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ATTEMPTS FOR *IN VIVO* INFLUENCE UPON THE RESISTANCE OF *ESCHERICHIA COLI* AFTER TREATMENT WITH FLAVOPHOSPHOLIPOL AND ECONOMICAL TRAITS OF ITS ADMINISTRATION UNDER CONDITION OF ARTIFICIAL INFECTION IN CHICKENS

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Summary

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In the conditions of experimental infection with a *E. coli* O2 strain, isolated from septic chickens and showing multiresistance to a variety of antimicrobial drugs, the effect of different doses of flavophospholipol (FPL) was surveyed. The possibilities for influencing the antibacterial resistance were followed up via monitoring the changes in growth inhibition zones in agar and the minimum inhibitory concentrations (MIC). The effect of the treatment on some economical traits – daily weight gain, feed conversion ratio etc. – was also determined.

It was found out that FPL had a relatively weak direct effect on parameters determining the resistance of the challenge strain. It was suggested that its inhibiting influence on the dissemination of the genetic determinants of resistance among microbial populations was rather more effective than that on the mechanisms of resistance, acting at the level of the microbial cell.

The features of FPL as growth promoter, acting effectively on weight gain and feed conversion ratio were confirmed.

Key words: feed conversion ratio, flavophospholipol, inhibition zones, minimum inhibitory concentrations (MIC), resistance, weight gain

INTRODUCTION

During the last two decades, the resistance of numerous bacterial species to various antimicrobial drugs has become extremely important. Often, the resistance is exhibited to more than two antimicrobial substances, i.e. the so-called multiple resistance is present. It is a major obstruction to the successful therapy and prophylaxis of bacterial diseases. Most commonly, this resistance is transferable and is mediated by various extrachromosomal determinants, particularly by the so-called R-plasmids. They could translate genes of resistance both within the species and among various microbial species and to disseminate this resistance among the mi-

crobial populations through conjugation (Van den Bogaard, 1999). The resident intestinal microflora is especially important in this connection. Most commonly, that is among it that resistant species are selected and mainly when antibiotics are used as growth promoters (Spring, 1978; Joint Expert Advisory Committee on Antibiotic Resistance, 1999) .

The importance of resident *E. coli* for the selection and dissemination of resistance is discussed as early as the 70-ties and the 80-ties years of the last century (Brana *et al.*, 1974; Corpet, 1984). Since the 70-ties years, there are data about the inhibiting role of some substances (including some antibiotics) on the selection and dissemination of microbial resistance (Sokol *et al.*, 1973; Karaivanov *et al.*, 1980). With this regard, a special attention is paid to flavophospholipol (bambermycin, FPL, Flavomycin) – a phosphate-containing glycolipid antibiotic that inhibits the peptidoglycan synthesis in the bacterial cell wall (Spring, 1975; 1978; Droumev, 2001; 2003).

In Bulgaria, Bineva (1985) has studied the effect of FPL upon the conjugative transfer of plasmids, determining a multidrug resistance in avian *E. coli* isolates. According to this study, FPL eliminated the markers of resistance, but the mechanism of this influence was not discussed. There are no data about the rate of this effect and its occurrence with time within and outside the population (Bineva, 1985).

With regard to the increasing importance of the problem with multiresistance, we aimed to determine whether the administration of FPL had an effect upon the parameters of resistance of a challenge *E. coli* strain in an experimental infection of growing chickens.

MATERIALS AND METHODS

Bacterial strain

A *E. coli* strain, serotype O2, isolated from chickens with septic form of colibacteriosis was used in the experiment upon the effect of various doses of FPL on the antimicrobial resistance. The strain was chosen on the following criteria: 1. Confirmed pathogenic potential; 2. Serological typability; 3. Phenotypically manifested multiresistance to aminopenicillins (ampicillin and amoxycillin), streptomycin, tetracycline; intermediate resistance pattern to enrofloxacin and preserved sensitivity to gentamicin and colistin (Table 1.)

