THE INFLUENCE OF A DIET SUPPLEMENTED WITH 20% RYE AND XYLANASE IN DIFFERENT HOUSING SYSTEMS ON THE OCCURRENCE OF PATHOGENIC BACTERIA IN BROILER CHICKENS*

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Abstract

Sanitary conditions and diet are important elements determining the occurrence of pathogens in animals. The aim of the research was to assess the effect of an experimental diet with rye and xylanase for broiler chickens in cages and in a free-range system on the intestinal microbiome. The study was carried out in two experimental stages, the first on 224 1-d-old male Ross 308 chickens with an initial weight of 41 g, and the second on 2000 1-d-old male chickens with an initial weight of 42 g. All birds were reared to 42 d of age and fed crumbled starter (1 to 21 d) and pelleted grower–finisher (22 to 42 d) isonitrogenous and isoenergetic diets, supplemented with 20% rye and/or 200 mg/kg xylanase. Directly after slaughter, bacteria were isolated from the cloaca of birds and identified using classical microbiological methods and MALDI-TOF mass spectrometry. The antibiotic susceptibility of the bacteria was assessed by the disc diffusion method. The study showed the presence of abundant bacteria in the gut microbiome of chickens kept in both housing systems. The most frequently isolated bacteria were *Escherichia coli*, *Enterococcus* spp., *Proteus* spp., *Campylobacter* spp., and *Staphylococcus* spp. Antibiotic resistance was significantly higher in *E. coli*, *Proteus* spp., and *Campylobacter* spp. obtained from chickens from the free-range farm, but in the case of *Enterococcus* and *Staphylococcus*, resistance was higher in bacteria from caged birds. The high antibiotic resistance among pathogens of the gastrointestinal tract necessitates the search

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for means to control the microbiome in favour of beneficial bacteria. The significant influence of rye and xylanase on the bacterial content may be the basis for the introduction of this method to support the control of pathogens.

Key words: microbiota, bacteria, feeding, broiler chickens, xylanase, rye

Environmental factors associated with livestock rearing conditions, the sanitary conditions of housing, and diet are important in determining the occurrence of pathogenic microorganisms in farm animals (Chmielowiec-Korzeniowska et al., 2020). In many cases environmental stressors may depress the immune system associated with the gastrointestinal tract (GIT), which may be critical to the growth of birds, the contents of the microbiome, and production, and can also lead to increased mortality rates. Environmental stress factors may increase the concentration of reactive oxygen species (ROS), causing lipid peroxidation and oxidative damage to cellular membranes. Disturbances in redox status can also significantly increase inflammation and colonization by pathogens (Lee et al., 2017; Huang and Lee, 2018)

At each step of poultry production these processes significantly reduce production parameters such as growth, average daily feed intake, health parameters, and the quality of food products. Moreover, economic losses associated with antibiotic therapy, the grace period, and high mortality have a significant influence on poultry production.

It should be emphasized that current EU legislation significantly limits the use of antibiotics as feed additives for poultry and other livestock raised for food. For this reason, in many cases alternative methods of improving animal health and welfare are sought, particularly diet modifications involving the use of feed additives, vitamins, minerals, or natural biostimulants contained in plants. Special attention is also paid to specific modification of the content of dry matter, protein and carbohydrates (Van et al., 2020).

Cereals such as wheat, triticale, barley, oat, and rye are commonly used in animal nutrition. However, they are not only a source of valuable nutrients, but also contain anti-nutritional factors such as non-starch polysaccharides (NSPs), which reduce digestion and the level of peptides that exert beneficial effects on gut physiology, including the microbiome (Iji et al., 2019).

Recent chemical analysis of various types of cereals carried out by Bach Knudsen (2014) and by Boros and Fraśm (2015) has shown that the content of soluble NSPs in rye, oat and triticale is much lower than in previous years, suggesting that their use in poultry nutrition could be increased. Therefore many studies have been performed on the use of a variety of feed additives as factors modifying the immune system of birds and preventing excessive inflammation and oxidative stress reactions, which will be beneficial in animal production (Arczewska-Włosek et al., 2019).

Numerous studies (Pan and Yu, 2014; Perween et al., 2016) have confirmed that modification of the animal feeding system through changes in the proportions of nutrients and nutrient supplementation can significantly affect the composition and activity of the gut microbiome and increase the activity of local or general immune

response mechanisms. For example, the use of Se and Zn in chicks significantly increases erythrocyte and leukocyte activity, which has been correlated with significant resistance to bacterial (*E. coli*) and viral (Newcastle) infection. In the case of application of β -carotene in one-day-old broiler chickens, a significant increase in T lymphocyte proliferation as well as neutrophils activity including chemotaxis has been observed (Haq et al., 1996; Cheng et al., 2004). In contrast, a reduction in the use of protein in broiler chicken diets from 18% to 14% significantly reduced the synthesis of antibodies against Newcastle disease. Other examples of feed supplements include phytochemicals, such as those contained in oregano, *Echinacea purpurea* L., cinnamon, turmeric (*Curcuma longa*) or thyme. Added to feed in the form of oil extract, essential oil, aqueous extract, pressed juice, or powder, they have varied immunomodulatory effects in broiler chickens, e.g. on the production of antibodies against various pathogens (bacteria and viruses) or white blood cell counts and activity. In many cases, significant suppression of pro-inflammatory cytokines has been observed, such as IL-1 β , IL-6, interferon- γ (IFN- γ), and TNF- α (Haq et al., 1996; Gadde et al., 2017).

