UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY

Jasna KOVAČ

ANTIMICROBIAL AND MODULATORY ACTIVITY OF SELECTED PHYTOCHEMICALS AND EPIDEMIOLOGICAL CHARACTERISTICS OF ANTIBIOTIC RESISTANCE IN *Campylobacter jejuni*

DOCTORAL DISSERTATION

Ljubljana, 2015

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EPIDEMIOLOŠKE ZNAČILNOSTI ANTIBIOTSKE ODPORNOSTI TER PROTIMIKROBNO IN MODULATORNO DELOVANJE IZBRANIH FITOKEMIKALIJ NA BAKTERIJE *Campylobacter jejuni*

DOKTORSKA DISERTACIJA

Ljubljana, 2015

“I am not apt to follow blindly the lead of other men.”
― Charles Darwin

On the basis of the Statute of the University of Ljubljana and by decisions of the Senate of the Biotechnical Faculty and the decision of University Senate, dated from January, 28th, 2013, the continuation to Interdisciplinary Doctoral Programme in Biosciences, field: Biotechnology, was approved. Prof. Sonja Smole Možina, PhD, as the supervisor, and Prof. Franz Bucar, PhD, as the co-advisor, were confirmed.

Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa Senata Univerze z dne 28.1.2013 je bilo potrjeno, da kandidatka izpolnjuje pogoje za opravljanje doktorata znanosti na Interdisciplinarnem doktorskem študijskem programu Bioznanosti, znanstveno področje biotehnologija. Za mentorico je bila imenovana prof. dr. Sonja Smole Možina ter za somentorja prof. Franz Bucar.

Doctoral dissertation was carried out at the Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, Slovenia, Department of Pharmacognosy, Institute of Pharmaceutical Sciences, University of Graz, Austria, Veterinary Medicine College, Iowa State University, USA, Institute of Food Research, Norwich, UK, and Department of Zoology, University of Oxford, United Kingdom.

Doktorska disertacija je bila opravljena na Oddelku za živilstvo na Biotehniški fakulteti na Univerzi v Ljubljani, na Oddelku za farmakognozijo na Institutu za farmacevtske znanosti univerzev Gradcu v Gradcu v Avstriji, na Koledžu za veterinarsko medicino Univerze Iowa State v Amesu v ZDA, na Institutu za raziskave hrane v Norwichu v UK in Oddelku za zoologijo na Univerzi v Oxfordu, Velika Britanija.

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Date of defense (datum zagovora): 16.4.2015

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Jasna Kovač

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AI *Campylobacter* spp. is the most prevalent bacterial pathogen associated with food borne diseases and *C. jejuni* causes the majority of cases. Despite its high prevalence observed all around Europe, the true incidence of campylobacteriosis is still thought to be significantly underreported, especially in the lower income countries, which do not have well established healthcare systems. In our study we have used the Bayesian approach to modelling the campylobacteriosis prevalence in Slovenia. We have used already existing, publicly available data to build models for key predictors influencing the reported prevalence rates. As expected, the predictions indicated substantial underreporting rate which is strongly influenced by the probabilities for non-bloody diarrhoea cases to visit a general practitioner and for general practitioner to submit their sample for analysis. In addition to high prevalence, also the antibiotic resistance has increased substantially in the last years, causing difficulties in clinical treatment of campylobacteriosis. Especially concerning is the quinolone resistance, due to the use of this class of antibiotics in veterinary, as well as in human medicine. In our study we have investigated the characteristics of quinolone resistance expansion among the *C. jejuni* isolates from different sources in Slovenia. Using the genotyping approach by employing the MLST and single nucleoide polymorphism analysis of quinolone resistance determining region withing *gyrA* gene we have confirmed the clonal spreading of the resistance with the most prevalent clonal complex, 21. Further analysis of quinolone resistance expansion on the whole genome sequences of a larger set of English clinical *Campylobacter* isolates revealed the clonal expansion in clonal complexes ST-354 and ST-464, as well. In order to tackle the problematic of increasing antibiotic resistance among *Campylobacter jejuni*, we aimed to identify novel resistance modulators with potential to restore bacterial susceptibility to antibiotics. We investigated the antimicrobial, resistance modulatory and efflux inhibitory potential of a Zingiberaceae *Alpinia katsumadai* used in traditional Chinese medicine. Its extracts, post-distillation residue extract and essential oil were confirmed as moderate to weak antimicrobials and essential oil was found to have high resistance modulatory and efflux inhibitory potential in *C. jejuni*. Furthermore, the pure terpen compound α-pinene was found as one of the constituents of this essential oil and one of its enantiomeres, (-)-α-pinene, was confirmed as a strong modulator of *C. jejuni* resistance against ciprofloxacin, erythromycin, triclosan and ethidium bromide. It has an efflux inhibitory activity, that is significantly better than that of the reference efflux inhibitors CCCP and reserpine. The efflux systems CmeABC and Cj1687 were identified as the main targets of its efflux inhibitory activity, along with enhanced permeability of the membrane. We have demonstrated that *C. jejuni* responds to treatment with (-)-α-pinene using the same regulatory set of genes as in heat and osmotic shock response and seems to be a type of general stress response system that protects bacteria from protein coagulation and cell death.

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AI Bakterije *Campylobacter* spp. so najpogostejši bakterijski vzrok okužb s hrano, vrsta *C. jejuni* pa je odgovorna za večino humanih kampilobakterioz. Kljub visoki poročani prevalenci v Evropi je realna pojavnost bolezni predvidoma močno podcenjena, še posebej v državah s slabše organiziranim zdravstvenim sistemom. V našem delu smo uporabili bajezijski pristop k modeliranju realne prevalence kampilobakterioz v Sloveniji, z uporabo javno dostopnih podatkov za postavitev modelov ključnih spremenljivk, ki prispevajo k poročanju prevalence. Po pričakovanju so napovedi pokazale visoko stopnjo podcenjenega poročanja, na katerega znatno vplivata verjetnost obiska zdravnika in verjetnost odvzema vzorca za analizo v primeru nekrvave diareje. Poleg visoke prevalence bolezni povzroča težave tudi naraščajoča bakterijska odpornost proti kliničnim antibiotikom, ki otežuje zdravljenje kampilobakterioz. Posebej zaskrbljujoča je odpornost proti kinolonskim antibiotikom, ki se uporabljajo v veterinarski in humani medicini. V našem delu smo raziskovali karakteristike širjenja odpornosti proti kinolonom med bakterijami vrste *C. jejuni* iz različnih virov po Sloveniji. Z uporabo tipizacije MLST in gena *gyrA* smo potrdili klonsko širjenje odpornosti z najpogostejšim klonskim kompleksom 21. Nadaljnja analiza širjenja kinolonske rezistence na podlagi sekvenc celotnih genomov večje skupine angleških kliničnih izolatov *Campylobacter* je razkrila klonsko širjenje odpornosti tudi v klonskem kompleksu ST-354 in ST-464. Za razreševanje problematike antibiotske odpornosti smo se osredotočili na raziskovanje novih modulatorjev odpornosti, ki bi lahko povrnili bakterijsko občutljivost na antibiotike. Preučevali smo protimikrobno, odpornostno-modulatorno in efluks-inhibitorno učinkovitost izvlečkov in eteričenga olja rastline *Alpinia katsumadai* iz družine ingverk, ki se v tradicionalni kitajski medicini uporablja za lajšanje želodčnih težav. Izvleček iz semen in odpadnega materiala po destilaciji in eterično olje smo potrdili kot srednje do šibko učinkovita protimikrobna sredstva. Eterično olje pa se je izkazalo kot zelo učinkovit modulator odpornosti z efluks-inhibitornim potencialom. Nadalje smo raziskovali modulatorno učinkovitost spojine (-)-α-pinen, ki je bila v neidentificirani enantiomerni sestavi prisotna v eteričnem olju iz semen *A. katsumadai*. (-)-α-pinen je močno modulatorno učinkovit pri bakterijah *C. jejuni*, in sicer v kombinaciji s ciprofloksacinom, eritromicinom, triclosanom in etidijevim bromidom. Njegova efluks-inhibitorna učinkovitost je bila izrazito boljša od učinkovitosti referenčnih efluksnih inhibitorjev, CCCP in reserpina. Efluksni črpalki CmeABC in Cj1687 sta bili poleg povečane prepustnosti membrane identificirani kot glavni tarči modulatornega delovanja. Pokazali smo tudi, da se *C. jejuni* odziva na stres tretiranja z (-)-α-pinenom z enakim setom genov kot na toplotni in osmotski stres, kar predstavlja tip splošnega stresnega odziva, ki bakterije ščiti pred koagulacijo proteinov in posledično smrtjo.

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Kovač J., Čadež N., Lušicky M., Nielsen E.M., Ocepek M., Raspor P., Možina S.S. 2014a. The evidence for clonal spreading of quinolone resistance with a particular clonal complex of *Campylobacter jejuni*. Epidemiology & Infection, 142: 2595-2603

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Kovač J., Šimunović K., Wu Z., Klančnik A., Bucar F., Zhang Q., Smole Možina S. 2015. Antibiotic resistance modulation and modes of action of (-)-α-pinene in *Campylobacter jejuni*. PLoS one, doi:10.1371/journal.pone.0122871 (accepted for publication on 5 Mar 2015)

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ANNEX A: Permission of Epidemiology and Infection for publishing the article entiteled 'Anti-*Campylobacter* and resistance-modifying activity of *Alpinia katsumadai* seed extracts' as part of the doctoral thesis.

