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Effects of probiotic feed supplement on antibiotic resistance of *E. coli* cultures in puppies

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Abstract. The purpose of this study was to evaluate the sensitivity of *Escherichia coli* to antibiotics in the context of supplementing the diet of three-month-old French bulldog puppies with a probiotic as a biologically active additive. The study employed standard clinical, microbiological, and bacteriological methods, specifically, the disk-diffusion method to assess the sensitivity of *Escherichia coli* cultures to antibiotics and the general bacteriological contamination of faeces samples of puppies of one control group and two experimental groups of puppies that received a probiotic preparation as a feed supplement to the main diet. The study of the effects of the probiotic on the intestinal microflora of puppies found that the use of the probiotic significantly influenced the level of bacterial contamination of the stool samples of the experimental groups. The results of determining the sensitivity of *Escherichia coli* culture to 14 antibiotics were provided. The sensitivity of the culture to the selected antibiotic was determined by the size of the diameter of the growth retardation zones of microorganisms. The findings of the study revealed that the cultures were reliably susceptible to fosfomycin, kanamycin, and azithromycin. The addition of a probiotic symbiotic preparation based on *Bacillus subtilis* and *Bacillus licheniformis* to the diet of the dogs had a significant effect on sensitivity in 78.6% of combinations of conditions and antibacterial substances. Considering the current findings of analogous studies of the preparation on poultry and data on the improvement of biosynthetic

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processes in the digestive tract, indicators of poultry productivity, as well as an increase in the weight of individuals and the intensity of metabolic processes in the body, there are significant prospects for further research in this area and a more detailed study on the use of probiotic therapy in healthy puppies. The findings of the present study can be useful for veterinarians, scientists, and kennel workers of the Kennel Club of Ukraine, the Canine Service of the Armed Forces of Ukraine, and other law enforcement agencies that directly maintain service dogs

Keywords: resistance; *Escherichia coli*; sensitivity of bacteria; probiotic therapy; probiotic

Introduction

Antibiotics were one of the most significant discoveries of the 20th century, saving millions of lives from infectious diseases. The high selective pressure resulting from the proliferation and misuse of antibiotics over the years has led to the development of antimicrobial resistance to many agents (Saha & Sarkar, 2021). Numerous interrelated factors in healthcare and agriculture contribute to the development of antimicrobial resistance through various mechanisms of drug resistance. This was largely facilitated by the spread of infection due to the uncontrolled use of antimicrobial drugs in livestock feed. The prevalence of antimicrobial resistance has reached unprecedented levels worldwide, becoming a silent pandemic that threatens global public health and requires immediate intervention (WHO, 2016). Infections brought on by bacteria resistant to antibiotics have few viable therapeutic choices. This results in serious economic repercussions as well as a marked rise in morbidity and death. According to M. Salam *et al.* (2023), the lack of discovery and availability of new antibiotics to treat life-threatening infections caused by resistant pathogens is in stark contrast to the demand. A. Parmanik *et al.* (2022) noted that the rapid spread of antibiotic-resistant pathogens requires the search for natural antibacterial agents. Studying the epidemiology, detection, and clinical management of drug-resistant bacteria is vital for monitoring, diagnosing, and treating drug-resistant bacterial colonies in hospital-acquired and community-acquired outbreaks (Van Camp *et al.*, 2020). Y. Zhu *et al.* (2022) highlighted the clinical implications of antimicrobial resistance in bacteria, focusing on its significant threat to public health. The researchers detailed the mechanisms by which bacteria develop resistance, including genetic mutations and horizontal gene transfer, and emphasised the challenges in treating infections caused by multidrug-resistant strains. The researchers also discussed the global burden of antimicrobial resistance, stressing the urgent need for coordinated efforts in antibiotic stewardship, infection prevention, and the development of novel antimicrobials.

According to L. Zhou *et al.* (2024), probiotics, prebiotics, and synbiotics are increasingly being used in modern animal feeding regimens as effective alternatives to antibiotics, with significant benefits for gut health and overall performance. Probiotics enhance the population of beneficial gut bacteria, regulate microbiota, reduce harmful bacteria, and improve digestion and nutrient absorption, contributing to better feed conversion ratios, growth rates, and carcass yields. Prebiotics, such as mannan oligosaccharides, provide substrates for beneficial bacteria, further supporting gut health and microbial balance. Synbiotics, combining prebiotics and probiotics, have shown even greater efficacy, improving broiler performance more effectively than either additive alone. Together, these feed additives improve the microbial composition of the gastrointestinal tract, boost resistance to harmful bacteria, and enhance metabolic functions, making them powerful tools for sustainable animal production.

