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*Article*

# Assessing of Virulent Factor Genes in Pig-Derived *Escherichia coli* from VOJVODINA region and Their Treatment with Postbiotic Substance and Herbal Essential Oils

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**Abstract**

Altogether 16 pigs-derived *Escherichia coli* (isolated pig farms in Vojvodina region, Serbia) were taxonomically identified using MALDI-TOF mass spectrometry; 14 (87.5%) with secure genus identification/probable species identification and two with highly probable genus identification. Testing virulence factor genes, the gene fimA was detected in 62.5% of strains. Crl gene was detected in 14 strains (87.5 %). Ec3419/2 contained 5 analyzed genes. Using Congo red agar, five E. coli were biofilm-forming which was not confirmed using the quantitative plate assay. The strains were mostly multiresistnt to antibiotics. Each E. coli was found with production of non-useful enzymes in values from 5 up to 30 nmol. However, they were susceptible to herbal essential oils (HEO-oregano, thyme, sage, and coriander) with average inhibitory zone from 15 up to 27 mm in diameter. Tey were also (6) susceptible to postbiotic substance (Ent) 412 (activity up to 6400 AU/ml. Postbiotic substances represent one of novel approaches to fight with virulence factor possessing E. coli. Moreover, their combination with HEO increases their antimicrobial effect. These treatment conditions correspond with One Health Concept.

**Keywords:** virulence factor; gene; *Escherichia coli*; postbiotic; essential oil; treatment

## 1. Introduction

In general, the prevalence of antibiotic-resistant bacteria is an increasing problem due to the possible transmission of resistant bacteria or their resistance genes between animals and humans via direct and/or indirect contact [1]. Antibiotic resistant *Escherichia coli* may constitute a reservoir of antibiotic-resistance determinants. The determinants can be spread to those bacteria which are pathogenic for animals and humans [1]. The biofilm-forming ability and so biofilm development represents also problem [2]. Although Gram-negative species *Escherichia coli* have been recognized to be a component of healthy intestinal microbiota [3], they can differ in their capacity to persist in the normal colonic microbiota. Resident strains may colonize an individual for months and years, while transient strains are found only once, or on a few occasions closely spaced in time in an individual intestine [3]. On the other side, diseases caused by some *E. coli* species are one of the most important diseases and they represent a public health risk [4,5]. *E. coli* synthesize a variety of adhesins which allow them to colonize, persist and/or cause disease. There are filaments called fimbriae that allow bacteria to adhere a variety of cells [6]. It enhances *E. coli* biofilm formation [7]. Curli regulatory gene (*crl*) is responsible for production of curli fibers in *E. coli* [6]. H*lyA* (α-hemolysin) is a toxin responsible for the strain pathogenicity [8]. The strains containing the *eae* gene (outer membrane protein-intimin)

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frequently cause diarrhea; and *espA* gene (superficial protein) as well. Therefore, it is very important to control and/or reduce growth of these type of *E. coli* strains.

Postbiotic substances and bacteriocins (to homogeneity or not purified antimicrobial substances of proteinaceous character with an inhibitory effect against more or less relative bacteria) represent one of these approaches [9]. Their inhibitory effect (e.g. Enterocin-like substances) was also presented in our several studies. Lauková et al. [10] reported effective treatment of contaminant strains *Enterococcus faecalis* (from raw goat milk) due to postbiotic active substances produced by autochthonous lactococci. Pogány Simonová et al. [11] reported reduction of fecal coliforms ($p< 0.001$) and pseudomonads ($p< 0.05$) in broiler rabbits after application of postbiotics, Ent M and Durancin ED26E/7. Postbiotic substance (PS-Ent) 412 is a thermo-stable antimicrobial proteinaceous substance produced by the horse strain *Enterococcus faecium* EF412 [12]. This strain is susceptible to antibiotics and it showed adhesion ability to human and canine mucus [13]. PS 412 was applied in horses with the following benefits noted: coliforms were significantly inhibited ($p<0.001$) after 3 weeks of PS 412 application; phagocytic activity was increased (69.0 ± 3.99 at day 21 to 67.89 ± 3.66 at start of experiment); biochemical parameters in blood were not negatively influenced (Lauková et al., unpublished data yet).

In addition, also herbal essential oils are known to have antimicrobial effect [14–16]. Herbal essential oils and their components as products from the secondary metabolism of plants have been known to have antimicrobial and antioxidant properties for a long time used in preservation, food and pharmaceutical industry [14]. E.g. carvacrol (2-methyl-5-(1-methylethyl)-phenol) is a major component of the oregano essential oil. It was reported to inhibit *E. coli, L. monocytogenes, Salmonella Enterica*, and *Campylobacter jejuni* [17]. Thymol (5-methyl-2-(1-methyl)-phenol) has been commercially used for hundred years [17]. Its essential oil has been shown to exhibit a range of biological activities, e.g. aflatoxin production inhibition. Its antimicrobial activity was also documented against *E. coli*, listeriae, campylobacters [17]. Sage (*Salvia officinalis L.;* family Labiatae) essential oil in combination with Enterocin M increased mucus production quantity in duodenum and jejunum ($p<0.001$, and $p<0.01$) in broiler rabbits after their 3 weeks application in comparison with control rabbits **[**18]. Moreover, *Coriandrum sativum* L. (Apiacae) essential oil has been reported to inhibit the growth of Gram-negative species such as *E. coli, Yersinia enterocolitica, Enterobacter* spp., *Klebsiella pneumoniae,* etc. [16**].** Therefore, carvacrol, thyme, oregano. containing oils have attracted research attention for further application.