Moreover, supplementation of the traditional broiler diet with feed enzymes may stimulate mechanisms of local immunity associated with gastrointestinal mucous membranes, in part through the activation of epithelial goblet cells and synthesis of class A immunoglobulins, which significantly reduces colonization by pathogenic bacteria that may pose a risk to humans (Neto et al., 2013).

In view of the above, the aim of the research was to assess the effect of an experimental feeding system with rye and xylanase in cage and free-range systems for broiler chickens on intestinal microbiome diversity.

Material and methods

Birds and experimental procedure

The experiment was conducted in two stages. The first stage was carried out in vivarium conditions using a cage housing system, and the second stage was carried out on a farm in a free-range housing system with fresh straw bedding, both in compliance with Council Directive 2007/43/EC of 28 June 2007 (2007).

A total of 224 1-d-old male Ross 308 chickens were used in the first experiment. The chickens were obtained from a commercial hatchery and had an average initial weight of 41 g. The birds were housed in wire-floored cages in an environmentally controlled room in the poultry house at the Experimental Station of the National Research Institute of Animal Production in Balice, Poland. In the second stage of the experiment, 2000 1-d-old male Ross 308 chickens with an average initial weight of 42 g were obtained from a commercial hatchery. The birds were housed in an environmentally controlled facility on a commercial farm in Silesia, Poland. During the first and second stages of the study, the temperature at the experimental facility and on the farm was initially 32°C (at 1 d of age) and was gradually decreased to 21°C (from 21 d of age). Relative humidity was cycled between 50% and 60%,

air exchange was 1 m³/kg of BWG/h, and the concentrations of CO₂ and NH₃ were maintained below 2000 and 20 ppm, respectively. The chickens from the first stage of the experiment were randomly assigned to four treatments, each comprising seven replicate cages, with eight birds per cage (7800 cm² total floor space in the cage). In the second stage of the experiment, the chickens were randomly divided into a control group and a group receiving a diet containing rye (maximum stocking density not exceeding 33 kg/m²).

Table 1. Composition and nutrient content of experimental diets, g/kg air-dry matter

Item	Starter (1–21 d)		Grower-finisher (22–42 d)	
	control	rye-containing diet	control	rye-containing diet
Rye	0	200	0	200
Corn	457.1	302.1	404.4	238.4
Wheat	100	40	200	150
Soybean meal	370	370	306	307
Rapeseed oil	33	48	52	67
Limestone	13.5	13.5	14	14
Monocalcium phosphate	15	15	13	13
NaCl	3	3	3	3
DL-Methionine	2.6	2.6	2.3	2.3
L-Lysine HCl	0.8	0.8	1.7	1.7
L-Threonine	—	—	0.6	0.6
Vitamin-mineral premix ¹	5	5	3	3
Calculated composition:				
Metabolizable energy (MJ/kg ²)	12.6		13.1	
Crude protein	225		205	
Lys	12.3		11.5	
Met	5.8		5.25	
Thr	8.5		8.1	
Ca	9.7		9.3	
Total P	7.1		6.6	
Available P	4.5		4.1	

¹The premix provided, per 1 kg of starter diet: vitamin A (retinol), 3.75 mg; vitamin D₃ (cholecalciferol), 1.25 mg; vitamin E (alpha-tocopherol), 125 mg; vitamin K₃ (menadione), 3 mg; vitamin B₁ (thiamine), 3 mg; vitamin B₂ (riboflavin), 8 mg; vitamin B₆ (pyridoxine), 4 mg; vitamin B₁₂ (cyanocobalamin), 0.02 mg; biotin, 0.2 mg; Ca-pantothenate, 16.3 mg; niacin, 50 mg; folic acid, 2 mg; choline chloride, 348 mg; manganese, 100 mg; zinc, 100 mg; iron, 50 mg; copper, 20 mg; iodine, 1 mg; selenium, 0.35 mg; coccidiostat: 100 ppm of total narasin/nicarbazin activity; per 1 kg of finisher diet: vitamin A (retinol), 3.0 mg; vitamin D₃ (cholecalciferol), 0.75 mg; vitamin E (alpha-tocopherol), 30 mg; vitamin K₃ (menadione), 2.5 mg; vitamin B₁ (thiamine), 2.5 mg; vitamin B₂ (riboflavin), 5 mg; vitamin B₆ (pyridoxine), 3.51 mg; vitamin B₁₂ (cyanocobalamin), 0.021 mg; biotin, 0.201 mg; Ca-pantothenate, 13 mg; niacin, 35 mg; folic acid, 1.0 mg; choline chloride, 300 mg; manganese, 80 mg; zinc, 90 mg; iron, 40 mg; copper, 20 mg; iodine, 0.5 mg; selenium, 0.2 mg; coccidiostat: narasin, 70 ppm.

²Calculated according to European Table (Janssen, 1989) as a sum of the ME content of components.

From 1 to 42 d of age, all chickens were provided with water and feed *ad libitum*. All birds were reared to 42 d of age and fed crumbled starter (1 to 21 d) and pelleted grower–finisher (22 to 42 d) isonitrogenous and isoenergetic diets, which were formulated to meet or exceed the nutrient requirements of broilers as described in an earlier study (Arczewska-Włosek et al., 2019; Janssen, 1989). The content of the starter and grower–finisher diets is shown in Table 1.

