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FRACTAL ANALYSIS OF FREQUENCY-TAXONOMIC PROFILE OF BROILER'S GUT MICROBIOTA FOR STUDYING THE INFLUENCE OF PROBIOTICS ON BIRD DEVELOPMENT

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Abstract

The article provides theoretical and empirical data about the probiotic effect on the microbiota bioconsolidation in the broiler intestines of the Smena cross. The results of such studies can be used to improve the quality and volume of meat products in large-scale production. The probiotics have been used to improve feed digestibility and accelerate bird development. To stimulate the transformation of plant substrates in the bird intestines, probiotics were used instead of antibiotics. The probiotics contained the bacteria *Lactobacillus plantarum* and *Lactobacillus fermentum*. The study goal is to develop a methodology for fractal analysis of the frequency-taxonomic profiles of operational taxonomic units (OTUs) of the microbiota into the bird intestines. Using the fractal methodology, the index of microbiom bioconsolidation of the bird intestines was calculated, which characterizes the biosystem self-organization of microflora and the efficiency of biochemical transformations of plant substrates in the bird intestines. In the experiment, the microflora was studied in one control and two experimental groups of birds. The OTUs profiles were obtained by the molecular genetic NGS method (Next Generation Sequencing). The key concept of fractal analysis of OTUs profiles was the concept of the elementary OTU fractal. The elementary OTU fractal is three OTUs, the frequencies of which form a geometric numerical sequence (for example, {0.5; 0.25; 0.125}). The OTU profiles may contain several elementary OTU fractals combined into one larger OTU megafractal. We assume that if the number of OTUs combined into the OTU megafractal increases, then biochemical transformations of plant substrate are carried out more efficiently and on a large scale, and the bird macroorganism receives more nutrients. Therefore, we define the bioconsolidation index of the broiler microbiome as the ratio of the number of OTUs in the OTU megafractal to the total number of OTUs in the OTU profiles. The fractal portraits of OTU profiles were used to identify elementary OTU fractals. The elementary OTU fractals were identified by the linear arrangement of three OTU images on fractal portrait. The fractal analysis confirmed that the bacterial probiotics increase the microbiom bioconsolidation in the bird intestines. The microbiome bioconsolidation index in the experimental groups of birds (0.82...0.86) was higher than this index in the control group of birds (0.55). According to the results of fractal analysis, probiotic No. 1 (with *Lactobacillus plantarum*) is not recommended for use, and probiotic No. 2 (with *Lactobacillus fermentum*) is recommended for use.

Keywords: frequency-taxonomic profile, fractal portrait, intestinal microbiota, biosystem consolidation index, broilers, dietary probiotics, *Lactobacillus*.

Annual volumes of pedigree and hybrid young poultry import to the Russian Federation poses a risk of the uncontrolled spread of infectious diseases of various etiologies. Currently, active studies are being conducted to replace imported chickens with domestic highly productive genetic crosses marked with slow-feathering (*K*) or rapid-feathering (*k*) gene alleles.

Feeding is equally important and should regard the response of the bird microbiome to individual dietary components as a factor to significantly increase the meat quality and broiler performance in commercial poultry.

The feed digestibility for poultry depends entirely on the enzymatic activity of the intestinal microbiota [1-4]. Change in keeping conditions or diets, dietary antibiotics, pro- or prebiotics [5-8] and feed contamination with mycotoxins [9-11] force gut microbiota to rearrange its destructive biosystems in the abundance and profiles of microbial genotypes. As a result, the bird intestine microbiota can maintain the biotransformation of different plant substrates at the most efficient level [12]. This allows most of the nutrient resources to be redirected to the development of the poultry.

Biosystemic self-organization of gut microbial communities is necessary for transformation of substrates into nutrient and their timely delivery to the macroorganism. Due to intestinal microbial biosystem, birds can gain live weight faster and reach maximum egg production. In addition, microbial biosystems can protect against pathogenic microflora [13]. Therefore, the biosystem self-organization of gut microbiota is a factor, ensuring better performance and high egg production in poultry.

The macroorganism and microbiota of the bird's intestines form an integral biosystem in which they interact to develop and survive together. As the feed composition changes, the configuration and genotype composition of gut microbial biosystem of a bird undergo changes to the most effectively transform a variety of plant substrates into nutrients [14-16]. Therefore, partial or complete participation of genotypes in the functioning of intestinal microbial biosystem can serve as a quantitative indicator of biochemical transformation of plant substrates and protection of the host organism from pathogenic microflora [17-19].

