Investigations on Effect of Bacillus *Licheniformis* BL11 Probiotic Formula on Antimicrobial Resistance in Commensal Poultry *E. Coli* Isolates

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Abstract. *The aim of the present study was to investigate phenotype resistance profiles and some genetic determinants in resident E. coli bacteria isolated from broilers whose ration was supplemented with a probiotic formula containing a Bacillus licheniformis BL11 strain (Huvepharma, Belgium).*

For bacteriological examination, cloacal swabs were collected at various intervals throughout the study: from day-old, 14-day-old and 28-day-old chickens. A total of 300 swabs were collected for bacteriological examination: 150 from control and 150 from probiotic-supplemented chickens. The total number of E. coli strains isolated from control and experimental broilers was 214: 107 strains from the control group and another 107 from birds that received a probiotic with the feed.

Among E. coli isolates from day-old broilers in the experimental group, the highest resistance rate was observed against gentamicin (69.0%), followed by that against ampicillin and amoxicillin/clavulanic acid (52.4%). Escherichia coli isolated from probiotic-supplemented broilers at 14 days of age demonstrated statistically significantly higher rate of resistance ($P \leqslant 0.05$) against cefotaxime (51.6%) and ceftazidime (38.7%) compared with isolates from the control group (12.0%). Also, the prevalence of E. coli strains resistant against amoxicillin/clavulanic acid in supplemented broilers (35.5%) was insignificantly lower than the respective rate in control chickens (48.0%). At 28 days of age, the resistance against ciprofloxacin in poultry E. coli isolates in probiotic-fed broilers was significantly higher ($P \leqslant 0.01$) than the resistance rate in non-supplemented birds (85.3% and 52.7%, respectively). The resistance to ampicillin among the isolates from experimental broilers was statistically significantly more common ($P \leqslant 0.01$), i.e., 82.3%, as well as against third generation cephalosporins (44.1%, 41.2%). The genetic analysis of resistance in commensal E. coli isolates revealed the presence of bla $_{CTX\text{-}M\text{-}1}$, tetA and QnrS genes. In conclusion, we should note that in our study related to the use of Bacillus licheniformis BL 11 strain, a probiotic formula in broilers, no basic differences were observed both in terms of the prevalence of resistance to chemotherapeutics and in terms of economic indicators in the broilers in the control and experimental groups.

Introduction

The spread of resistance to antimicrobial drugs is a serious public health concern of our time. Resistant bacteria isolated from animals and the genetic determinants carried by them may be transferred to people via various mechanisms, direct contact, contaminated food, and from the environment (Thorsteinsdottir et al., 2010; Dolejska et al., 2013; Huijbers et al., 2014; Aun et al., 2021; Rousham et al., 2021). The use of third generation cephalosporins and fluoroquinolones in intensive livestock operations (poultry farming, pig farming) is a subject of thorough monitoring and analysis due to their critical importance in the treatment of severe bacterial infections in humans (Costa et al., 2011). According to Costa et al. (2011), the administration of enrofloxacin in farm animals may increase resistance rates to other classes of chemotherapeutics as well. Szmolka et al. (2013) has mentioned that the broader use of fluoroquinolones in poultry farming is a recognized prerequisite for the increased prevalence of resistant *E. coli* bacteria. Furthermore, the transfer of resistant animal *E. coli* isolates producing extended spectrum beta-lactamases is also deemed as a risk for the therapy of some human bacterial infections (Leverstein van Hall et al., 2011; Dahms et al., 2015), having in mind that poultry meat is very often contaminated with such strains (Overderest et al., 2011; Kluytmans et al., 2013; Ceccarelli et al., 2019). According to the criteria of the WHO (2017) and OIE (2015), the aminoglycosides, third generation cephalosporins, macrolides and penicillins are defined as critically important chemotherapeutics for human and veterinary medicine.

The acknowledged necessity about restriction of the use of antimicrobial drugs in farm animals implies the investigation of alternative products for infectious diseases control, animal welfare improvement, selection of more resistant breeds, etc. (Gadde et al., 2017).

