall results of performance showed that chickens supplemented with DFM had a significant body weight (BW) higher than PC. Furthermore, the body weight gain (BWG) and feed conversion ratio (FCR) were 59 g higher and 17 points lower, respectively, in the DFM group than PC (**Table 8**).

This enhancement in the performance of chickens supplemented with *Bacillus*-DFM could be due to better digestibility of nutrients, maintenance of the beneficial gut microbiota, and promotion of a healthy intestinal integrity [48, 87, 91]. Furthermore, these results could relate to the low-serum FITC-d concentration, bacterial translocation (BT), ileal lesion (IL), and total intestinal IgA levels in the DFM group compared to the PC group given the low impact of EM and CP challenge since DFM could produce beneficial chemical compounds, has immunoregulatory capacity, and stimulates the homeostasis of the intestinal microbiota, resulting in a proper intestinal health status [92].

Microbiota analysis confirms that DFM played a vital role in restoring gut dysbiosis. Although only the phylum *Proteobacteria* was significantly lower in DFM group than PC group, it could be explained due to the antimicrobial properties of DFM against ST [25], a predisposing factor in the NE model. In contrast, the genus *Lactobacillus* was significantly predominant in both NC and DFM groups with respect to PC, but it was higher in the DFM group than NC group (**Figure 2**). It has been reported that DFM is capable of increasing the genus *Lactobacillus*, which plays a crucial role in preventing dysbiosis and maintaining gut integrity (homeostasis) [36, 93].

Diet	Control diet	*Bacillus*-DFM
Corn-based	6.44 ± 0.19^{a}	6.68 ± 0.08^{a}
Wheat-based	7.12 ± 0.07^{a}	5.20 ± 0.18^{b}
Barley-based	7.50 ± 0.13^{a}	6.86 ± 0.11^{b}
Rye-based	7.15 ± 0.09^{a}	6.68 ± 0.12^{b}
Oat-based	6.96 ± 0.13^{a}	5.76 ± 0.07^{b}

[a,b] *Different superscripts within a row indicate significant differences $p < 0.05$.*
[1] *Inoculum used 10^5 cfu of CP.*
[2] *Data expressed in $\log_{10}$ cfu/mL.*

Table 7.
Concentration of Clostridium perfringens (CP)[1] in different digested diets with or without inclusion of Bacillus-DFM candidate spore[2].

Item	Negative control	Positive control	DFM
BW, g/broiler			
d 0	46.88 ± 0.64[b]	46.54 ± 0.64[b]	49.23 ± 0.68[a]
d 7	127.14 ± 2.90[a]	115.58 ± 3.27[b]	123.05 ± 3.80[ab]
d 14	273.80 ± 11.02[b]	295.78 ± 12.10[ab]	318.08 ± 13.57[a]
d 18	457.79 ± 18.97[ab]	456.32 ± 19.39[b]	525.58 ± 17.92[a]
d 21	603.81 ± 24.32[a]	445.96 ± 18.50[c]	507.77 ± 20.60[b]
BWG, g/broiler			
d 0–7	80.39 ± 3.06[a]	67.74 ± 3.24[b]	75.08 ± 3.64[ab]
d 7–14	147.01 ± 9.51[b]	182.60 ± 9.48[a]	196.22 ± 10.56[a]
d 14–18	183.99 ± 9.85[ab]	160.55 ± 9.02[b]	198.31 ± 9.61[a]
d 14–21	325.78 ± 15.58[a]	152.13 ± 9.67[b]	185.27 ± 10.52[b]
d 0–21	552.72 ± 24.35[a]	399.42 ± 19.79[b]	458.58 ± 20.48[b]
FI, g/broiler			
d 0–21	808.21 ± 29.86[a]	772.34 ± 10.66[a]	805.21 ± 71.07[a]
FCR			
d 0–21	1.46 ± 0.04[b]	1.93 ± 0.10[a]	1.76 ± 0.18[ab]

[1]*Data expressed as mean ± SE from 40 chickens (four replicates with 10 chicks each pen). $p < 0.05$.*
[a–c]*Values within columns with different superscripts differ significantly ($p < 0.05$).*

Table 8.
Evaluation of body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) in chickens supplemented with or without DFM on a necrotic enteritis challenge model[1].