Only a part of the gut microbiota is incorporated into the biosystem. In the self-organization of biosystems, only those genotypes are selected that are capable of performing the required biochemical transformations of forage substrates most efficiently and with the lowest energy and resource consumption [4, 18, 19]. Therefore, the self-organization and bioconsolidation of microorganisms ensure the implementation of required biochemical transformations of organic substrates with maximum intensity and in a certain order, that is, in an organized manner, in stages and with the lowest energy and resource costs on the part of the macroorganism. In such conditions, the bird receives all the necessary nutrients and develops rapidly.

In response to feed composition, the gut microbial biosystem rearranges its enzymatic profiles, metabolic pathways and connections between components [3, 13]. To stimulate the activity of microbial biosystems and increase the efficiency of transformation of plant substrates in the bird's intestines, various feed additives are used, e.g., antibiotics, exogenous enzymes, prebiotics, probiotics, synbiotics, and phytobiotics [4]. However, the ability of pathogenic microorganisms to acquire antibiotic resistance [2] led to the ban on their use in poultry feed in the EU countries since 2006. In this regard, the use of other types of feed stimulating and protective additives replacing antibiotics becomes relevant [6].

Molecular methods provide more information about gut microbiota of birds. To date, detailed frequency-taxonomic profiles of operational taxonomic units (OTU profiles) comprising thousands of OTUs are available [21]. For the 16S rRNA gene, the OTU profiles are only 10 % identified to genus and species according to international taxonomic information databases, and the rest OTUs add to the list of unidentified genotypes, possibly representing new species and genera [21-23]. OTU profiles provide taxonomic information on cultured and uncultured microorganisms and quantitative estimates for each microbial genotype, from minor to major member of the intestinal microbiota. For multivariate statistical analysis of quantitative and taxonomic information of the OTE profile, fractal analysis is best suited.

Fractal analysis of biological data is a class of multivariate statistical analyzes. Therefore, with its help, from the entire set of actual molecular genetic data, it is possible to extract information about the features of the biosystem organization of microbiomes in birds and to study the effect of probiotics on the self-organization of microbial biosystems. As applied to OTU profiles, the key concept of fractal analysis is an elementary fractal OTU, taking into account the special power-law ratio of the frequencies of three OTUs [24, 25]. This key concept stems from fundamental power-law quantitative relationships that are reflected in the relative sizes of elements in nature, including in plants (for example, tree fractals) and in microbiological biosystems.

Obviously, in birds, any changes in feeding and keeping are reflected in changes in the OTU profiles and their fractal characteristics. We believe that a decrease in the number of elementary OTU fractals and in the number of microbial genotypes in OTU fractals is a sign of weaker biosystem interactions of microorganisms and less effective biochemical transformations they carried out. Therefore, using the fractal analysis of the OTU frequency-taxonomic profiles and determining the bioconsolidation index of the gut microbiota in birds, it will be possible to assess the influence of probiotics on the biosystem self-organization of microbial communities and on the poultry grow and development.

The aim of the study was to develop a methodology for fractal analysis of frequency-taxonomic profiles of operational taxonomic units (OTUs) of the gut microbiota in birds. The fractal technique will allow calculation of the bioconsolidation index for the microbiota, and this index, in turn, is supposed to be used to study the effect of probiotics on the biosystem self-organization of microflora and the efficiency of biochemical transformations of plant substrates in the intestines of birds.

Our goal was to develop methods for fractal analysis of the frequency-taxonomic profiles of operational taxonomic units (OTUs) to characterize gut microbiota of birds. The fractal technique will allow calculation of the gut microbiota bioconsolidation index to assess it as a tool in investigation of how probiotics affect biosystem self-organization of microflora and biochemical transformations of plant substrates in the intestines of birds.