Having in mind this information, the aim of the study was to study if four selected essential oils (oregano, thyme, sage and coriander) and postbiotic substance PS (Ent) 412 can inhibit virulence factor genes possessing pig-derived *E.* coli to find another new tool for prevention and/or elimination of those type strains of *E. coli*.

## 2. Materials and Methods

### *2.1. Taxonomical Identification of Isolates*

The isolates were selected from 26 rectal swabs and 9 samples from dead pigs obtained from eight pig farms from Vojvodina region in Serbia in the framework of co-operation with our colleague [19]. Sampling was provided according to the rules for Animals handling with acceptance of farmers and veterinarians and appropriate veterinary administration in Serbia. The colleagues from Serbia immersed the swabs into sterile saline solution (pH 7.5) and transported to the laboratory in cooling boxes at the same day [19]. The samples were inoculated onto Mac Conkey agar (Biokar Diagnostics, Allone, France), XLD agar (Biokar, France) and Trypticase soy agar (Biokar, France) enriched with 5% of defibrinated sheep blood [19]. Particular identification of grown colonies was performed phenotypically using biochemical testes (oxidase and catalase tests, indole, methyl red, urea and citrate) by colleagues in Serbia. After supplying identified strains into our laboratory (Laboratory of Animal Microbiology, Department of Animal Physiology, Centre of Biosciences of the Slovak Academy of Sciences in Košice, Slovakia), the strains were pre-inoculated using Trypticase soy broth

(Difco, Sparks, MD, USA) and Mac Conkey agar (Difco, USA) and analyzed using MALDI-TOF mass spectrometry (Matrix-Assisted-Laser Desorption/Ionization Time of Fight Mass Spectrometry; Bruker Daltonics, San Jose, CA, USA).

The MALDI-TOF analysis was performed as previously described by Lauková et al. [20,21**]**. This method is based on protein detection "fingerprints" (Bruker Daltonics, Biotyper 2.2.) [20–23] performed using a Microflex MALDI-TOF mass spectrometer. Briefly, a solitary colony of each isolate from Trypticase soy agar (Difco) with 5% of defibrinated sheep blood was mixed with a matrix (α-cyano-hydroxycinnamic acid and trifluoroacetic acid). This suspension was spotted onto a MALDI plate and ionized with a nitrogen laser (wave length of 337 mm; frequency of 20 Hz). The evaluation score was assessed according to the MALDI Biotyper 3.0 (Bruker Daltonics, Billerica, MA, USA) identification database. The taxonomy of strains was classified as highly probable species identification (score range 2.300-3.000), secure genus identification/probable species identification (2.000-2.299), and on the basis of highly probable genus identification (1.700-1.999). The positive controls were involved in the identification database. Identical colonies evaluated using the score were excluded. Finally, 16 strains were used for the next analyses. The Microbank system (Pro-Lab Diagnostic, Richmond, BC, Canada) was used for the identified strains storage.

### *2.2. Virulence Factor Genes Detection of Pig-Derived Escherichia coli*

The following virulence factor genes were screened: *fimA* (fimbriae), *crl* (curli regulatory gene), *hlyA* (hemolysin), eaeA (outer membrane protein-intimin), and *espA* (superficial protein). The primers used are summarized in Table 1. The single PCR was performed using Thermocycler Techgene KRD, Techne, the United Kingdom) and protocol for *fimA* gene detection reported previously Nowrouzian et al. [3]: an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of DNA denaturation at 94 °C for one minute, primer annealing at 63 °C one min, a primer extension at 72 °C (one min) and a final extension step at 72 °C for 7 min. In case of *hlyA* gene, the PCR condition included an initial denaturation at 94 °C (2 min), then 30 cycles at 94 °C (one min), annealing at 58 °C for one min, primer extension at 72 °C (one min) and a final extension at 72 °C (7 min) [24]. The *eaeA* gene was determined under the PCR protocol including and initial denaturation at 94° C (one min), 30 cycles at 94 °C for 30 s, annealing at 55° C for 30 s, an extension at 72 °C (one min), and a final extension at 72 °C for 10 min [25]. The conditions 94 °C for 5 min, 40 cycles at 94 °C for 30 s, annealing at 50 °C (one min) and extension at 72 °C (one min) and at 72 °C (10 min) were used for e*spA* gene detection [26]. In case of *crl* gene, an initializing denaturation was performed at 94 °C for 2 s, followed by 30 cycles at 55 °C for 3 s, an annealing at 55° C, an extension at 72° C for 15 s [6]. The amplifications were carried out in a single tube with a volume of 25 μL, utilizing Taq polymerase (0.2 μL, Promega, Madison, WI, USA) with 10 x buffer (2.5 μL), and $MgCl_2$ (1.6 μL), primers (0.2 μL each, Lambda Life, Bratislava, Slovakia), deoxynucleotide triphosphate (dNTP, Promega, USA) and water (17.4 μL) mix. Specific gene products were analyzed using electrophoresis on 1.5% agarose gel (Sigma, Germany), 1x TAE (Tris-acetate-EDTA, Merck, Germany) gel at 70 V. The products were visualized with GelRed (Biotium Inc., Hayward, CA, USA) under UV light. The 100 bp ladder (Promega, Madison, WI) served as molecular weight standard to determine molecular weight of the products. Positive controls were *E. coli* Ec A/Zn/2021 and EcK212Tr (our strains).