Prior to the experiment, the strain was tested for pathogenicity via inoculation in 5-day old chickens at a dose of 1 mL with bacterial suspension density of 1.10^8 cfu/mL once daily for 3 consecutive days. At the 4th day, the strain was re-isolated from the liver, spleen and ventricular blood of infected chickens.

FPL

The commercial product Jivet/Pharmastim – 8% premix, produced by Biovet – Peshtera, containing 8.0 g active substance bambermycin in 100.0 g product, was used.

Birds

Seventy five broiler chickens, Cobb-500 hybrids, obtained at the age of 1 day from a hatchery with a guaranteed healthy status were used. They were housed in a common cage, on a hard floor with litter and had a constant access to drinking water and food. A starter concentrated forage (Provimi Ltd) was fed. The chickens were reared under these conditions up to their

Table 1. Diameters of the inhibition zones (DIZ) of the challenge *E. coli* strain prior to the beginning of the experiment

Antimicrobial drug	Code	DIZ (mm)	Type
Ampicillin	Am	6	R
Amoxycillin	Ax	6	R
Streptomycin	S	6	R
Gentamicin	G	24	S
Tetracycline	T	6	R
Colistin	Cs	16	S
Enrofloxacin	Enr	21	I

R– resistant; S–sensitive, I–intermediate.

separation according to the experimental design (age of 7 days).

Experimental design

At the age of 7 days, the chickens were divided into 3 experimental and 2 control groups (positive and negative controls), with 15 chickens in each.

The experimental and positive control birds were challenged with the chosen microbial isolate, applied orally by oesophageal tube as bacterial suspension with a density of 1.10^6 cfu/mL for 5 consecutive days. The treatment was individual and the total amount of suspension administered over the entire period was 0.7 mL per chicken. Thus, a colonization of the intestinal tract and formation of a carriership of the challenge strain without development of clinical signs was attempted.

By the day of the last inoculation (age of 11 days), feeding with forage containing FPL was initiated as followed: 2 mg/kg FPL for group I; 4 mg/kg FPL for group II and 8 mg/kg FPL for group III.

The chickens from the negative control group (uninfected) and those from the positive control group (infected) remained untreated with FPL.

In order to reisolate the challenge strain from the intestinal tract of chickens, 3 chickens from each experimental group and the infected but FPL-untreated controls were euthanized by days 7, 14 and 21 after the beginning of the FPL treatment.

The feeding with the forage containing FHL continues 30 days or up to the age of 41 days, when the euthanasia of birds was performed.

Determination of the behaviour of reisolated strains to antimicrobial drugs

The sensitivity of reisolated coliform strains was tested by the disk diffusion method of Bauer-Kirby according to the criteria of NCCLS (2002). A Mueller-Hinton agar was used (National Centre of Infectious and Parasitic Diseases, Sofia).

The used antibiotic disks were selected depending on the preliminary study on the phenotype of strain's antibiotic sensitivity: ampicillin (10 μg), amoxycillin (25 μg), tetracycline (30 μg), streptomycin (10 μg), enrofloxacin (5 μg), gentamicin (10 μg), colistin (50 μg). The disks with ampicillin, gentamicin, tetracycline and streptomycin were obtained from the National Centre of Infectious and Parasitic Diseases Sofia, those with enrofloxacin – from Bayer, Bulgaria and disks with amoxycillin and colistin – from Ceva Animal.

The behaviour of reisolates to tested antibiotics was interpreted by the three-degree system of Bauer-Kirby as sensitive (S), intermediate (I) and resistant (R).

Due to differences in the observed diameters of inhibition zones (DIZ) prior to and after a certain period of treatment with FPL, the behaviour to tetracycline and streptomycin was tested also by determination of the minimum inhibitory concentrations (MIC). A macro method in broth was employed according to NCCLS criteria (NCCLS, 1997) – Approved Standard M7 –A3, Villanova, PA., 1997.