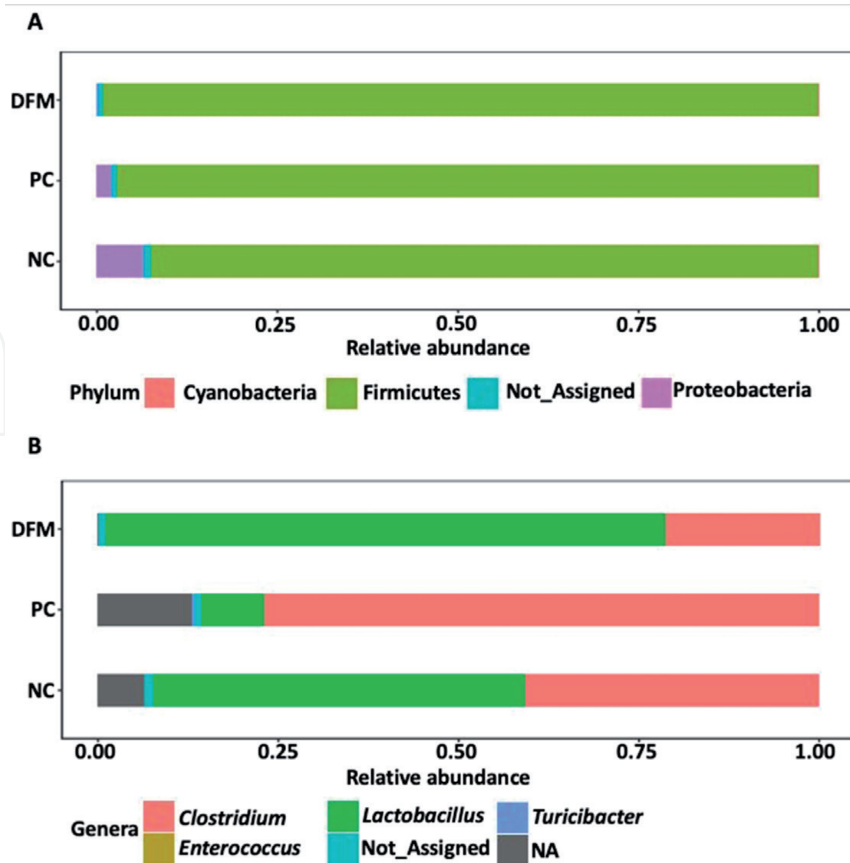

Figure 2.
Relative abundance of different phyla (A), families (B), and genera (C) in different treatment groups (NC, PC, and DFM). NA refers to those reads that were not assigned to the respective taxonomic levels.

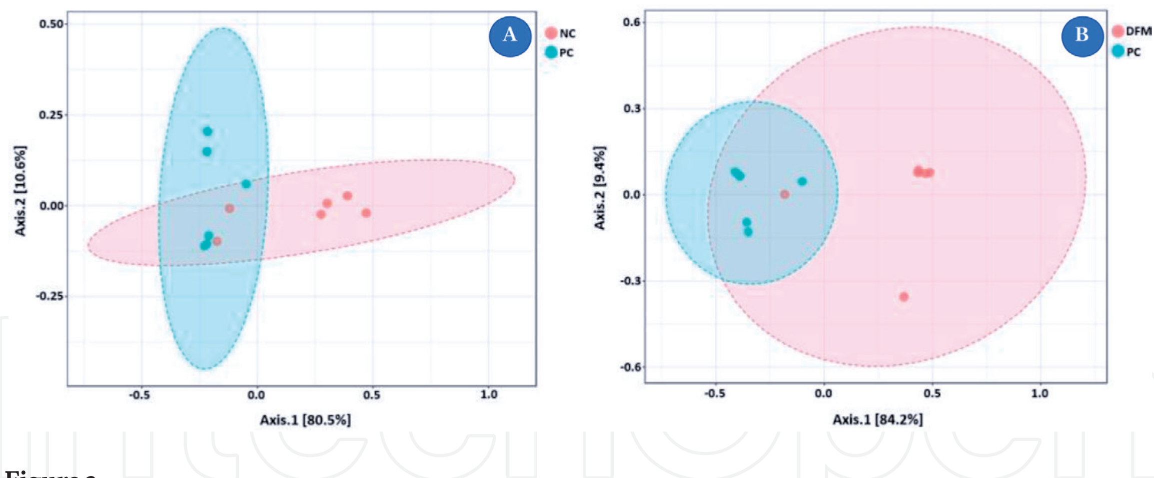

Figure 3.
PCoA plot showing difference in microbial community structure between (A) NC and PC (ANOSIM; R = 0.40 and p < 0.05) and (B) DFM and PC (ANOSIM; R = 0.73 and p < 0.01).

Furthermore, *Clostridium* was significantly higher in PC group due to the change in the ileum microbiota caused by NE [94], whereas the genera *Lactobacillus* and *Bacillus* were more abundant in the DFM group, suggesting that these genera could alleviate the negative impacts caused by CP [95].