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Probiotic products based on members of *Bacillus* spp. are resistant and can be stored for a long time due to specificity of spore forming bacteria (Gao et al., 2007). They possess an inhibitory effect on microbial pathogens due to production of organic acids, reducing the gastrointestinal pH. Cladera-Olivera et al. (2004) discussed the hypothesis that the production of bacteriocins was one of the important features of bacilli in probiotic products, influencing gastrointestinal microbial pathogens in poultry. Also, they support the gastrointestinal microbiota via competitive exclusion, improve the activity of digestive enzymes, reduce the amount of toxic products, ammonia and aflatoxins, produce antimicrobial substances and, last but not least, modulate the immune system by promoting the production of secretory immunoglobulins A (Xu et al., 2012; Latorre et al., 2017; Mingmongkolchai and Panbangred, 2018). These features of probiotic formulas with *Bacillus* spp. reduce the possibility for emergence of infections and mortality rates in poultry, along with improvement of feed conversion. Rajput et al. (2020) affirmed that probiotics containing *B. licheniformis* strains may alleviate oxidative stress and influence gene expression associated with lipid metabolism. As outlined by Mingmongkolchai and Panbangred (2018), some representatives of the genus *Bacillus*, e.g., *B. clausii*, *B. cereus*, *B. subtilis*, *B. licheniformis* possess plasmids conferring antimicrobial drug resistance that may be transferred into the gastrointestinal tract of birds. This gave a reason to EFSA experts (2015) recommending a preliminary testing of strains in probiotic formulas for their sensitivity to various classes of chemotherapeutics.

The aim of the present study was to investigate phenotype resistance profiles and some genetic determinants in resident *E. coli* bacteria isolated from broilers whose ration was supplemented with a probiotic formula containing a *Bacillus licheniformis* BL11 strain (Huvepharma, Belgium).

Material and methods

Birds, rearing conditions and treatment

The experiment was performed in a poultry plant in South Bulgaria. The farm had two production facilities. In each facility, 17 500 day-old unsexed Ross 308 chicks were housed with free access to feed and water. The chickens in both facilities received the same feed from the first day of life until slaughter. Three types of compound feeds were fed: starter, grower and finisher; their composition is presented in Table 1. Halofuginone hydrochloride was added to the starter and the grower as coccidiostat. The drinking water was supplied via nipple drinking systems. The results of the investigation of water quality are presented in Table 2. During the experiment, broilers were not treated with chemotherapeutics. The two groups were reared under equal conditions. The environmental conditions in facilities (ventilation rate, heating, lighting and relative humidity) were in line with technological norms for the hybrid (Aviagen, 2018). The chickens were reared on the floor on ground straw bedding. During the experiment, the birds had free access to fresh water and feed. At the beginning of the study, the ambient temperature was 32°C, then it gradually decreased to 22°C, and was maintained at that level until the end. The relative humidity in

Table 1. Composition of broiler chicken feed (%)

Ingredients	Starter (0–17 days)	Grower (18–27 days)	Finisher (28 day to slaughter)
Maize	23	20	26
Wheat	36	34	31
Maize gluten meal	4	1.5	
Soybean meal	29	27	22
Sunflower meal		6	7
Sunflower oil	3.00	6.75	5.50
L-valine	0.035	0.03	0.06
Lysine	0.39	0.34	0.26
Methionine	0.29	0.28	0.28
Threonine	0.16	0.13	0.12
Limestone	1.16	1.19	1.05
Monocalcium phosphate	1.16	0.66	0.54
Sodium chloride	0.17	0.17	0.19
Sodium hydrogen carbonate	0.33	0.33	0.32
Mineral vitamin premix	0.55	0.55	0.55
Vitamin C	0.01	0.01	0.01
Coccidiostat – halofuginone hydrochloride	0.05	0.05	

Table 2. Results from the laboratory analysis of drinking water

Parameter	Units	Test result*	Reference values
Calcium	mg/L	108.72	150
Manganese	µg/L	< 10.00	50
Nitrates	mg/L	27.10	50
Nitrites	mg/L	< 0.05	0.50
Hydrogen ions activity (pH)	pH	7.35	6.5–9.5
Electroconductivity	µS/cm	847.00	up to 2000
Cations – ammonium	mg/L	< 0.06	0.50
Turbidity	FNU	0.14	up to 1.5
Free chlorine residual	mg/L	0.321	0.3–0.4
Enterococcus spp.	cfu/100 mL	< 1	0
Escherichia coli	cfu/100 mL	< 1	0
Clostridium perfringens	cfu/100 mL	< 1	0
Coliforms	cfu/100 mL	< 1	0
Total viable counts	cfu/mL	23	up to 100
Total viable counts	cfu/mL	15	up to 20
Salmonella spp.	detectable / undetectable in 250 mL	not detected	undetectable

* The water sample for laboratory analysis was collected from the last nipple of the drinking system.

facilities was 60–70%. A 6-hour dark period was provided for the night, and light intensity was subject to dawn-to-dusk light control. The health of birds was monitored three times per day.