Description of the technique. Materials and methods of the verification test. To verify the method of fractal analysis of OTE profiles, a feeding trial was carried out (the Zagorskoe EPH, Moscow Province, 2018). Broiler chickens of the Smena cross were assigned for three feeding groups, 25 birds each. Control group 1c fed a basal diet (BD, Table 1), experimental group 2e fed the BD supplemented with *Lactobacillus plantarum*-based probiotic No. 1 (10^7 CFU/g, 1 kg/t feed), and experimental group (3e) fed the BD supplemented with *Lactobacillus fermentum*-based probiotic No. 2 (10^7 CFU/g, 1 kg/t). The chickens were kept in

AviMax cage batteries (Big Dutchman, Germany). The poultry was raised up to 36 days of age in accordance with the recommendations (FSC VNITIP RAS).

1. Composition (%) of basal diet (BD) supplemented with potential probiotic preparations for Smena cross broiler chickens in feeding groups (n = 25, Zagorskoe EPH, Moscow Province, 2018)

Ingredient	Days of age					
	1-21			22-36		
	1c	2e	3e	1c	2e	3e
Corn	60.00	–	–	60.00	–	–
Wheat	–	63.58	65.59	–	60.00	61.92
Soybean meal	26.21	16.63	16.52	17.12	17.52	17.43
Corn gluten	3.50	7.17	6.87	7.52	5.44	5.18
Fish flour	4.59	4.00	4.00	–	–	–
Sunflower meal	–	–	–	6.98	4.92	4.86
Sunflower oil	2.00	4.46	2.76	3.83	7.53	5.92
Limestone	1.46	1.58	1.58	1.49	1.53	1.53
Monocalcium Phosphate	0.91	0.82	0.82	1.35	1.23	1.23
Lysine monochlorohydrate	0.23	0.50	0.50	0.50	0.50	0.50
DL-methionine	0.29	0.31	0.31	0.25	0.30	0.30
Threonine	0.09	0.17	0.17	0.12	0.15	0.15
Salt	0.22	0.28	0.28	0.34	0.38	0.38
Cellobacterin-T	–	–	0.10	–	–	0.10
Premix	0.50	0.50	0.50	0.50	0.50	0.50

N o t e. 1c — control group, 2e, 3e — experimental groups. For 2e, BD was supplemented with probiotic No. 1 (10^7 CFU *Lactobacillus plantarum* per g), for 3e, BD was supplemented with probiotic No. 2 (0^7 CFU *Lactobacillus fermentum* per g), both at a dosage of 1 kg/t feed. Dashes indicate that the ingredient was removed from the diet.

On day 36, the broilers were weighed. Weighing results were processed by standard methods of analysis of variance using Microsoft Excel 2010 software. Parametric (Student's t-test) and nonparametric (Wilcoxon-Mann-Whitney method) statistical methods were used. The mean values (M) and standard errors of the means (±SEM) were calculated.

The caecum contents were aseptically collected after slaughter of chickens aged 36 days (in three replicates for each group) with strict adherence to sampling techniques and immediately frozen. The cecal microbiota was studied using next generation sequencing (NGS) technique [26] (the International Laboratory of Molecular Genetics and Poultry Genomics, Moscow). Total DNA was extracted using Genomic DNA Purification Kit (Fermentas, Inc., Lithuania). In PCR reaction, eubacterial primers 343F 5'-CTCCTACGGRRSGCAGCAG-3' and 806R 5'-GGACTACNVGGGTWTCTAAT-3' were used (Verity DNA amplifier, Life Technologies, Inc., USA). Metagenomic sequencing of amplified 16S rRNA gene fragments was performed (MiSeq Reagent Kit v3, MiSeq device, Illumina, Inc., USA). The resulting reads were subjected to bioinformation processing (CLC Bio GW 7.0 platform, Qiagen N.V., the Netherlands). Taxonomic analysis was performed using the RDP Classifier program (https://rdp.cme.msu.edu/classifier/classifier.jsp) using information databases.

Table 2 shows the taxonomic groups of microorganisms in the obtained OTU profiles of the intestinal microbiota in broiler chickens.