Together with the study of the bacterial sensitivity to antimicrobial drugs, the following economical traits were also investigated: total feed intake, average daily weight gain, feed intake per unit weight gain and feed conversion ratio (FCR).

The data were statistically processed by means of one way analysis of variance (ANOVA) using the StatMost software package.

RESULTS

The data from the studies of the effect of FPL on the behaviour of the challenge *E. coli* strain to a number of antimicrobial drugs are presented in Tables 2–6. Table 2 presents the data about the changes in the inhibition zones for *tetracycline* as well as the observed MIC for *E. coli*, reisolated from the different experimental groups and the control group, untreated with FPL.

The table shows that by the end of the first week of FPL treatment, there were no changes in the inhibition zones around the tetracycline disks. There were neither changes in the MIC values – they were maintained within the limit of 256 mg/L.

By day 14, the sterile zones increased from 6 to 9 mm in chickens, treated with 4 and 8 mg/kg FPL (groups II and III, respectively).

By day 21, the reisolated strain showed a continuous increase in the inhibition zone. An increasing diameter of the inhibition zone (from 9 to 11 mm) was detected in the group with 4 mg/kg FPL. However, this tendency was not observed in the 8 mg/kg FPL group. In the group that received feed with 2 mg/kg FPL there was an increased inhibition zone (9 mm) but without change in MIC values.

Table 2. Diameters of the inhibition zones (DIZ) and minimum inhibitory concentrations (MIC) of tetracycline in *E. coli* strain isolated from experimentally infected chickens and treated with different doses of flavophospholipol (FPL)

Groups	Group I 2 mg/kg FPL		Group II 4 mg/kg FPL		Group III 8 mg/kg FPL		Control group –infected and untreated	
Days	DIZ (mm)	MIC (mg/L)	DZI (mm)	MIC (mg/L)	DZI (mm)	MIC (mg/L)	DZI (mm)	MIC (mg/L)
Day 0	6	≥256	6	≥256	6	≥256	6	≥256
Day 7	6	≥256	6	≥256	6	≥256	6	≥256
Day 14	6	≥256	9	≥256	9	≥256	6	≥256
Day 21	9	≥256	11	≥256	9	≥256	6	≥256
Day 30	11	≥256	12	≥256	11	≥256	6	≥256

Table 3. Diameters of the inhibition zones (DIZ) and minimum inhibitory concentrations (MIC) of streptomycin in *E. coli* strain isolated from experimentally infected chickens and treated with different doses of flavophospholipol (FPL)

Groups	Group I 2 mg/kg FPL		Group II 4 mg/kg FPL		Group III 8 mg/kg FPL		Control group –infected and untreated	
Days	DIZ (mm)	MIC (mg/L)	DZI (mm)	MIC (mg/L)	DZI (mm)	MIC (mg/L)	DZI (mm)	MIC (mg/L)
Day 0	6	≥32	6	≥32	6	≥32	6	≥32
Day 7	6	≥32	6	≥32	6	≥32	6	≥32
Day 14	6	≥32	11	≥32	9	≥32	6	≥32
Day 21	9	≥32	12	≥32	11	≥32	6	≥32
Day 30	9	≥32	13	≥32	11	≥32	6	≥32

Table 4. Diameters of the inhibition zones (DIZ) of amoxycillin in *E. coli* strain isolated from experimentally infected chickens and treated with different doses of flavophospholipol (FPL)

Groups	Group I 2 mg/kg FPL	Group II 4 mg/kg FPL	Group III 8 mg/kg FPL	Control group –infected and untreated
Days	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)
Day 0	6	6	6	6
Day 7	6	6	6	6
Day 14	6	6	6	6
Day 21	6	6	6	6
Day 30	6	6	6	6

The last check up, performed at the day of euthanasia (day 30 of FPL administration) revealed a continuous increase, although at a lower rate. Up to the end of the treatment however, MIC values of reisolated strains did not change in all groups of chickens.