The birds in the experimental group received a probiotic containing Bacillus licheniformis BL11. The probiotic was applied via drinking water (100 g per 20 000 birds, with a dose of *B. licheniformis* in sachet $1.6X10^6$ cfu/mL). The probiotic treatment was done twice: from day 1 to day 7 of age and from day 20 to day 26. The birds from the control group were not treated with the probiotic.

Samples and bacteriological examinations

Cloacal swabs were collected from broilers at 1, 14 and 28 days of age. For each age period, 50 swabs were collected from control broilers and another 50 swabs from probiotic-supplemented broilers. The total number of samples was 300 (150 from control birds and 150 from experimental birds). For primary isolation of *E. coli*, swabs were inoculated on McConkey agar and incubated at 37°C for 24 hours. The algorithm of *E. coli* identification included cultivation on Kligrer iron agar (Himedia, India), IMViC test (production of indole, methyl red test, Voges-Proskauer test, growth on Simmons citrate agar) and a kit for identification of enterobacteria (Erba Lachema, Czech Republic).

Tests of *E. coli* sensitivity to antimicrobial drugs

The sensitivity of *E. coli* isolates to chemotherapeutics was initially determined by the disk diffusion method using the following antibiotic disks: ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), cefotaxime (10 µg), ceftazidime (5 µg), gentamicin (10 µg), tetracycline (30 µg), and ciprofloxacin (5 µg) (Himedia Biosciences, India). Minimum inhibitory concentrations (MIC) of chemotherapeutics were determined by the E-test, (Hi Comb™, Himedia, India). For evaluation of the inhibitory effect of clavulanic acid on some ESBL producers, gradua-ted MIC strips loaded with combinations of *cef-tazidime+clavulanic acid* (0.064–4 µg/mL) and *cefotaxime+clavulanic acid* (0.016–1 µg/mL (Hi Comb™ , Himedia, India) were used. *Escherichia coli ATCC 25922* was used for control of the methods for phenotypic determination of antimicrobial resistance of *E. coli* strains. The interpretation of results was done on the basis of epidemiological cut-off values (ECOFFs). Categorization of MIC_{90} was done by determining the cumulative percentage of resistant isolates.

Genetic tests

The extraction of DNA from pure cultures was done with DNeasy Blood Tissue kit (Qiagen, Germany). The identification of genes conferring resistance to beta-lactams ($bla_{CTX\text{-}M\text{-}1}$,$bla_{SHV,}$ catalog number BBAR00377A and catalog number BBAR00387A), tetracyclines (*tetA*, catalog number BBAR00449A) and ciprofloxacin (*qnrS*, catalog number BBAR00441A) was performed with commercial Microbial DNA qPCR assay kits, Qiagen, Germany. The thermal profile of the protocol included an initial denaturation step at 95°C for 10 min, 40 cycles with initial denaturation at 95°C for 15 sec and annealing/elongation at 60°C for 2 min. Amplification reactions were done in a Stratagene Mx3000P qPCR system (Agilent Technologies, USA).

Economic performance

During the experiment, the live body weight was

determined on 1, 14 and 28 days of age on 1003 randomly selected broiler chickens from each group. The average daily weight gain (ADG) was determined for the periods 1–16, 17–36 and 1–36 days. For the same periods, the average daily feed intake (ADFI) and feed conversion rate (FCR) were calculated. The European Production Efficiency Factor (EPEF) for these periods was calculated using the formula:

EPEF = [(livability, % × BW, kg) / (age, days × FCR)] × 100.

Statistical analysis

The statistical analysis of sensitivity rates of isolates to chemotherapeutics was done with GraphPad Instat 3.

The parameters of economic performance in both groups were subjected to the following tests: Kolmogorov-Smirnov test for normality of sample distribution; descriptive statistics with calculation of arithmetic mean, standard error of the mean, minimum and maximum values; and the t-test for independent samples for between-group comparison. The minimum level of statistical significance was $P < 0.05$. The calculations were done with the statistical software MedCalc v 10.2.0.0, Belgium.

Results

The total number of *E. coli* isolates from the control and experimental group of broilers was 214: 107 from control birds and another 107 from probiotic-supplemented birds, and from the remaining 86 samples no bacteria belonging to the species *Esherichia coli* were isolated.

Tables 3, 4 and 5 present the results of the prevalence of resistance in poultry *E. coli* isolates in both groups at 1, 14 and 28 days of age.