T. Uddin *et al.* (2021) noted that novel approaches like whole genome sequencing, bacteriophage therapy, monoclonal antibodies, and quorum-quenching mechanisms offer promising alternatives. One particularly fascinating insight is the resurgence of bacteriophage therapy, which predates antibiotics and is now being revisited as a targeted and environmentally friendly solution to combat resistant bacterial strains. This analysis underscores the urgency of innovation and collaboration to address the antimicrobial resistance crisis and protect global health. According to C. Brives & J. Pourraz (2020), phage therapy, an alternative to treating bacterial infections using bacteriophage viruses, has been around for over a century. Despite gaining support over the past 15 years from researchers who have utilised it as a promising treatment in the face of rising resistance, this treatment has been challenging to develop.

According to O. Shkromada *et al.* (2022), modern feeding strategies based on the use of probiotic preparations are aimed at reducing the colonisation or population of pathogenic bacteria in the intestines of animals and increasing the amount of beneficial microflora for example in piglets. At the same time, the ban on the use of antibiotics as animal growth stimulators and their limited use for preventive or therapeutic purposes led to an increase in the use of probiotic preparations in compound feed (Yue *et al.*, 2020). The range of probiotics available on the market is growing every year. Moreover, the newly created categories of probiotics differ from each other in terms of properties and mode of action. In the countries of Europe, Asia, and in Ukraine, probiotics are widely used as an alternative to antibiotics in the poultry industry, particularly for growing broiler chickens and the subsequent production of meat and poultry slaughter by-products. The use of probiotic preparations in poultry organisms increases the stimulation of biosynthetic processes in the digestive tract and increases poultry productivity, including livestock preservation, poultry growth, a reduction in the amount of time that poultry are fed, an increase in the weight of poultry carcasses and offal, and improved metabolic processes in the body of grill chickens (Bohatko, 2023). The probiotic is used as a biologically active additive to feed poultry, pigs, cattle, and rabbits to form the normal microflora of gastrointestinal diseases, stabilising the body's defences, increasing productivity, and preserving livestock (Tian *et al.*, 2021). According to A. Hasan *et al.* (2022), the use of additives in the early spring feeding of bee families helps to increase the wax-making activity of bees, as well as honey productivity and honey collection in the spring-summer period. Notably, there is no data that would detail the results of the use of probiotics in puppies. In this regard, the study of the effect of probiotics on the dog population was conducted. Thus, the purpose of the present study was to investigate the effects of probiotics on the antibiotic resistance of the microflora of healthy puppies at varying dosages of the drug.

Materials and Methods

Preliminary analysis of literary sources necessitated the study of the effects of the drug "Subtiform" on the body of the bird, which improved the bird's overall output and the activation of biosynthetic processes in the digestive system. The use of probiotics contributed to the improvement of metabolic processes in the body, the preservation of livestock, the growth of poultry, and at the same time, the reduction of the duration of poultry feeding, the increase in the weight of the carcass and offal of broiler chickens (Bohatko, 2023). The experiment was conducted in October-November 2024, based on the nursery in Bila Tserkva, Ukraine. The study was conducted in dogs of the French bulldog breed (n = 15). Groups were formed from dogs of the same age and weight characteristics (puppies aged 3 months). The biochemical indicators were weighed and analysed before the start of the experiment and weekly. The dose per dog was adjusted according to the baseline age, weight, and final weight at the end of the study. Probiotic "Subtiform" (TC U 10.9-30165603-027:2023, Ukraine) is a preparation of a symbiotic nature, containing bacteria of the genus *Bacillus subtilis* and *Bacillus licheniformis* in the amount of 2.5×10^9 CFU/g and a filler-dry milk whey. For dogs, the dosages of the probiotic in the experimental groups were as follows: 0.5 g per 10 kg of live weight and 2.0 g per 10 kg of live weight. Probiotic was not used in the control group. The duration of the study was 30 days. Sampling was performed in individuals during sedation with the drug "Madison" subcutaneously, in the amount of 0.3 mL per 10 kg of live weight, faeces were collected directly from the rectum, in sterile gloves, and in sterile containers that were sent to the Control and Testing Laboratory of Food and Agricultural Products of the Kharkiv Regional Consumer Union (Kharkiv, Ukraine) in refrigeration units. The weight of each sample was 10 g.