**Table 1.** Oligonucleotide primer pairs to detect virulence factor genes in *Escherichia coli strains.*

| Gene | Oligonucleotide primer pairs | Size of PCR product (bp) | Reference |
|---|---|---|---|
| *crl (curli regulatory gene), M571, M570* | F:5-TTTCGATTGTCTGGCTGTATG-3 | 250 | [6] |
| | R:5-CTTCAGATTCAGCGTCGTC-3 | | |
| *eaeA (outer membrane protein-intimin,* | F:5-CTGAACGGCGATTACGCGAA-3 | 917 | [25] |

| | | | |
|---|---|---|---|
| *non-fimbrial adhesin), eaeA 1, eaeA 2* | R:5-CCAGACGATACGATCCAG-3 | | |
| *espA (protein secreted by enteropathogenic E. coli), espA1, espA2* | F:5-GCGAGTACTTCGACATC-3<br>R:5-TTATTTACCAAGGGATAT-3 | 579 | [26] |
| *hlyA (α-hemolysin), hly1, hly2* | F:5-GTCTGCAAAGCAATCCGCTGCAAATAAA-3<br>R:5-CTGTGTCCACGAGTTGGTTGATTAG-3 | 1177 | [24] |
| *fimA (fimbrial adhesin), fimA 1, fimA 2* | F:5-ACGTTTCTGTGGCTCGACGCATCT-3<br>R:5-ACGTCCCTGAACCTGGGTAGGTTA-3 | 721 | [3] |

*2.3. Biofilm-Forming Ability Testing*

Two methods were used for this testing. The qualitative method based on biofilm-forming ability using Congo red agar [27]. The components of this medium include TS broth (Difco, MI, USA, 37 g/L) with 30 g/L pure agar, 36 g/L sucrose and Congo red dye (0.8 g/L, Merck, Germany). *E. coli* were inoculated on Congo red agar and incubated at 37 °C for 24 h. Biofilm-forming ability of *E. coli* was demonstrated by black colonies with a dry crys talline consistency. The non- biofilm-forming strains remained pink. The color was also checked after 48 h and 72 h placing plates at room temperature [28].

The quantitative microtiter plate assay [29] was also applied for biofilm testing. An individual colony of the *E. coli* strain grown on Trypticase soy agar overnight (TSA, Difco, Sparks, MD, USA) was transferred into 5 mL of Ringer solution (pH 7,0; Merck, Darmstadt, Germany) to reach 1.0 x $10^8$ CFU/L. The, 100 μL of this suspension was transferred into 10 mL of Trypticase soy broth/infusion (BHI). A 200 μL of their dilution was inoculated in microtiter plate wells (Greiner ELISA 12 Well Strips, 350 μL (Greiner ELISA 12 Well Strips flat bottom, Frickenhausen GmbH, Germany) as previously reported by Lauková et al. [30]. After incubation at 37 °C overnight, the biofilm forming in the microtiter wells was washed twice with 200 μL of deionized water and dried at 25 °C for half hour. The next step was to

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stain the remaining bacteria for 30 min at 25 °C with 200 µL of 0.1 % (w/v) crystal violet in deionized water. The second drying of plates was provided (half hour at room temperature). The dye bound to the adhered biofilm was extracted using 200 µL of 95% ethanol and stirred. A 150 µl aliquot was again transferred to a microplate well and absorbance $A_{570}$ (Synergy TM4 MALDI mode Microplate reader, Biotek, Seattle, WA, USA) was measured. Two independent analyses with 12 replicates were performed for each *E. coli* strain. A sterile BHI was included in each test as a negative control. The positive control was the strain *Streptococcus equi* subsp. *zooepidemicus* CCM 7316 (kindly provided by Dr. Eva Styková from the University of Veterinary Medicine and Pharmacy in Košice, Slovakia). The biofilm-forming ability of the strains E. *coli* was evaluated as reported Chaieb et al. [29]. The following classification was used: highly-positive (A570 ≥ 1, low grade positive (0.1 ≤ A570 < 1.0, or negative (A570 < 1.0).

