Praca oryginalna ***Original paper***

In vitro antibacterial effect of enrofloxacin combined with lavender essential oil on selected *Salmonella* serotypes isolated most commonly in poultry

MICHALINA ADASZYŃSKA-SKWIRZYŃSKA, SŁAWOMIR ZYCH, DANUTA SZCZERBIŃSKA

Faculty of Biotechnology and Animal Husbandry, West Pomeranian University of Technology, Szczecin, 29 Klemensa Janickiego Street, 71-270 Szczecin, Poland

Received 08.09.2022 *Accepted 28.12.2022*

Adaszyńska-Skwirzyńska M., Zych S., Szczerbińska D.

***In vitro* antibacterial effect of enrofloxacin combined with lavender essential oil on selected *Salmonella* serotypes isolated most commonly in poultry**

Summary

The present study investigated the antimicrobial activity of enrofloxacin in combination with lavender (*Lavandula angustifolia*) essential oil against three serotypes of *Salmonella*: *Salmonella* Enteritidis ATCC 13076, *Salmonella* Gallinarum biovar Pullorum ATCC 13036, *Salmonella* Typhimurium ATCC 14028 and *Salmonella* Typhimurium – a moderately sensitive monophasic strain. The analysis of the interaction of enrofloxacin with lavender essential oil was carried out using the checkerboard method. The obtained FICI values were interpreted as follows: synergistic when ≤ 0.5; additive when > 0.5 but ≤ 1; non-interactive when > 1 but ≤ 4; antagonistic when > 4. When analysing individual combinations of enrofloxacin with lavender oil, a positive additive effect was found in all cases (*S.* Enteritidis ATCC 13076 FICI – 0.85; *S.* Gallinarum biovar Pullorum ATCC 13036 FICI – 1.0; *S.* Typhimurium ATCC 14028 and *S.* Typhimurium – a moderately sensitive monophasic strain FICI – 0.7). Antagonistic interaction was not recorded in any case. When analysing individual combinations of enrofloxacin with lavender oil against the serotypes tested, an additive effect was demonstrated in all cases (FIC indices: 0.7-1.0). There was no antagonistic effect in any of the analysed combinations.

Keywords: lavender essential oil, enrofloxacin, antibacterial activity

Salmonella are members of the family Enterobacteriaceae and are facultative anaerobic rods (11). Salmonelloses are among the most widespread zoonotic diseases worldwide that can be transmitted from animals to humans (11, 17). The main sources of infections with bacteria of the genus *Salmonella* are products of animal origin, especially poultry and eggs (12). The genus *Salmonella* includes two species: *Salmonella enterica* (divided into six subspecies) and *Salmonella bongori* (within which 20 serotypes are distinguished) (8). Non-motile *Salmonella* Gallinarum, comprising *Salmonella* Gallinarum biovar Gallinarum and *Salmonella* Gallinarum biovar Pullorum responsible for fowl typhoid and pullorum disease, respectively, is a specific invasive serotype occurring in farmed poultry (25). The non-specific though equally invasive serotypes include *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Infantis (17). Bacteria of the genus *Salmonella* spp. may be present in farmed poultry's gastrointestinal tracts and reproductive systems while contaminating the rearing environment. An important factor causing direct infections in animals is the use of feed mixtures contaminated with pathogens, which may result from non-compliance with certain rules during their preparation. A frequent cause of *Salmonella* occurrence in poultry farms are inadequate housing conditions, e.g., suboptimal temperature, humidity, air exchange, lack of light and too high density, which affects the health and condition of the birds. Despite the undoubted progress in treating bacterial diseases in humans and animals, largely dependent on properly selected therapy, there is still a need for more effective and less toxic drugs. The effectiveness of antimicrobial substances applied to date is limited due to, among other things, the development of drug resistance in bacteria. Hence, the possibility of using, for instance, various plant products for veterinary purposes is attracting increasing interest. Their search includes

not only the identification of compounds with direct antimicrobial activity but also those that modulate drug resistance or inhibit the expression of virulence factors (21, 26). The search for complementary/alternative treatments is required in light of the constantly increasing microbial drug resistance and depletion of effective antimicrobial agents. It is desirable not only to find new agents with biocidal activity but also such ones that can show synergism or an additive effect with available classical antimicrobials.