The sensitivity of the cultures isolated from the samples to antibiotics was determined according to the method of determining the sensitivity of microorganisms to antibacterial drugs (Harkavenko *et al.*, 2015). The general level of tank contamination and the presence of microorganisms of the genus *Proteus* were determined following the methodological recommendations for the bacteriological analysis of animal feed (ISO 4831:2006). Enteropathogenic *Escherichia coli* in 1 g was determined according to the method of DSTU 8680:2016. The level of colonisation of the medium by enterococci was carried out by the general principles and methodical instructions of DSTU 8534:2015. All the studies were conducted following the European Convention on the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes (1986) and the Law of Ukraine No. 3447-IV "On the Protection of Animals from Cruelty" (2006). Statistical processing was performed using the software Statistica 6.0 and Microsoft Excel.

Results and Discussion

The study of the effects of the probiotic on the intestinal microflora of puppies found that the use of the probiotic significantly influenced the level of bacterial contamination of the stool samples of the experimental groups (Table 1).

Table 1. Effects of the probiotic on the total bacterial contamination of the samples

	Control		**Experimental group 1**		**Experimental group 2**	
Total bacterial contamination	before	2.43×10^8 $\pm0.12\times10^8$ CFU	before	2.17×10^8 $\pm0.11\times10^8$ CFU	before	2.46×10^8 $\pm0.12\times10^8$ CFU
	after	2.48×10^8 $\pm0.12\times10^8$ CFU	after	$*1.91\times10^8$ $\pm0.09\times10^8$ CFU	after	$*2.15\times10^8$ $\pm0.11\times10^8$ CFU

Note: ** – the difference is significant at $P < 0.05$; CFU – colony-forming unit*
Source*: developed by the authors of this study based on the findings*

General examination showed that before the start of probiotic therapy, organisms of the genus *Proteus* were detected in three samples, and no causative agents of diplococcal infection were isolated from the material. On the other hand, it was not possible to establish the presence of microorganisms of the genus *Proteus* and isolate the causative agents of diplococcal infection in the samples isolated for the 4th week of the experiment. The presence of enteropathogenic *Escherichia coli* was established in all samples.

It was found that even the use of a lower dosage of the drug helped to significantly reduce the number of bacteria in the samples. The effectiveness of the drug in the experimental group with a greater concentration of the drug in the feed was lower compared to the indicators of the experimental group 1 on the 4th week of the study. Despite this, the indicators of both groups were significantly lower than the values obtained in the control group. Such a regularity may suggest the absence of a cumulative effect from the use of probiotics as a biological supplement to the main diet of dogs. However, this implies the possibility of using the probiotic as an auxiliary component to increase the effectiveness of empiric therapy with antimicrobial drugs. It is known that the use of probiotics allows making the treatment of sick individuals more qualitative, safer, and economically profitable (Pentylyuk, 2006; WHO, 2016). This was confirmed by analogous studies of Ukrainian scientists, which indicated that the use of probiotic preparations in the composition of granulated compound feed contributed to increasing the productivity of young pigs, reducing feed costs by 0.15-0.25 energy feed units (Hutsol *et al.*, 2023). Other scientists, such as P. Markowiak & K. Śliżewska (2017), also obtained analogous findings.

Notably, the obtained findings may also be related to the possibility of reducing the spread of antimicrobial resistance and other undesirable phenomena from the use of antimicrobial drugs. In this regard, the effect of probiotics on the sensitivity of *E. coli* cultures to antibiotics was investigated (Table 2).