Finally, significant differences in beta diversity were found between NC versus PC and PC versus DFM (**Figure 3**), which agrees with another study where NE causes significant changes in the intestinal microbiota [96]. Interestingly, there was no difference in bacterial community structure between NC and DFM. It confirms again that DFM played a vital role in restoring the gut dysbiosis in this study.

Item	NC	AFB1	DFM	SEM2	*p*-value
BW, g/broiler					
d 0	46.23 ± 0.68^{a}	47.92 ± 0.72^{a}	48.12 ± 0.74^{a}	0.4174	0.1275
d 7	133.29 ± 4.64^{a}	129.92 ± 2.78^{a}	137.02 ± 4.19^{a}	2.2763	0.4502
d 14	320.92 ± 17.53^{a}	272.06 ± 8.54^{b}	318.42 ± 14.65^{a}	8.4215	0.0263
d 21	640.10 ± 31.51^{a}	474.81 ± 15.57^{b}	571.60 ± 25.47^{a}	16.2361	0.0001
BWG, g/broiler					
d 0–7	87.06 ± 4.24^{a}	82.00 ± 2.71^{a}	88.90 ± 4.15^{a}	2.1705	0.4103
d 7–14	187.63 ± 13.82^{a}	142.13 ± 7.06^{b}	181.40 ± 11.38^{a}	6.7337	0.0097
d 14–21	319.17 ± 16.08^{a}	202.75 ± 9.77^{c}	253.17 ± 14.89^{b}	9.5832	<0.0001
d 0–21	593.87 ± 31.21^{a}	426.88 ± 15.66^{c}	523.48 ± 25.42^{b}	16.2105	0.0001
FI, g/broiler					
d 0–21	750.55 ± 17.23^{a}	775.93 ± 3.51^{a}	731.97 ± 82.35^{a}	25.1292	0.8193
FCR					
d 0–21	1.27 ± 0.06^{b}	1.82 ± 0.06^{a}	1.40 ± 0.06^{b}	0.0875	0.0016

a–c Superscripts within rows indicate significant difference at p < 0.05.

Table 9.
Evaluation of body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) in broiler chickens consuming a corn-soybean-based diet contaminated with aflatoxin B1 (2 ppm) supplemented with or without DFM.

3.3 The use of *Bacillus*-DFM candidate to prevent the toxic effects of aflatoxin B1 (AFB1) in poultry

Aflatoxin B1 (AFB1) is the predominant mycotoxin produced by several species of *Aspergillus* [97]. This mycotoxin has hepatotoxic and hepatocarcinogenic effects [98]. It has been reported that AFB1 has detrimental effects on performance parameters, which can cause serious economic problems in the poultry industry [99]. Therefore, the control of AFB1 is critical for producers. In this sense, the use of probiotics has proven effective in preventing and controlling the toxic effects of AFB1.

An *in vitro* study performed in our laboratory showed that 3 of 69 *Bacillus* spp. candidates were capable of biodegrading AFB1 since they reduced the fluorescence and area of clearance around each colony [100]. However, when these *Bacillus* spp. were tested in broiler chickens, no significant differences in performance parameters were observed when the groups were compared [101].

Despite the previous results, the *Bacillus*-DFM candidate spores formed by the isolate of *Bacillus subtilis* and the two of *Bacillus amyloliquefaciens* were included in the diets containing AFB1 to determine their effect on performance in broiler chickens fed with 2-ppm AFB1-contaminated diet [unpublished work from our laboratory]. The results are promising since the *Bacillus*-DFM improved performance of broilers, and even, there were no significant differences between the negative control (NC) and DFM group. It was due to the capacity of DFM to produce certain essential nutrients, extracellular enzymes, and growth factors to promote host growth [99, 102] (**Table 9**).

4. Conclusions

As it can be seen, probiotics could be considered a potential alternative to the use of antibiotics in poultry since it has been reported that they can improve the performance, as well as prevent and control enteric pathogens in poultry. However, their applications depend on the type of microorganism. In this regard, since lactic acid bacteria (LAB) are very sensitive to pelletizing processes for feed production (heating), environmental factors, and the low pH of the stomach, as well as the presence of bile salts in the small intestine, their administration in a single dose could be the most viable application especially to prevent bacterial diseases in both *in ovo* and broiler chickens. In contrast, *Bacillus* spp. direct-fed microbials (DFM) can be a better alternative since they are more stable because they can form spores. Therefore, DFM can be included in the feed, and, in addition, the production costs are lower than the microencapsulation and freezing or lyophilization processes that are used to maintain the viability of LAB. Finally, probiotics as *Bacillus*-DFM have also shown beneficial effects in preventing and controlling toxic effects of AFB1. Although the mechanisms by which the DFM reduce the effect of AFB1 are still known, our laboratory is working to elucidate the mechanism.

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