*2.4. Antibiotic Disc Diffusion Test and Enzyme Activity Detection*

To test susceptibility to antibiotics of *E. coli*, agar disc diffusion method [31] was applied. The antibiotics (13) used were supplied by Oxoid Ltd. (Basingstoke, UK) as follows: clindamycin (DA-2 µg), penicillin (P-10 IU), ampicillin (AMP-10 µg), gentamicin (CM-10 µg), erythromycin (E-15 µg), azithromycin (AZM-15 µg), amikacin (AK-30 µg), chloramphenicol (C- 30 µg), tetracycline (T-30 µg), mezocillin (MEZ-75 µg), ticarcillin (TIC-75 µg), carbenicillin (Car-100 µg), and piperacillin (PRL-100 µg). Briefly, broth cultures (in Trypticase soy broth, Difco) of tested strains (100 µL) were spread onto Mueller-Hinton agar (Bio-Rad, Bratislava, Slovakia) as well as on Trypticase soy agar (Difco). When antibiotic discs were applied on the agar surface, the agar plates were incubated at 37 °C for 18 h. After incubation the inhibitory zones were evaluated and expressed in mm. They were assessed according to the guidelines of the Clinical and Laboratory Standards Institute [21].

The API ZYM panel (Bio Merieux, Marcy lEtoile, France) was used for the enzyme detection according to the manufacturers instructions as previously reported by Lauková et al.

[32]. Briefly, a volume of 65 μL of McFarland standard inoculum was transferred into each well of the tested panel plate. After incubation of the panel plate at 37 °C for 4 h in incubator, reagents Zym A and Zym B were added in each one well. Enzyme activity was evaluated based on color intensity values (0-5) and their relevant values in nanomoles (nmol). They were assigned for each reaction according to the color chart supplied with the kit. The enzymes tested included in panel were as follows: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, Naphtol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidae, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase.

### *2.5. Susceptibility to Herbal Essential Oils of E. coli*

The essential oils used were obtained from Calendula a.s. Nová Ľubovňa (Slovakia). In testing were included: Salviae aetheroleum from sage (*Salvia officinalis L.;* family Labiatae) contained cineol 15.0 ± 10.0.5%), thujon (24.0 ± 1.0%), and borneol 18.0 ± 1.0). Oregano aetheroleum from *Origanum vulgare* L. (Lamiaceae) contained 55.0 ± 3.0 % of carvacrol. Coriandri aeteheroleum from *Coriandrum sativum* L. (Apiacae) contained 53. 0 ± 2.0 % of the main component linalool. Thymi aetheroeum from thyme (*Thymus vulgari L.,* Lamiacae) contained 40.0 ± 3.0 % of p-cymene, and 32. 0 ± 2.0 % of thymol. The components of these oils were measured using gas chromatography. The agar spot test [33] was used to test essential oils effect against the strains *E. coli.* For this purpose, *E. coli* were grown in TSY (overnight). The volume 4 mL of soft agar (w/v 0.7 %) was inoculated with 200 μL of each tested strain and TSY (w/v, 1.5%) was overlaid with this mixture. Then 10 μL of each the essential oil (EO) was dropped on the plate surface. The plates were incubated at 37 °C and after 4 h first check was done. The second check was provided after overnight incubation. The susceptibility of *E. coli* was evaluated as an average size of inhibitory zone (in mm). *Escherichia coli* ATCC 2592 was positive control.

*2.6. Postbiotic Substance (Enterocin-like) 412 Preparation and Susceptibility to PS 412 of Pig-Derived E. coli*

The PS 412 was prepared following the procedure according to Mareková et al. [34]. The volume 200 mL of MRS broth (de Man-Rogosa-Sharpe) (pH 7, Merck, Germany) was inoculated with 14 h-18 h culture of *Enterococcus faecium* EF412 (0.1% inoculum) at 37 °C. The culture was centrifuged (10 000 *x g*) for 30 min. The supernatant was adjusted to have pH 5.5 and precipitated with ammonium sulfate (40% saturation) in the plastic jar by stirring at 4 °C for 1 h and/or more regarding the precipitation process. Then followed second centrifugation (10 000 *x g*) for 30 min. The pellets from centrifuging tubes were re-suspended in minimal volume of 10 mM phosphate buffer (pH 6.5). The inhibitory activity (volume 2.0 mL) of precipitate (postbiotic substance) was tested against *E. avium* EA5 (the principal indicator, our strain) using agar spot test [33] and expressed in AU/mL after dilution in the phosphate buffer. The activity reached 51 200 AU/ml. Then *E. coli* strains were treated with PS as formerly indicated.

## 3. Results

*3.1. Strains Taxonomy Using MALDI-TOF Mass Spectrometry, Virulence Factor Genes and Biofilm-Forming Ability of Escherichia coli*

Identified *E. coli* strains with evaluated score are summarized in Table 2. Altogether 16 strains were taxonomically allotted and confirmed to the species *Escherichia coli.* Fourteen strains (87.5%) reached score in range 2.000-2.299. It responds with secure genus identification/probable species identification. The strains *E. coli* Ec3419/4 and Ec3298/4 were evaluated with range score 1.700-1.999 meaning highly probable genus identification (Table 2). The strain Ec3419/1 reached the highest score (2.238).