In the first stage of the experiment, the chickens were divided into four groups with seven replicate cages containing eight birds per cage, and in the second stage, conducted in free-range farm conditions, the birds were divided into two groups – a control group (n =1000) and a rye group (n =1000), which received 20% rye and xylanase. At the end of the first stage of the experiment, and after 12 h of fasting, all chickens were individually weighed, and eight representative birds (one bird each from six replicate cages and two from the last replicate cage) with live body weights close to the group average were chosen from each group, tagged with numbers, and decapitated after electrical stunning (150 mA, frequency of 200 Hz for 4 s). At the end of the second stage of the experiment, 56 birds were randomly chosen from each group and individually weighed. Ten representative birds (from each group) with live body weights close to the group average were selected for sampling. The design of the experiment is presented in Table 2.

Table 2. Experimental groups of broiler chickens

Group of chickens	Diet and supplementation
Group 1 a – control	Starter and grower–finisher without supplements rye and xylanase
Group 2 a	Starter and grower–finisher with 20% rye
Group 3 a	Starter and grower–finisher with 200 µg/kg xylanase
Group 4 a	Starter and grower–finisher with 20% rye and 200 µg/kg xylanase
CON Group 1 b – control	Starter and grower–finisher without supplements
Group 2 b	Starter and grower–finisher with 20% rye and 200 µg/kg xylanase

Legend: a – laboratory conditions using a cage housing system; b – free-range housing system.

Rye grain was ground using a sieve with 5 mm mesh size. A 2 x 2 factorial design was used, with two dietary levels of the Brasetto variety of ground rye (0% and 20%). All diets were either supplemented with xylanase (200 mg/kg of feed; Ron-ozyme WX, (CT) with minimum xylanase activity of 1000 FXU/g (DSM Nutritional Products Sp. z o.o., Mszczonów, PL) or not supplemented with xylanase.

At the end of the experiment, all chickens were individually weighed, and eight representative birds from all replicates with live body weights close to the group average (2.63 ± 0.27 kg for control groups and 2.64 ± 0.32 for experimental groups) were chosen from each group and decapitated following electrical stunning (150 mA, frequency of 200 Hz for 4 s). The entire intestines were removed, and samples were taken from the cloaca contents. In the second stage of the experiment, cloacal swabs were taken before slaughter. Samples were frozen in liquid nitrogen, transported to the laboratory, and kept at -80 °C until microbiome analysis.

Isolation and morphological examination of bacteria

Bacteria were isolated directly from chicken faeces samples after slaughter in a slaughterhouse. First the samples were plated on enrichment agar with 5% sheep blood or without blood and on selective differential media and incubated at 37°C in various aerobic or anaerobic conditions. Anaerobic bacteria were isolated on 5% sheep blood agar and anaerobically incubated at 37°C for 48 hours.

Enterococcus strains were isolated on the selective differential media Bile Esculin Azide Lab-Agar (BIOCORP, PL) and Blood Lab Agar (BIOCORP, PL), supplemented with 5% defibrinated horse blood, at 37°C for 24–48 h under microaerophilic conditions. The bacterial isolates were initially characterized based on their colony morphology, Gram-stained morphology, the presence and type of haemolysis, and catalase production.

Campylobacter spp. strains were isolated on Bolton Broth Base (Oxoid Ltd., UK) with Bolton Broth Selective Supplement (Oxoid Ltd., UK) and 5% haemolysed horse blood (ProAnimali, PL). The bacterial cultures were incubated at 37°C for 48 h in microaerophilic conditions (CampyGen system, Oxoid Ltd., UK). Next, the cultures were transferred to selective mCCDA agar (Campylobacter Blood-Free Selective Agar Base, Oxoid Ltd., UK) with CCDA Selective Supplement (Oxoid Ltd., UK) and incubated at 41.5°C for 48 h in microaerophilic conditions. Then, single colonies resembling *Campylobacter* spp. were transferred to Karmali enrichment medium (Campylobacter Agar Base, Oxoid Ltd., UK) with Campylobacter Selective Supplement and incubated at 41.5°C for 24 h in a microaerophilic atmosphere. *Campylobacter* isolates were stored at –80°C in Microbank kits for storing microbes (PL.170/M, Biocorp, PL).

Staphylococcus spp. strains were isolated on two types of substrate: mannitol agar (Chapman medium) and Columbia agar with 5% sheep blood, at 37°C for 24 h. The resulting cultures were then incubated in TSB broth (BTL, PL) at 37°C for 24 h to obtain optimal growth of pure strains in liquid medium. Phenotypic identification of the bacterial isolates was carried out by Gram staining and with STAPH API biochemical tests (Marek et al., 2016).

The total number of bacteria was expressed as colony-forming units per millilitre (CFU/mL). The samples obtained from chickens were diluted from 10^{-1} to 10^{-9} in duplicate and incubated in various media specific for a given bacterial species.

