tehnologija mesa UDK: 637.54.03/.07 : 579.67 : 615.28
Osnivač i izdavač: Institut za higijenu i tehnologiju mesa, Beograd BIBLID: 0494-9846

PREVALENCE AND RESISTANCE AGAINST DIFFERENT ANTIMICROBIAL COMPOUNDS OF C*ampylobacter* spp. IN/FROM RETAIL POULTRY MEAT*

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A b s t r a c t: *The increasing antimicrobial resistance rates of microorganisms is an urgent world-wide problem, especially microbial multidrug resistance phenotypes, dispersed also among food-related bacteria. A case study could be the resistance of campylobacters, usually transmitted in the food chain by contaminated poultry meat. We tested the resistance of Campylobacter chicken meat isolates against a) selected antibiotics used in human and veterinary medicine - erythromycin, ciprofloxacin, tetracycline, b) selected disinfectants used in food processing - benzalkonium chloride (BC), chlorhexidine diacetate (CHA), cetylpyridinium chloride (CPC) and c) alternative group of antimicrobial compounds - phenolic extracts from grape skins of different grape varieties. Campylobacter was isolated from retail chicken meat samples by standard (ISO 10272) isolation procedure. Beside classical phenotyping methods of species identification and resistotyping, polymerase chain reaction (PCR) and/or restriction fragment lenght polymorphisms of specific amplicons (PCR-RFLPs) were used for species identification and determination of mutations in target genes. Antibiotic resistance phenotypes were studied by disk diffusion, agar dilution and broth microdilution method with CellTiter-Blue® reagent and automated fluorescence signal detection. The resistance to disinfectants and phenolic extracts was tested by broth microdilution method and expressed as minimal inhibitory concentrations (MICs). The involvement of efflux pumps in antibiotic and disinfectant resistance was assessed by measurements of MICs with and without addition of chemical efflux pump inhibitors, phenylalanine-arginine ß-naphthylamide (PAßN) and 1-(1-naphthylmethyl)-piperazine (NMP) and by testing Campylobacter jejuni cmeB mutant strain. High prevalence of antibiotic resistant strains was found among chicken meat isolates. Regarding ciprofloxacin, 66.5 % of tested strains were found resistant, including C. jejuni and C. coli strains, but resistance to erythromycin was much more frequent among C. coli isolates (34.5 %, but 13.9 % in average among 158 tested isolates. Tetracycline resistance was relatively rare, but multidrug resistant strains were found. No significant difference in biocide susceptibility between antibiotic resistant and sensitive Campylobacter isolates was confirmed. Finally, the results of initial screening of susceptibility of Campylobacter meat isolates against grape skin phenolic extracts tested are promising for further study of such antimicrobial compounds (or their mixtures) for potential use in assuring safety of poultry meat and other products.*

Key words: *food safety, poultry meat, Campylobacter, antibiotic resistance, disinfectant resistance, phenolic extracts*

Zastupljenost *Campylobacter spp* i rezistencija na različita antimikrobna jedinjenja u živinskom mesu iz prometa

S a d r ž a j: povećani nalaz antimikrobne rezistencije kod mikroorganizama je rasprostranjen svetski problem, naročito rezistencija fenotipova mikroorganizama na više antimikrobnih lekova koja se javlja i kod bakterija hrane. Jedna od studija mogla bi da bude rezistencija Campylobacter mikroorganizama koja se prenosi putem lanca hrane preko kontaminiranog živinskog mesa. Ispitivali smo rezistenciju izolata Campylobacter vrsta iz pilećeg mesa na: a) odabrane antibiotike koji se koriste u humanoj i veterinarskoj medicini – eritromicin, ciprofloksacin, tetraciklin, b) odabrane dezinficijense koji se koriste u preradi hrane – benzalkonijum hlorid (BC), hlorheksidin diacetat (CHA), cetilpiridin hlorid (CPC) i c) alternativnu grupu antimikrobnih jedinjenja – fenolne ekstrakte iz kožice različitih vrsta grožđa. Campylobacter je izolovan iz uzoraka pilećeg mesa iz prometa standardnom metodom (ISO 10272). Pored klasičnih metoda identifikacije vrsta, fenotipizacije i tipizacije rezistencije, za identifikaciju i utvrđivanje mutacija selektovanih gena korišćene su PCR i/ili PCR-RFLP metoda specifičnih amplikona. Fenotipovi koji pokazuju antimikrobnu rezistenciju su proučavani metodama disk difuzije, agar dilucije i bujon mikrodilucije sa CellTiter-Blue reagensom i automatskom detekcijom fluorescentnog signala. Rezistencija na dezinficijense i fenolne ekstrakte je ispitivana korišćenjem bujon mikrodilucije i izražena je kroz minimalne inhibitorne koncentracije (MIC). Uloga efluks pumpi kod rezistencije na antibiotike i dezinficijense je ispitivana merenjem MIC, sa i bez dodavanja hemijskih inhibitora efluks pumpi kao što su fenilalanin-arginin, β-naftilamid (PAβN) i 1-(1-naftilmetil)-piperazin (NMP), kao