Table 2. Sensitivity of selected cultures of *E. coli* to antibiotics

Variant name of antibiotic	1st week			4th week		
	Control	Experimental group 1	Experimental group 2	Control	Experimental group 1	Experimental group 2
Levofloxacin	24±1.20	24.2±1.21	*20.8±1.04	21.8±1.09	*16.6±0.83	*24.0±1.20
Cefepime	26.4±1.32	26.6±1.33	26.8±1.34	27.4±1.37	*22.0±1.10	27.0±1.35
Gentamicin	24.2±1.21	*21.0±1.05	23.2±1.16	23.8±1.19	23.4±1.17	23.0±1.15
Norfloxacin	24.0±1.20	24.0±1.20	25.0±1.25	25.4±1.27	*21.2±1.06	25.0±1.25
Ceftriaxone	23.4±1.17	21.8±1.09	*21.0±1.05	21.8±1.09	21.8±1.09	20.0±1.0
Ofloxacin	24.6±1.23	25.4±1.27	25.6±1.28	24.6±1.23	24.8±1.24	25.0±1.25
Kanamycin	22.4±1.12	21.8±1.09	23.4±1.17	25.0±1.25	*22.0±1.10	*20.0±1.0
Nitrofurantoin	22.0±1.10	23.8±1.19	*26.6±1.33	26.8±1.34	*20.0±1.0	*20.0±1.0
Azithromycin	23.8±1.19	24.4±1.22	25.2±1.26	26.0±1.30	*21.2±1.06	*20.0±1.0
Chloramphenicol	23.8±1.19	*26.6±1.33	24.4±1.22	23.8±1.19	25.8±1.29	25.0±1.25
Doxycycline	3.8±0.19	*8.2±0.41	*10.8±0.54	10.0±0.5	10.2±0.51	10.0±0.50
Cefazolin	2.0±0.10	*9.0±0.45	*11.0±0.55	11.2±0.56	*13.4±0.67	10.0±0.50
Fosfomycin	–	*1.8±0.09	*2.4±0.12	2.4±0.12	*–	*–
Enrofloxacin	2.2±0.11	*2.0±0.10	*–	–	*18.0±0.90	–

Note: ** – the difference is significant at P < 0.05*
Source: *developed by the authors based on the research*

The significant differences were established in indicators of sensitivity to antibiotics of *E. coli* cultures isolated from the intestines of puppies at the beginning of the study and the end of the experiment. Table 2 lists the names of 14 antibiotics whose diameters of growth zones were the largest: cultures that formed growth zones with a diameter of 1-10 mm were considered weakly sensitive, 10-20 mm – sensitive, and over 20 mm – highly sensitive. The absence of zones of growth retardation indicated the resistance of *Escherichia coli* to the antibiotic.

The indicators of sensitivity of *E. coli* to fluoroquinolone antibiotics (levofloxacin, norfloxacin, and enrofloxacin), as well as to doxycycline, a representative of the tetracycline group of antibiotics, are significant, as the issue of the development of resistance to them is quite acute since the phenomenon is developing quite rapidly. The sensitivity of the cultures to the action of the representative of nitrofurans, nitrofurathion, was significantly higher in experimental group 2 at the beginning of the study, but these indicators significantly decreased after long-term use of the probiotic. This was also confirmed by the findings of other scientists who used a multi-strain probiotic to treat the consequences and symptoms of gastroenteritis, where the improvement occurred as early as on the 7th day of using the drug (Molina *et al.*, 2023). B. Han *et al.* (2024) demonstrated an analogous effect for *Lactobacilli*, which could be a good choice to improve the gut health and immune functions of cats, being associated with the lipid mechanism of cats. The effect of the probiotic on the sensitivity of microorganisms to kanamycin, which is typically active against *Escherichia coli*, and fosfomycin, which rarely develops resistance due to its unique mechanism of action, was expected (O'Brien, 1997; Nunan, 2017). The effects of probiotic on the sensitivity of selected cultures to chloramphenicol, which has a bacteriostatic effect, was analogous, although it was characterised by the rapid development of resistance of pathogenic microorganisms to it, including due to the natural origin of the antibiotic.

As a result of the study, the sensitivity to antibiotic drugs fosfomycin, kanamycin, and azithromycin increased significantly. As for the increased sensitivity to the latter, it was related to the macrolide adsorption site and, accordingly, the synergistic effect of the antibiotic with the symbiotic probiotic. Notably, the sensitivity of *Escherichia coli* stayed high under the action of antibiotics in 78.6% of cases. Thus, the use of probiotics contributed to the formation of fairly high resistance indicators in puppies, even during a fairly short period of use.