Species identification of bacterial isolates by MALDI-TOF MS

Measurements were performed using an UltrafleXtreme MALDI-TOF MS mass spectrometer (Bruker, GER) with a 1000 Hz neodymium-doped yttrium aluminium garnet (Nd:YAG) laser. Single bacterial colonies grown on agar were re-suspended in 1.2 ml of 75% ethanol. After centrifugation at 13,000 g for 2 min at 20°C and removal of the supernatant, cells were extracted with 50 µl of formic acid (Sigma-Aldrich, PL) and 50 µl of acetonitrile (Sigma-Aldrich, PL). After centrifugation, each sample was transferred onto a spot of the 384 MTP AnchorChip TF stainless steel MALDI target plate (Bruker, GER). Then the bacterial sample was overlaid with 1 µl of matrix solution containing 10 mg/ml HCCA (α-cyano-4-hydroxycinnamic acid, Sigma-Aldrich, PL) resolved in 50% acetonitrile and 2.5% TFA (trifluoroacetic acid, Sigma-Aldrich, PL) and air-dried. The MALDI plate was then placed in the spec-

trometer for automated measurement and data interpretation. Prior to the analyses, calibration was performed with a bacterial test standard (Bruker, GER) containing extract of *Escherichia coli* DH5 alpha (Dec et al., 2014). The mass spectra were processed with the MALDI Biotyper 3.0 software package (Bruker, GER), containing 3995 reference spectra corresponding to different types of bacteria.

The results were shown as the top 10 identification matches with confidence scores ranging from 0.00 to 3.00. A log (score) < 1.70 does not allow for reliable identification; a log (score) of 1.70–1.99 allows for identification to the genus level; a log (score) of 2.00–2.29 means highly probable identification to the genus level and the species level; and a log (score) > 2.30 indicates highly probable identification at the species level (according to the manufacturer's instructions).

Evaluation of the antibiotic susceptibility of bacteria by the disc diffusion method

The drug resistance profile of isolates identified as *E. coli* was assessed by the disc diffusion method on Mueller-Hinton agar (Oxoid Ltd.) as described in CLSI, 2015 (2015).

For *Campylobacter* spp., the test was performed on Mueller-Hinton agar supplemented with 5% sheep blood according to the manufacturer's instructions. Inocula were prepared by incubating the strains for 24 h at 42°C under microaerobic conditions in trypticase soy broth.

The following discs were used: enrofloxacin (ENR 30 µg), ampicillin (AMP 10 µg), amoxicillin (AML 25); amoxicillin/clavulanic acid (AMC 30 µg), cephalixin (CT 30 µg), sulfamethoxazole/trimethoprim (SXT 25 µg), tetracycline (TE 30 µg), nalidixic acid (NA 30 µg), ciprofloxacin (CIP 5 µg), erythromycin (E 15 µg), gentamicin (CN 10 µg), streptomycin (S 10 µg), tylosin (TY 30 µg), neomycin (N 30 µg), clindamycin (DA 2 µg); florfenicol (FFC 30 µg), vancomycin (VA 30 µg), lincomycin/spectinomycin (LS 109 µg), kanamycin (K 30), tobramycin (TOB 10), colistin sulphate (CT 25), doxycycline (DO, 30), and amikacin (AK, 30).

The choice of antibiotic discs used to test antibiotic susceptibility depended on the bacteria, according to the CLSI. For quality control, reference strains for antibiotic disc control were used: *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212 and *C. jejuni* ATCC 33560. An isolate was considered multi-drug resistant if it showed resistance or intermediate resistance to two or more antimicrobials (Capita, 2007; Marek et al., 2015).

Zones of growth inhibition were evaluated as susceptible or resistant according to the CLSI Guidelines for Susceptibility Testing. The procedures of Marek et al. (2015, 2016), Dudzic et al. (2016), Nowaczek et al. (2019) and Stępień-Pyśniak et al. (2016) were followed.

Statistical analysis

Statistical analysis of the results was performed using Statistica 10.0 software. First, the non-parametric chi-square test was used. The relationship between the diet and the content of the microbiota was determined using Pearson's correlation coefficients and non-parametric tests at $P \leq 0.05$.

Results

The research confirmed the presence of abundant bacteria in the gastrointestinal tract of broiler chickens kept in experimental laboratory conditions as well as in the standard conditions of a large farm. The total bacterial content ranged from 1.1×10^2 CFU/mL to 4.6×10^9 CFU/mL. The bacteria most frequently isolated from the samples were *E. coli*, *Enterococcus* spp., *Proteus* spp., *Campylobacter* spp., and *Staphylococcus* spp. (Table 3). The most abundant bacteria were isolated from the chickens raised in farm conditions. In most cases, the same bacteria, i.e. strains of *E. coli*, *Enterococcus* spp., and *Proteus* spp., were found in all test samples in similar concentrations. By far the most strains were isolated from the birds from the control groups, while in the other groups the numbers of various bacteria were significantly varied (Table 3).