*Plenary paper on International 55th Meat Industry Conference held from June 15-17th 2009 on Tara mauntain
*Plenarno predavanje na Međunarodnom 55. savetovanju industrije mesa, održanom 15-17. juna 2009. na Tari

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i ispitivanje cmeB mutiranog soja Campylobacter jejuni. Među izolatima pilećeg mesa utvrđen je veliki broj sojeva rezistentnih na antibiotike. 66.5% ispitivanih sojeva su pokazali rezistenciju na ciprofloksacin, uključujući C. Jejuni i C. Coli dok je rezistencija na eritromicin bila zastupljenija kod izolata (34.5%, ali prosečno 13.9% od 158 ispitivanih izolata). Rezistencija na tetraciklin je bila relativno retka, ali su pronađeni sojevi sa multiplom rezistencijom. Nije potvrđena značajna razlika u prijemčivosti na biocide iznmeđu rezistentnih i osetljivih izolata Campylobacter vrsta. Konačno, rezultati inicijalnog skrininga prijemčivosti mesnih izolata Campylobacter vrsta na fenolne ekstrakte iz kožice grožđa su obećavajući za izvođenje daljih studija ovih antimikrobnih jedinjenja (ili njihovih smeša) i ispitivanje mogućnosti njihove upotrebe u osiguranju bezbednosti živinskog mesa i drugih proizvoda.

***Ključne reči:** bezbednost hrane, živinsko meso, Campylobacter, antimikrobna rezistencija, rezistencija na dezinficijense, fenolni ekstrakti*

Introduction

Thermotolerant campylobacters as gram-negative, non-sporeforming microaerophilic microorganisms are currently recognized as theleading cause of foodborne illnesses in many developed countries worldwide (*Tauxe,* 2002; *EFSA,* 2006; 2008). Identified by case-control studies, the major route of infection in humans is thought to be the consumption of undercooked, contaminated broiler chicken meat, because of the high prevalence of contamination of chicken carcasses with *Campylobacter* and the frequency of poultry consumption. Foodborne exposure is frequent also via cross--contamination during preparation of meat and/or other food. Although most reports based on molecular typing have shown a major contribution of chicken meat to human *Campylobacter* infections (*Zorman et al.*, 2006), there is still much unknown in epidemiology of campylobacters. Since most infections occur sporadically, the sources usually remain unidentified.

Additional problem to frequent contamination of some food types and water is the emergence and spread of antimicrobial resistance among campylobacters from different sources, including multidrug resistant isolates. Macrolides, fluoroquinolones and tetracyclines are the most commonly used antimicrobial agents for the treatment of severe human *Campylobacter* infections, as well as for veterinary purposes including treatment of food animals. The prevalence of resistant *Campylobacter* strains is increasing worldwide and is becoming a major concern for public health (*Moore et al*., 2006).

The mechanisms of macrolide and fluoroquinolone resistance in *Camyplobacter* spp. have been described recently (*Gibreel et al*., 2005; *Moore et al*., 2006). Mutations in target genes and efflux pumps activity are both important. For example, mutations in domain V of the 23S rRNA gene at positions 2074 and 2075 have been attributed to high-level erythromycin resistance (Vacher et al., 2003; Payot et al., 2004). The presence of *tetO* gene contributes to tetracycline resistance. In addition, recent studies demonstrated the involvement of CmeABC efflux pump in both intrinsic and acquired resistance to erythromycin in *C. jejuni* and *C. coli*, mostly by the use of the efflux pump inhibitor (EPI), phenylalanine-arginine ß-naphthylamide (PAßN) (*Payot et al.,* 2004; *Mamelli et al.*, 2005; *Gibreel et al.,* 2007; *Kurinčič et al.,* 2007).