The highest resistance rates among *E. coli* isolates from control day-old broilers were shown against gentamicin (86.9%), ampicillin (67.4%), tetracycline (60.9%) and ciprofloxacin (52.1%). For isolates from the experimental birds, the resistance against gentamicin was also the most frequent (69.0%), followed by resistance against ampicillin and amoxicillin/clavulanic acid (52.4%). The prevalence of tetracycline-resistant strains was lower as compared with the control group (19.0%). *E. coli* isolates from the control group which were resistant to cefotaxime were 6.5% vs 7.1% among isolates from the experimental group. A lower rate of resistance

Table 3. Resistant *E. coli* isolates from day-old broilers

	Control group (n = 46)		Experimental group (n = 42)	
Chemotherapeutics	Resistant *E. coli* isolates (n/%)	CL	Resistant *E. coli* isolates (n/%)	CL
Ampicillin	31/67.4*	53.4÷79.9	22/52.4*	37.5÷67.0
Amoxicillin/clavulanic acid	21/45.6	31.7÷59.9	22/52.4	37.5÷67.0
Cefotaxime	3/6.5	1.3÷15.2	3/7.1	1.4÷16.5
Ceftazidime	0	-	1/2.4	0÷9.0
Gentamicin	40/86.9*	75.9÷94.4	29/69.0*	54.5÷81.8
Tetracycline	28/60.9***	46.5÷74.3	8/19.0***	8.8÷31.9
Ciprofloxacin	24/52.1	37.9÷66.1	20/47.6	32.9÷62.5

Legend: $P < 0.05$*; $P < 0.01$**; $P < 0.001$ ***

Table 4. Resistant *E. coli* isolates from 14-day-old broilers

	Control group (n = 25)		Experimental group (n = 31)	
Chemotherapeutics	Resistant *E. coli* isolates (n/%)	CL	Resistant *E. coli* isolates (n/%)	CL
Ampicillin	17/68.0	49.8÷84.4	23/74.2	57.6÷87.8
Amoxicillin/clavulanic acid	12/48.0	29.0÷67.2	11/35.5	19.9÷52.9
Cefotaxime	0	-	16/51.6	32.4÷70.6
Ceftazidime	3/12.0*	2.4÷27.2	12/38.7*	20.9÷58.1
Gentamicin	12/48.0	29.0÷67.2	19/61.3	41.8÷79.0
Tetracycline	21/84.0**	67.4÷95.4	14/45.2**	26.5÷64.6
Ciprofloxacin	17/68.0	49.8÷84.4	26/83.9	67.5÷95.3

Legend: $P \leqslant 0.05$*; $P \leqslant 0.01$**; $P \leqslant 0.001$***

Table 5. Resistant *E. coli* isolates from 28-day-old broilers

	Control group (n = 36)		Experimental group (n = 34)	
Chemotherapeutics	Resistant *E. coli* isolates (n/%)	CL	Resistant *E. coli* isolates (n/%)	CL
Ampicillin	19/52.7**	36.6÷68.5	28/82.3**	67.9÷93.0
Amoxicillin/clavulanic acid	11/30.5	16.7÷46.2	15/44.1	28.1÷60.8
Cefotaxime	4/11.1**	3.0÷23.2	15/44.1**	28.1÷60.8
Ceftazidime	4/11.1**	3.0÷23.2	14/41.2**	25.4÷58.9
Gentamicin	21/58.3	42.1÷73.6	19/55.8	39.1÷71.8
Tetracycline	24/66.7	50.7÷80.8	27/79.4	64.4÷91.0
Ciprofloxacin	19/52.7**	36.6÷68.5	29/85.3**	70.3÷95.0

Legend: $P \leqslant 0.05$*; $P \leqslant 0.01$**; $P \leqslant 0.001$***

against ceftazidime was observed in poultry from the experimental group (2.4%).

The prevalence of resistance to cefotaxime (51.6%), respectively to ceftazidime (38.7%), in *E. coli* isolates from 14-day-old experimental broilers statistically significantly exceeded ($P \leqslant 0.05$) the proportion of resistant isolates from the control group (12.0%). The tetracycline resistant strains from control chickens (84.0%) were significantly more prevalent ($P \leqslant 0.01$) than those isolated from the supplemented experimental group (45.2%). Higher yet statistically insignificant rates of resistance in both groups were demonstrated against ampicillin (68.0%, 74.2%), gentamicin (48.0%, 61.3%) and ciprofloxacin (68.0%, 83.9%). The percentage of *E. coli* strains resistant against amoxicillin/clavulanic acid in the probiotic-treated group was insignificantly lower (35.5%) as compared with isolates from control birds (48.0%).