Table 3. The differences between the number of bacteria (CFU/g) isolated from experimental groups of broiler chickens

Kind of bacteria and total numbers CFU/mL	Experimental groups						P-value $P \leq 0.05$
	CON a	XYL a	RYE a	XYL+RYE	CON b	XYL+RYE b	
<i>Proteus</i> spp.	4.5×10^7	1.1×10^7	2.5×10^7	2.6×10^7	4×10^8	4.5×10^8 b	0.043
<i>E. coli</i>	4.5×10^8	4.2×10^8	4.5×10^8	4.4×10^8	4.6×10^8	4.5×10^8	0.56
<i>Staphylococcus</i> spp.	4.2×10^8	2.5×10^6 a	3.2×10^2 a	1.2×10^2 a	3.2×10^6	1.2×10^2 ab	0.01
<i>S. aureus</i>	2×10^4 ab	nd	nd	nd	nd	nd	0.003
<i>C. perfringens</i>	1.2×10^4	nd	nd	nd	2.4×10^6	2.1×10^2 ab	0.04
<i>Enterococcus</i> spp.	4.4×10^7	4.5×10^7	3.2×10^7	4×10^7	4.5×10^8	4×10^8 b	0.05
<i>E. faecalis</i>	3×10^6	4×10^4 a	4×10^4 a	4×10^4 ab	nd	nd	0.008
<i>E. faecium</i>	nd	nd	nd	nd	3.2×10^4	3×10^4 b	0.003
<i>E. durans</i>	1×10^2	1×10^1 a	2×10^2	2×10^2	2×10^2	5×10^1 ab	0.001
<i>E. hirae</i>	2×10^3	3×10^3	1×10^1 a	1×10^2	1×10^1	1×10^1 b	0.001
<i>Campylobacter</i> spp.	2.5×10^6	2×10^2	4×10^2	nd	4×10^7	2×10^2 ab	0.016
<i>C. jejuni</i>	3×10^4	1.2×10^2 a	1.2×10^2	nd	2×10^2 ab	nd	0.049
<i>C. coli</i>	2×10^1 a	nd	2.2×10^1	nd	3×10^6	2.5×10^2 ab	0.033

Legend: Experiment in cage systems. CON a – control – chickens received standard diet (starter continued with grower-finisher without rye and xylanase; XYL a – chickens received starter continued with grower-finisher with 20% rye; RYE a – chickens received starter continued with grower-finisher with 200µg/kg xylanase; XYL+RYE – chickens received starter continued with grower-finisher with 20% rye and 200µg/kg xylanase. Experiment in free-range housing system. CON b – control group of chickens in farm conditions; XYL+RYE b – the experimental group of chickens in a free-range farm system feeding with experimental diet with supplement of rye and xylanase both.

Nd – not detected (no bacterial growth was observed).

a – values within a row with different feeding significantly at $P < 0.05$.

b – values within a row with different breeding system differ significantly at $P < 0.05$.

The chicken diet had no significant ($P \leq 0.05$) effect on the number of isolated *Proteus* spp., *E. coli* or *Enterococcus* spp. strains, but the number of *Staphylococcus*, *Clostridium* and *Campylobacter* spp. strains isolated from groups whose diet was supplemented with rye and xylanase was significantly lower than in the control groups. Analysis of the correlation between these two parameters showed a moderate

relationship or none at all between the diet and bacterial content, with coefficients of $r = 0.39, 0.35, 0.42, 0.49, 0.52$ and 0.48 , respectively. However, there was a significant correlation between the housing system and bacterial content, with correlation coefficients of ≥ 0.55 for all pathogens except the *E. coli* strains, for which the correlation coefficient was $r = 0.32$ and was not statistically significant. Examples of selected bacterial strains grown on agar plates are shown in Figure 1.

A – *Escherichia coli* strains grown on MacConkey agar, B – *Staphylococcus* spp. strains grown on Chapman agar, C – *Enterococcus* spp. grown on Bile Esculin Azide Agar.

Figure 1. Examples of selected isolates of bacterial species grown on agar plates

The most numerous group of bacteria comprised strains of *E. coli*, with 52 isolates identified based on the morphological characteristics of colonies grown on selective media. The total number of *E. coli* isolates was 10^8 CFU/mL. It should be emphasized that these bacterial strains were present in similar concentrations in all animals from which material was obtained in the form of faeces or cloaca contents. Species identification by MALDI-TOF MS mass spectrometry also confirmed 52 *E. coli* isolates. The probability of correct identification in the MALDI Biotyper 3.0 system is expressed as a score: 2.000–2.999 – reliable identification to the genus level and probable identification to the species level; 1.700–1.999 – probable identification to the genus level; 0–1.699 – unreliable identification. For all *E. coli* strains ($n = 52$), the identification score was higher than 2.300, which indicated a very high probability of correct identification to the species level.

A similarly high number of bacteria were isolated in the case of *Enterococcus* spp., with a total of 50. MALDI TOF MS identified four species of enterococci: *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae*. The predominant *Enterococcus* species among strains from poultry raised in the cage system was *E. faecalis* ($n = 18$), which was isolated only from birds raised in laboratory cage conditions, irrespective of diet. There was also a high number of strains of *E. faecium* ($n = 11$), which was isolated only from poultry raised in free-range farm conditions. In the case of *E. durans*, the number of isolates was comparable in the caged and farm groups, with six and seven, respectively (Table 4). MALDI TOF MS spectrometric analysis identified a high percentage of strains in the log (score) range of 2.000–2.299, with 86% of all isolates initially classified as *Enterococcus* spp., 89% of *E. faecalis* strains, 82% of *E. faecium*, 80% of *E. durans*, and 88% *E. hirae* strains in the range of the highest spectral values (Table 4).