ANNEX B: Permission of Journal of Applied Microbiology for publishing the article entiteled 'The evidence for clonal spreading of quinolone resistance with a particular clonal complex of *Campylobacter jejuni*' as part of the doctoral thesis.

ANNEX C: Permission of Food Technology and Biotechnology for publishing the article entiteled 'Antimicrobial and resistance modulatory activity of *Alpinia katsumadai* seed extract, essential oil and post-distillation extract' as part of the doctoral thesis.

ABBREVIATIONS AND SYMBOLS

AP	Alpha pinene
CC	Clonal Complex
CHL	Chloramphenicol
CIP	Ciprofloxacin
cj1876	Gene encoding putative efflux protein Cj1876
cmeABC	Gene encoding efflux pump CmeABC
cmeDEF	Gene encoding efflux pump CmeDEF
cmeG	Gene coding for efflux pump CmeG
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ERY	Erythromycin
EtBr	Ethidium Bromide
EU	European Union
GEN	Gentamicin
GP	General Practicioner
gyrA	Gene encoding gyrase subunit A
MAMA PCR	Mismatch amplification mutation assay
mfd	Gene encoding Mutation Frequency Decline
MIC	Minimal Inhibitory Concentration
MLST	Multilocus Sequence Typing
NAL	Nalidixic Acid
QRDR	Quinolone Resistance Determining Region
RFU	Relative Flourescence Units
rMLST	Ribosomal Multilocus Sequence typing
ST	Sequence Type
STR	Streptomycin
wgMLST	Whole Genome Multilocus Sequence Typing
WHO	World Health Organization
MS	Member State

1 INTRODUCTION AND RESEARCH AIMS

1.1 INTRODUCTION

1.1.1 The general introduction to the *Campylobacter* genus and a brief overview of the research challenges

Bacteria of *Campylobacter* genus are small, gram negative, spirally shaped organisms with a single polar flagellum at one or both sides, but some are also non-motile. Campylobacters are thermophilic microorganisms with optimal growth at 37 to 42 ºC. Due to absence of cold shock response they are incapable of growth below 30 ºC. Almost all species have active oxidase. Because they do not metabolize majority of carbohydrates, they tend to obtain energy from utilizing amino acids or Krebs cycle intermediates. *C. jejuni* hydrolyses hippurat and indoxyl acetate and reduces nitrate, which is exploited in its biochemical differentiation from other *Campylobacter* species (Vandamme, 2000). One of the specialities of these bacteria is the requirement of microaerophilic environment for optimal growth. They are also known to change the physiological and morphological state to so called viable but non-culturable state in unfavourable environment and when exposed to stress. This is also one of the factors which make them difficult to isolate (Kassem et al., 2013).

The history of *Campylobacter* discovery is thought to go back to the far year of 1886, when Theodor Escherich, the famous German-Austrian paediatrician and also a professor at university in Graz, has first described it as non-culturable bacteria of spiral shape (Shulman, 2007; Vandamme, 2010). Later on the bacteria were frequently found in sheep uterus, aborted bovine fetuses, cattle and pig faeces and were named *Vibrio jejuni* and *V. coli* (Silva et al., 2011). It was not recognized as *Campylobacter* genus (campylos meaning curved in Greek) until 1963, when this group of bacteria was distinguished from *Vibrio* based on the smaller genome size, non-fermentative metabolism and microaerophilic respiration (Silva et al., 2011).

Campylobacters started to draw attention in the early seventies, when Butzler and co-workers started a systematic search for vibrio related microorganisms in stools of children and adults (Butzler et al., 1973). With this pioneer study and invention of the first *Campylobacter* selective agar, they have laid the foundation of *Campylobacter* research. Soon after, new studies with successfully isolated *Campylobacter* spp. from children with characterized clinical background have followed (Bokkenheuser et al., 1979). In those times *C. fetus* specific bacteriophages were also successfully introduced for the first time as the potential antimicrobials. At the same time this was the ground-breaking era of recognizing *Campylobacter* genus as a causative agent of human disease, which has increased an interest in this pathogen and accelerated the research in the field of campylobacteriosis. However, it was not known at that time, that bacteria can be transmitted with food, so the mystery of its origin was yet to be unravelled.

Today we know that campylobacteriosis is a zoonotic disease that can be transmitted to humans directly from animals, or via foodstuffs. While it usually does not cause any disease symptoms in animals, the human infection reflects in elevated body temperature, diarrhoea and abdominal cramps. In some cases also post-infectious disorders, such as Guillain-Barré syndrome occur (EFSA, 2014a).

Campylobacter spp. is acknowledged as the most common bacterial foodborne cause of gastroenteritis in EU, as well as in other parts of the developed world (EFSA, 2014a). The trend of its increasing prevalence is recorded ever since the European Food Safety Authority (EFSA) was established and started to monitor the trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in the EU. Moreover, the officially reported numbers are thought to be significantly underestimated, due to different levels of underdiagnosis and underreporting among different countries (Haagsma et al., 2012). It is therefore no surprise, that tremendous efforts are invested, economic- as well as research-wise, to elucidate the epidemiological characteristics of campylobacters. The sound scientific results on this subject could namely contribute to efficient policy making in the field, resulting in decrease of the disease burden.

With the effective use of molecular tools in the last decade, it has become clear that meat, especially poultry, is the major source of *Campylobacter* infections, although other, especially environmental sources are thought to be underestimated (Sheppard et al., 2010). A great contribution to this endeavour was the establishment of multi locus sequence typing scheme for *Campylobacter jejuni* in 2001 (Dingle et al., 2001), as well as other source-tracing genotyping methods (Stabler et al., 2013). This has triggered a new wave of studies in *Campylobacter* molecular epidemiology with focus on tracing the pathogen along the food production chain and exploiting the available techniques for investigation of foodborne outbreaks and following the spreading of highly pathogenic clones (Muhammad et al., 2011; Sahin et al., 2012; Kwan et al., 2014). These techniques are lately advantageously used also in studying the antibiotic resistance expansion (Kittl et al., 2013, Wimalarathna et al., 2013; Kovač et al., 2014).

The resistance against ciprofloxacin, one of the first drugs of choice for treatment of gastrointestinal diseases, including campylobacteriosis, is substantially increasing among campylobacters, with the increasing trend being more pronounced in southern European countries, including Slovenia (Smole Možina et al., 2011; EFSA, 2014b). Due to the acute nature of infectious diseases, the antimicrobial drug discovery is not a priority in pharmaceutical industry. In order to solve this problem, therefore a lot of academic research is focused on finding new antimicrobial compounds. In this aspect plant kingdom represents a valuable source of bioactive constituents that is not yet fully exploited. In the last years several new anti-*Campylobacter* natural products have been identified, some possessing a direct antimicrobial activity, while others having a resistance-modulatory potential, meaning they can potentiate the activity of existing antibiotics when administered together (Klančnik et al., 2012a; Klančnik et al., 2012b; Kurinčič et al., 2012; Kovač et al., 2014b). The most promising strategy of resistance modulation approach seems to be inhibition of bacterial efflux and will be presented more in detail later on.

1.1.2 *Campylobacter* prevalence and its underreporting issues

In 2002 the EU established the independent agency called The European Food Safety Authority (EFSA) to monitor and collect information on zoonoses. Today all EU member states are obliged to collect and report relevant data on zoonoses, zoonotic agents and antimicrobial resistance and food-borne outbreaks according to the Zoonoses Directive 2003/99/EC. One of the food-borne pathogens included in the monitoring scheme is also *Campylobacter* spp., which is followed in animals, foodstuffs and humans. Among campylobacters, *C. jejuni* is the species most commonly associated with human disease (EFSA, 2014a).

The notification rate of campylobacteriosis in 2012 was 55.49/100,000, which was for 4.3% less than the year before that. The prevalence of campylobacteriosis in Slovenia was somewhat lower with 47.83 cases per 100,000. This was the first decrease in reported cases on the EU level in the last five reporting years. In the years 2008-2012 the clear seasonal trend with peak of the reported cases in summer months was observed, as well as the clear increase in the reported cases of human campylobacteriosis. In contrast to high hospitalization rate (47.7%), low mortality (0.03%) is reported, probably due to selflimiting nature of the disease and often mild symptoms. *Campylobacter* was also on the fourth place of known causes of outbreaks with weak and strong evidence altogether. Outbreaks, which are less common than sporadic cases, are usually caused by ingestion of contaminated milk or water (EFSA, 2014a).

To protect consumers from health risks associated with food-borne pathogens, EFSA has implemented an integrated "farm to fork" approach to study the food safety. Part of this approach is risk assessment based on the collected data, as well as risk management, which is based on the sound output of the scientific analyses. The risk management is performed in cooperation of EU member states with European commission, European Parliament, EFSA and the European Centre for Disease Prevention and Control, in order to ensure efficient risk communication and protect the consumers (EFSA, 2014a).