In comparison to antibiotic resistance, *Campylobacter* resistance to biocides has been described more recently and much less studied. In contrast to antibiotics and bacterial resistance to antibiotics, resistance to disinfectants is thought unlikely to occur because most disinfectants are complexes of antimicrobials that inactivate several target sites in bacterial cells (*McDonnell and Russell,* 1999; *Russell,* 2002). Due to multiplicity of cellular targets, bacterial biocide resistance results from changes of envelope permeability or enhanced biocide efflux (*Poole,* 2002). Antimicrobial resistance in *Campylobacter* may be mediated by different resistance-nodulation-cell division (RND) efflux or non-RND efflux pumps, which could be involved in the extrusion of toxic compounds (*Pumbwe et al.,* 2005). The involvement of efflux mechanisms in bacterial resistance is mostly studied by the use of efflux pump inhibitors (EPIs) like phenylalanine-arginine beta-naphthylamide (PAβN) and 1-(1-naphthylmethyl)-piperazine (NMP) which enhance drug accumulation inside the bacterial cell, thereby increasing bacterial susceptibility to antimicrobials (*Marquez,* 2005). Those studies can be confirmed by using mutants lacking functional genes of efflux pump proteins (*Lin et al.,* 2002). A possible linkage of biocide and antibiotic resistance in different enteric bacteria via efflux related mechanisms has been recently reported (*Thorrold et al.,* 2007; *Karatzas et al*., 2007; 2008).

Because of the increasing problem of antimicrobial resistance of bacteria in general and in the food chain, many different types of alternative bioactive compounds have been screened recently for their potential antibacterial effects. Among others, phenolic extracts from different plant materials gave promising results (*Cowan*, 1999; *Moreno et al.,* 2006).

We studied the prevalence of thermotolerant *Campylobacter* spp. in retail poultry meat samples and included the isolates of *Campylobacter jejuni* and *C. coli* in further testing of their antimicrobial resistance and the mechanisms involved in resistance. In this study, erythromycin, ciprofloxacin, tetracycline as well as benzalkonium chloride, chlorhexidine diacetate and cetylpyridinium chloride resistance is presented and discussed. In addition, an alternative group of antimicrobial compounds - phenolic grape skin extracts of fourteen *Vitis vinifera* varieties grown in Dalmatia (Croatia) have been screened for antimicrobial activity against selected multiresistant chicken meat isolate.

Materials and methods

Isolation of bacterial strains from poultry meat and species identification

A hundred and fifty eight (158) samples of chicken meat from different suppliers on Slovenian market were investigated with the ISO 10272 guideline for the presence of thermotolerant campylobacters in the periods 2002-2003 and 2008-2009. A hundred and eighteen (118) strains isolated in the first period were long-term stored at –80°C in culture collection ZIM, BF, Ljubljana, for further studies.

C. jejuni and *C. coli* were identified by standard phenotyping (ISO) methods and polymerase chain reaction (PCR) procedures with amplification of hippuricase gene in *C. jejuni* and aspartokinase gene in *C. coli* in multiplex PCR as well as with the genus specific primers, as described previously (*Zorman and Smole Možina*, 2002). Additionally, the identity of most chicken meat isolates from the period 2002-2003 was confirmed by PFGE typing using *Sma*I restriction endonuclease and CHEF mapper XA System (Bio-Rad) (*Zorman et al.,* 2006).