In recent years, the discovery of new antibiotics has slowed, and the management of existing substances has become crucial to the rational use of antibiotics. Considering this, well-known substances, such as antibiotics and essential oils, should be better utilised. The essential oil obtained from lavender (*Lavandula angustifolia*) is particularly noteworthy and characterised by a strong antiseptic effect (1, 2, 4, 28). The antimicrobial properties of essential oils, including lavender oil, are proven; however, their interaction with synthetic antibiotics is poorly understood (1, 4, 20). Essential oils can be safely combined with antimicrobials if the effect of this combination is synergism (potentiation of the effect) or addition (summation of the effect) (3, 4).

Enrofloxacin belongs to the fluoroquinolone group of antibiotics, which was the first to be approved for use in the treatment of domestic animals and is now applied as a chemotherapeutic for many infections found in poultry. Quinolones and their currently used second-generation derivatives – fluoroquinolones, which include enrofloxacin – are a group of chemotherapeutics with a broad spectrum of applications. Enrofloxacin is characterised by its efficacy against many Gram-positive and Gram-negative pathogens, including strains of *Salmonella*; however, increasing resistance has been detected in recent years. Fluoroquinolone resistance is associated chiefly with some chromosomal mutations in the genes encoding targeted enzymes, mainly DNA gyrase and topoisomerase IV. In most *Salmonella* strains resistant to quinolones, the so-called QRDR (quinolone resistance-determining region) containing *gyrA*, *gyrB*, *parC* and *parE* genes has been identified (9). However, plasmid-encoded genes forming so-called plasmid-mediated quinolone resistance can also acquire fluoroquinolone resistance (PMQR). Among PMQR gene groups, the qnr family (qnrA, qnrB, qnrC, qnrD, qnrE, qnrS, and qnrVC), antibiotic efflux pump-coding genes (qepA and oqxAB), antibiotic modification enzyme gene (aac(6) Ib-cr) and phosphorylase gene (crpP) can be distinguished (15). Studies have indicated that the use of only strong doses of enrofloxacin (even 100 mg/kg body weight) for seven days could give positive results in the treatment of salmonellosis with minimal risk of the emergence of resistant strains (16). A practical research direction is to search for synergism or additivity of enrofloxacin with natural antimicrobial agents such as essential oils. This is because it allows the reduction of the dose of the drug below its toxicity threshold (26). Therefore, the study aimed to investigate the interaction of lavender oil with enrofloxacin against selected *Salmonella* serotypes.