As the prevalence of foodborne diseases is thought to be significantly underestimated, there is a need to evaluate the effectiveness of the reporting process in order to assess the real burden of disease. The prevalence is underestimated due to underreporting and underdiagnosis associated with passive surveillance, incorrect diagnosis and inefficient communication with authorities, as well as with the unregistered cases with mild symptoms that do not seek medical help. Prevalence is normative, characterizing the rate of cases in a population at a specific time and is easy to visualize and compare. It is measured from finite samples and the reported cases behave according to a binomial process; therefore uncertainty in prevalence can be described by beta distributions. Prevalence can be considered as the probability for a binomial process and the uncertainty about prevalence is the probability density of the binomial parameter which has a beta distribution. Existing belief about the prevalence can therefore be systematically updated given the data from successive finite samples. Bayes' theorem can be used to express how to rationally change a subjective belief by taking evidence into account. Model parameters affecting the prevalence reporting by general practitioners (GPs) can be defined in order to construct a probabilistic

graphical model. Since different parameters affecting the process of reporting are involved in cases of hospitalization, these have to be acknowledged with a separate branch of the model. Because of the differences in symptoms and severity of the disease caused by different pathogens, as well as the differences in medical and reporting practice among EU countries, some of the parameters are dependent of the country and others of the pathogen (Haagsma et al., 2012).

Most of the studies approach to reconstruction of the surveillance pyramid for foodborne diseases by evaluating few vital fractions, which estimate the underdiagnosis and underreporting rate of the diseases. These include the probability for a gastroenteritis patient to visit a GP, for a GP to submit a stool, for a positive result and for a positive result to be reported. The odds for diarrhoea case to seek medical help and for a GP to submit the patient's stool sample for microbiological analysis are the most important components contributing to the overall notification fraction according to the data from previous studies (Michel et al., 2000; Scallan et al., 2006; Hall et al., 2008; Scallan et al, 2011; Haagsma et al., 2012). Moreover, the medical help seeking behaviour, as well as GP decision making is influenced by the severity of symptoms, thus the cases experiencing bloody and non-bloody diarrhea were often analysed separately (Scallan et al., 2006; Scallan et al., 2011; Haagsma et al., 2012). Although some studies report similar proportions of diarrhoea cases seeking medical health despite the differences in structure and access to health care systems in Australia, Canada, Ireland and United States (Scallan et al., 2005), other reveal significant differences in medical help seeking behaviour among countries within EU (Haagsma et al, 2012).

In our study, parameters were modelled using linear regression based on existing data for seven foodborne pathogens in seven EU countries and only common statistical data available for EU countries was used to model the parameter uncertainty distributions. Monte Carlo simulations of modelled parameters were used to estimate beliefs about prevalence of foodborne pathogens in additional EU countries. Furthermore, the sensitivity of parameters comprizing the prevalence model was examined.

1.1.3 Tracing *Campylobacter* along the food chain and detecting the source of infection

Modern molecular tracing techniques, like pulsed field gel electrophoresis (PFGE) and multi locus sequence typing (MLST) have been widely used to trace the *Campylobacter* along the food chain and to identify the sources of sporadic and outbreak cases (Roux et al., 2013; Kovač et al., 2014a; Kittl et al., 2014; Kwan et al., 2014). Although PFGE is known as a golden standard among genotyping methods, the MLST begins to take over the lead. There are several reasons for its successful establishment in molecular epidemiological studies of *Campylobacter,* although it is not competitive concerning the cost of analysis. Firstly, the method is portable, completely reproducible and the results are easily comparable among different laboratories, secondly a user-friendly publicly available database called PubMLST was build by the University of Oxford molecular epidemiology research group, which currently has over 29000 entries (Cody et al., 2010a). The MLST community is constantly increasing and public health authorities like European Centre for Disease Control and Prevention (ECDC) are starting to implement it in their epidemiological studies (Kwan et

al., 2014). Since the price of sequencing is rapidly decreasing due to the implementation of the highly efficient next generation sequencing, MLST is expected to become more affordable and widely accepted as the method of first choice also in the routine laboratories. Even more, the advanced laboratories are already beginning to implement the extended MLST schemes like ribosomal multi locus sequence typing (rMLST) and whole genome MLST (Jolley et al., 2012; Cody et al., 2013). The rMLST examines the variation of 53 genes, instead of only seven housekeeping genes and it includes ribosomal genes. Ribosomal MLST is integrating typing and genealogy, while whole genome MLST (wgMLST) provides definitive characterization of *C. jejuni* and *C. coli* and is proposed to be used in a routine as a practical and rapid exploitation of whole-genome sequencing (Jolley et al., 2012; Cody et al., 2013).

1.1.4 *Campylobacter* antibiotic resistance prevalence and spreading

The increasing incidence of antibiotic resistant bacteria reflects in the impaired efficacy of clinical treatments, prolonged illness and greater mortality due to infectious diseases. The decline in new antibiotic discovery in the last decades, with only two antibiotics from new classes being brought on the EU market since the 1970s, poses serious economic and public health threat. Not only 25,000 patients dying due to incurable infections with multidrug-resistant (MDR) bacteria, but also the European healthcare budget being burdened for at least 1.5 billion additional euros each year because of the prolonged treatments of MDR infections is concerning (ECDC, 2009). *C. jejuni*, being the most prevalent bacterial cause of gastroenteritis and with high rates of ciprofloxacin resistance, is an important part of this issue (EFSA, 2014a; EFSA 2014b). Drug resistant *Campylobacter* is on the CDC's list of serious threats, causing 120 deaths per year in the US alone, and its threat is expected to worsen without ongoing public health monitoring and preventing activities (CDC, 2013).

According to EFSA (2014b), high resistance of campylobacters to ciprofloxacin, nalidixic acid and tetracycline exists in EU countries. On the other hand, they are susceptible to the first drug of choice for campylobacteriosis treatment, erythromycin, as well as gentamicin. Especially resistance against fluoroquinolones, which is commonly observed in isolates from broilers, chicken meat, pigs and cattle, is of great concern. It is ranging from 32 to 83% among different EU Member states (EFSA, 2014b). Fluoroquinolones are usually the first drug of choice for treatment of gastrointestinal infections with unknown causative agent; this is why the increasing resistance can seriously impair effective clinical treatment. In Slovenia, the ciprofloxacin resistance of human *Campylobacter* spp. isolates is extremely high, with 68% resistance rate (N=981), which is significantly higher than the EU average (47%, N=36,172). When considering *C. jejuni* alone, the rate is by more than half lower (31%; N=1,044) and is below the EU average (54%, N=11,551). Alarming is also the high rate of multidrug resistance (24%, N=695), while co-resistance to ciprofloxacin and erythromycin is kept at a low level (0.7%). The ciprofloxacin resistance of human *C. coli* is higher (75%, N=55) than of *C. jejuni*, but *C. coli* represents only a minority of total human *Campylobacter* infections (EFSA, 2014a; EFSA, 2014b).

The rapidly increasing resistance of campylobacters against clinically used antibiotics raises a lot of interest in how the antibiotic resistance is acquired and spread among bacterial

population. It is known that bacteria of *Campylobacter* genus are naturally competent and can take up the genes from the environment via horizontal gene transfer (Vegge et al., 2012; Gaasbeek et al., 2010). The horizontal gene transfers also present one of the ways for the antibiotic resistance genes to spread. For example *tetO*, which encodes ribosomal protection protein and provides resistance against tetracycline is one of the typical examples of resistance genes in *Campylobacter* spp. (Pérez-Boto et al., 2014). Based on GT content, sequence homology, codon usage and hybridization it is suggested that *Campylobacter tetO* gene was acquired by horizontal transfer from *Streptomyces, Streptococcus* or *Enterococcus* spp. (Luangtongkum et al., 2009). The gene is in most strains plasmid-encoded, although it can also be found on the chromosome of some species (Luangtongkum et al., 2009). It is therefore possible that the *Campylobacter* can uptake the *tetO* resistance gene from other bacteria present in the gut of the colonized animals via horizontal gene transfer or recombination events.

On the other hand, there are antibiotics to which resistance is provided by point mutations in the genes which usually code for the target of antibiotic action. Examples are erythromycin with mutation A2075G in 23S rRNA and ciprofloxacin with mutation Thr86Ile in quinolone resistance determining region (QRDR) of gyrase gene *gyrA* (Iovine, 2013; Kurinčič et al., 2007). Quite some time ago it was found that acquirement of point mutation in *gyrA* QRDR not only provides *C. jejuni* with resistance to quinolones, but also enhanced their survival fitness when colonizing chickens in competition with ciprofloxacin susceptible strains. Surprisingly, this feature is preserved even in the absence of antibiotic selective pressure (Hyytiäinen et al., 2013; Luo et al., 2005). The opposite was observed in case of erythromycin resistant strains, which were outcompeted in transmission and host colonization by susceptible *C. jejuni* strains (Luangtongkum et al., 2012). The latter somewhat explains the low reported prevalence of erythromycin resistance among campylobacters, however it is important to note that high resistance to fluoroquinolones exists also due to the extensive use of this class of antibiotics in veterinary medicine, while use of macrolides in veterinary is more rational since the EU banned its use for growth promotion of farm animals (EMA, 2011a; EMA, 2011b). The long-term exposure to sub-inhibitory concentrations of macrolides namely results in higher frequency of resistant isolates compared to treatment with therapeutic doses (EMA, 2011b). The resistance of these types of antibiotics can expand by acquisition of individual mutations in new strains or by clonal spreading of resistant strains with increased survival fitness.