By day 28 of age, the prevalence of ciprofloxacin-resistant *E. coli* isolates from experimental broilers exceeded significantly ($P \leqslant 0.01$) that of control strains (85.3% vs 52.7%). Furthermore, the spread of resistance against ampicillin (82.3%) and third generation cephalosporins (44.1%, 41.2%) among isolates from experimental birds was substantially higher. In both groups, high resistance rates were found out against gentamicin (58.3%, 55.8%) and tetracycline (66.7%, 79.4%).

Tables 6 and 7 present the minimum inhibitory concentrations in *E. coli* isolates from control and experimental broilers.

Among *E. coli* isolates from both groups, the highest detected MIC_{90} values were for ampicillin and tetracycline (32 µg/mL). MIC_{90} values of 16 µg/mL were found for amoxicillin/clavulanic acid and tetracycline. MIC_{90} for third generation cephalosporins was 1 µg/mL for *E. coli* isolates from the control group, whereas MIC_{90} for ceftazidime in isolates from the experimental group was 2 µg/mL. In isolates from both groups, MIC_{90} for gentamicin was 2 µg/mL, and for ciprofloxacin, it was 1 µg/mL.

Table 8 presents phenotype resistance profiles and some of the genetic determinants of resistance in multi-resistant *E. coli* isolated from control and probiotic-supplemented broilers.

Among multi-resistant *E. coli* strains isolated from control broilers, the phenotype profile including resistance to ampicillin, amoxicillin/clavulanic acid and gentamicin (33.6%) was the most common one, followed by the profile determining resistance

Table 6. Minimum inhibitory concentrations in commensal *E. coli* isolates from the control group of broilers at 1, 14, and 28 days of age (n = 107)

	MIC_{90} µg/mL												
Chemotherapeutics	MIC_{90}	0.06	0.125	0.5	1	2	4	8	16	32	64	128	256
Ampicillin	32			1	3	9	5	22*	39	18	7	3	
Amoxicillin/clavulanic acid	16		4		1	1	20	37*	21	14	9		
Cefotaxime	1.0		13	12	75*	7							
Ceftazidime	1.0		17	21	62*		7						
Gentamicin	4.0			24		10*	58	13	2				
Tetracycline	32					2	3	29*	42	17	11	3	
Ciprofloxacin	1		47	42*	18								

Legend: MIC thresholds are marked with asterisks

to gentamicin, tetracycline and ciprofloxacin (27.1%). *E. coli* isolates from the experimental group demonstrated a higher prevalence of multi-resistance to ampicillin, amoxicillin/clavulanic acid, cefotaxime and gentamicin (15.9%), as well as to ampicillin, cefotaxime, ceftazidime, gentamicin and ciprofloxacin (15.9%). The presence of the *bla* $_{CTX-M-1}$ gene but not of the *bla* $_{SHV}$ gene was confirmed in strains resistant to beta-lactams. Fifty strains resistant to ciprofloxacin (37%) carried the *Qnr*S gene.

Economic performance

Data about the economic performance of broilers are presented in Table 9. According to the results, the treatment with a probiotic containing *Bacillus licheniformis* BL11 strain improved ($P < 0.05$) growth performance in broilers (BW, FCR and EPEF) in comparison to the same indices in the control

Table 7. Minimum inhibitory concentrations in commensal *E. coli* isolates from the experimental group of broilers at 1, 14, and 28 days of age (n = 107)

	MIC$_{90}$ µg/mL												
Chemotherapeutics	MIC$_{90}$	0.06	0.125	0.5	1	2	4	8	16	32	64	128	256
Ampicillin	32			2	1	1	2	28*	41	15	12	5	
Amoxicillin/clavulanic acid	16			7	4	4	2	42*	7	41			
Cefotaxime	1	2	2	37	32*	34							
Ceftazidime	2	3	1	17	57*	2	25	2					
Gentamicin	4.0		1	4	2	33*	55	12					
Tetracycline	32				4	4	6	44*	31	7	11		
Ciprofloxacin	1		32	21*	51	3							

Legend: MIC thresholds are marked with asterisks

Table 8. Phenotype resistance profile of *E. coli* isolates from broilers and genes conferring resistance to antimicrobial drugs