Table 4. The mean log (score) results of MALDI-TOF MS analysis for bacteria isolated from broiler chickens

Log (score)	Description	Symbol	Number of test strains											
			Ec	Cp	St	Pr	En	Ef	Efa	Ed	Eh	Cam	Cj	Cc
2.000–2.299	Secure genus identification and species identification	+++	52	12	12	28	43	16	9	11	7	16	6	10
1.700–1.999	Genus identification and medium species identification	++	0	3	2	6	5	2	1	1	1	3	2	1
< 1.700	Genus identification and low species identification	+	0	–	1	5	2	0	1	1	0	2	0	2

Legend: Ec – *Escherichia coli*; Cp – *Clostridium perfringens*; St – *Staphylococcus* spp.; Pr – *Proteus* spp.; En – *Enterococcus* spp.; Ef – *Enterococcus faecalis*; Efa – *Enterococcus faecium*; Ed – *Enterococcus durans*; Eh – *Enterococcus hirae*; Ca – *Campylobacter jejuni*; Cc – *Campylobacter coli*.

Table 5. Antibiotic resistance profiles of examined bacteria isolates

Kind of antibiotic	Numbers of resistant and susceptible bacteria													
	<i>E. coli</i> (n=52)		<i>Staphylococcus</i> spp. (n = 16)		<i>Enterococcus</i> spp. (n = 50)		<i>Campylobacter</i> spp. (n = 22)		<i>Clostridium perfringens</i> (n = 15)					
	R	S	R	S	R	S	R	S	R	S	R	S		
Amoxycillin AML25 Amoxycillin/clavulanic acid AMC30 Ampicillin AMP10 Amikacin AK30 Doxycyline DO30 Enrofloxacin ENR5 Florfenicol FFC30 Colistin Sulphate CT25 Lincomycin/spectinomycin LS109 Sulphamethoxazole/trimethoprim SXT25 Tylosin TY30 Cefaloxim CT Erythromycin E 15 Gentamycin CN 10 Tetracycline TE Clindamycin Streptomycin S 25 Vancomycin VA 30 Tobramycin TOB 10 Neomycin N 30 Kanamycin K 30 Ciprofloxacin CIP 5	28 (53.8%) 4 (7.69%) 11 (21.15%) 23 (44.23%) 15 (28.84%) 42 (80.76%) 11 (21.15%) 2 (3.84%) 1 (1.92%) 8 (15.38%) 52 (100%) 0 — 20 (38.46%) 32 (61.5%) — — — — — — —	24 48 41 29 37 10 41 50 50 46 0 52 — 32 20 — — — — — — —	13 (81.25%) 11 (68.75%) 14 (87.5%) 3 (18.75%) 2 (12.5%) — 6 (37.5%) — — 13 (81.25%) 15 (93.75%) 11 (68.75%) 3 (18.75%) 12 (75%) 9 (56.25%) 2 (12.5%) 1 (6.25%) 7 (43.75%) 4 (25%) 15 (93.75%) 4 (25%)	3 5 2 13 14 — 10 — — 3 — 1 5 13 3 6 14 15 9 12 1 12	0 0 2 (4%) 2 (4%) 37 (54%) 29 (58%) 1 (2%) 50 (100%) 30 (60%) 50 (100%) — 50 (100%) 20 3 42 (84%) 39 (78%) — 1 (2%) — — — —	50 50 48 48 13 21 0 0 20 0 0 0 8 11 11 — — 49 — — — —	7 (72.3%) 5 (22.7%) 8 (36.4%) — — 4 (18.2%) — — — 3 (13.63) — — — 6 (27.3%) — 6 (27.3%) — — — — —	15 17 14 — — 18 — — — 19 — — 10 16 — 16 — — — — —	3 (20%) 1 (6.66%) 6 (40%) — — 11 (73.3%) 2 14 (93.3%) 15 (100%) 14 (93.3%) — 8 (53.3%) 6 (74.66%) 15 (100%) 11 (50%) 6 (27.3%) — 6 (27.3%) — — — —	— —				

Legend: R – resistance, S – susceptibility, „—“ – not detected.

Figure 2. Examples of MALDI TOF MS spectra images from the detection of bacteria. In the case of *Proteus* spp., significantly higher ($P \leq 0.05$) numbers were observed in broilers kept in the free-range system

The statistical analysis of the results showed significant differences ($P \leq 0.05$) in the content of all *Enterococcus* spp. strains in birds kept in the free-range system.

In the case of *Campylobacter* spp., *Clostridium perfringens*, and *Staphylococcus* spp., the number of isolates obtained from the chickens raised on the farm was higher than in birds raised in experimental conditions. The results also confirmed a significantly lower ($P \leq 0.05$) total number of *Clostridium* pathogens in broilers from group 2b, which received feed with rye and xylanase, in comparison to the control.

Among *Campylobacter* spp. isolated from chickens reared in cages, the dominant species was *C. jejuni* (about 3×10^4 CFU/mL), while in the case of birds raised in farm conditions, *C. coli* (about 3×10^6 CFU/mL) was the most common. MALDI

TOF MS spectrometric analysis confirmed a high percentage of strains identified to the species level, with a log (score) of 2.000–2.299 for 76% of both *C. jejuni* and *C. coli* (Table 4). Examples of spectra images from the detection of bacteria are shown in Figure 2.

Legend: A – *Enterococcus* spp. strains, B – *Staphylococcus* spp. strains, C – *E. coli* strains, D – *Campylobacter* spp. strains.

Figure 3. Examples of antibiograms of selected strains grown on Mueller-Hinton agar

Analysis of the antibiotic susceptibility of the bacterial strains showed a significant ($P \leq 0.05$) percentage of strains resistant to the antibiotics. Selected examples of antibiograms of strains grown on Mueller-Hinton agar are shown in Figure 3.

