Chapter 15
Analysis of Changes in Broiler Microbiome Biodiversity Parameters Due to Intake of Glyphosate and Probiotic Bacillus Sp. Gl-8 Using Next-Generation Sequencing

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Abstract In recent years, there have been more data that the nonselective herbicide glyphosate (GLY) can negatively impact gut bacterial communities. The aim of our study was to investigate the composition of broiler caecal microbiome under chronic exposure to GLY and the introduction of a probiotic microorganism strain into the diet. 120 broilers were divided into three groups: Group 1 of control birds fed the basic diet (BD); Group 2 of experimental birds fed BD supplemented with GLY; and Group 3 of experimental birds fed BD supplemented with GLY and a probiotic strain of the microorganism *Bacillus* sp. GL-8. For analysis, we used the next-generation sequencing (NGS) technique. Due to the GLY administration, there was a trend of lowering the biodiversity of normal microflora representatives, along with intestinal colonization by undesirable forms of microorganisms. In particular, when adding GLY (Group 2), we observed a decreased number of *Tepidimicrobium* representatives ($0.001 \pm 0.00006\%$) that ferment indigestible polysaccharides, while in Group 1 their

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content was greater (0.3 ± 0.02%; $P \leq 0.05$). In Group 3 with probiotic, there was a lower number of Firmicutes (by 16.7%) and a rise in the number of Bacteroidetes (by 19.1%) as compared to Group 2 ($P \leq 0.05$).

15.1 Introduction

Glyphosate (GLY), known as an active ingredient of Roundup®, is the most widely used nonselective herbicide in the world. It has a broad spectrum of activity and application *including* weed control in agriculture, vegetation control in non-agricultural areas, and usage as a desiccant. The use of GLY in agriculture has expanded significantly due to the development of GLY-tolerant varieties of GM crops [1]. Its ubiquitous use has raised public concern and initiated a scientific debate about the true extent of the compound's toxicity [2]. GLY residues' content has been the subject of systematic assessments by national and international regulators [3], although they found that it has a relatively low toxicity to mammals. However, recent studies by the International Agency for Research on Cancer concluded that the herbicide is likely to be carcinogenic to humans [4]. Swanson et al. [5] demonstrated a significant correlation between an increased usage of Roundup® and a rise in the number of US citizens suffering from one or more of 22 chronic diseases, which included obesity, hypertension, senile dementia, and several types of cancer.

Currently, the GLY safety in farm animals, including poultry, remains a controversial issue, as there is no unequivocal evidence of its toxicity. Behind the development of diseases associated with GLY, there may be both direct pathological changes and disturbances in the composition of microbiomes. After all, GLY blocks 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme of the shikimate pathway, which is involved in the synthesis of three essential aromatic amino acids (phenylalanine, tyrosine and tryptophan) not only in plants, but also in most prokaryotes. Until recently, the importance of the shikimate pathway and the EPSPS enzyme diversity in many microorganisms were not considered critical. Changes in the composition of microbial communities cannot but affect the host organism's metabolism. It is well known that normal microbiome functioning plays a significant role in maintaining basic body functions.

In recent years, a number of publications have appeared suggesting that GLY can negatively affect intestinal bacterial communities in several model macroorganisms, as well as in cultures in vitro [6]. However, in vivo experiments targeting the poultry microbiome composition under the GLY impact using the next-generation sequencing (NGS) technology have practically not been carried out.

Developing methods to counter the toxic GLY effects on the microbiomes of farm animals, including poultry, is thought to be important. Probiotics are of increasing interest compared to other methods of feed detoxification due to their biological nature and safety, as well as their complex properties to influence the biodegradation of toxins, restore microbiome balance and participate in the digestive process.

In this respect, an ecological strategy for restoring microbial balance using probiotic microorganisms is extremely promising. In addition, some microorganisms, in particular *Bacillus* species (e.g., [7]), have a pronounced biodegrading ability.