Material and methods

Four microorganisms were selected for the susceptibility analysis to enrofloxacin: *Salmonella* Enteritidis ATCC 13076, *Salmonella enterica* serovar Gallinarum biovar Pullorum ATCC 13036; *Salmonella* Typhimurium ATCC 14028 (KwikStik™, Microbiologics, St. Cloud, MS, USA); *Salmonella* Typhimurium – monophasic strain with the 1,4,[5],12:i:-antigene pattern (source: Proficiency Testing, National Reference Laboratory for Salmonellosis, National Veterinary Research Institute, National Research Institute, Puławy, Poland). The disc-diffusion method was used to determine the preliminary classification of the tested strains for susceptibility to enrofloxacin (commercial disc 5 µg, OXOID, Argenta, Poznan, Poland). Interpretive criteria of zone diameter (mm) was used according to CLSI VET01S 5th edition (*Enterobacteriaceae*, pets and poultry; ≤ 16 (R) resistant, 17-22 (I) intermediate, ≥ 23 (S) susceptible). Baytril 10% (Bayer Animal Heath, Leverkusen, Germany) – a commercial drug containing enrofloxacin 100 mg/ml – was used for microdilution testing as the active agent, and a commercial lavender oil (Avicenna-Oil, Wrocław, Poland). The lavender essential oil ingredients were described in our previous study (2). In lavender essential oil, 26 chemical compounds were identified, of which linalool acetate (46.25%) and linalool (35.17%) were the main compounds. At lower concentrations (< 5%): borneol, bornyl acetate, camphene, camphor, 3-carene, carvacrol, caryophyllene, copaene, p-cymene, eucalyptol, β-farnesene, geraniol acetate, hexyl acetate, lavandulol acetate, linalool oxide, p-menth-1-en-8-ol, nerol acetate, nerol oxide, cis-β-ocimene, trans-β-ocimene, α-pinene, β-pinene, 4-terpineol and α-terpineole were also present. Chromatographic analysis showed that the relative percentages of the individual classes of compounds in the studied essential oil were as follows (%): oxygenated monoterpenes (87.79), monoterpene hydrocarbons (8.17), sesquiterpene hydrocarbons (2.93). Dimethylsulfoxide (POCH, Gliwice, Poland) was used as an organic solvent to dilute the oil. In order to determine the susceptibility of the tested microorganisms to the analysed antimicrobial agents, the determination of minimum inhibitory concentration (MIC). All identifications were made on individually packed, sterile, divided, 96-wells polystyrene titration plates with flat bottoms. Again, interpretive criteria of MIC (µg/mL) was used according to CLSI VET01S 5th edition (*Enterobacteriaceae*, dogs and cats ≥ 4 (R), 1-2 (I), ≤ 0.5 (S) and poultry; ≥ 2 (R), 0.5 – 1 (I), ≤ 0.25 (S). The Clinical and Laboratory Standard Institute (CLSI) recommendations were followed (6, 7). Each test was repeated three times. The medium was used as a ready-to-use cation-adjusted Mueller-Hinton broth (CAMHB) (Graso, Starogard Gdański, Polska). The final volume of broth in each well was 100 µL. For enrofloxacin, a series of two-fold dilutions were made in the range of 0.001÷100 mg/mL, and for essential oil, a concentration gradient was prepared in

the range of 0.005÷50% v/v. Colonies of each serotype were selected from 18- to 24-hour nonselective blood agar plates. The inoculum of 1-2 × 10^8 CFU/mL, corresponding to 0.5 McFarland standard measured in a DEN-1 densitometer (BioSan, Józefów, Poland) were employed in the tests (according to CLSI, the final concentration in each well was approximately 5 × 10^5 CFU/mL). The microtiter plate was incubated in an incubator at + 35.0 ± 2°C for 18 h ± 2 h. Due to the natural clouding of the medium at high concentrations of the essential oil and DMSO, the result was read macroscopically, taking this into account. Both antimicrobial agents were considered inefficient when clouding characteristic only for bacteria growth was present. The study of the effect of enrofloxacin in combination with lavender oil was carried out using the checkerboard method, according to the methodology described in the previous article (3). MIC data of the lavender essential oil and enrofloxacin were converted into Fractional Inhibitory Concentration (FIC) and defined as the antimicrobial concentration in an inhibitory concentration with a second compound to the concentration of the antimicrobial by itself (27). In the combination assays, the checkerboard procedure described by Rosato et al. (23) was followed to evaluate the interaction of the essential oil with an antibiotic. The substance combinations were analysed in our experimental protocol by calculating the FIC index (FICI).

Results and discussion

The sensitivity of the tested *Salmonella* strains to lavender oil and enrofloxacin was determined using the microdilution method. The tested bacterial strains differed in their susceptibility to enrofloxacin. MIC for *Salmonella* spp. was determined in the present study as % v/v. It fluctuated significantly in the range of 0.5--0.625% v/v for serotypes from group D (*S.* Enteritidis and *S.* Gallinarum biovar Pullorum) and as much as 10% v/v for serotypes from group B – *S.* Typhimurium (Tab. 1). In contrast, Hossain et al. (10) carried out a study on six *Salmonella* spp. isolates and obtained a MIC for lavender oil of 0.5-1.0% v/v. The reported increase in the frequency of bacterial infections and drug resistance is forcing the search for new solutions to improve the effectiveness of therapies (21). Publications have presented studies on the application of essential oils (4, 9, 21, 23), which exhibit broad-spectrum antimicrobial properties. They exhibit broad-spectrum antimicrobial properties while being less toxic than most chemotherapeutics (26). It should be emphasised that it is possible to improve clinical efficacy by combining the action of chemotherapeutics and substances of natural origin in search of a synergistic or additive effect to broaden the spectrum or enhance the potency of already known and used antibiotics. In our study, the evaluation of the effects of lavender oil in combination with enrofloxacin was carried out using the checkerboard method. The calculated FICI values were used to demonstrate the presence or absence of synergism, addition or antagonism.