The highest resistance was displayed by strains of the genus *Enterococcus*, which showed 100% resistance to four of 16 antibiotics tested (sulfamethoxazole/trimethoprim, tylosin, cephalexin and colistin). In addition, 100% of *Clostridium perfringens* strains showed resistance to another four antibiotics (lincomycin/spectinomycin, erythromycin, gentamicin and streptomycin) and 93.3% to another three antibiotics (doxycycline, colistin and sulfamethoxazole/trimethoprim). High multi-drug resistance was also found for *Staphylococcus* spp. strains, which showed a high level of resistance (88–99%) to three of 18 antibiotics. Two bacterial strains (one *Enterococcus* spp. and one *Staphylococcus* spp.) were resistant to vancomycin. In the case of *E. coli* strains, 100% resistance to tylosin was observed. A detailed analysis of the antibiotic resistance of the tested bacterial strains is presented in Table 5.

The research also found significant variation in the antibiotic resistance of the test strains depending on the rearing system. The isolates obtained from birds raised under farm conditions showed significantly higher resistance (*Campylobacter* spp., *Clostridium* spp. and *E. coli*), while in the case of *Enterococcus* spp. and *Staphylococcus* spp., strains from birds raised under laboratory conditions were more resistant to the antibiotics.

Discussion

The occurrence of abundant bacteria posing a threat to the health and life of humans and animals is a very important health and economic problem in poultry production. Effective control of infections and carriage, in particular of *Staphylococcus* spp., *Enterococcus* spp., *Campylobacter* spp., *Clostridium* spp., and pathogenic

strains of *E. coli*, seems impossible. Legislative restrictions on the use of feed antibiotics in EU countries since 1 January 2006 (Regulation 1831/2003/EC) and the increasingly common phenomenon of drug resistance in bacteria necessitate the search for alternatives to antibiotics or supportive preventive methods to control the gut microbiome of poultry. In many cases, numerous feed additives are used, including additives of plant origin, vitamins, or feed enzymes, which increase the resistance of birds to colonization by infectious agents by modifying the intestinal environment and immune status.

According to Perween et al. (2016), different levels of energy and protein in the diet of one-day-old chicks have a significant influence on growth parameters, such as body weight gains and feed conversion ratio. The authors also achieved a significant gradual increase in antibody titre against Newcastle disease virus. Improvement in the immune status of animals translates into increased resistance to infection, increased weight gains resulting from better feed conversion, and lower production costs. Many studies (Carter et al., 2009; Chee et al., 2010; Chambers and Gong, 2011; Mead, 2000; Timbermont et al., 2010) have shown that various diet supplements, such as probiotics (live microorganisms), prebiotics (substances that stimulate beneficial microbial activity in the digestive system), and phytobiotics (primary or secondary components of plants with bioactive compounds) can regulate microbial diversity and have a beneficial effect on the growth and health of animals.

The present study showed that the addition of feed enzymes to poultry feed, in this case xylanase, especially in combination with 20% rye, had a significant effect on the content of pathogenic or conditionally pathogenic bacteria in the gut of poultry. Moreover, differences were observed in the concentrations of specific bacterial species between the cage and free-range housing systems. This confirms that the housing system is another important factor determining the occurrence of certain bacteria, such as *Proteus* spp., *Staphylococcus* spp., *Campylobacter* spp., *Enterococcus* spp., and *Clostridium perfringens*. The present study showed a much higher number of microorganisms in the case of the free-range system. On the other hand, diet supplementation with 20% rye and 200 mg/kg xylanase significantly reduced the presence of these bacteria. Such a significant (about 2 or 3 log₁₀ CFU) reduction in the occurrence of pathogenic or conditionally pathogenic bacteria (for humans as well) is a very promising preventive element in poultry production. Control of the intestinal microbiome is the most important factor in poultry production.

According to Aland and Madec (2009), bacteria in the host can be divided into dominant bacteria (>10⁶ CFU/g sample), subdominant bacteria (10³ to 10⁶ CFU/g sample), and residual bacteria (<10³ CFU/g sample). Enteric pathogens present in the environment may attach to and damage the intestinal mucosal layer and cause infection in new hatchlings, which lack a normal gut microbiome. In older birds, an increase in pathogens altering the normal microbiome affects feed intake, average weight gains, health, and immune status. Modification of the quality of poultry feed to control colonization of the gut microbiome, in order to protect consumer health and reduce production losses, has been the subject of many studies. Pan and Yu (2014) suggest that bacterial synthesis of vitamins, especially B vitamins, may prevent coprophagy in chickens housed in wire cages, which have greater vitamin

requirements than chickens raised on hard floors. This was also correlated with differences in intestinal microbiome content. For example, diets with high percentages of protein (>19%) obtained from animal-based feed such as fishmeal stimulate the growth of *C. perfringens* strains in the hind-gut of chickens, which is the main predisposing factor of necrotic enteritis (Drew et al., 2004). Knarreborg et al. (2002) found that *C. perfringens* was more abundant in the ileum of broiler chickens fed a diet with animal fat (a mixture of lard and tallow) than in chickens fed a diet with soybean oil.