Thus, the potential impact of GLY residues on the gut microbiome still needs to be studied in more detail. After all, the indirect GLY impact on the health of birds through changes in their microbiomes may be no less important than its direct impact on the physiology of macroorganisms. Feed quality control and safety remain crucial aspects of the modern animal husbandry, including efficient poultry meat production [8–11].

In this regard, the aim of our investigation was to explore the caecal microbiome composition under chronic exposure to GLY and the introduction of a probiotic strain into the diet in broiler chickens (*Gallus* L.) of a broadly exploited Ross 308 cross (e.g., [12]).

15.2 Materials and Methods

The experiments were carried out in the BIOTROF + Ltd. vivarium on Ross 308 broilers from 1 to 35 days of age in 2022. A total of 120 birds were divided into three equal groups: Group 1 of control birds fed the basic diet (BD) without the GLY administration; Group 2 of experimental birds fed BD supplemented with GLY at a dose of 20 ppm, which conformed to 1 maximum residue level (MRL) in foods (according to SanPiN 1.2 0.3685–21 "Hygienic standards and requirements to ensure safety and/or harmlessness for humans of environmental factors"); and Group 3 of experimental birds fed BD supplemented with GLY at 20 ppm and a probiotic strain *Bacillus* sp. GL-8 (from the BIOTROF + Ltd. collection).

As a source of GLY in this experiment, the Agrokiller preparation (CJSC Avgust, Russia) was used that contained 500 g/l GLY acid (isopropylamine salt). For this purpose, the Agrokiller working solution was made and applied on the compound feed by the spraying method followed by mechanical mixing with observance of personnel safety requirements. After the GLY application, its concentration in the feed was monitored using the enzyme immunoassay (ELISA; e.g., [13]). In addition, BD contained almost no background GLY amounts, which indicates the experiment's purity. For the ELISA analysis of GLY content in the feed, a STAT FAX 303 + strip immunoassay (Awareness Technology, LLC, USA) and a GLY ELISA Microtiter Plate test system (Abraxis, USA) were used.

At the experiment's end, the caecal chyme was sampled from three birds per group. Total DNA was extracted using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., USA). The cecal bacterial community was assessed by NGS using the MiSeq platform (Illumina, Inc., USA) and the following primers for the V3-V4 region of the 16S rRNA gene: forward primer, 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; and reverse primer, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. PCR was

performed under the following conditions: 25 cycles of 3 min at 95 °C; 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C (as required to elongate an amplified sequence) followed by 5 min at 72 °C (final elongation). Sequencing was performed using Nextera® XT IndexKit reagents (Illumina, Inc., USA) for library preparation, Agencourt AMPure XP (Beckman Coulter Inc., USA) for purification of PCR products, and MiSeq® ReagentKit v2 (500 cycles) (Illumina, Inc., USA) for sequencing.

Bioinformatic data analysis was performed using the QIIME2 ver. 2020.8 software (https://docs.qiime2.org/2020.8/). Noise sequences were filtered using the DADA2 method built into the QIIME2 package, which included quality information in its error model and made the algorithm robust to lower quality sequences, while using a maximum trimming sequence length of 250 bp (https://benjjneb.github.io/dada2/tutorial.html). To build a de novo phylogeny, multiple sequence alignment was performed using the MAFFT software package. The reference database Silva 138.1 (https://www.arb-silva.de/documentation/release-138.1/) was used for taxonomy analysis.