2. OTU frequency-taxonomic profiles and taxonomic groups of gut microbiota in 36-day-old Smena cross broiler chickens under probiotic feeding trials (n = 25, Zagorskoe EPH, Moscow Province, 2018)

No. OTU	Microorganisms
1	Bacillus
2	Lactobacillus*
3	Bifidobacteria
4	Cellulolyticus. Lachnospira
5	Cellulolyticus. Ruminococcus
6	Cellulolyticus. Clostridium
7	Cellulolyticus. Bacteroides

Continued Table 2

8	Cellulolyticus. Eubacterium
9	Conditionally pathogenic. Enterobacteriaceae*
10	Conditionally pathogenic. Actinomycetes*
11	Staphylococcus
12	Campylobacter
13	Pseudomonas
14	Proteobacteria
15	Tenericutes
16	Eripsipelotrichs
17	Uncultivated

N o t e. OTU — operational taxonomic unit. Lactobacilli (OTU No. 2), opportunistic Enterobacteriaceae (OTU No. 9), and Actinomycetes (OTU No. 10) are indicator groups of microorganisms to assess feed digestibility and the protection of poultry from pathogens.

Fractal analysis of OTU profiles. To minimizing energy and resource consumption, it is more profitable to decompose plant substrates by several microbial genotypes combined into a biosystem. This ensures the timely and sufficient enzymatic flows generated by microorganisms for destruction of plant substrates. In this case, both the number of enzymes and the number of microbial genotypes that generate enzymes will presumably be in one-to-one correspondence with the number of target restriction sites in decomposed organic molecules. Therefore, in an optimized destructing biosystem, there should be the same ratios between the numbers (frequencies) of microbial genotypes and the numbers (frequencies) of target restriction sites in organic molecules. This means that, in optimal microbial biosystems, the frequencies of genotypes and the frequencies of OTUs in OTU profiles, as well as restriction sites in decomposed organic molecules should be described by fundamental fractal power relations. On the basis of this statement, the definition of an elementary fractal OTU was formulated and a fractal analysis of the OTU profiles was carried out.

Determination of an elementary OTU fractal. If three OTE frequencies are ordered in a geometric numerical sequence, for example {0.5; 0.25; 0.125}, or in the logarithmic form the arithmetic numerical sequence{$log_2(0.5) = -1$; $log_2(0.25) = -2$; $log_2(0.125) = -3$}, then these OTUs represent an elementary OTU fractal, and the corresponding microbial genotypes belong to the biosystem part of the microbiota. In this case, it is allowed to combine several elementary OTU fractals into a larger OTU mega-fractal.

OTU mega-fractals give an idea of the genotypic composition of biosystems in the gut microbiota of birds and of the number of microbial genotypes realizing individual development strategies outside the biosystem. The ratio of fractal to out-of-fractal OTUs characterizes the microbial resource of the bird microflora involved in the biochemical transformation activity. We assume that a decrease in the number of OTUs in OTU mega-fractals, and hence, of genotypes in microbial biosystems, signals a decrease in the productivity of gut biochemical transformations in birds and, as a consequence, a delay in their development.

Fractal portrait of the OTE profile. For visual detection of OTE mega-fractals and their mathematical accounting, you can use the fractal portrait of the OTU profile. The construction of OTU fractal portraits greatly facilitates the detection of elementary OTU fractals.

Before constructing a fractal portrait, it is necessary to arrange the OTU profile in descending order of frequency values (Table 3). This is necessary to determine the OTU with the maximum frequency (p_{max}), that is, the OTU ranked first in the ordered OTU profile. After that, the procedure for constructing a fractal portrait of an OTU profile will be reduced to placing points (or any geometric figures) on the coordinate plane, which represent each OTU by their position on the portrait.

3. OTUs frequencies ranked in descending order in feeding groups of 36-day-old Smena cross broiler chickens under probiotic feeding trials (n = 25, Zagorskoe EPH, Moscow Province, 2018)

Group					
1кс		2e		3e	
No. OTU	frequency, %	No. OTU	frequency, %	No. OTU	frequency, %
6	26.7	5	39.4	5	31.7
7	25.3	6	20.8	6	22.3
5	24.5	7	19.3	7	19.3
17	14.8	17	13.8	17	13.6
2	3.2	2	2.3	2	4.1
14	2.0	4	1.2	14	3.2
4	1.8	14	1.2	9	1.3
10	0.6	10	0.6	4	1.0
16	0.5	3	0.4	1	0.9
9	0.4	16	0.3	10	0.8
8	0.2	8	0.2	13	0.4
		9	0.2	16	0.4
		15	0.2	11	0.3
		1	0.1	15	0.3
				3	0.2
				8	0.1
				12	0.1