Six out of 16 *E. coli* were *fimA* gene absent; however, *fimA* genes were found in 10 strains (62.5%, Table 2). *Crl* gene was detected in 14 strains (87.5 %); only two strains (Ec7676/4 and Ec3477/1) were *crl* gene absent. The *E. coli* tested were mostly *hlyA*, *eae* and *espA* genes absent (11 strains in each, Table 2). These genes were detected altogether in 5 strains. *E. coli* Ec3419/2 possessed all 5 analyzed genes. The strain Ec3298/1 possessed four genes (e*spA* gene was not detected). Mostly three genes were found in tested strains. In the strain Ec3419/4 was present only *crl* gene. The strains Ec7676/4 and Ec3477/1 were virulence factor genes absent.

Using Congo red agar to test biofilm-forming ability in *E. coli*, this ability was found in five strains (31.2%, Table 2); dubious reaction was noted in three strains, and 11 strains did not show biofilm-forming using Congo red agar. In dubious reaction, negative production was confirmed using the quantitative plate assay. The values less than 0.1 were measured (Table 2) almost in all tested strains, even in those with positive reaction on Congo red agar. In three negative strains tested using Congo red agar, no biofilm-forming ability was also confirmed by the plate agar assay (Table 2). In general, measurements ranged from 0.006 up to 0.050.

**Table 2.** The MALDI-TOF evaluating score, virulence factor genes and biofilm -forming ability of *E. coli*

| Strain | MALDI-TOF | *fimA* | *crl* | *hlyA* | *eaeA* | *espA* | Congo/72 h | Biofilm ± SD |
|---|---|---|---|---|---|---|---|---|
| Ec3298/1 | 2.067 | + | + | + | + | - | - | 0.011 ± 0.009 |
| Ec3298/2 | 2.127 | - | + | - | + | + | ± | 0.006 ± 0.002 |
| Ec3298/3 | 2.209 | - | + | - | + | + | - | 0.005 ± 0.003 |
| Ec3298/4 | 1.897 | + | + | - | - | - | + | 0.050 ± 0.01 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Ec3098/1 | 2.081 | + | + | + | - | - | + | 0.007 ± 0.003 |
| Ec3098/2 | 2.265 | - | + | - | + | + | - | - |
| Ec3152 | 2.135 | + | + | + | - | - | - | - |
| Ec3276 | 2.222 | + | + | + | - | - | - | 0.027 ± 0.02 |
| Ec3419/1 | 2.238 | + | + | - | - | - | - | - |
| Ec3419/2 | 2.197 | + | + | + | + | + | ± | 0.013 ± 0.004 |
| Ec3419/3 | 1.988 | + | + | - | - | - | ± | 0.025 ± 0.004 |
| Ec3419/4 | 2.216 | - | + | - | - | - | - | 0.013 ± 0.003 |
| Ec3419/6 | 2.213 | + | + | - | - | - | + | 0.018 ± 0.005 |
| Ec3717/1 | 2.016 | + | + | - | - | + | + | 0.015 ± 0.006 |
| Ec7676/4 | 2.060 | - | - | - | - | - | + | 0.048 ± 0.012 |
| Ec3477/1 | 2.204 | - | - | - | - | - | - | 0.039 ± 0.014 |

Virulence factor genes: *fimA* (fimbriae), *crl* (curli regulatory factor), *hlyA* (hemolysin), *eaeA* (outer membrane protein-intimin), *espA* (superficial protein). Growth of *E. coli* strains on Congo red agar (biofilm formation); + means positive reaction; - means negative reaction; ± means dubious reaction;.

*3.3. Antibiotic Disc Diffusion Test and Enzyme Activity Detection*

The strains *E. coli* were resistant to clindamycin and penicillin. Oppositelly, they were all susceptible to amikacin (AK30) with the inhibitory zones up to 13 mm. Eleven strains out of 16 (Table 3, 68.8%) were resistant to chloramphenicol (C30), five strains were susceptible to this antibiotic (Table 3). Even, 13 strains (81.3%) were resistant to tetracycline (T30), 12 strains (75%) were found to have resistance to ampicillin (AMP10). Surprisingly, 8 strains were gentamicin (CM10) resistant and 8 strains were CM10 susc eptible. Fifteen *E. coli* (15) were resistant to erythromycin (E15, 93.8%). To azithromycin (AZM15) were *E. coli* mostly resistant (11 strains, 68.8%) as well as to mezlocillin (MEZ75,) 10 strains of *E. coli* (62.5%). The same amount of *E. coli* (10 strains, 62.5 %) were resistant to ticarcillin (TIC75, Table 3). The tested *E. coli* (16) were also mostly resistant to Car100 carbenicillin- 15 out of 16 strains (93.8 %). Opposite condition was noted in case of pipe-

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racillin (PRL100) when almost 12 strains were susceptible and six strains were resistant this antibiotic. In general, the strains were mostly resistant to antibiotics tested.