15.3 Results and Discussion

Using NGS for the microbiome examination, we generated a total of 57,234 16S rRNA gene sequences (with a median read number of 6,134, minimum of 5,123, and maximum of 7,795). GLY without the probiotic *Bacillus* sp. GL-8 strain administration (i.e. in Group 2) had no effect on the number of OTUs (Fig. 15.1), as well as on the Shannon and Simpson α-biodiversity indices (Fig. 15.2; $P > 0.05$) as compared to Group 1. At the same time, adding the *Bacillus* strain sp. GL-8 (Group 3) was associated with a decline in OTU values (Fig. 15.1) and α-biodiversity indices ($P \leq 0.05$; Fig. 15.2). The decreased biodiversity may be due to the microbiome stabilization as influenced by the probiotic strain, since a large number of interacting species often tend to have a destabilizing effect on the microbiome [14]. It was also previously shown that prebiotics, by inhibiting the growth of pathogenic microorganisms, contributed to the formation of more stable microbial communities of the intestine with a lower biodiversity [15].

The intestinal microbiome of birds from all groups contained a total of 34 bacterial phyla, with Firmicutes, Bacteroidetes, Proteobacteria and Tenericutes being dominant among them (Fig. 15.3). The most numerous was the phylum Firmicutes (63.9 ± 4.20−88.5 ± 5.34%).

The GLY introduction in the feed in Group 2 led to a rise in the bacteria of the phylum Proteobacteria by 1.6 times as compared to Group 1 and the lower number of Firmicutes ($P \leq 0.05$). The data seemed plausible, since the microorganisms of the phylum Proteobacteria have long been known for their ability to degrade xenobiotics, including complex aromatic compounds, using various electron acceptors [16]. Therefore, these microorganisms, in the presence of GLY, could gain a competitive advantage over other forms. With respect to Firmicutes, GLY administered as

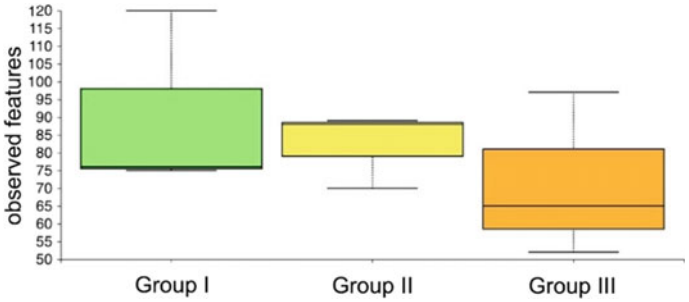

Fig. 15.1 Values of operational taxonomic units (OTU) in the gut microbiome of Ross 308 broilers fed GLY and the *Bacillus* sp. GL-8 strain

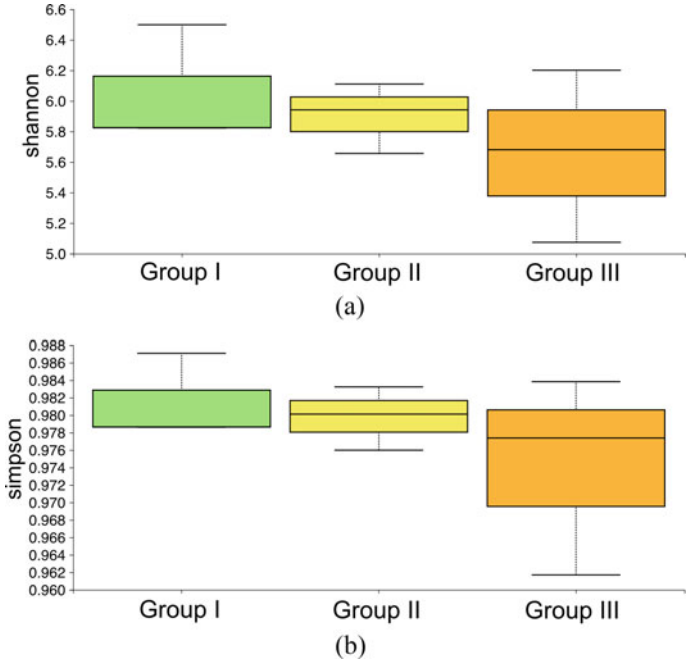

Fig. 15.2 Absolute values of α-biodiversity indices (**a**, Shannon and **b**, Simpson) in the gut microbiome of Ross 308 broilers fed GLY and the *Bacillus* sp. GL-8 strain. Calculated using QIIME2 ver. 2020.8 plugins

Roundup® orally or intravenously in mice at higher concentrations (250 or 500 ppm per day) for 6 or 12 weeks was also shown to reduce Firmicutes [6].