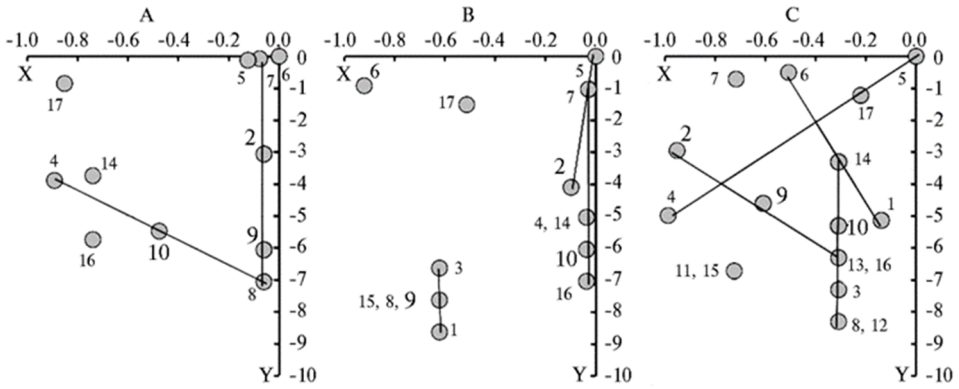
N o t e. OTU — operational taxonomic unit. OUT numbering corresponds to Table 2. 1c — control group, 2e, 3e — experimental groups. For 2e, BD was supplemented with probiotic No. 1 (10^7 CFU *Lactobacillus plantarum* per g), for 3e, BD was supplemented with probiotic No. 2 (0^7 CFU *Lactobacillus fermentum* per g), both at a dosage of 1 kg/t feed.

To determine each OTU position on the portrait (each OTU point), it is necessary to calculate their Y- and X-coordinates using the following formulas:

$$Y_i = log_2(p_i/p_{max}),\ X_i = \text{fractional part } log_2(p_i/p_{max}), \quad (1)$$

where p_i is the frequency OUT with serial number i in the OTU profile (see Table 3).

In accordance with the formulas (1) and the Table 3, we calculated the coordinates of the OTU points and constructed fractal portraits of the OTU profiles for the intestinal microbiota of the three groups of broilers under study (Fig.).



Fractal portraits of OTU (operational taxonomic unit) **profiles of gut microbiota in three feeding groups of 36-day-old Smena cross broiler chickens under probiotic feeding trials:** A — control group 1c (basal diet BD, see Table 1), B — experimental group 2e (BD + probiotic No. 1, *Lactobacillus plantarum*, 10^7 CFU/g), C — experimental group 3e (BD + probiotic No. 2, *Lactobacillus fermentum*, 10^7 CFU/g) (n = 25, Zagorskoe EPH, Moscow Province, 2018).

Y and X coordinates of OTU were calculated (1). The numbers near the circles correspond to the OTU numbering from Table 2. The numbers of lactobacilli (OTU No. 2), opportunistic Enterobacteriaceae (OTU No. 9) and Actinomycetes (OTU No. 10) are enlarged, since these are indicator groups of microorganisms to assess feed digestibility and poultry protection from pathogens. Segments of straight lines connecting the points indicate elementary OTU fractals.

In fractal portraits (see Fig.), some points are connected by segments of

straight lines. In this way, elementary OTU fractals are distinguished in the portraits, which does not contradict the definition of the elementary OTU fractal of the. For example, the logarithms of the frequencies for OTU No. 3, 9, 1 (see Fig., B) make an arithmetic series {–6.62; –7.62; –8.62}. Therefore, they represent an elementary OTU fractal and in the portrait should be connected by a straight-line segment. For similar reasons, the elementary OTU fractals are highlighted by straight-line segments, namely, the OTU Nos. 4, 10, 8 (see Fig., A) with frequencies logarithm series {–3.89; –5.48; –7.06}, OTU Nos. 10, 13, 3, 8 (see Fig., C) with {–5.31; –6.31; –7.31; –8.31}, etc. In the portraits, the elementary OTU fractals are combined into one OTU mega-fractal (see Fig. A, C) and into two OTU mega-fractals (see Fig., B).