Conclusions

During the long-term use of the probiotic, the indicators of the total contamination of the stool samples of the French bulldog puppies significantly decreased, while the indicators of the sensitivity of the *Escherichia coli* culture to the antibiotics fosfomycin, kanamycin, and azithromycin increased, which is associated with the synergism of the action of the probiotic and antibacterial substances. It is known that the use of probiotics allows making the treatment of sick individuals more qualitative, safer, and economically profitable. Furthermore, such an effect on microbiological indicators, specifically, the reduction of microbiological contamination, can be achieved even during a short period of use of the supplement. In addition, the indicators of sensitivity of cultures selected according to bacteriological methods of research suggest the significant synergism in the action of probiotics and antibiotics, which is currently quite significant in the global strategy of combating the emergence of antibiotic resistance in pathogenic microorganisms. Reducing the level of contamination of faeces is a prerequisite for reducing the probability of the developing a more stable immunity and own intestinal microbiota. It also allows reducing potentially dangerous risks for human health, other animals, and the environment. Such features of the effect of the studied probiotic will be of great interest to specialists in the kennels of the Ukrainian Kennel Service and structures where the well-being of animals is not only a generally accepted area of work, but also a basis for the national security of the country.

Analogous studies testify to the effects of probiotics on metabolic processes, weight gain, and biochemical indicators, which gives grounds for further research. Building on these findings, future studies could focus on exploring the long-term effects of probiotics on broader aspects of animal health, including their role in modulating immune responses and enhancing resistance to various environmental pathogens. Investigating the specific mechanisms underlying the synergistic effects of probiotics and antibiotics could provide valuable insights for optimising combined therapeutic approaches. Additionally, further studies are required to assess the effects of probiotics on metabolic functions, growth performance, and the prevention of antibiotic-resistant infections in various breeds and age groups.

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Conflict of Interest

None.

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Вплив пробіотичної кормової добавки на антибіотикорезистентність культур *E. coli* у цуценят

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Анотація. Метою дослідження було оцінити чутливість *Escherichia coli* до антибіотиків у контексті додавання пробіотика як біологічно активної добавки до раціону тримісячних цуценят французького бульдога. Робота виконана за допомогою загальноприйнятих клінічних, мікробіологічних та бактеріологічних методів дослідження, зокрема дискодифузійного методу, з метою оцінки чутливості культур кишкової палички до антибіотиків та загальної бактеріологічної забрудненості зразків калу цуценят однієї контрольної групи та двох експериментальних груп цуценят, що отримували в якості кормової добавки до основного раціону пробіотичний препарат. Під час дослідження впливу пробіотика на мікрофлору кишечнику цуценят було встановлено, що використання пробіотика достовірно впливало на рівень бактеріальної забрудненості зразків калу цуценят експериментальних груп. Наведено результати визначення чутливості культури кишкової палички до 14 антибіотиків. За розміром діаметра зон затримки росту мікроорганізмів визначали чутливість культури до обраного антибіотика. Результати досліджень засвідчили, що достовірно високочутливими культури були до фосфоміцину, канаміцину та азитроміцину.

Додавання до раціону собак пробіотичного симбіотичного препарату на основі *Bacillus subtilis* і *Bacillus licheniformis* мало суттєвий вплив на чутливість у 78,6 % комбінацій умов та антибактеріальної речовини. Зважаючи на сучасні результати аналогічних досліджень препарату на поголів'ї птиці та дані щодо поліпшення біосинтетичних процесів у травному тракті, показники продуктивності птиці, а також збільшення ваги особин та інтенсивність обмінних процесів в організмі, існує значна перспектива подальших досліджень у цьому напрямі та перспектива більш детального вивчення питання використання пробіотикотерапії у здорових цуценят. Дослідження може стати корисним для ветеринарів, науковців та працівників розплідників Кінологічної спілки України, Кінологічної служби ЗСУ та інших силових структур, що безпосередньо утримують службових собак

Ключові слова: резистентність; кишкова паличка; чутливість бактерій; пробіотикотерапія; пробіотик