When adding the probiotic *Bacillus* sp. GL-8 strain into the diet in Group 3, a 2.6-fold decline in the phylum Proteobacteria was observed as compared to Group 2 ($P \leq 0.05$). It is possible that the introduced strain reduced the toxic load of GLY due to the supposed biodegradation properties. In Group 3, a decrease in the number

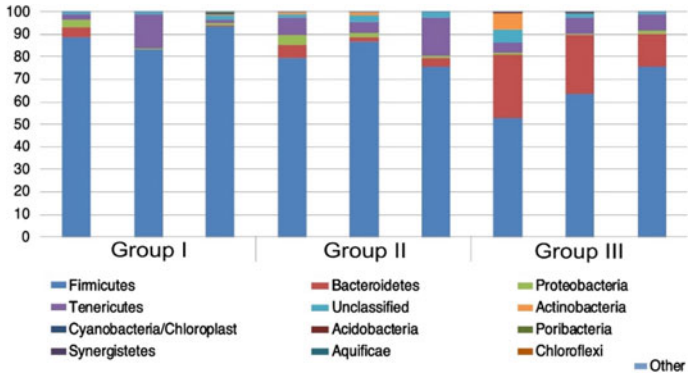

Fig. 15.3 Composition of the gut microbiome at the level of bacterial phyla according to the NGS data of 16S rRNA amplicons in Ross 308 broilers fed GLY and the *Bacillus* sp. GL-8 strain

of Firmicutes by 16.7% and an increase in the number of Bacteroidetes by 19.1% were also noted as compared to Group 2 ($P \leq 0.05$). In recent years, the ratio of Firmicutes and Bacteroidetes (F/B) in the gastrointestinal tract (GIT) has received much attention [17]. It is widely recognized that this ratio has an important impact on the maintenance of normal intestinal homeostasis. An elevated or declined F/B ratio is considered dysbiosis. In our experiment, the phenomenon of a decrease in Firmicutes observed, along with an increased number of Bacteroidetes and was estimated by many researchers as a favorable condition for the health of the macroorganism [17]. In Group 3, the content of the Bacillaceae family bacteria rose by 1.7 times as compared to Groups 1 and 2 ($P \leq 0.05$). This may be indicative of a successful GIT colonization with the introduced probiotic strain.

In Groups 2 and 3 (i.e., with the GLY introduction), dysbiotic disturbances in the intestines of birds were observed in the composition of microorganisms fermenting plant polysaccharides (Lachnospiraceae, Ruminococcaceae) [18] ($P \leq 0.05$).

In addition, in Group 2, as compared to Group 1, the number of other families elevated, among which there were many pathogenic and opportunistic microorganisms. Particularly, the content of Staphylococcaceae increased by 5.0 times, and that Enterobacteriaceae by 1.5 times ($P \leq 0.05$). The fact is that intestinal prokaryotes vary significantly in their sensitivity to GLY depending on the type of EPSP enzyme. Previously, it was demonstrated in vitro [19] that highly pathogenic bacteria in poultry such as *Salmonella enteritidis, S. gallinarum, S. typhimurium, Clostridium perfringens* and *C. botulinum* were highly tolerant to GLY. However, most useful members of the normal flora, such as *Enterococcus faecium, Bacillus badius, Bifidobacterium adolescentis* and *Lactobacillus* spp. were sensitive to GLY [19]. The use of the *Bacillus* sp. GL-8 strain in Group 3 contributed to a decreased number of bacteria of the families Staphylococcaceae (by 1.75 times) and Enterobacteriaceae (by 3.3 times; $P \leq 0.05$).