Determination of antibiotic and disinfectant resistance and mechanisms involved

Antimicrobial resistance testing was first performed using disc diffusion method as described previously (*Kurinčič et al.,* 2005). Minimal inhibitory concentrations (MICs) of ciprofloxacin (Fluka Biochemika), erythromycin and tetracycline (both from Sigma-Aldrich) were determined by E-test as well as with broth microdilution method (*Kurinčič et al*., 2007). CellTiter-Blue® reagent and automated fluorescence signal detection by a microplate reader (Tecan, Mannedorf/Zurich, Switzerland) were used. Mutations in bacterial target genes (*23S rRNA, tetO*) were studied by PCR-RFLP and PCR, as described previously (*Kurinčič et al*., 2007). The resistance of selected 25 strains was tested against benzalkonium chloride (BC), chlorhexidine diacetate (CHA) and cetylpyridinium chloride (CPC), (Sigma-Aldrich, Saint Luis, USA) with broth microdilution method on the same principle as described for antibiotic resistance testing. The involvement of efflux pumps in antibiotic and biocide resistance mechanisms were evaluated by measurements of antimicrobial MICs values with or without chemical efflux pump inhibitors, phenylalanine-arginine ß-naphthylamide (PAßN), (Sigma-Aldrich Saint Louis, USA) or 1-(1-naphthylmethyl)-piperazine (NMP), (Chess GmbH, Mannheim, Germany) as described by *Kurinčič* et al. (2007). For this purpose, the Müller Hinton (MH) broth was supplemented with PAßN (20 µg/mL) or NMP (80 µg/mL). Two independent experiments were conducted to confirm the reproducibility of MIC data and the ATCC 33559 and ATCC 33560 strains were included as quality control strains. Additionaly, another strategy was used to study the mechanisms of resistance. We determined MIC values of selected antibiotics and disinfectants also for *Campylobacter jejuni* NCTC 11168 and its c*meB* mutant strain, kindly provided by dr. Payot (Institut National de la Recherche Agronomique, UR086 BioAgresseurs, Santé, Environnment, Nauzilly, France).

Determination of antimicrobial activity of phenolic grape skin extracts

Phenolic extracts of native or introduced* grape varieties (White: Debit, Kuć, Kujundžuša, Maraština, Medna, Rkaciteli*, Zlatarica; Red: Babić, Lasin, Merlot*, Plavina, Rudežuša, Trnjak, Vranac*) were extracted from homogenized grape skins using conventional solvent extraction procedure. They were characterized with determination of total phenols (TPC), total flavonoids (TFLO), total flavanols (TFA) and total anthocyanins (TA), HPLC analysis of phenolic compounds and with different procedures for antioxidant activity as described by *Katalinić et al.* (2009). Determination of the minimum inhibitory concentration (MIC) of fourteen different extracts was performed for selected multiresistant poultry meat isolate *C. coli* 137 and two susceptible reference strains, *Campylobacter coli* ATCC 33559 and *Staphylococcus aureus* ATCC 25923, with broth microdilution method by a Microplate Reader (Tecan, Mannedorf/Zurich, Switzerland) as described by *Klančnik et al*. (2009a). Minimum inhibitory concentrations (MICs) of tested phenolic extracts are expressed in mg of gallic acid equivalents (GAE) per mL of growth medium (*Katalinić et al.,* 2009). All measurements of MIC values were repeated three times and the most representative values were used.

Results and discussion

Isolation and identification of thermotolerant *Campylobacter* spp. from chicken meat

In total, 86.7% (137/158) of Slovene fresh retail chicken meat samples were found positive for thermotolerant campylobacters. The rate has not changed significantly during the six-year period (90.0% of tested samples were found positive in the years 2002-2003 and 84.7% in the years 2008-2009). Such results indicate the high extent of chicken meat contamination with campylobacters on retail market in Slovenia. Similar observations were reported from other European countries like Italy, France, Great Britain and Poland (*Pezzoti in sod*., 2003; *Meldrum in Wilson*, 2007, *Maćkiw in sod*., 2008), while reports from Scandinavian countries show much lower contamination level (NORM/NORM-VET, 2006; EFSA, 2007). The prevalence of thermotolerant *Campylobacter* in retail poultry meat in reports of official monitorings of food safety in Slovenia in recent years is constantly increasing. In the years 2006 and 2007, the officially tested poultry meat samples were found positive for thermotolerant campylobacters in 59.0 and 67.1%, respectively (*EFSA,* 2009).

A hundred and twelve (112) chicken meat isolates collected in 2002-2003 have survived long--term freezing and were included in PCR species identification. We found high proportion of *C. coli* among thermotolerant campylobacters from chicken meat (64/112), not reported from other European countries, except in some Balkan countries (*Uzunović-Kamberović et al*, 2007). This unusual result of classical and molecular identification of strains with species specific PCR primers was confirmed also by molecular typing of strains with macrorestriction analysis with *Sma*I and PFGE typing (*Zorman et al.*, 2006). However, in recently tested chicken meat samples *C. jejuni* was much more frequently isolated than *C. coli* (Table 1).