Microbiota bioconsolidation index. Fractal portraits of OTU profiles (see Fig.) represent OTU mega-fractals of different genotype and quantitative composition. We believe that the number of OTUs in the of OTU mega-fractals (and hence the number of genotypes in the microbial biosystem) reflects the intensity of biochemical transformations of organic substrates in the bird's intestines performed by the part of microbiota arranged in a biosystem. Consequently, the more genotypes of the microbiota form the biosystem for biochemical transformation of plant substrates, the more efficient and large-scaled these transformations are, and the more nutrients the macroorganism receives. Hereof, we define the bioconsolidation index of the intestinal microbiota *Ind* in broilers as the ratio of the OUT number in the mega-fractal to total OUT in the OUT profiles:

$$Ind = N_F/N_0, \quad (2)$$

where N_F, N_0 are the number of OTUs in OUT mega-fractals and total OUT in the OUT profiles. Table 4 shows the results of bioconsolidation index calculations.

4. The mean live mass and bioconsolidation index of microbiota in 36-day-old Smena cross broiler chickens under probiotic feeding trials (n = 25, Zagorskoe EPH, Moscow Province, 2018)

Parameter	Group		
	1c	2e	3e
Average body weight, kg	2.15±0.02	2,05±0,02	2,14±0,02
Bioconsolidation index *Ind*	0.55±0.02	0,86±0,02	0,82±0,02

N o t e. 1c — control (basal diet BD, see Ttable 1), 2e, 3e — experimental groups. For 2e, BD was supplemented with probiotic No. 1 (10^7 CFU *Lactobacillus plantarum* per g), for 3e, BD was supplemented with probiotic No. 2 (0^7 CFU *Lactobacillus fermentum* per g), both at a dosage of 1 kg/t feed.

Discussion. Fractal analysis of the OTU frequency-taxonomic profiles of the broiler intestinal microbiota provides information on the size of OTU mega-fractals and on the number of microbial genotypes in the intestines that are arranged into a biosystem. The microbiota bioconsolidation index (*Ind*) is calculated as the ratio of the number of OTUs in OTU mega-fractals to the total number of OTUs in the frequency-taxonomic profiles of the intestinal microbiota. Equality to 1 of the microbiota bioconsolidation index (*Ind* = 1) means that all microbiota genotypes form biosystem to provide biotransformation, and equality to 0 (*Ind* = 0) means the absence of the biosystem organization of the microbiota in broilers. Based on the fractal analysis of the OTU frequency-taxonomic profiles, it was found that in broilers from the experimental groups the value of the microbiota bioconsolidation index (*Ind* = 0.86 in group 2e and *Ind* = 0.82 in group 3e) exceeds the microbiota bioconsolidation index in the control (*Ind* = 0.55). This means that the use of *L. plantarum* and *L. fermentum* bacteria as probiotics promotes better self-organization of the poultry intestinal microbiota.

However, the average body weight of broilers in the control and experimental groups (see Table 4) do not correlate with microbiota bioconsolidation

indices. In addition, the average body weight of broilers in the experimental group 2e (2.05±0.02 kg) was even less than in the control group 1c (2.15±0.02 kg). Perhaps this is a consequence of the unsuccessful self-organization of microbial biosystems in the intestines of birds from group 2e, which was provoked by the probiotic preparation No. 1. In contrast to the fractal portrait B (see Fig.), portraits A and C (see Fig.) contain one OTU mega-fractal each. In addition, attention should be drawn to the fact that the indicative OTUs No. 2, 9, 10 (lactobacilli, enterobacteria and actinomycetes) are separated in two unrelated OTU mega-fractals (see Fig., B) while in fractal portraits A and B (see Fig.) these OTUs are grouped in one OTU mega-fractal. Probably, the location of indicative OTUs in different OTU mega-fractals, and hence the location of indicative genotypes in different biosystems, affects the decrease in the efficiency of conversion of organic substrates and delayed development of broilers in group 2e.

Thus, here we propose methods for fractal analysis of the frequency-taxonomic profiles of operational taxonomic units (OTUs) of the intestinal microbiota in birds to assess the influence of food factors on the biosystemic self-organization of microflora and the efficiency of biochemical transformations of plant substrates. Based on the calculated bioconsolidation index of the intestinal microbiota for broilers fed diets supplemented with various experimental probiotics, *Lactobacillus fermentum* is recommended for use, since these bacteria contribute to the better self-organization of microorganisms into functional biosystems, promote the development of birds and may reduce incidence of diseases.

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