When analysing individual combinations of enrofloxacin with lavender oil, a positive additive effect was found in all cases (*S.* Enteritidis ATCC 13076 FICI – 0.85; *S.* Gallinarum biovar Pullorum ATCC 13036 FICI – 1.0; *S.* Typhimurium ATCC 14028 and *S.* Typhimurium – a moderately sensitive monophasic strain FICI – 0.7). Antagonistic interaction was not recorded in any case. Enrofloxacin used in the study was a formulation administered in drinking water to poultry at a daily dose of 10 mg/kg body weight for 3-5 days. In this form, enrofloxacin is rapidly absorbed and reaches in poultry a serum concentration of 1.81-2.44 µg/ml after just 1.5-2 h (22). Some strains of *Salmonella* at classical dosing can survive treatment in most organs and/or tissues, with the exception of the intestines, where the highest concentrations of enrofloxacin are usually recorded, creating a risk of recurrence of the disease (16, 22). In this case, usually double doses of the drug (2-25-fold) or extended treatment are used, but as shown by Randall et al. (22), it could also be ineffective with intermediate sensitive strains to enrofloxacin. Unfortunately, as already mentioned, in recent years, an increase in resistance of *Salmonella* to enrofloxacin has been observed, and *Salmonella* Typhimurium is particularly predisposed to drug resistance (12, 18). Other researchers also found a positive effect of the combination of enrofloxacin with cinnamon essential oil (FICI ≤ 0.5 in 60% of tests) against 15 strains of *S. enterica* (24). In contrast,

Tab. 1. Lavender essential oil in combination with enrofloxacin – fractional inhibitory concentration (FIC) and FIC indices (FICI)

Strain	*Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076				*Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028				*Salmonella enterica* subsp. *enterica* serovar Typhimurium monophasic strain 1.4,[5],12:i:-				*Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Pullorum ATCC 13036			
Mean zone diameter (enrofloxacin 5 µg)	30.3 mm susceptibility				28.3 mm susceptibility				22.3 mm intermediate				30.7 mm susceptibility			
	MIC_0	MIC_c	FIC	FICI	MIC_0	MIC_c	FIC	FICI	MIC_0	MIC_c	FIC	FICI	MIC_0	MIC_c	FIC	FICI
Lavender Essential oil (LEO) (% v/v)	0.625	0.5	0.8	0.85	10.0	2.0	0.2	0.7	10.0	2.0	0.2	0.7	0.5	0.25	0.5	1.0
Enrofloxacin (ENRO) (µg/mL)	0.04	0.002	0.05		0.08	0.04	0.5		0.16	0.08	0.5		0.04	0.02	0.5	

Explanations: MIC – Minimal Inhibitory Concentration; FIC – Fractional Inhibitory Concentration; FICI – Fractional Inhibitory Concentration Index; MIC_0 = individual MIC (LEO or ENRO); MIC_c = MIC in combination (LEO × ENRO or ENRO × LEO); FIC = MIC_c/MIC_0; FIC index = FIC of LEO + FIC of ENRO FICI ≤ 0.5 synergistic; FICI > 0.5-1.0 additive; FICI > 1.0-4.0 no interaction; FICI > 4.0 antagonistic (2, 22)

a study on one of the main components of lavender oil – linalool – in combination with nisin showed no interaction against *S.* Typhimurium (MTCC 3224), FICI = 1.25 (5). Lauteri et al. (14) tested the combination of ciprofloxacin and essential oils from *Thymus vulgaris*, *Eugenia caryophyllata* and *Coridothymus capitatus* against 36 resistant strains of *Salmonella* and obtained FICIs ranging from 0.5 to 4.1.