Genes conferring resistance to beta-lactams, tetracyclines and quinolones (n/%)				
Phenotype resistance profile of *E. coli* isolates	*bla* $_{CTX-M-1}$	*bla* $_{SHV}$	*tet*A	*Qnr*S
Control group (n = 107)				
T (20)	-	-	16 (14.9%)	
CIP (1)	-	-	-	-
AMP, AMC, G (36)	-	-	-	-
AMP, AMC, CIP (8)	-	-	-	2 (1.9%)
G,T, CIP (29)	-	-	27 (25.2%)	14 (13.1%)
AMP, T, CIP (15)	-	-	11 (10.3%)	3 (2.8%)
AMP, G, T (1)	-	-	-	-
AMP, CTX, CAZ, G, T, CIP (7)	7 (6.5%)	-	3 (2.8%)	1 (0.9%)
Experimental group (n = 107)				
AMP (3)	-	-	-	-
AMP, G (3)	-	-	-	-
AMP, AMC (7)	-	-	-	-
G, T (14)			4 (3.7%)	-
T, CIP (25)	-	-	9 (8.4%)	9 (8.4%)
AMP, G, CIP (2)	-	-	-	-
AMP, AMC, G, CIP (14)	-	-	-	3 (2.8%)
AMP, AMC, CTX, CIP (17)	11 (10.3%)	-	-	7 (6.5%)
AMP, CTX, CAZ, G, CIP (17)	17 (15.9%)	-	-	11 (10.3%)
AMP, AMC, CTX, CAZ, G, T (10)	10 (9.3%)	-	10 (9.3%)	-

Legend: AMP – ampicillin, AMC – amoxicillin/clavulanic acid, CTX – cefotaxime, CAZ – ceftazidime, G – gentamicin, T – tetracycline, CIP – ciprofloxacin

Table 9. Economic performance of chickens from control and experimental group (n = 1003)

	Control group	Experimental group	*P value*
Initial body weight, g	41.00 ± 0.08	48.00 ± 0.06	< 0.0001
BW 16 day, g	570.00 ± 0.69	580.00 ± 0.60	< 0.0001
BW 36 day, g	1886.00 ± 0.44	1811.00 ± 0.22	< 0.0001
ADG 1–16 days, g/ day	33.06 ± 0.04	33.25 ± 0.04	= 0.0008
ADG 17–36 days, g/ day	69.26 ± 0.23	64.79 ± 0.03	< 0.0001
ADG 1–36 days, g/ day	51.25 ± 0.12	48.97 ± 0.01	< 0.0001
FCR 1–16 days, g/g	0.872 ± 0.001	0.769 ± 0.001	< 0.0001
FCR 17–36 days, g/g	1.422 ± 0.001	1.560 ± 0.001	< 0.0001
FCR 1–36 days, g/g	1.631 ± 0.001	1.753 ± 0.001	< 0.0001
EPEF 1–16 days	400.96 ± 0.96	463.34 ± 0.88	< 0.0001
EPEF 17–36 days	673.74 ± 0.31	591.35 ± 0.15	< 0.0001
EPEF 1–36 days	310.08 ± 0.14	277.80 ± 0.07	< 0.0001
ADFI 1–16 days, g/ day	30.36	27.36	
ADFI 17–36 days, g/ day	136.30	144.06	
ADFI 1–36 days, g/ day	85.43	88.19	
Mortality 1–16 days, %	2.15	1.84	
Mortality 17–36 days, %	1.34	1.35	
Mortality 1–36 days, %	3.49	3.19	

Legend: BW – body weight; ADG – average daily weight gain; ADFI – average daily feed intake; FCR – feed conversion rate; EPEF – European production efficiency factor.

group during the starter stage (from days 1–14). During the grower stage (days 14–36) the broilers treated with the probiotic demonstrated reduced performance compared with controls. Thus, FCR of the experimental group was worse than that of control birds. The same trend was observed for the average daily feed intake and the average daily weight gain. For the entire duration of the trial (days 1–36), average values of economic parameters of controls were better than those in the experimental group. At the same time, the mortality rate of probiotic-supplemented broiler chickens was lower than the rates of the control group.

Discussion

According to Arif et al. (2021), probiotics based on *Bacillus* spp. increase the counts of spore-forming bacteria from this genus between days 21 and 35, and on the other side, reduce the counts of *Clostridium perfringens*, *Salmonella* spp., *Escherichia coli* in the small intestinal compartment of broilers reared in farms with poor biosecurity parameters. Cladera-Oliveraet al. (2004) discussed the ability of *Bacillus* spp. representatives to produce bacteriocins, which inhibit the development of other bacterial species in the avian gut.