1.1.5 Overcoming the antibiotic resistance in *Campylobacter jejuni*

Search for alternative antimicrobials derived from plants seems to be a viable solution for mitigation of existing bacterial antibiotic resistance (Savoia et al., 2012). In addition to searching for new antimicrobially active plant formulations, the antibiotic resistance combating strategies are focused also on resistance modulators. These are not necessarily antimicrobially active, but can decrease the resistance of pathogens when administered together with other antimicrobials. The microbial sensitization using resistance modulators is mostly due to efflux inhibition, increased membrane permeability, increased porin production or change in porin profile (Bolla et al., 2011; Tegos et al., 2011). Active efflux is

one of the most important contributors to the bacterial multidrug resistance, since it helps bacteria to extrude antimicrobials of broad specificity out of the cells. The most important efflux pump in *C. jejuni* is CmeABC, along with CmeDEF and CmeG (Lin et al., 2002; Akiba et al., 2006; Jeon et al., 2011). These efflux pumps alone or in combination with specific point mutations and antibiotic resistance genes provide increased resistance of bacteria against several different classes of antibiotics.

Plant extracts rich in phenolic compounds, and essential oils have long been shown to possess antimicrobial activity and were frequently studied and reviewed (Cowan, 1999; Burt, 2004; Negi, 2012; Klančnik et al., 2012a). In order to restore the activity of already existing antibiotics on the market, a lot of research is therefore focused on finding new compounds from plant sources with ability to inhibit bacterial efflux (Klančnik et al., 2012a; Klančnik et al., 2012b; Kurinčič et al., 2012). To the date, no efflux pump inhibitor has been licensed for clinical use, although some of the drugs like calcium ion influx inhibitor verapamil, which is licensed for arrhythmia treatment, possess also efflux inhibitory activity (Mahamoud et al., 2007). Still, many new compounds, especially from the natural sources, have recently been identified as potential efflux pump inhibitors in the *in vitro* assays (Stavri et al., 2007; Gröblacher et al., 2012a; Gröblacher et al., 2012b; Shiu et al., 2013). They are especially interesting as they have a potential to be used as preservatives in food products. One of the plants interesting from this aspect is *Alpinia katsumadai* Hayata (syn. *A. katsumadae* Hayata; Zingiberaceae) (Gröblacher et al., 2012a). It is widely used in traditional Chinese medicine as an anti-emetic remedy and to increase the appetite, but also in animal feed, to facilitate rapid growth of domestic animals (Klančnik et al., 2012a).

In our studies we have focused on investigating anti-*Campylobacter*, resistance modulatory and efflux inhibitory potential of an *A. katsumadai* seed extract, essential oil and waste material that remains after essential oil production (Klančnik et al., 2012; Kovač et al., 2014b). Such residual materials are often disposed of and may present an environmental problem. Their high phenolic content and the potential to provide an economically feasible source of natural antioxidants and antimicrobials is still unused (Moure, 2000).

One of the natural compounds, which were proven to have antimicrobial activity against various microorganisms, is a terpen compound α-pinene, naturally found in several essential oils, including that of *A. katsumadai* (da Silva et al., 2012; Wang et al., 2012; Thu et al., 2013; Sieniawska et al., 2013). Alpha pinene naturally exists in two enantiomers, (+)-α-pinene and (-)-α-pinene. The latter was investigated in one of our studies for its antimicrobial, resistance-modulatory and efflux-inhibitory potential on a set of antibiotic resistant and susceptible *Campylobacter jejuni* isolates from different sources.

1.1.6 *Campylobacter jejuni* adaptational response to treatment with antimicrobial and resistance modulatory compounds

In the last years several expressional studies of *Campylobacter* response to treatment with different antimicrobials have been carried out. Two of those have analyzed the bacterial adaptation to antibiotics erythromycin (Xia et al., 2013) and ciprofloxacin (Han et al., 2008) and one to natural antimicrobial product (Dufour et al., 2013). It was found that *C. jejuni*

adapts to erythromycin treatment by up-regulation of motility genes and down-regulation of energy metabolism genes, which helps the bacteria to survive this chemical stress (Xia et al., 2013). Similar analysis of *C. jejuni* response to treatment with ciprofloxacin revealed that the gene *mfd* plays an important role in bacterial adaptation to this antibiotic. It was demonstrated, that the mutation of these gene results in 100-fold reduction in the rate of spontaneous mutation to ciprofloxacin resistance, while overexpression elevates the ciprofloxacin resistance conferring mutation rate (Han et al., 2008). The treatment of *C. jejuni* with phytochemical benzyl isothiocyanate resulted in up-regulation of heat shock response genes and impact on energy metabolism. The consequence of treatment was the impaired oxygen consumption and increased ATP content, as well as increased intracellular protein aggregation (Dufour et al., 2013).

We have investigated transcriptomic response of *C. jejuni* to treatment with resistance modulatory efflux inhibitor (-)-α-pinene, in order to elucidate the mode of its action.

1.2 THE RESEARCH AIMS

- To evaluate the underreporting of *Campylobacter* spp. prevalence in Slovenia;
- To investigate the correlation between antibiotic resistance and certain *Campylobacter jejuni* genotypes;
- To investigate the anti-*Campylobacter,* resistance modulatory and efflux inhibitory activity of *Alpinia katsumadai* seed extract and essential oil, as well as the extract from residual material left after the distillation of the essential oil;
- To evaluate the potential of (-)α-alpha-pinene to inhibit the efflux systems CmeABC and Cj1687 of *Campylobacter jejuni*;
- To evaluate the bacterial response to treatment with (-)-α-pinene.

1.3 THE RESEARCH HYPOTHESES

- Prevalence of *Campylobacter* spp. in Slovenia is underreported;
- Antibiotic resistant *Campylobacter jejuni* are more prevalent in specific clonal complexes;
- Extracts of *Alpinia katsumadai* poses antimicrobial, resistance-modulatory and efflux-inhibitory activity;
- (-)-α-pinene is inhibitor of *Campylobacter jejuni* efflux systems CmeABC and Cj1687;
- *Campylobacter jejuni* uses multiple mechanisms of adaptation to (-)-α- pinene.

2 SCIENTIFIC PUBLICATIONS

2.1 PUBLISHED ARTICLES

2.1.1 The evidence for clonal spreading of quinolone resistance with a particular clonal complex of *Campylobacter jejuni*

Dokaz za klonsko širjenje odpornosti proti kinolonom z določenim klonskim kompleksom bakterij *Campylobacter jejuni*

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Epidemiology & Infection (2014), 142: 2595–2603

Campylobacter is the most prevalent cause of bacterial gastroenteritis worldwide and it represents a significant public health risk, severity of which is becoming even higher due to its increasing resistance to clinically important quinolone and macrolide antibiotics. As a zoonotic pathogen *Campylobacter* is transmitted along the food chain and naturally cycles from environmental waters, feedstuff, animals and food to humans. We have determined antibiotic resistance profiles, as well as MLST and *flaA*-SVR types for 53 *C. jejuni* isolated in Slovenia from human, animal, raw and cured chicken meat and water samples. Twenty-eight different sequence types, arranged in ten clonal complexes, three new allele types and five new sequence types were identified, indicating the relatively high diversity among a small group of strains. The assignment of strains from different sources to the same clonal complexes indicates their transmittance along the food supply chain. The most prevalent clonal complex (CC) among isolates was ST-21, which was also the genetic group with 95% of quinolone resistant strains. Based on the genetic relatedness of these quinolone resistant strains found with MAMA PCR and *gyrA* QRDR sequencing, we conclude that high resistance prevalence observed in our study indicates the local clonal spreading of quinolone resistance with the CC ST-21.

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The evidence for clonal spreading of quinolone resistance with a particular clonal complex of *Campylobacter jejuni*

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SUMMARY

Campylobacter is the most prevalent cause of bacterial gastroenteritis worldwide and it represents a significant public health risk of increasing severity due to its escalating resistance to clinically important quinolone and macrolide antibiotics. As a zoonotic pathogen *Campylobacter* is transmitted along the food chain and naturally cycles from environmental waters, feedstuff, animals and food to humans. We determined antibiotic resistance profiles, as well as multilocus sequence types and *flaA*-SVR types for 52 *C. jejuni* isolated in Slovenia from human, animal, raw and cured chicken meat and water samples. Twenty-eight different sequence types, arranged in ten clonal complexes, three new allele types and five new sequence types were identified, indicating the relatively high diversity in a small group of strains. The assignment of strains from different sources to the same clonal complexes indicates their transmission along the food supply chain. The most prevalent clonal complex was CC21, which was also the genetic group with 95% of quinolone-resistant strains. Based on the genetic relatedness of these quinolone-resistant strains identified by polymerase chain reaction with a mismatch amplification mutation assay and sequencing of the quinolone resistance-determining region of the *gyrA* gene, we conclude that the high resistance prevalence observed indicates the local clonal spread of quinolone resistance with CC21.