Antimicrobial resistance and mechanisms involved in *Campylobacter* chicken meat isolates

We tested the occurence of antimicrobial resistance to ciprofloxacin and erythromycin among 158 chicken meat isolates and tetracycline resistance among 61 isolates identified as *C. jejuni* or *C. coli.* Resistance to ciprofloxacin was most frequent (66.5%) and almost equally distributed among *C. jejuni* and *C. coli* isolates (Table 1). In our previous report, including the chicken meat isolates from the years 2002-2003, the rate of resistant *C. jejuni* isolates was only 38.5% (Kurinčič et al., 2005), but among isolates from the period 2008-2009, 74.6% of *C. jejuni* isolates were resistant to ciprofloxacin. This indicates still increasing rate of ciprofloxacin resistance. The prevalence of erythromycin resistance was much lower, but in fact very high, in comparison with the reports from some other European countries, USA or Canada (EFSA, 2007; Gyles, 2008). A significant difference in resistance rates was found among *C. jejuni* and *C. coli* isolates. Similarly, in food producing animals, the prevalence of erythromycin resistance is generally reported to be higher in *C. coli* than in *C. jejuni*, particularly among *C. coli* isolates from swine (*Belanger and Shryock*, 2007). In our study three groups of strains were observed concerning erythromycin resistance: susceptible with MICs, from 0,25 to 2 μg/mL, low-level resistant (LLR) with MICs, from 4 to 16 μg/mL, and high-level resistant (HLR) with MICs, higher than 32 μg/mL.

PCR-RFLP procedure has been used to test the presence of the A2075G mutation in the 23S rRNA gene. Seven HLR *C. coli* strains exhibited the A2075G mutation. Conversely, the A2075G mutation was not identified in any of LLR and susceptible strains. Other studies have also indicated that the mutation at position 2075 is usually responsible for high-level erythromycin resistance (*Payot et al.,* 2004; *Mamelli et al.,* 2005; *Gibreel et al.,* 2005). Interestingly, no A2075G mutation was identified in one HLR *C. coli* isolate originated from chicken meat.

Eight, out of 61 isolates from retail chicken meat samples (13.1%), were found resistant to tetracycline. PCR procedure confirmed the presence of *tetO* gene in all tetracycline resistant strains. No strains susceptible to tetracycline were found to have *tetO*. This is in agreement with other reports, but much more prevalent resistant strains have been found in different countries and from different sources, including farm animal isolates (*Alfredson and Korolik*, 2007; *Mazi et al*., 2008; *Uzunović-Kamberović et al*., 2007; 2009).

Our recent study of antibiotic resistance of *Campylobacter* isolates from animals, food and environmental sources (surface and drinking water) and different geographical regions also revealed interesting differences in resistance patterns of strains from different sources. For comparison with Slovene chicken meat isolates (Table 1), Table 2 includes strains from different sources and two different geographical regions. Beside Slovene samples, animal, food, environmental (water) and human clinical *Campylobacter* isolates from Zenica-Doboj canton, collected during two bilateral research projects in the years 2002-2007, are included. Comparison of these results with our recent testing of antibiotic resistance of chicken meat isolates reveals again the increasing rate of ciprofloxacin resistant isolates.

Table 1. Frequency of isolation of *Campylobacter jejuni* and *C. coli* from retail chicken meat samples and percentage (resistant/tested,%) of resistant isolates against erythromycin, ciprofloxacin and tetracycline
Tabela 1. Učestalost izolovanja *Campylobacter jejuni* i *C. coli* iz uzoraka pilećeg mesa iz prometa i procenat (rezistentnih/ispitanih,%) rezistentnih izolata na eritromicin, cipoflokacin i tetraciklin

Species isolated from retail chicken meat	**Frequency of isolation**	**Erythromycin -R**		**Ciprofloxacin -R**		**Tetracycline –R**	
C. coli	65 (33.5%)	10/29	34.5%	18/30	60.0%	4/28	14.3%
C. jejuni	129 (66.5%)	12/129	9.3%	87/128	68.0%	4/33	12.1%
∑	194 (100%)	22/158	13.9%	105/158	66.5%	8/61	13.1%