Another study (Mitsch et al., 2004) has shown that plant-derived trans-cinnamaldehyde and eugenol were effective in reducing *Salmonella* Enteritidis colonization in 20-d-old broiler chickens. Supplementation with essential oils containing thymol, carvacrol, eugenol, curcumin, and piperine has also been shown to reduce colonization and proliferation of *C. perfringens* in the gut of broiler chickens. The use of prebiotics in chickens provides evidence of positive effects by inhibiting colonization by pathogenic *Salmonella* and *E. coli* (Chambers and Gong, 2011; Stanley et al., 2013).

Alfalfa used as a diet supplement in hens has been found to reduce *Salmonella* colonization and modify bacterial profiles in caecal or faecal samples, which is crucial in the control of bacterial contamination (Dunkley et al., 2007).

Phytobiotics (or essential oils) have been reported to modulate the microbiota, with the potential to replace antibiotics as growth promoters (Franz et al., 2010; Hashemi and Davoodi, 2011). According to Yadav and Jha (2019), feed ingredients and additives such as endogenous enzymes (e.g. xylanase or β -glucanase) modulate the gut microbiota and immune system activity of poultry, resulting in better growth performance and making it possible to reduce or eliminate the use of antibiotics in feed. Yang et al. (2008) confirmed the growth-promoting effects of enzymes, including xylanase, linking it to the mucosal morphology of the small intestine. The results also showed a reduction in the crypt depth in the jejunum together with the increase in membrane enzyme activity.

Tellez et al. (2014) reported that rye supplementation significantly increased the total number of *Lactobacillus* strains in the duodenum, ileum, and caecum relative to chickens fed maize.

In the present study, a significant percentage (>75%) of strains were resistant to more than three antibiotics. The bacteria were resistant to different groups of antibiotics, including aminoglycosides, fluoroquinolones, lincosamides, and tetracyclines. By far the highest percentage of resistant strains was found in birds from the two control groups and in the chickens raised in free-range farm conditions whose feed was supplemented with rye.

A worrisome finding in this study is the occurrence of 100% resistance among *Enterococcus* spp. and *Clostridium perfringens* strains to four chemotherapeutic agents belonging to different groups, including sulphonamides with trimethoprim, tylosin, cephalosporin and colistin. It should be emphasized that *Enterococcus* spp. are among the most numerous species in the intestinal microbiome in poultry. Stępień-Pyśniak et al. (2016) obtained a similarly high percentage of resistance of *Enterococcus* spp. isolated from various poultry species (including broiler chickens)

to tylosin (75%), sulphonamides (88%) and lincomycin/spectinomycin (56%). Gad et al. (2011) observed 100% resistance to colistin in *Clostridium* strains. However, the percentage of resistance to other antibiotics was lower, or the strains were susceptible, as in the case of lincomycin/spectinomycin.

Enterococcus spp. and *Clostridium* spp. could pose a threat to consumers as potential pathogenic agents. The presence of two vancomycin-resistant bacterial isolates of the genera *Enterococcus* and *Staphylococcus* is very dangerous as well. Resistance of *Enterococcus* spp. strains to vancomycin was also observed in 2013 in a study by Sánchez Valenzuela et al. (2013). In the case of *E. coli* isolates, the results of the present study showed that all strains were resistant to tylosin, and a high percentage (80.76%) of the strains were resistant to enrofloxacin. The high resistance of *E. coli* to tylosin observed in the study confirms results reported by Talebiyan et al. (2014). Bakhshi et al. (2017) have also shown significant resistance (78%) of *E. coli* isolates to enrofloxacin and 100% resistance to tylosin.

The results of the present study confirm the significant problem of antibiotic resistance of pathogens isolated from broilers and show that a diet modified by the addition of rye and xylanase has a positive effect on microbiome content, which could make it possible to reduce the use of antibiotics in poultry production.

Conclusions

To sum up, the study confirmed that the experimental diets and housing systems for broiler chickens significantly affected the diversity of the intestinal microbiome. The high antibiotic resistance among pathogens of the gut microbiome in poultry and the widespread threat to humans and animals necessitates the search for methods to control the microbiological composition of the gut of poultry to favour beneficial, probiotic microbiota. The significant influence of the feed additives used in the study, i.e. 20% rye and 200 mg/kg xylanase, on the content of the microbiome may be the basis for the introduction of this feeding method to support the control of pathogens in poultry. This will undoubtedly help to reduce the use of antibiotics in poultry production, thus improving the consumer value of the raw materials.

Abbreviations

ATCC – American Type Culture Collection; CFU – colony-forming unit; CLSI – Clinical and Laboratory Standards Institute; cm – centimeter; CO₂ – carbon dioxide; d-old – day old; EU – European Union; FXU – Fungal Xylanase Unit; g – gram; GIT – gastrointestinal tract; Hz – hertz; IL – interleukin; IFN – interferon; kg – kilogram; log – logarithm; m – meter; mA – milliamperes; MALDI-TOF MS – matrix assisted laser desorption and ionization mass spectrometry; µl – microlitre; NH₃ – ammonia; NSPs – non-starch polysaccharides; ppm – parts per million; ROS – reactive oxygen species; TNF – tumour necrosis factor; TSB – tryptic soy broth.

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Ethics approval

All procedures with animals received prior approval from the Local Ethics Committee 71/2011.

Conflict of interest

We declare that we have no financial or personal relationships with other people or organizations that can inappropriately influence our work and that there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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