Interpretation and comparison of the results of such studies are complicated; however, in the current combat against the growing problem of drug resistance, the correlations detected may contribute in the future to the introduction and use of combination therapy in veterinary medicine. In light of the results presented in the current study, research on the interactions between combinations of essential oils and synthetic antibiotics should be continued. Considering the demonstrated lavender essential oil *in vitro* effect with enrofloxacin towards *Salmonella* serotypes, further studies are necessary to confirm the efficacy of the combination in *in vivo* tests.

References

1. *Adaszyńska M., Swarcewicz M., Dzięcioł M., Dobrowolska A.*: Comparison of chemical composition and antibacterial activity of lavender varieties from Poland. Nat. Prod. Res. 2013, 27, 1497-1501.
2. *Adaszyńska-Skwirzyńska M., Szczerbińska D.*: The antimicrobial activity of lavender essential oil (Lavandula angustifolia) and its influence on the production performance of broiler chickens. J. Anim. Physiol. Anim. Nutr. 2018, 102, 1020-1025.
3. *Adaszyńska-Skwirzyńska M., Szczerbińska D., Zych S.*: Antibacterial activity of lavender essential oil and linalool combined with gentamicin on selected bacterial strains. Med. Weter. 2020, 76, 2, 115-118.
4. *Adaszyńska-Skwirzyńska M., Szczerbińska D., Zych S.*: The use of lavender (Lavandula angustifolia) essential oil as an additive to drinking water for broiler chickens and its in vitro reaction with enrofloxacin. Animals 2021, 11, 6, 1535.
5. *Bag A., Chattopadhyay R. R.*: Synergistic antibacterial and antibiofilm efficacy of nisin in combination with p-coumaric acid against food-borne bacteria Bacillus cereus and Salmonella typhimurium. Lett. Appl. Microbiol. 2017, 65, 5, 366-372.
6. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 5th Edition. CLSI supplement VET01S, Wayne, PA, USA 2020.
7. Clinical Laboratory Standards Institute: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, approved standard: M7-A6, 2002, vol. 23-n 2.
8. *El-Saadony M. T., Salem H. M., El-Tahan A. M., Abd El-Mageed T. A., Soliman S. M., Khafaga A. F., Swelum A. A., Ahmed A. E., Alshammari F. A., Abd El-Hack M. E.*: The control of poultry salmonellosis using organic agents: an updated overview. Poult. Sci. 2022, 101, 4, 101716.
9. *Hopkins K. L., Davies R. H., Threlfall E. J.*: Mechanisms of quinolone resistance in Escherichia coli and Salmonella: recent developments. Int. J. Antimicrob. Agents 2005, 25, 5, 358-373.
10. *Hossain S., Heo H., De Silva B. C. J., Wimalasena S. H. M. P., Pathriana H. N. K. S., Heo G. J.*: Antibacterial activity of essential oil from lavender (Lavandula angustifolia) against pet turtle-borne pathogenic bacteria. Lab. Anim. Res. 2017, 33, 3, 195-201.
11. *Hossain M. J., Attia Y., Ballah F. M., Islam M. S., Sobur M. A., Islam M. A., Ievy S., Rahman A., Nishiyama A., Islam M. S., Hassan J., Rahman M. T.*: Zoonotic significance and antimicrobial resistance in *Salmonella* in poultry in Bangladesh for the period of 2011-2021. Zoonotic Diseases 2021, 1, 3-24.
12. *Iwiński H., Wódz K., Chodkowska K, Nowak T, Różański H.*: In vitro evaluation of antimicrobial effect of phytobiotics mixture on Salmonella spp. isolated from chicken broiler. Antibiotics 2022, 11, 7, 868.
13. *Janda J. M., Abbott S. L.*: The family enterobacteriaceae. In Practical Handbook of Microbiology 3rd ed.; CRC Press: Boca Raton, FL, USA 2015, 307.