Key words: Antibiotic resistance, *Campylobacter jejuni*, epidemiology, *flaA*, food supply chain, genotyping, MLST, transmittance.

INTRODUCTION

Campylobacteriosis is a leading zoonotic foodborne disease in Europe and around the world. Its latest yearly incidence in Europe and Slovenia is 50.28 and 48.7/100 000, respectively [1, 2]. These numbers are still believed to be strongly underreported, since most campylobacteriosis cases remain unrecognized by surveillance systems due to the mild self-limiting symptoms or undetermined cases of gastroenteritis. In Slovenia, as well as in other countries, there is a trend of increasing campylobacteriosis incidence [2, 3].

Of the thermotolerant campylobacters, *Campylobacter jejuni* is the most infectious, causing around 90% of reported human campylobacteriosis cases in

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Slovenia [2]. Besides the high prevalence of infections, antibiotic resistance of *C. jejuni* against quinolones (ciprofloxacin and enrofloxacin) and to a lesser extent, macrolides (erythromycin and azithromycin) is problematic [4]. In Slovenia in 2010, 78% ($n=60$) of *C. jejuni* strains isolated from meat were resistant against ciprofloxacin, while all of them were susceptible to erythromycin. Of the human *C. jejuni* clinical isolates from the same year, 67.2% and 1.0% ($n=882$) were resistant against ciprofloxacin and erythromycin, respectively [3]. This degree of resistance and the emergence of multidrug-resistant strains can seriously hinder clinical treatment of campylobacteriosis [5].

Campylobacter colonizes the gut of many production animals, especially broilers, in which it rarely causes disease symptoms. The meat of these animals is therefore often contaminated during slaughtering and serves as a vehicle of transmission of this pathogen via the food production chain [6]. *Campylobacter* is widely known for its rapid adaptive ability and genomic instability [7–9]. When different reservoirs harbour strains with specific genotypes, it is possible to determine the source of the infection by pulsed-field gel electrophoresis, multilocus sequence typing (MLST) and/or *fla* gene typing [10–12]. Nevertheless, MLST has become the method of choice for *Campylobacter* genotyping. Moreover, in combination with sequencing of the short variable region within the flagella-encoding *flaB* gene MLST allows further strain differentiation within the same sequence type (ST) [13]. This approach has allowed the correlation of specific genotypes with specific antimicrobial resistance profiles [13, 14]. It was shown that resistance to quinolones and macrolides are mostly associated with point mutations in *gyrA* and 23S rRNA, respectively, acting together with efflux mechanisms [15, 16]. The RND-type efflux pumps of some Gram-negative bacteria are able to extrude different types of antibiotics and can also be induced by their substrates [17]. This indicates that environmental selective pressures play an important role in acquiring non-specific (via active efflux), or specific (via point mutation), resistance against antimicrobial drugs. Specific STs have already been associated with quinolone resistance in strains with point mutations, but it is not yet known whether this is because these genotypes are more prone to mutations conferring resistance or because they are clonal [13].

We investigated the potential correlation between MLST/*fla*A genotypes, source of the isolates and resistance against seven antibiotics of 52 Slovenian *C. jejuni* isolates, in order to elucidate the spread of antibiotic resistance. This is the first report on MLST/*flaA* characterization of Slovenian *C. jejuni* isolates from humans, animals, water, and food.

MATERIALS AND METHODS

Campylobacter strains and growth conditions

Fifty-two *C. jejuni* strains isolated from different sources (human, raw and cured chicken meat from retail, animal, water) and regions in Slovenia were used in our study. Control reference strains included *C. jejuni* ATCC 33 560 and *C. coli* strains 158 and 01378 as negative controls. The majority of meat and animal isolates, and all water isolates from Slovenian rivers were obtained through national monitoring in 2008/2009. Five chicken meat isolates were obtained from a laboratory collection and human isolates were from reported campylobacteriosis cases in 2009. *C. jejuni* was confirmed by *hipO* gene species-specific polymerase chain reaction (PCR) [18]. Isolates were grown on Columbia blood agar (Oxoid, UK) under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 42 °C for 24 h and maintained at −80 °C in brain heart infusion broth (Oxoid) with 5% horse blood (Oxoid) and 20% glycerol.

MLST and *flaA* short variable region sequence typing

DNA was extracted from 24-h cultures with Prep-Man® (Applied Biosystems, USA) according to the manufacturer's instructions and MLST was performed as described by Dingle *et al.* [19]. Seven housekeeping genes, *aspA, glnA, gltA, glyA, pgm, tkt, uncA* were amplified using *Taq* polymerase (Promega, USA) with primers detailed in Korczak *et al.* [20]. The short variable region of the *flaA* gene (*flaA*-SVR) was amplified by primers flaA_Cjc-L and flaAB_Cjc-R [20] according to the protocol of Josefsen *et al.* [21]. The purified PCR products were sequenced with the BigDye terminator v. 3.1 ready reaction cycle sequencing kit (Applied Biosystems) on an ABI3130XL genetic analyser (Applied Biosystems).

The sequences of the seven MLST loci and *flaA* were compared with sequences in the MLST database (www.pubmlst.org/campylobacter) to determine the allele number. The STs, clonal complexes (CCs) and *flaA* type of each strain were assigned from the profiles of the seven alleles in the MLST database with an integrated automated link in the computer program

Table 1. *Prevalence of* Campylobacter jejuni *isolates from different sources in identified clonal complexes in this study*

		Source (no. of isolates)				
CC	No. of STs	Human	Animal	Meat	Water	Total
21	4	4	10 (53%)	5		19
45	5		1	6 (75%)	1	8
48	3	1	1	1		3
206	1		1			1
353	2	3		3		6
354	3	1	3	1		5
403	1		1			1
464	2		1	1		2
607	1	1				1
658	2				2	2
Not defined	4		1		3	4
Total	28	10	9	11	6	52

CC, Clonal complex; STs, sequence types.

BioNumerics v. 6.6 (Applied Maths NV, Belgium). Relationships between strains were determined by the MLST-based network creation method, minimum spanning tree (MST) and by the unweighted pair-group method with arithmetic mean cluster analysis based on *flaA*-SVR in BioNumerics. New alleles and STs were submitted to the PubMLST database.

Antibiotic susceptibility

The susceptibility of isolates to seven antibiotics (chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nalidixic acid, streptomycin, tetracycline) was determined by a broth microdilution method using a commercial diagnostic test for *Campylobacter* minimum inhibitory concentrations (MICs) (Sensititre *Campylobacter* plate – EUCAMP; Trek Diagnostic Systems, USA) according to the manufacturer's instructions. Resistance against antibiotics was expressed as MICs based on cut-off values recommended by European Food Safety Authority (EFSA) [22]. Multidrug resistance was defined as resistance to at least three non-related antibiotics.

Mutation assay PCR and *gyrA* sequencing

Mismatch amplification mutation assay (MAMA) PCR for detection of the Thr^{86}-Ile mutation, which confers resistance against ciprofloxacin was performed on all phenotypically ciprofloxacin-resistant isolates. Additionally, the quinolone resistance-determining region (QRDR) of *gyrA* was sequenced (Macrogen, South Korea) to study the genetic relatedness of ciprofloxacin-resistant isolates from different CCs. Both procedures were as described previously [23]. Thirty-two sequences of *gyrA* QRDR were deposited in GeneBank (accession numbers KF831198–KF831229).

Statistical analysis

The discriminatory index of the typing methods was calculated by Simpson's index of diversity according to Hunter & Gaston [24]. Statistical significance ($P < 0.05$) of association of specific genotypes with quinolone resistance and source of the strains was tested using IBM SPSS Statistics v. 20 (IBM, USA), with Fisher's exact two-tailed test and Pearson's χ^2 test.