At the level of lower taxonomic ranks (genera), there were also marked differences between the groups in terms of the number of microorganisms ($P \leq 0.05$; Fig. 15.4).

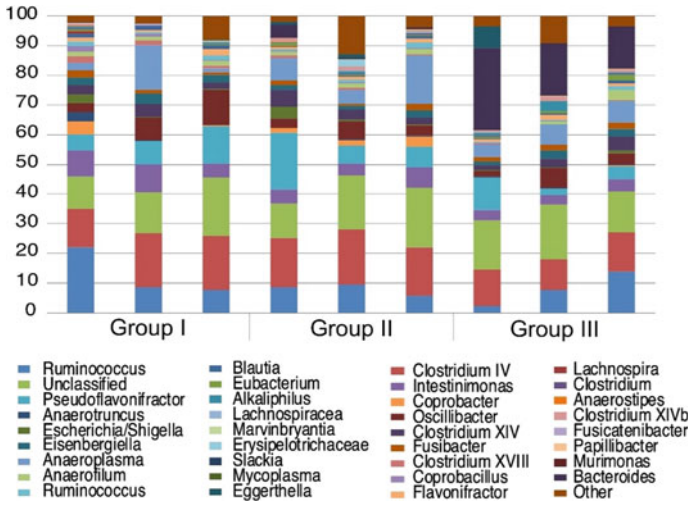

Fig. 15.4 Composition of the gut microbiome at the level of bacterial genera according to the NGS data of 16S rRNA amplicons in Ross 308 broilers fed GLY and the *Bacillus* sp. GL-8 strain ($P \leq 0.05$)

The main trend continued to be a decline of some representatives of the normoflora, along with an increased number of undesirable forms of microorganisms.

In particular, in the presence of GLY in Groups 2 and 3, the numbers of the phylum Firmicutes representatives and the genus *Oscillibacter* bacteria were 4.1 ± 0.24 and 4.1 ± 0.32%, respectively, versus 7.5 ± 0.48% in Group 1 ($P \leq 0.05$; Fig. 15.4). *Oscillibacter* spp. representatives are active producers of valeric acid as the main end product of glucose metabolism. Valeric acid is valuable for the metabolism of birds, as it is involved in preventing the consequences of oxidative stress and reducing the expression of pro-inflammatory cytokines and α-synuclein, with a subsequent increase in vital antioxidant enzymes.

When administering GLY in Group 2, *Tepidimicrobium* bacteria were also almost completely excluded from the microbiome. The number of these microorganisms declined to 0.001 ± 0.00006%, while in Group 1, it was 0.3 ± 0.02% ($P \leq 0.05$; Fig. 15.4). This microorganism is capable of fermenting indigestible polysaccharides, including xylan and cellobiose, to such important products as acetate, butyrate, propionate, etc. The probiotic strain intake in Group 3 contributed to a slight increase in the abundance of this genus (up to 0.004 ± 0.0003%) as compared to Group 2 ($P \leq 0.05$).

In Group 2 fed GLY, the displacement of the described useful species was accompanied by an increased number of undesirable taxa of microorganisms. For example, the number of *Pseudoflavonifractor* representatives rose in this group up to 10.7 ± 0.82 as compared to Group 1 (8.7 ± 0.39%; $P \leq 0.05$; Fig. 15.4). Elevation in numbers *Pseudoflavonifractor* was linked to metabolic disorders [20]. In Group 3,

there was a lower number of *Pseudoflavonifractor* representatives (5.8 ± 0.45%) as compared to Group 2 ($P \leq 0.05$).