Table 2. Percentage (%) of resistant strains of *C. jejuni* and *C. coli* against erythromycin, ciprofloxacin and tetracycline among the isolates from farm animals, retail chicken meat, surface and drinking water and human clinical samples, collected in the period 2002-2007 in Slovenia and Zanica-Doboj canton (taken from *Kurinčič et al.,* 2009).
Tabela 2. Procenat (%) rezistentnih sojeva *C. jejuni* i *C. coli* na eritromicin, cipofloksacin i tetraciklin u izolatima sa farmskih životinja, pilećeg mesa iz prometa, sa površina, iz pijaće vode i iz humanih kliničkih uzoraka sakupljenih u periodu od 2002-2007. u Sloveniji u kantonu Zanica-Doboj (preuzeto *Kurinčič i sar.*, 2009).

Source and number of tested isolates / **Antimicrobial agent**	**Farm animal** (n = 15)	**Chicken meat** (n = 112)	**Water** (n = 50)	**Human clinical isolates** (n = 179)
Erythromycin -R (%)	38.5	21.4	44.0	10.6
Ciprofloxacin -R (%)	30.8	43.8	26.0	31.8
Tetracycline - R (%)	61.5	18.8	6.0	6.8

Concerning possible cross-resistance we selected twenty five chicken meat isolates and reference strains *C. jejuni* ATCC 33560 and *C. coli* 33559 to be tested for their resistance against three antibiotics (erythromycin, ciprofloxacin, tetracycline) and three disinfectants (benzalkonium chloride (BC), cetylpyridinium chloride (CPC), chlorhexidine diacetate (CHA). For presentation, the results for sixteen thermotolerant *Campylobacter* chicken meat isolates are included in Table 3.

Strains susceptible and resistant to antibiotics (also multidrug resistant strains like *C. coli* 137, *C. coli* 140, and *C. coli* 171, Table 3) were tested. Two different BC and CPC resistance phenotypes were observed and classified, as described previously for *Listeria monocytogenes* (Aase et al., 2000). Irrispective of antibiotic resistance, all chicken meat isolates were sensitive to BC at concentration 1 µg/ml, or below, and were considered as BC sensitive, but three (12%) of the isolates were considered CPC resistant (MIC 4 µg/mL). Most of the strains were sensitive to CHA concentration 1 µg/ml or below, but four strains were tolerant to CHA concentration 2 µg/ml. However, no significant difference in biocide susceptibility between antibiotic resistant and sensitive *C. coli* and *C. jejuni* isolates was found. The isolates from the same meat samples usually shared the same resistotype (like strains 53/1 and 53/4 in Table 3).

With the aim to study the mechanisms involved, the resistance of isolates to antibiotics and disinfectants was studied in the absence and presence of efflux pumps inhibitors (EPIs), PAßN and NMP. Both EPIs increased erythromycin susceptibility significantly, wherein PAβN had greater effect than NMP, although both affected the main efflux pump, CmeABC, in *Campylobacter* cells. Both EPIs had much greater effect in *C. coli* than in *C. jejuni.* The results confirm that efflux mechanism mediated by efflux pumps plays an active role in resistance to erythromycin in *Campylobacter*. The presence of efflux pumps activity in HLR isolates with 23 rRNA mutations suggests that the synergistic activity of these two drug resistance mechanisms exist in *Campylobacter*. BC susceptibility was significantly increased by both EPIs. There was no significant difference between NMP and PAβN effect on BC susceptibility. The smaller effect of EPIs was observed when used in the presence of ciprofloxacin, tetracycline, CHA and CPC (data not shown).