Some investigators discussed the emergence of multi-resistant *E. coli* strains from the resident microflora resistant against beta-lactams, fluoroquinolones, aminoglycosides, tetracyclines even in newly hatched chickens (Baron et al., 2014; Olsen et al., 2017; Projahn et al., 2017; Roth et al., 2017; Saliuet al., 2017). In their opinion, birds become infected with resistant strains from eggshells and from the environment; a vertical transmission of such strains from breeding flocks is also possible.

Possibly, the spread of antimicrobial resistance among commensal *E. coli* is a multifactorial process including both the selective pressure from the application of these drugs in intensive livestock husbandry, as well as the spread of resistant bacterial clones, dissemination of plasmids conferring resistance to various classes of chemotherapeutics and, last but not least, the co-selection among resistant bacterial strains.

For example, the last report of EFSA (2023) on the prevalence of resistance among commensal *E. coli* from broilers in EU member states affirms that the resistance against ciprofloxacin was the most common (52.7%). The report discusses the fact that the proportion of *E. coli* isolates from broilers resistant to ciprofloxacin and cefotaxime from 2020 to 2021 was low (1%) while the proportion of strains resistant only against third generation cephalosporins was higher (7.1%). According to the analysis, multi-resistant strains were frequently encountered among commensal *E. coli* from broilers (37.7%). In Belgium, as reported by De Koster et al. (2021), the resistance to third generation cephalosporins and ciprofloxacin in resident poultry *E. coli* was high (70%) and co-

resistance was determined in 33.4% of strains. In Austria, Galleret al. (2021) observed a high incidence of resistance against third generation cephalosporins (cefotaxime 100%; ceftazidime 93.8%) and tetracycline (93.8%) but not against ciprofloxacin among commensal *E. coli* isolated from broilers. In the present study, multi-resistant strains including resistance against third generation cephalosporins were more prevalent among broilers from the experimental group (41.1%) at a lower rate than that reported by De Koster et al. and Galler et al.

As already acknowledged, beta-lactam antibiotics are among the most commonly used in human and veterinary medical practice, which creates prerequisites for colonization of the intestinal tract with extended spectrum beta-lactamase (ESBL) producing *E. coli* (Ferreira et al. 2022). The incidence of ESBL producing *E. coli* in the gastrointestinal tract of broilers is important for the spread of multi-resistant strains to people along the food chain, as well as among animals and in the environment (Dierikx et al., 2018; Subramanya et al., 2021). In the Netherlands, Van Hoek et al. (2018) found 30% of ESBL-producing strains among *E. coli* isolates from day-old chickens. According to the authors, as early as during the first 48 hours after population of premises with birds, the rate of ESBL producing *E. coli* strains increased without application of selective pressure. In their view, one of the factors determining the distribution of *E. coli* ESBL producers was the spread of specific clones ST88, ST10, ST58, ST155, and the horizontal transfer of such strains was discussed as a risk factor. A number of European researchers affirmed that beta-lactamases of the CTX-M-1 subtype were the most commonly encountered among resistant *E. coli* bacteria (Girlich et al., 2007; Chauvin et al., 2013; Dierikx et al., 2018; Apostolakos et al., 2019). In Germany, Laube et al. (2013) determined a broader prevalence of *bla* $_{CMY}$ (21.73%), followed by *bla* $_{SHV-12}$ (13.2%) and *bla* $_{CTX-M}$ (10.59%) genes in a similar survey on ESBL-producing isolates from broiler chickens. Furthermore, they reported a rather elevated percentage of ESBL producers (51%) in day-old chicks. Valentin et al. (2004) affirmed that, in Germany, *E. coli* strains from livestock and birds that produced beta-lactamases from the CTX-M-1 group were the most prevalent. Galler et al. (2021) presented data from Austria associated with the predominant spread of *bla* $_{SHV-12}$ (81.3%), followed by *bla* $_{CTX-M-1}$ (12.5%) among commensal *E. coli* isolated from broilers. In Portugal, Ferreira et al. (2022) also observed dominance of the *bla* $_{SHV-12}$ gene among commensal ESBL-producing isolates from broiler chickens. Saliu et al. (2017) reported the fact that the *bla* $_{CTX-M-1}$, *bla* $_{TEM-52}$, and *bla* $_{SHV-12}$ were the most common resistance genes in intensive livestock operations. Due to the genetic resemblance between ESBL-producing enterobacteria isolated from humans and broiler chickens, the authors suggested that broilers may be a primary reservoir of plasmids carrying the *bla* $_{CTX-M-1}$ gene. In our study, the resistance against third generation cephalosporins among commensal *E. coli* isolates was determined by the presence of *bla* $_{CTX-M-1}$, but not by the presence of *bla* $_{SHV}$.