Each one tested *E. coli* was found to produce enzymes but with various values (Table 4). The values of esterase (C4), lipase (C14), leucin-arylamidase, valin- arylamidase, cystin-arylamidase, trypsin, α-glucosidase, β-glucosidase, α- mannosidase, and α-fucosidase reached 5 nmol in each tested *E. coli* (Table 4). Higher values (10-20 nmol) were measured for alkalic phosphatase. Regarding esterase-lipase, and α-galactosidase, the most strains reached 5 nmol, except Ec3152 and Ec3419/6 with value 10 nmol and Ec3298/4 as well. The enzyme α -chymotrypsin was also produced by the tested *E. coli* in amount 5 nmol except Ec3419/6 (10 nmol). The values for acidic phosphatase and Naftol-AS-BI-phosphohydrolase were almost the same; the values 10, 20 nmol dominated, except in the strain Ec7612/4 for acidic phosphatase (30 nmol) and for the strains Ec3298/3 3298/4 in case of Naftol-AS-BI-phosphohydrolase (30 nmol). The volumes 5 nmol were found for α-galactosidase, except Ec3298/4 (10 nmol. For β-galactosidase, the values 5 nmol up to strains 10 nmol were noted. In case of N-acetyl-β-glucosaminidase only Ec7612/4 reached 30 nmol, the others produced 5 nmol of this enzyme.

**Table. 3.** Antibiotic profile of tested *E. coli.*

| **Strain** | **C30** | **T30** | **Amp10** | **CM10** | **E15** | **AZM15** | **AK30** | **Mez75** | **Tic75** | **Car100** | **Prl100** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ec3298/1 | R | R | R | +14 | R | R | +12 | R | R | R | +10 |
| Ec3298/2 | +22 | R | R | +12 | R | +11 | +10 | R | R | R | +11 |
| Ec3298/3 | +20 | R | R | +11 | R | +10 | +10 | R | R | R | +10 |
| Ec3298/4 | R | R | R | R | R | +10 | +11 | R | R | R | R |
| Ec3098/1 | +20 | R | R | +11 | R | R | +12 | R | R | R | +13 |
| Ec3098/2 | R | R | R | R | R | R | +12 | R | R | R | R |
| Ec3152 | +20 | R | R | +11 | R | +10 | +10 | R | R | R | +10 |
| Ec3276 | R | +12 | R | +11 | +13 | +10 | +11 | +10 | +10 | R | +10 |
| Ec3419/1 | R | R | +15 | R | R | +9 | +10 | +15 | +15 | R | +9 |
| Ec3419/2 | R | R | R | +10 | R | R | +10 | R | R | R | +12 |
| Ec3419/3 | +23 | +17 | R | +11 | R | +10 | +10 | R | R | R | R |
| Ec3419/4 | R | R | +15 | R | R | R | +10 | +13 | +14 | R | +16 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ec3419/6 | R | R | R | R | R | +10 | +13 | +15 | +15 | +10 | +20 |
| Ec7162/2 | R | +8 | +17 | R | R | +8 | +10 | +10 | +11 | R | +17 |
| Ec7612/4 | R | R | +12 | R | R | R | +10 | +10 | +10 | R | +10 |
| Ec3477/1 | R | R | R | R | R | R | +10 | R | R | R | R |

C30, chloramphenicol (30 μg); T30, tetracycline (30 μg); AMP10, ampicillin (10 μg); CM10, gentamicin (10 IU);
E15, erythromycin (15 μg); AZM15, azithromycin (15 μg); AK30, amikacin (30 μg), MEZ75, mezlocillin (75 μg)
TIC75, ticarcillin (75 μg); Car100, carbenicillin, (100 μg); PRL100, piperacillin (100 μg); R-resistant; + (no.) means
the inhibitory zone size in mm; The strains *E. coli* were resistant to clindamycin and penicillin.

### *3.4. Susceptibility to Herbal Essential Oils and Postbiotic Substance of E. coli*

All tested strains *E. coli* were susceptible to oregano, thyme, sage, and coriander as well (Table 5). The average value of inhibitory zone after *E. coli* treatment with oregano reached 27.0 ± 0.0 mm. *E. coli* Ec3419/1 was the least susceptible to oregano with an inhibitory zone size 16 mm. The most susceptible was *E. coli* Ec3276 with an inhibitory zone size 36 mm. Treatment with oregano lead to the broadest inhibitory zones (Table 5). As second essential oil to which *E. coli* were susceptible was thyme. There average inhibitory zone size measured 18 mm. The strain Ec3419/2 could be supposed resistant because small inhibitory zone (5 mm). The broadest zone 25 mm reached Ec3419/3. In case of sage and coriander, the average inhibitory zones reached 15 mm. The strains Ec3298/1, Ec3298/2, 3298/3, 3298/4 belonged to the most susceptible to essential oils and also to PS 412 (Table 5) with inhibitory activity from 100 up to 6 400 AU/ml. Ec3298/4 was the most susceptible to PS 412 (6 400 AU/ml). Ten strains were not inhibited using PS 412; however, their growth was inhibited using herbal essential oils.