RESULTS AND DISCUSSION

The 52 *C. jejuni* isolates were recovered from animals (chicken, bovine, turkey skin and faeces), raw and cured chicken meat from retail, human patients and environmental water samples during 2008–2009 in different regions of Slovenia. The isolates were grouped by MLST into 28 STs, four of which were novel additions to the PubMLST database; one of these new STs (ST5207 – *C. jejuni* 1603) was a novel combination of previously known alleles but three, ST5205, ST5206, ST6309, included novel allele sequences in *tkt, aspA* and *glyA* loci, respectively. Each of the isolates with

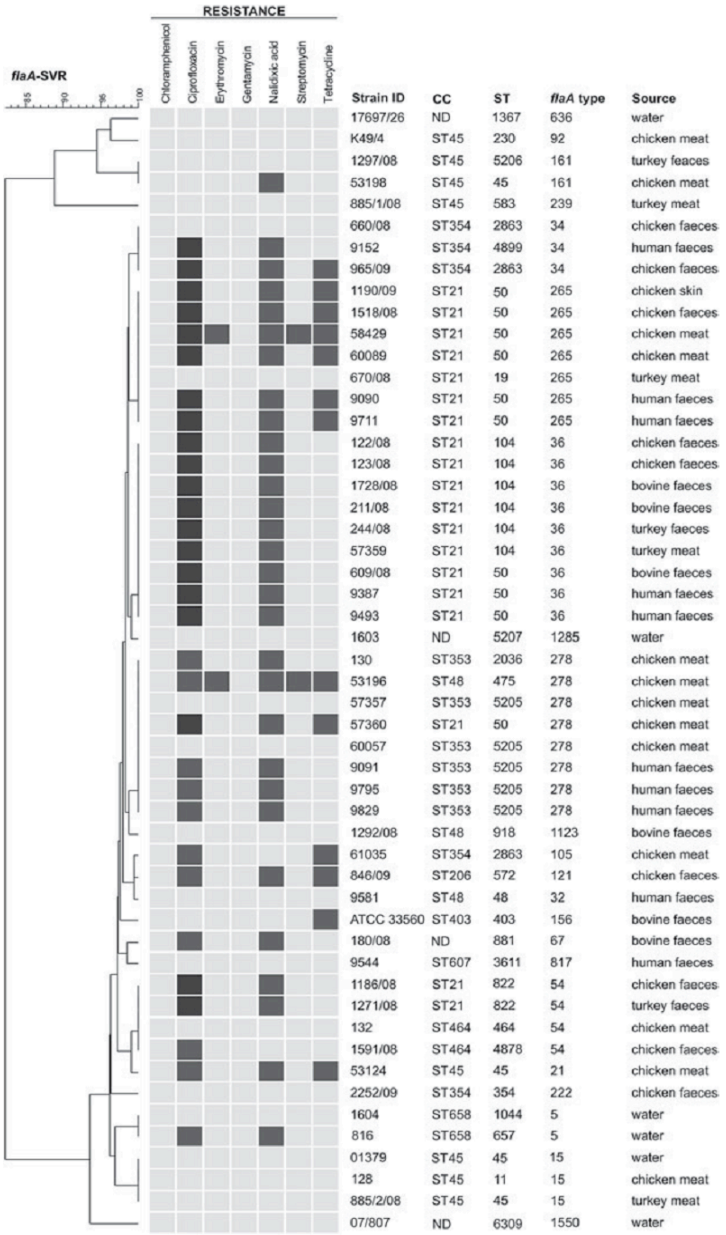

Fig. 1. Phylogenetic tree constructed on the basis of the short variable repeat of the *flaA* region showing correlation between antibiotic resistance, origin of the strains and MLST type. Light grey squares represent antibiotic-sensitive phenotypes; dark grey squares represent antibiotic-resistant phenotypes; black squares represent ciprofloxacin-resistant phenotypes of clonal complex ST21. CC, Clonal complex; ST, sequence type; ND, not determined.

the novel *tkt* allele type originated from the same region in Slovenia (data not shown) and were isolated from raw and cured chicken meat and human faeces, which is suggestive of chicken being the most probable source of infection.

As shown in Table 1, 48 *C. jejuni* isolates were assigned to ten pre-defined CCs and 24 STs by MLST. Four isolates could not be assigned to a CC. CC21 and CC45 predominated and accounted for 19 and eight, respectively, of the total 52 isolates typed.

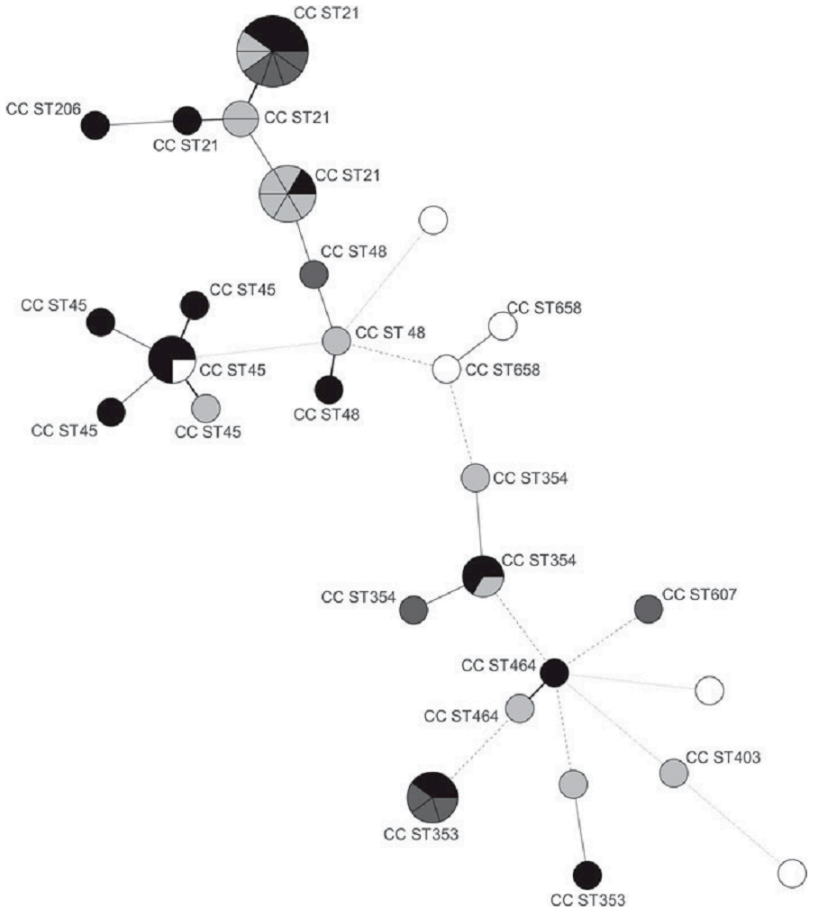

Fig. 2. Minimum spanning tree generated from MLST comparisons of *Campylobacter jejuni* strains isolated from animals, humans, meat, and water in Slovenia. Each circle represents one sequence type (ST), the clonal complexes (CC STs) are indicated by numbers. Node sizes represent higher strain numbers within one ST. Node colour indicates the origin of the strains: black (meat), dark grey (human), light grey (animal), white (water). The connecting lines between STs depict the number of allelic differences between them: one allele difference (black bold lines), two alleles difference (grey bold lines), three alleles difference (grey dashed lines), more than three alleles difference (grey dotted lines).

ST50 and ST104 were the most frequent STs accounting for 10 and six, respectively, of the total (Fig. 1, Table 1). Twenty-three (79%) STs appeared only once, indicating high genetic diversity. STs belonging to CC21 and CC45 were previously shown to be predominant in Europe and typical for poultry [13, 20, 25, 26]. In our study, 12/19 isolates assigned to CC21 and 6/8 assigned to CC45 were isolated from poultry meat or faeces.

The distribution of isolates in the MST analysis (Fig. 2) indicates some correlation between specific genotypes and their main source of isolation. The majority (71%) of human isolates was distributed among CC21 and CC353 and most (67%) of the meat isolates among CC21 and CC45, while animal isolates were mostly (67%) assigned to CC21 and CC354. Water isolates were genetically the most diverse group and 2/5 could not be assigned to any existing CC. Their distinct STs present an interesting group with little in common with isolates from human and animal sources. Water as an abiotic source, represents a different environment to which bacteria need to adapt phenotypically and genetically. Similar genetic differences between *C. jejuni* strains originating from biotic and abiotic sources have been found in the past and resulted in the identification of specific genetic markers able to distinguish between isolates from biotic and abiotic sources [27].

Table 2. *Distribution of antibiotic resistant strains in clonal complexes*

CC	No. of isolates	STR	CIP	TET	ERY	NAL	MDR
21	19	1	18 (95%)	7 (37%)	1	18 (95%)	1
45	8		1	1		2	
353	6		4 (67%)			4 (67%)	
354	5		3 (60%)	2		2 (40%)	
206	1		1	1		1	
403	1			1			
464	2		1				
48	3	1	1	1	1	1	1
607	1						
658	2		1			1	
Other	4		2			1	
Total	52	2	7	6	2	6	2

CC, Clonal complex; STR, streptomycin; CIP, ciprofloxacin; TET, tetracycline; ERY, erythromycin; NAL, nalidixic acid; MDR, multidrug-resistant strain (resistant against $\geqslant 3$ unrelated antibiotics).

flaA-SVR typing revealed 22 different genotypes in the *C. jejuni* isolates (Fig. 1), and was thus a less discriminatory method than MLST (28 STs). Indeed, *flaA*-SVR typing additionally discriminated isolates within only one ST (ST50), whereas MLST distinguished between isolates in more *flaA* types. This was reflected by the higher discriminatory power of MLST (0.942) over *flaA*-SVR (0.928). The combination of both typing methods increased the discriminatory power to 0.967.