Table 3. Antimicrobial activity (expressed as MICs , µg/mL) of antibiotics (erythromycin, ERI, tetracycline, TET, ciprofloxacin, CIP), and disinfectants (benzalkonium chloride, BC), cetylpyridinium chloride (CPC), chlorhexidine diacetate (CHA)) against reference strains and sixteen thermotolerant *Campylobacter* chicken meat isolates

Tabela 3. Antimikrobna aktivnost (izražena kao MICs, µg/mL) antibiotika (eritromicin, ERI), tetraciklin, TET, ciprofloksacin, CIP) i dezinficijenasa (benzaalkonijum hlorid, BC), cetilapiridin hlorid (CPC), hlordexin diacetat (CHA) na referentne sojeve i 16 termotolerantnih izolata *Campylobacter* iz pilećeg mesa

Species	Strain	ERI	TET	CIP	BC	CPC	CHA
C. coli	**ATCC 33559**	2	0.25	0.063	0.125	2	1
C. jejuni	**ATCC 33560**	0.5	1	0.125	<0.016	0.125	0.5
C. jejuni	**203**	0.5	1	0.125	0.25	2	1
C. jejuni	**K29/3**	0.25	0.5	0.063	0.5	4	2
C. jejuni	**K45/4**	0.5	0.5	8	0.063	4	1
C. jejuni	**K49/4**	0.5	0.125	0.063	1	2	0.125
***C.* spp.**	**K31/2**	2	0.5	8	0.25	1	2
***C.* spp.**	**K37/4**	128	0.5	0.25	0.25	2	0.5
***C.* spp.**	**K40/2**	0.25	1	8	0.125	2	1
C. coli	**128**	0.25	0.25	32	0.25	4	1
C. coli	**137**	> 512	256	16	1	1	0.25
C. coli	**140**	512	128	16	0.063	1	0.25
C. coli	**171**	512	32	16	1	0.5	0.125
C. coli	**K31/4**	1	0.5	8	0.125	1	2
C. coli	**K32/3**	2	128	16	0.25	2	0.5
C. coli	**K39/3**	1	1	64	0.125	1	2
C. coli	**K53/1**	1	128	16	0.063	0.5	0.5
C. coli	**K53/4**	1	128	16	0.063	0.5	0.5

In the study of efflux involvement in antibiotic and disinfectant resistance of *Campylobacter* strains, the reference strain *C. jejuni* NCTC 11168 and its *cmeB* mutant were also assessed - by using the EPIs PAßN and NMP. Both inhibitors, PAßN and NMP, increased the susceptibility of the wild-type strain to erythromycin by 4-fold. Additionally, PAßN was also able to reduce the MIC of ciprofloxacin, BC, CPC and CHA by 2-fold. In case of tetracycline, no effect of the inhibitors was observed. Insertional inactivation of *cmeB* gene increased the susceptibility to antibiotics erythromycin, ciprofloxacin and tetracycline by 8-fold, 4-fold, 8-fold, respectively, and to disinfectant BC by 4-fold. Additionally, both inhibitors were also able to reduce the MIC of ciprofloxacin and all disinfectants tested by at least 2-fold in *cmeB* mutant, suggesting that resistance mechanisms are very complex and another efflux pump(s) are involved in *Campylobacter jejuni* resistance to these antimicrobials.

Antimicrobial activity of phenolic grape skin extracts against antibiotic resistant chicken meat isolate

In our recent studies of antimicrobial activity of phenolic extracts from different plant sources (*Klančnik et al*., 2009a,b;) we got an evidence that campylobacters, although gram-negative organisms, could be quite sensitive to different phenolic compounds or their mixtures (*Katalinić et al.,* 2009). For this reason we tested also the extracts from grape skins of white and red cultivars against different antibiotic resistant chicken meat isolates. The results for the strain *C. coli* 137 (MDR meat isolate, see Table 3) and two antibiotic susceptible reference strains *Campylobacter coli* ATCC 33559 and *Staphylococcus aureus* ATCC 25923 are collected in Table 4. Staphylococci are known as susceptible microorganisms to different antimicrobials and thus usually used as reference material in screening tests of antimicrobial activity of new compounds, so they were used for comparison also in these experiments. We found very low MICs for both *Campylobacter* strains, although the outer membrane surrounding the cell wall in gram-negative bacteria could restrict diffusion of compounds through its lipopolysaccharide covering. In our previous work gram-positive bacteria were more sensitive than gram-negative bacteria, especially for oil-soluble extracts with carnosic acid as the major phenolic compound (*Klančnik et al.,* 2009a). However, in this initial screening of the activity of grape skin phenolic extracts against *Campylobacter* chicken meat isolates we got very promising results, especially in antimicrobial activity of grape skin extracts of white cultivars, which on average gave even better results. It is important that they leave the wine processing still rich in biologically valuable components. Further tests are needed to confirm the screening results in *in vitro* and *in vivo* assays to confirm the potential use as additives for reduction of bacterial load of fresh chicken meat and products and thus in lowering the risk of bacterial transmission via this important route.