In addition, in Group 2, the abundance of the *Staphylococcus* and *Terrisporobacter* genera and the species *Mycoplasma conjunctivae* increased as compared to Group 1 ($P \leq 0.05$). *Mycoplasma conjunctivae* is the causative agent of several diseases, such as infectious keratoconjunctivitis in various species of farm animals. *Terrisporobacter* is an anaerobic pathogen. It has been proven that an elevation in its content contributes to an increase in oxidative stress in animals. It has long been known that xenobiotics induce oxidative stress, with the formation of a number of metabolites that lead to the release of superoxide, provided that the ability to restore the formed diones (quinones) to hydroquinones is retained. Hydroxyl radicals are highly reactive, but short-lived molecules known to cause DNA damage. Mutagenesis is a major contributing factor to the risk of carcinogenesis associated with conditions of increased oxidative stress. The results of our studies would suggest that there is indeed a possible association between changes in the gut microbiota and carcinogenesis in the GLY presence [4].

It is important that under the influence of the probiotic *Bacillus* sp. strain in Group 3, there were lower numbers of such clostridial clusters as *Clostridium*_III, *Clostridium*_IV, *Clostridium_sensu_stricto Clostridium*_XlVa, *Clostridium*_XlVb and *Clostridium*_XVIII as compared to Group 2 ($P \leq 0.05$; Fig. 15.4). In sum, this difference reached a total 6.4%. *Clostridium* spp. bacteria are known to have a higher capacity to form toxic or carcinogenic metabolites among the gut microbiota. Metabolites of *Clostridium* spp. can lead to an excess of dopamine quinones, generating reactive oxygen species and leading to oxidative stress and mitochondrial dysfunction. In addition, it was previously shown in monogastric animals that exposure to GLY resulted in severe *Clostridium* bacteremia [21]. Previously, chicks exposed to GLY (370 ± 92 ppm) had typical clostridial symptoms associated with increased levels of intestinal clostridia [22]. The symptoms of clostridial infections were suppressed by humic acids, which bind to GLY molecules in the GIT.

It is essential that, among other bacteria observed in our experiment, the number of species such as *C. bolteae* and *C. leptum* decreased due to the probiotic *Bacillus* sp. strain in Group 3 respectively by 2.2 and 1.7 times as compared to Group 2 ($P \leq 0.05$). It is known that *C. bolteae* has a highly conserved glycerol uptake accelerator and a related protein, aquaporin (pp 59-71), which shares sequence homology with the AQP4 peptide (pp 92-104) located at the immunodominant (AQP4-specific) T cell epitope (pp 91-110). The presence of *C. bolteae* correlates with the expression of inflammatory genes associated with both innate and adaptive immunity, and specifically involved in plasma cell differentiation, B cell chemotaxis, and Th17 activation [23]. In turn, *C. leptum* created an immunosuppressive environment for pneumonia in mice. An increase in a $CD4^+$ $CD25^+$ $FOXP3^+$ subset of regulatory T cells impaired the production of T effector cells, including Th1, Th2, Th9, and Th17, and secretion of cytokines in vivo [24].

15.4 Conclusion

Our studies have shown that GLY, when being present in the contaminated bird feed even at minimal concentrations that are several times lower than feed maximum allowable concentration levels, can adversely affect gut microbial communities under chronic exposure to this toxicant. Pathogenic and opportunistic microorganisms, which are likely to be less sensitive or even insensitive to GLY, may increase in numbers, replacing normal microflora. Displacement of the symbiotic normobiota can adversely affect the processes of fiber digestion and the synthesis of metabolites important for birds. An increase in the proportion of pathogens can cause the spread of infectious diseases. Therefore, we suggest that assessing the problems associated with the large-scale and heavy use of GLY and other pesticides is a much more difficult task than was originally envisaged by regulators.

Since the exact causes of differential sensitivity to GLY in macroorganisms are unknown, this remains to be elucidated in the future.

The introduction of the probiotic strain into the diet in the GLY presence in the feed had a positive effect on the diversity and abundance of microorganisms of various taxa. This is probably due to the antimicrobial activity of the bacterial strain in the composition of the probiotic preparation and/or the presence of genes responsible for the biodestruction of xenobiotics, which suggests future research in this direction.

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