14. *Lauteri C., Maggio F., Serio A., Festino A. R., Paparella A., Vergara A.*: Overcoming multidrug resistance in Salmonella spp. isolates obtained from the swine food chain by using essential oils: an in vitro study. Front. Microbiol. 2022, 9, 12, 808286.
15. *Lee S., Park N., Yun S., Hur E., Song J., Lee H., Kim Y., Ryu S.*: Presence of plasmid-mediated quinolone resistance (PMQR) genes in non-typhoidal Salmonella strains with reduced susceptibility to fluoroquinolones isolated from human salmonellosis in Gyeonggi-do, South Korea from 2016 to 2019. Gut Pathog. 2021, 13, 35.
16. *Li J., Hao H., Cheng G., Wang X., Ahmed S., Shabbir M. A. B., Liu Z., Dai M., Yuan Z.*: The effects of different enrofloxacin dosages on clinical efficacy and resistance development in chickens experimentally infected with Salmonella Typhimurium. Sci. Rep. 2017, 7, 11676.
17. *Mazurkiewicz M., Borzemska W. B.*: Choroby drobiu. Praca zbiorowa, Wydawnictwo Akademii Rolniczej, Wrocław 2005. (In Polish).
18. *Michael G. B., Schwarz S.*: Antimicrobial resistance in zoonotic nontyphoidal Salmonella: an alarming trend? Cli. Microbiol. Inf. 2016, 22, 12, 968-974.
19. *Morales-Gutiérrez F. J., Barbosa J., Barrón D.*: Metabolic study of enrofloxacin and metabolic profile modifications in broiler chicken tissues after drug administration. Food Chem. 2015, 1, 172, 30-39.
20. *Moussaoui F., Alaoui F.*: Evaluation of antibacterial activity and synergistic effect between antibiotic and the essential oils of some medicinal plants. Asi. Pacif. J. Tropi. Biomed. 2016, 6, 1, 32-37.
21. *Munita J. M., Ariasa C. A.*: Mechanisms of antibiotic resistance. Microbiol. Spectrum 2016, 4, 2, 1-37.
22. *Randall L. P., Cooles S. W., Coldham N. C., Stapleton K. S., Piddock L. J., Woodward M. J.*: Modification of enrofloxacin treatment regimens for poultry experimentally infected with Salmonella enterica serovar Typhimurium DT104 to minimise selection of resistance. Antimicrob. Agents Chemother. 2006, 50, 12, 4030-4037.
23. *Rosato A., Piarulli M., Corbo F., Muraglia M., Carone A., Vitali M. E., Vitali C.*: In vitro synergistic action of certain combinations of gentamicin and essential oils. Curr. Med. Chem. 2010, 17, 3289-3295.
24. *Solarte A. L., Astorga R. J., Aguiar F., Galán-Relaño Á., Maldonado A., Huerta B.*: Combination of antimicrobials and essential oils as an alternative for the control of Salmonella enterica multiresistant strains related to foodborne disease. Foodborne Pathog. Dis. 2017, 14, 10, 558-563.
25. *Vaid R. K., Thakur Z., Anand T., Kumar S., Tripathi B. N.*: Comparative genome analysis of Salmonella enterica serovar Gallinarum biovars Pullorum and Gallinarum decodes strain specific genes. PLOS ONE 2021, 16, e0255612.
26. *Veras H. N. H., Rodrigues F. F. G., Botelho M. A., Menezes I. R. A., Coutinho H. D. M., Costa J. G. M.*: Enhancement of aminoglycosides and β-lactams antibiotic activity by essential oil of Lippiasidoides Cham. and the thymol. Arab. J. Chem. 2017, 10, 2, 2790-2795.
27. *Vuuren S. van, Viljoen A.*: Plant-based antimicrobial studies. Methods and approaches to study the interaction between natural products. Planta Med. 2011, 77, 1168-1182.
28. *Yap P. S., Yiap B. Ch., Ping H. C., Lim S. H. E.*: Essential oils, a new horizon in combating bacterial antibiotic resistance. Open Microb. J. 2014, 8, 6-14.

Corresponding author: Michalina Adaszyńska-Skwirzyńska, PhD, 29 Klemensa Janickiego Street, 71-270 Szczecin, Poland; e-mail: madaszynska @zut.edu.pl