Conclusion

The emergence and dissemination of resistant bacteria is an inevitable side effect of the use of antimicrobials. We need a monitoring system of the prevalence and antibiotic resistance of zoonotic bacteria from human, animal, food and environmental samples to understand the epidemiology of resistant strains to assure food safety and consumers health. In our studies we confirmed high prevalence and also antibiotic resistance of *C. jejuni* and *C. coli* in/from retail chicken meat. Ciprofloxacin is one of the critically important antimicrobial agents in human medicine. It is often used for treatment of human gastroentiritis because of its activity against enteric bacterial pathogens. However, agricultural use of some fluoroquinolones, including food producing animals, contribute to selection of resistant *Campylobacter* spp. which are transmitted into the food chain. We have confirmed high and still increasing rate of ciprofloxacin resistant isolates

Table 4. Antimicrobial activity of phenolic extracts (expressed as MICs of total phenols, e.g. mg GAE* per mL of growth medium in broth microdilution test) from grape skins of white and red cultivars against *C. coli* 137 (MDR chicken meat isolate) and two reference strains

Tabela 4. Antimikrobna aktivnost fenolnih ekstrata (izražena kao MIC ukupnih fenola, npr. GAE* po mL podloge u bujonskom mikrodilucionom testu) iz kožice grožđa belih i crvenih sorti na *C. coli* 137 (MDR izolat iz pilećeg mesa) i dva referentna soja

Testing organisms / **Cultivars**	***C. coli* 137 (MDR chicken meat isolate)**	***Campylobacter coli* ATCC 33559 (susceptible reference strain)**	***Staphylococcus aureus* ATCC 25923 (susceptible reference strain)**
Kujundžuša	0.076 ± 0.020	0.032 ± 0.005	0.15 ± 0.02
Rkaciteli	0.023 ± 0.000	0.014 ± 0.002	0.20 ± 0.03
Zlatarica	0.051 ± 0.020	0.042 ± 0.005	0.21 ± 0.03
Medna	0.024 ± 0.005	0.014 ± 0.002	0.21 ± 0.03
Kuč	0.023 ± 0.010	0.019 ± 0.002	0.26 ± 0.04
Maraština	0.036 ± 0.010	0.015 ± 0.002	0.21 ± 0.03
Debit	0.060 ± 0.010	0.025 ± 0.005	0.25 ± 0.05
Average for white cultivars	*0.042 ± 0.02*	*0.023 ± 0.01*	*0.22 ± 0.04*
Vranac	0.10 ± 0.03	0.20 ± 0.03	0.23 ± 0.03
Trnjak	0.21 ± 0.04	0.14 ± 0.03	0.22 ± 0.04
Rudežuša	0.13 ± 0.02	0.25 ± 0.06	0.29 ± 0.04
Merlot	0.07 ± 0.01	0.13 ± 0.03	0.44 ± 0.08
Babić	0.10 ± 0.02	0.08 ± 0.01	0.42 ± 0.08
Lasin	0.03 ± 0.01	0.04 ± 0.01	0.34 ± 0.07
Plavina	0.05 ± 0.01	0.09 ± 0.02	0.29 ± 0.06
Average for red cultivars	*0.10 ± 0.05*	*0.13 ± 0.07*	*0.32 ± 0.09*

*Total phenols are expressed as gallic acid equivalents (GAE) in grape skin extract from retail chicken meat. However, so far we have not found any evidence suggesting that tolerance to disinfectants or other potential antimicrobials (like plant phenolic extracts) is connected to antibiotic resistance of *Campylobacter* isolates.

Acknowledgement

The authors would like to thank the Ministry of Higher Education, Science and Technology of the Republic of Slovenia for financing their projects, especially PhD grants for M.K. and A.K., to dr. Tina Zorman for her contribution in early testing of *Campylobacter* in Laboratory for Food Microbiology, BF, and to dr. Alenka Štorman for enabling the part of experimental work that was performed at ZZV Celje in 2009.

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Paper recieved: 28.04.2009.