**Table 4.** Enzyme production by tested *E. coli* expressed in nmol

| Strain | 1 | 3 | 9 | 10 | 11 | 12 | 13 | 17 |
|---|---|---|---|---|---|---|---|---|
| Ec3298/1 | 10 | 5 | 5 | 20 | 20 | 5 | 5 | 5 |
| Ec3298/2 | 10 | 5 | 5 | 20 | 20 | 5 | 10 | 5 |
| Ec3298/3 | 10 | 5 | 5 | 20 | 30 | 5 | 10 | 5 |
| Ec3298/4 | 20 | 5 | 5 | 20 | 30 | 10 | 10 | 5 |
| Ec3098/1 | 10 | 5 | 5 | 20 | 20 | 5 | 5 | 5 |
| Ec3098/2 | 10 | 5 | 5 | 10 | 20 | 5 | 5 | 5 |
| Ec3152 | 20 | 10 | 5 | 20 | 20 | 5 | 5 | 5 |
| Ec3276 | 20 | 5 | 5 | 20 | 20 | 5 | 20 | 5 |
| Ec3419/1 | 20 | 5 | 5 | 20 | 20 | 5 | 5 | 5 |
| Ec3419/2 | 20 | 5 | 5 | 10 | 20 | 5 | 10 | 5 |
| Ec3419/3 | 10 | 5 | 5 | 10 | 20 | 5 | 10 | 5 |
| Ec3419/4 | 10 | 5 | 5 | 10 | 10 | 5 | 5 | 5 |
| Ec3419/6 | 20 | 10 | 10 | 20 | 20 | 5 | 5 | 5 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Ec7162/2 | 20 | 5 | 5 | 10 | 20 | 5 | 5 | 5 |
| Ec7612/4 | 20 | 5 | 5 | 30 | 30 | 5 | 5 | 30 |
| Ec3477/1 | 20 | 5 | 5 | 20 | 20 | 5 | 5 | 5 |

1-alkalic phosphatase, 3-esterase lipase (C8), 9-*α* chymotrypsin, 10-acidic phosphatase, 11-Naftol-AS-BI-
phospho-hydrolase, 12-*α*-galactosidase, 13-β-galactosidase, 14-β-glucuronidase, 15-*α*-glucosidase,
17-N-acetyl-β-glucosaminidase. In case of the enzymes 2-esterase (C4), 4-lipase (C14),5-leucine arylamidase,
6- valine arylamidase, 7-cystine arylamidase, 8-trypsin, 14-β-glucuronidase, 15-*α*-glucosidase, 16-β-glucos
idase,18-*α* mannosidase, and 19-*α*-fucosidase the reached values were 5 nmol.

## 4. Discussion

The first important step to work with selected microbiota is their identification. In this study after first phenotyping, MALDI-TOF MS was applied. It is an accurate method for laboratory use because of simple approach designed for protein detection. It represents a reliable technology for the precise identification of cultured microbiota [20]. This method looks as sufficient identification tool regarding the species *E. coli* reaching high score. It was also successfully applied in identification of fecal *E. coli* from ostriches [**35**]. As it is known, the species *E. coli* belongs to the family Enterobacteriacae, to order Enterobacterales, to class Gammaproteobacteria and phylum Pseudomonata (Proteobacteria). As formerly mentioned, *E. coli* is a common commensal bacterial species of animals and humans that may become a troublesome pathogen causing diseases [36].

Regarding the virulence factors, the curli gene *crl* is mostly present in avian *E. coli* [6]. Eae protein (intimin) contribute to cells microvilli damaging. It is a key virulence factor associated with diarrheal disease [36]. Intimin is critical for the bacterial ability to adhere to intestinal cells. The strains containing the *eae* gene are responsible for causing diarrhea [37]. In our strains 5 were found possessing eaeA gene. The *fimA* gene represents a crucial virulence factor in *E. coli* for adhesion to host cells and colonization of tissue. It is adhesin helping bacteria to attach to host tissues, allowing them to colonize and establish infections [3]. The e*spA* gene encodes protein essential for activating epithelial signal transduction, intimate contact and formation of attaching and effacing lesions, processes which are central to pathogenesis [26]. *HlyA* gene is a member of the RTX toxin family. It is believed that the persistence of α-hemolytic *E. coli* strains in the host may be a reason for the emergence of intestinal and/or extra-intestinal infections [38]. In this study, mostly *crl* gene was detected.

Here studied *E. coli* shown multi-drug resistance which can be problem in Serbian pig farming. Transfer of microbial resistance genes is a problem which lead to permanent control of antimicrobial resistance. As Stojanov et al. [19] previously reported, 100% resistance to penicillin of *E. coli* from piglets at grower stage, we also found the same results in pigs-derived *E. coli* from Vojvodina region**.** Roderová et al**.** [36] reported 97% resistance to ampicillin and 96% resistance to PRL of *E. coli* from hospitalized patients. It is in opposite to by us noted 100% susceptibility to PRL. Gentamicin represents an older member of the aminoglycosides to which half of our tested *E. coli* was resistant and half strains (8) susceptible. Similarly, Kerluku et al. [39] observed CM resistance in *E. coli* with low resistance level from cattle and sheep. Similarly, as by Kerluku et al. [39], also in our strains moderate resistance toward AZM was noted.