The most common *flaA* alleles were 36, 278 and 256 with prevalences of nine (17%), eight (15%) and seven (13%) out of a total of 52 alleles. All *flaA* allele types 256 and 36 were segregated into CC21, while most of the strains with allele type 278 were assigned to CC353, as well as to CC21 and CC48. Cluster analysis of *flaA*-SVR sequences revealed six major clusters at a similarity level of 95%. Statistical analysis confirmed *flaA* allele type 36 (8/9) as typical for animal isolates and type 278 (5/8) as typical for meat isolates.

The highest frequency of antibiotic resistance was recorded for the quinolones ciprofloxacin and nalidixic acid (61% and 58%), followed by tetracycline (25%), streptomycin and erythromycin (4%) (Table 2). All *C. jejuni* isolates were susceptible to gentamicin and chloramphenicol. These results are in accordance with trends reported by the EFSA [3]. Antibiotic resistance in *C. jejuni* is becoming a significant issue, not only in Slovenia, but also in other European countries [3] with the most problematic being resistance against quinolones, which are the second group of drugs of choice for campylobacteriosis treatment. In Slovenia quinolones are widely used for the non-specific treatment of bacterial gastroenteritis as well as for enterococcal and *E. coli* infections in poultry flocks. The prevalence of ciprofloxacin-resistant animal *Campylobacter* isolates in Europe is reported to range from 37% to 84%, depending on the country [3] which is consistent with the results reported here, where 72% of animal and 44% of meat isolates were ciprofloxacin resistant and this increased to 80% for human isolates. Almost all (94%) ciprofloxacin-resistant strains from our study were cross-resistant to nalidixic acid. Interestingly, tetracycline resistance occurred almost exclusively (92%) in ciprofloxacin-resistant strains. Although erythromycin resistance was rare, it is of concern that both erythromycin-resistant isolates were from raw chicken meat and exhibited multidrug resistance to ciprofloxacin, nalidixic acid, streptomycin and tetracycline, which seriously compromises the choice of treatment for infections caused by the ingestion of these organisms.

The high incidence of quinolone resistance compared to erythromycin resistance was previously demonstrated to be due to the faster development of ciprofloxacin-resistant mutants during exposure to antibiotics, improved biological fitness of resistant mutants and their ability to persist in the environment after the selective pressure is removed [28–30]. By contrast, macrolide resistance appears to require longer exposure to antibiotics to develop, and resistant strains are less likely to survive in the absence of selective pressure [31–33]. We also observed persistence of ciprofloxacin-resistant strains of the same genotypes (e.g. ST50) which were isolated from animals, meat, and human samples.

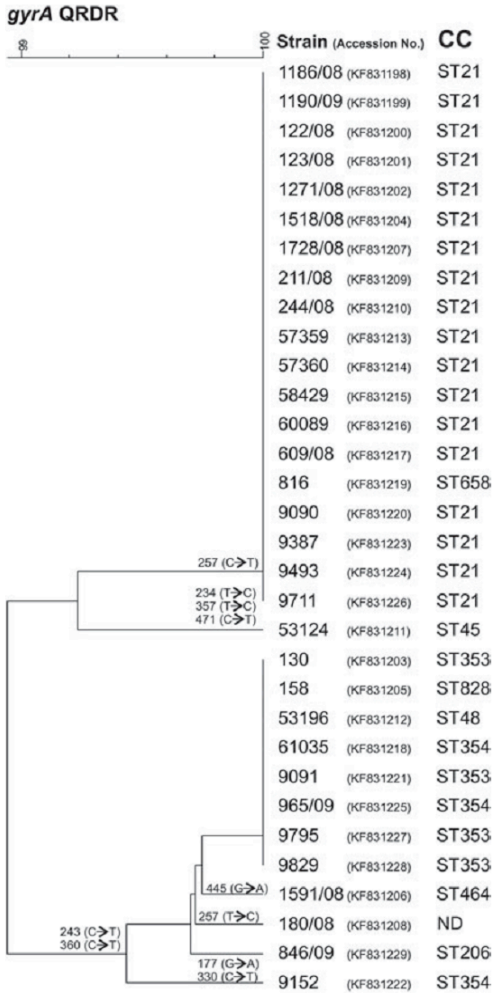

Fig. 3. Relationship between *Campylobacter jejuni* strains based on the sequences of quinolone resistance-determining region (QRDR) of the *gyrA* gene in comparison to clonal complex (CC). The position of silent mutations in relation to the reference strain *C. jejuni* NCTC 11158 are shown on the branches. The accession numbers of the sequences are shown in parentheses after the strain designation. ND, Not determined.

Analysis of antibiotic resistance and MLST genotypes revealed that all but one of the 19 strains of *C. jejuni* belonging to the predominant CC21 were quinolone resistant ($P < 0.05$). Over half of ciprofloxacin-resistant genotypes within CC21 belonged to ST50 and over 30% to ST104 and only 13 (42%) of these resistant strains were identified in all other CCs ($P < 0.05$). A similar pattern was observed with *flaA* types, where 47% of ciprofloxacin-resistant CC21 genotypes were assigned to *flaA* 36 and 32% to *flaA* 265. All ciprofloxacin-resistant strains in CC21 also showed cross-resistance to nalidixic acid. An association between CC21 and quinolone resistance has already been found in Belgium [34] and Switzerland [13] accounting for resistance rates in these countries of 66% and 30%, respectively.

In order to determine whether the association of quinolone resistance with CC21 was due to individual mutational events or an increased ability to spread clonally [24], we investigated the most common ciprofloxacin resistance conferring mutation Thr^{86}-Ile and analysed the QRDR by sequence typing. The results show that all phenotypically ciprofloxacin-resistant isolates, with the exception of strain 180/08, have the mutation ACA to ATA in the 86th codon. Six additional silent mutations in the QRDR were identified, and on the basis of these results we constructed the dendrogram shown in Figure 3, which clearly indicates the high degree of genetic relatedness of ciprofloxacin-resistant isolates from CC21, which together with one strain from CC658 and two strains with unassigned CCs form a separate group compared to the ciprofloxacin-resistant strains from all other CCs. As quinolone resistance is quickly developed under antibiotic selective pressure and can persist long after cessation of treatment, we conclude that the high incidence of such strains within CC21 found here is not due to high genetic plasticity of this particular CC [24], but rather to acquired efficiency of clonal spreading. This hypothesis is additionally supported by the fact that not all investigations from other countries [35] found a correlation between quinolone resistance and CC21.

In conclusion, this study is the first report on the genetic variability of *C. jejuni* isolates from Slovenia based on MLST, which contributes new allele types and STs to the PubMLST database. CC21 was the predominant genetic group with representative isolates from a variety of different sources and this group was highly associated with quinolone resistance, confirming its clonal spreading along the food supply chain. We also identified distinct genotypes of strains from water sources with high antibiotic susceptibility.

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DECLARATION OF INTEREST

None.

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2.1.2 Anti-*Campylobacter* and resistance-modifying activity of *Alpinia katsumadai* seed extracts

Protimikrobno in odpornostno modulatorno delovanje izvlečkov semen rastline *Alpinia katsumadai* na bakterije *Campylobacter*

Anja Klančnik, Barbara Gröblacher, Jasna Kovač, Franz Bucar, Sonja Smole Možina

Journal of Applied Microbiology (2012), 113, 5: 1249-1262

Aims: We tested extracts from Alpinia katsumadai seeds for anti-Campylobacter activity and investigated the roles of the CmeABC and CmeDEF efflux pumps in *Campylobacter* resistance to these natural phenolics. Additionally, we investigated an *A. katsumadai* ethanolic extract (AlpE) and other plant extracts as putative efflux pump inhibitors on *Campylobacter* isolates and mutants in efflux pump genes.
Methods and Results: AlpE showed antimicrobial activity against sensitive and multidrug-resistant *Campylobacter* isolates. *CmeB* inactivation resulted in the greatest reduction in resistance, while *cmeF* and *cmeR* mutations produced only moderate effects on minimal inhibitory concentrations (MICs). The chemical efflux pump inhibitors additionally reduced MICs in isolates and mutants, confirming that active efflux is an important mechanism in resistance to AlpE, with additional contributions of other efflux systems. A notable decrease in resistance to tested antimicrobials in the presence of subinhibitory concentrations of AlpE confirms its modifying activity in *Campylobacter* spp.
Conclusions: AlpE is important anti-*Campylobacter* source of antimicrobial compounds with resistance-modifying activity. At least two of the efflux systems are involved in the resistance to *A. katsumadai* antimicrobial seed extracts.
Significance and Impact of the Study: This is the first report of antimicrobial and resistance modifying activity of AlpE from *A. katsumadai* seeds, demonstrating its potential in the control of *Campylobacter* in the food chain.

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ORIGINAL ARTICLE

Anti-*Campylobacter* and resistance-modifying activity of *Alpinia katsumadai* seed extracts

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Keywords
Alpinia katsumadai, antimicrobial activity, *Campylobacter* spp., efflux pump inhibitors, resistance-modifying activity.