Reisolates from the positive control group (infected and untreated with FPL) did not manifest any differences either in diameters of inhibition zones (DIZ), or in MIC values.

The data of the behaviour to *streptomycin* are presented in Table 3. In this case, the changes in DIZ were observed during the control study on the sensitivity of reisolates by the 14th day of FPL treatment and onwards. The changes occurred in all three experimental groups but the most significant increase in DIZ was found out in strains reisolated from the second group of chickens (treated with 4 mg/kg FPL).

By day 21, the tendency toward increase was preserved only for isolates obtained from chickens treated with FPL at 4 and 8 mg/kg. Similarly to tetracycline, no change in MIC values were observed.

During the last examination (by day 30), the trend of increasing DIZ was preserved in reisolates from group II (treated with 4 mg/kg FPL). For the first time, MIC values decreased from 32 to 16 mg/L.

The data about *amoxycillin* and *enrofloxacin* are shown on Tables 4 and 5 respectively. The supplementation of feed

with FPL did not influence the DIZ of the challenge *E. coli* strain in both treated groups.

Tables 6–9 present the data of the economical traits: live body weight, average daily weight gain, total feed intake and feed conversion ratio (FCR).

The changes in the live body weight of chickens treated with different FPL doses and both control groups – challenged with *E.coli* but untreated (positive controls) and non-infected and untreated (negative controls) (Table 6) showed statistically insignificant differences in the total average live body weight in the favour of groups that received FPL. The differences between FPL-supplemented groups were also insignificant (P>0.05).

Table 7 presents the changes in the average weight gain. Again, better results although statistically insignificant (P>0.05) were obtained in treated chickens. The differences among groups treated at various FPL doses and between both control groups were also not significant.

The feed intake per one chicken (Table 8) was lower in birds treated with FPL and higher in chickens, non-supplemented with the preparation. The differences between both categories were not significant as well as those among experimental and control groups.

The experimental chickens, treated with FPL exhibited a tendency towards lower feed conversion ratio (FCR) (Table 9), especially during the first 4 weeks of

Table 5. Diameters of the inhibition zones (DIZ) of enrofloxacin in *E. coli* strain isolated from experimentally infected chickens: untreated (controls) and treated with different doses of flavophospholipol (FPL)

Groups	Group I 2 mg/kg FPL	Group II 4 mg/kg FPL	Group III 8 mg/kg FPL	Control group –infected and untreated
Days	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)
Day 0	21	21	21	21
Day 7	21	21	21	21
Day 14	21	21	21	21
Day 21	21	21	21	21
Day 30	21	21	21	21

Table 6. Dynamics of the body weight (g) in chickens from experimental (infected with *E. coli* and treated with flavophospholipol , FPL) and control groups during the experiment

Age (days)	Group I 2 mg/kg FPL	Group II 4 mg/kg FPL	Group III 8 mg/kg FPL	Control group –infected and untreated	Control group – non-infected and untreated
7	146.0	146.0	146.0	146.0	146.0
11	283.0	283.0	283.0	283.0	283.0
18	627.0	640.0	632.0	603.0	602.0
25	1078.0	1096.0	1073.0	1040.0	1038.0
32	1590.0	1636.0	1585.0	1525.0	1528.0
41	2134.0	2184.0	2128.0	2052 .0	2050.0

Table 7. Dynamics of the daily weight gain (g) in chickens from experimental (infected with *E. coli* and treated with flavophospholipol , FPL) and control groups during the experiment

Age (days)	Group I 2 mg/kg FPL	Group II 4 mg/kg FPL	Group III 8 mg/kg FPL	Control group –infected and untreated	Control group – non-infected and untreated
7	–	–	–	–	–
11	34.3	34.3	34.3	34.3	34.3
18	49.1	51.0	49.8	45.7	45.5
25	64.4	65.1	63.0	62.4	62.2
32	73.1	77.1	73.1	69.2	70.0
41	60.4	60.8	60.3	58.5	58.0