In this study, strains resistant to cefotaxime and ceftazidime were more commonly isolated from broilers, supplemented with the probiotic formula. For example, 7.1% of isolates from day-old experimental broilers were resistant against cefotaxime, and 2.4% to ceftazidime, whereas in control chickens, the prevalence of cefotaxime-resistant strains was 6.5%, and none of the strains were resistant against ceftazidime. An interesting fact was that ampicillin-resistant (52.4%), amoxicillin/clavulanic acid-resistant (52.4%), and gentamicin-resistant (69.0%) strains from day-old probiotic-supplemented birds were also more numerous. During the next study period at 14 days of age, the prevalence of cefotaxime-resistant (51.6%) and ceftazidime-resistant (38.7%) *E. coli* isolates among experimental birds was also higher than that in controls. None of the commensal *E. coli* isolates from controls was resistant against cefotaxime and those resistant against ceftazidime were 12.0%. In 28-day-old birds, a variable resistance against third generation cephalosporins was noted: 44.1% and 41.2% of strains were resistant against cefotaxime and ceftazidime, respectively, whereas the resistance rates to these chemotherapeutics among control group isolates was once again lower: 11.1% for both drugs. In connection with these findings, the statement of Clemente et al. (2021) about the possible association of the broad spread of commensal *E. coli* poultry strains producing ESBL with some risk factors in industrial poultry farming co-selection of genes and mobile genetic platforms, plasmids, transposons, integrons resulting from the use of other chemotherapeutic classes, tetracyclines, sulfonamides and fluoroquinolones, may be cited. Saliu et al. (2017) also pointed out the environment as a factor influencing the spread of ESBL-producing enterobacteria in intensive poultry operations and discussed the effect of the ration on these processes. According to the investigations of Blaak et al. (2015), the spread of *E. coli* producing extended-spectrum beta-lactamases from poultry farms to the environment is an important risk factor for the transfer of such strains to people.

With respect to the prevalence of *E. coli* isolates resistant to aminopenicillins, a statistically significant reduction of strains resistant against amoxicillin/clavulanic acid was observed among isolates from the probiotic-supplemented group by day 14 of the experiment. The resistance rate among isolates from the experimental group was 35.5% vs 48.0% for isolates from untreated controls.

From the beginning of the trial to day 28, a tendency towards the increased number of tetracycline- and ciprofloxacin-resistant strains from the experimental group of birds was established. In strains from day-

old broilers, the prevalence of strains resistant against tetracycline was 19.0%, and against ciprofloxacin, it was 47.6%; by day 28 of the trial, the respective rates increased to 79.4% and 85.3%. The resistance to tetracycline and ciprofloxacin was conferred by the *tetA* and *QnrS* genes. Thus, our findings may be commented in the light of the statement of Ribeiro et al. (2023) that both among pathogenic and commensal *E. coli* isolates from broilers, the strains expressing resistance to ampicillin, tetracycline, ciprofloxacin, nalidixic acid and sulfamethazole-trimethoprim were the most numerous. Similar results were reported by Roth et al. (2019) affirming that, in some European countries, the highest resistance rates among commensal *E. coli* strains from broilers were those against ampicillin (91%), ciprofloxacin (90%), tetracycline (73%) and sulfonamides (60%).

Conclusion

The demonstrated broader prevalence of *E. coli* strains, resistant against third generation cephalosporins, ciprofloxacin and tetracycline, isolated from broilers receiving a probiotic formula was probably related to several specific factors, such as the exchange of certain bacterial clones, the presence of plasmids carrying the respective genetic determinants. Providing that scientific literature reports regarding the possible effects of alternative products of antibiotics on the spread of resistance among commensal intestinal microbiota are still few, the probiotic formula containing *Bacillus licheniformis* BL11 could possibly affect these events through the classic characteristics of probiotics, namely modulation of the immune system, antibacterial activity of probiotic strains and competitive exclusion. On the other hand, this product could also have a better effect in implementing strict control on biosecurity measures affecting the welfare of birds and the status of the environment. We should note that in our study, no basic differences were observed both in terms of the prevalence of resistance to chemotherapeutics and in terms of economic indicators in the broilers in the control and experimental groups.

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