The tested *E. coli* produce damaging enzymes although not in huge amount. The production of enzymes such as α-chymotrypsin, β-glucuronidase or alkalic phosphatase contribute to their pathogenic character. E. g. alkalic phosphatase serves as a marker in case of hepatitis diagnosis. The enzyme β-glucuronidase as microbial source can play role in disease related mainly with estrogen metabolism [40].

Naturally occurring substances were found effective to inhibit/reduce *E. coli* infections *[14,15*]. Among those natural belongs also lantibiotic bacteriocin nisin which was effectively used in combination with cinnamaldehyde and EDTA to control swine origin *E. coli* [41]. In our case, postbiotic substance, Enterocin-like 412 inhibited the growth of 6 out of 16 strains. It is interesting that in experimental in vivo application using various animals, but most frequently in broiler rabbits, poultry and/or horses, reduction of *E. coli* was noted and beneficial influence using this and also other Enterocins, Enterocin-like substance and/or postbiotics [42]. There was shown beneficial preventive use of Ent7420 in improving the growth and immunity of rabbits and also protective effect against infection caused by methicillin-resistant staphylococci. Underlining postbiotic substances effectivity, the completely new achievements were reported by Petrová et al. [43]. The direct effect of Enterocins (Ent M and Duranci ED26E/7) on *Trichinella spiralis* fecundicity was documented by in vitro test. There Durancin-like showed a reducing effect (40-60%). The reducing activity of *T. spiralis* infection induced by Ent M was also noted [43]. Moreover, Al Atya et al**.** [44] reported inhibition of swine-derived *E. coli* grown in both planktonic and biofilm cultures using LAB-bacteriocins (nisin + Enterocin DD14) in combination with colistin. The author even outlines advantages of antibiotic-LAB bacteriocin combinations and merits further development as potential novel treatments for *E. coli* infections. Here tested *E. coli* did not show biofilm-forming ability. Postbiotic 412 use approach was supported by the use of HEO. In some experiments [15] authors also indicated that dietary administration of a combination of thyme and oregano essential oils in appropriate concentrations can reduce the production of pro-inflammatory cytokines and attenuate the degree of colonic tissue injury and ameliorate colitis in mice. In our case, the strains Ec3298/1, Ec3298/2, 3298/3, 3298/4 possessing virulence factor genes were belonged to the most susceptible to essential oils and also to PS 412**.** Therefore, this study contributes to indicate a possible strategy for protection and/or elimination of non-requested microbiota, such virulence factor genes containing *E. coli* belong. Although it is necessary to continue in this testing, it is indication to solve problem with multidrug resistant *E. coli* also on farms in Vojvodina region.

## 5. Conclusions

The pig-derived *Escherichia coli* with virulence factor genes, multi-drug resistance and production of damaging enzymes were susceptible to essential herbal oils (HEO: oregano, sage, thyme and coriander). Moreover, 6 strains out of 16 strains were susceptible to postbiotic (Enterocin-like) substance 412. These results are contribution showing another new tool such as postbiotic substance in combination with HEO to reach the final elimination of virulence factor genes possessing *E. coli*. To control those strains is requested because of One Health concept.

**Table 5.** Susceptibility to postbiotic substance PS (Ent-like) 412 in arbitrary unit per ml) and herbal essential oils of pig-derived *E. coli* (inhibitory zone in diameter - mm).

| Strain | Oregano | Thyme | Sage | Coriander | PS (Ent) 412 |
|---|---|---|---|---|---|
| Ec3298/1 | 35 | 21 | 15 | 21 | 800 h |
| Ec3298/2 | 22 | 27 | 24 | 31 | 100 |
| Ec3298/3 | 19 | 10 | 20 | 10 | 1600 |
| Ec3298/4 | 30 | 21 | 19 | 19 | 6400 |
| Ec3098/1 | 30 | 21 | 21 | 15 | - |
| Ec3098/2 | 30 | 15 | 13 | 10 | - |
| Ec3152 | 30 | 24 | 13 | 19 | - |
| Ec3276 | 36 | 14 | 10 | 15 | - |
| Ec3419/1 | 16 | 20 | 9 | 10 | 3200 |
| Ec3419/2 | 30 | 5 | 10 | 12 | - |
| Ec3419/3 | 23 | 25 | 10 | 7 | 1600 |
| Ec3419/4 | 27 | 20 | 15 | 10 | - |
| Ec3419/6 | 20 | 20 | 10 | 12 | - |

| | | | | | |
|---|---|---|---|---|---|
| Ec7162/2 | 20 | 15 | 10 | 10 | - |
| Ec7612/4 | 21 | 14 | 10 | 10 | - |
| Ec3477/1 | 35 | 13 | 10 | 10 | - |

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