Table 8. Dynamics of the average daily feed intake per chicken in experimental (infected with *E. coli* and treated with flavophospholipol , FPL) and control groups during the experiment

Age (days)	Group I 2 mg/kg FPL		Group II 4 mg/kg FPL		Group III 8 mg/kg FPL		Control group –infected and untreated		Control group – non-infected and untreated	
7	15	26.0	15	26.0	15	26.0	15	26.0	15	26.0
11	15	51.0	15	51.0	15	51.0	15	51.0	15	51.0
18	12	83.0	12	84.0	12	84.0	12	79.0	15	79.0
25	9	114.0	9	112.0	9	115.0	9	121.0	15	122.0
32	6	142.0	6	147.0	6	148.0	6	151.0	15	151.0
41	6	169.0	6	168.0	6	171.0	6	172.0	15	171.0

Table 9. Dynamics of the feed intake per weight gain (feed converision ratio, FCR) in chickens from experimental (infected with *E. coli* and treated with flavophospholipol, FPL) and control groups during the experiment

Age (days)	Group I 2 mg/kg FPL	Group II 4 mg/kg FPL	Group III 8 mg/kg FPL	Control group –infected and untreated	Control group – non-infected and untreated
11	1.48	1.48	1.48	1.48	1.48
18	1.68	1.64	1.68	1.72	1.73
25	1.76	1.71	1.82	1.93	1.95
32	1.94	1.90	2.02	2.17	2.15
41	2.79	2.75	2.83	2.93	2.94

life, when the growth of broiler chickens was intensive and the highest FCR values were expected. The differences between chickens, treated and untreated with the growth promoter were not statistically significant.

DISCUSSION

Our results showed that the *in vivo* effect of FPL upon the sensitivity of the experimental challenge *E. coli* strain was manifested by a low influence on diameters of inhibition zones only with respect to streptomycin and tetracycline. Such effect was not however detected in MIC values, if the lowering from 32 mg/L to 16 mg/L against streptomycin at day 30 of FPL treatment at 4 mg/kg is not taken into account.

These results confirmed our preliminary expectations, that FPL supplementation could hardly influence the intrinsic mechanisms of microbial resistance – i.e. the production of inactivating or modifying enzymes, changes in the permeability, drug efflux pump systems etc. Rather, an inhibiting effect on the transfer and dissemination of genetic determinants of resistance within the microbial populations has occurred. A similar interpretation is provided also by van den Bogaard *et al.*, (2002) in an analogous study with coli strains from the resident intestinal microflora in pigs.

The hypothesis of Riedl *et al.* (2000) in connection with the observed capacity of FPL to decrease the resistance to vancomycin of the type Van A in *Enterococcus faecium* strains was comparable. The authors explained the effect with inhibition of the transfer of conjugational plasmids, determining the synthesis of lytic transglycosylases – enzymes that catalyze the cleavage of β-1,4 glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid of the bacterial peptidoglycan.

George *et al.* (1984) confirmed the effect of inhibition of conjugational plasmids *in vitro* in Gram negative bacteria and assumed that the conjugation process itself was probably inhibited (George *et al.*, 1982).

The results obtained about the effect of FPL as growth promoter showed that although statistically insignificant, the differences found in body weight gain and FCR confirmed the stimulating effect of the product even in cases when the birds were challenged with subinfective doses of *E. coli* with confirmed pathogenic properties. The lack of a significant difference between the average daily weight gain in untreated birds from both infected and non-infected controls was interesting. It could be explained by the lack of clinical expression of the colibacteriosis, because an induction of a non-symptomatic carriership was only attempted.

In conclusion, the necessity of further studies on the influence of FPL upon the different stages of microbial resistance appearance and especially in multiresistance patterns is definitely present. In case that its inhibiting effect upon the conjugational transfer of plasmids, determining production of enzymes related to the antimicrobial activity or other genetic determinants of resistance is confirmed, this would be an indicator that the tested growth promotor should be applied in population treatments of animals, especially against dissemination of the bacterial resistance.

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