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Abstract

Aims: We tested extracts from *Alpinia katsumadai* seeds for anti-*Campylobacter* activity and investigated the roles of the CmeABC and CmeDEF efflux pumps in *Campylobacter* resistance to these natural phenolics. Additionally, we investigated an *A. katsumadai* ethanolic extract (AlpE) and other plant extracts as putative efflux pump inhibitors on *Campylobacter* isolates and mutants in efflux pump genes.

Methods and Results: AlpE showed antimicrobial activity against sensitive and multidrug-resistant *Campylobacter* isolates. *CmeB* inactivation resulted in the greatest reduction in resistance, while *cmeF* and *cmeR* mutations produced only moderate effects on minimal inhibitory concentrations (MICs). The chemical efflux pump inhibitors additionally reduced MICs in isolates and mutants, confirming that active efflux is an important mechanism in resistance to AlpE, with additional contributions of other efflux systems. A notable decrease in resistance to tested antimicrobials in the presence of subinhibitory concentrations of AlpE confirms its modifying activity in *Campylobacter* spp.

Conclusions: AlpE is important anti-*Campylobacter* source of antimicrobial compounds with resistance-modifying activity. At least two of the efflux systems are involved in the resistance to *A. katsumadai* antimicrobial seed extracts.

Significance and Impact of the Study: This is the first report of antimicrobial and resistance-modifying activity of AlpE from *A. katsumadai* seeds, demonstrating its potential in the control of *Campylobacter* in the food chain.

Introduction

Campylobacteriosis is the leading bacterial food-borne illness throughout the world and the most frequently reported zoonosis in humans. Campylobacters frequently enter the food chain via infected food production, animals and/or cross-contamination of thermally untreated foods. Their antimicrobial resistance is constantly growing, including resistance to fluoroqinolones and macrolides, which are the drugs of choice for campylobacteriosis treatment, as well as for multidrug resistance (MDR) (Luangtongkum *et al.* 2009). Therefore, new antimicrobials for reducing the prevalence of *Campylobacter* spp. in the food chain are needed. In our previous studies, we showed that although they are Gram-negative organisms, campylobacters are relatively sensitive to phenolic compounds from different plant sources, both *in vitro* (Klančnik *et al.* 2009, 2010) and in poultry meat (Piskernik *et al.* 2011). Additionally, agents from natural sources that might reverse or decrease the resistance of campylobacters have been investigated (Smole Možina *et al.* 2011). Most phytochemicals can only modulate the activities of Gram-positive micro-organisms, while little or no activity is shown towards Gram-negative micro-organisms. Recently, there have been reports of new potential efflux pump inhibitors (EPIs) from various natural extracts, like of *Levisticum officinale*, *Mirabilis jalapa* and *Artemisia absinthium* (Michalet *et al.* 2007; Fiamegos *et al.* 2011; Garvey *et al.* 2011). Indeed, natural products provide unlimited opportunities, because of their

unmatched range of chemical diversity (O'Bryan *et al.* 2008).

While the target-site activities of most antibiotics are known, the mechanisms of natural antimicrobials in *Campylobacter* spp. remain largely unknown. *Campylobacter* spp. have multiple resistance mechanisms that are associated with target mutations and resistance-nodulation-cell division (RND) and non-RND efflux pumps (Lin *et al.* 2005b; Pumbwe *et al.* 2005; Akiba *et al.* 2006). CmeABC is involved in the extrusion of structurally diverse antimicrobials and contributes to intrinsic and acquired resistance to various antimicrobials (Lin *et al.* 2002; Guo *et al.* 2010). Its function has also been defined for pure phenolic compounds and extracts of plant phenolics (Klančnik *et al.* 2012). In addition, CmeDEF, which has different substrate-binding properties, interacts with CmeABC and has a secondary role in conferring intrinsic antimicrobial resistance (Akiba *et al.* 2006).

To our knowledge, compounds from *A. katsumadai* have not been studied yet for application as food additives, although the seeds have a long tradition in herbal medicine, and as a flavouring agent and animal-feed additive. In traditional Chinese medicine, the seeds are applied for fullness and distending pain in the abdomen, to increase appetite, and against nausea and vomiting (Zhongzhen 2004; Chinese Pharmacopoeia 2009; Zhao 2011). *Alpinia* seeds are ingredients in a preparation to treat dysentery in piglets and in animal feed as an additive to facilitate rapid growth of domestic animals (Zhang 2010). In addition, our recent evidence has shown that compounds isolated from *A. katsumadai* seeds can act as potential EPIs in *Mycobacterium smegmatis* (Gröblacher *et al.* 2012).

The aim of this study was first to prepare and chemically characterize new bioactive plant materials from *A. katsumadai* by extraction of seeds with solvents of different polarities. We determined antimicrobial activity of these extracts in different *Campylobacter* spp., including antibiotic resistant isolates. By comparing the sensitivity of wild-type *C. jejuni* 11168 and its mutants in specific efflux pump genes (*cmeB, cmeR, cmeF*), we evaluated the roles of the CmeABC and CmeDEF efflux pumps in the resistance to these natural phenolics in *Campylobacter* spp. Moreover, we examined the role of efflux using different EPIs, for example, phenylalanine-arginine β-naphthylamide (PAβN), 1-(1-naphthylmethyl)-piperazine (NMP), verapamil, reserpine and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), to determine whether they can potentiate the anti-*Campylobacter* activity of AlpE, the ethanolic extract from *A. katsumadai* seeds. Finally, we investigated AlpE and two additional plant extracts as putative EPIs on the susceptibility to different antibiotics, biocides, ethidium bromide (EtBr) and plant phenolic extracts, for the first time in *Campylobacter* isolates and following mutation of the efflux pump genes.

Materials and methods

Chemical characterization of the extracts

Preparation of A. katsumadai seed extracts

The *A. katsumadai* seeds were bought from a commercial source (Plantasia; Cat. no. 680381, Oberndorf/Salzburg, Austria). Their identity was defined according to the Chinese Pharmacopoeia (2009). A voucher specimen is kept at the Institute of Pharmaceutical Sciences, University of Graz. To cover a broader range of polarity, the crushed seeds (900 g) were extracted in a Soxhlet apparatus, successively with hexane, dichloromethane and methanol, for 24 h each. These solvents were evaporated to dryness, which resulted in dried crude extracts, as 36·7 g from hexane (AlpH), 31·4 g from dichloromethane (AlpD) and 48·8 g from methanol (AlpM), respectively. For comparison, an ethanolic extract, a solvent of less toxicity compared with the others, was prepared by stirring 100 g of crushed seeds with 1 l of EtOH (96%) at room temperature for 72 h. After evaporation of the solvent, 9·8 g of dried crude ethanolic extract (AlpE) was obtained. Owing to its ease of preparation and the reduced toxicity of the solvent, which could be relevant for *in vivo* application, the ethanolic extract was used in the full spectrum of assays.

Phytochemical analysis of extracts

Liquid chromatography–photo diode array-mass spectrometry (LC-PDA-MS) analysis was conducted with a Thermo Surveyor LC system coupled to a Thermo Finnigan LCQ DECA XP plus mass detector [electrospray injection (ESI), negative mode] and a 2·6 μm C18 100A Kinetex column (100 × 2·1 mm; Phenomenex, Torrance, CA, USA). Elution was performed with an acetonitrile (A) and water (B) gradient system. The major compounds **1–5** present in the ethanolic extract, which was used for most experiments, were identified based on their UV spectra and their ESI mass spectra, in comparison with the literature (Kuroyanagi *et al.* 1983; Huang *et al.* 2007; Li *et al.* 2010). Pinocembrin (**2**), (5*R*)-trans-1, 7-diphenyl-5-hydroxy-6-hepten-3-one (**4**) and 1,7-diphenyl-4,6-heptadien-3-one (**5**) were previously isolated from the hexane extract of the seeds (Gröblacher *et al.* 2012), and they were used as reference compounds (purity >90% according to HPLC and ^{1}H NMR analysis). The major compounds **1–5** of the ethanolic extract were quantified by HPLC using a Merck Hitachi LaChrome HPLC System and a Zorbax SB C-18, 3 μm, 2·1 × 150 mm column (Agilent, Santa Clara, CA, USA).

Gradient elution was with acetonitrile (A) and water (B), with both containing 0·1% trifluoroacetic acid. This started with 10% A and increased to 90% A in 25 min at a flow rate of 250 μl min^{-1} and a column temperature of 30°C. Compounds **1**, **2** and **4** were quantified at 215 nm, with **2** as the external standard, whereas compounds **3** and **5** were quantified at 320 nm using purified **5.** The samples were analysed in triplicate.

Bacterial strains and growth conditions

Twenty-two food, animal, water and human *Campylobacter* strains were used in this study (Table 1). They were isolated and identified phenotypically and by multiplex polymerase chain reaction (mPCR), as described previously (Zorman and Smole Možina 2002). *Campylobacter jejuni* NCTC 11168 was used as the reference strain, and its knockout *cmeB*, *cmeR* and *cmeF* mutants were also used (obtained from Prof. Qijing Zhang, Iowa State University, USA). Purified DNA from the *cmeB* (Lin *et al.* 2002), *cmeF* (Akiba *et al.* 2006) and *cmeR* (Lin *et al.* 2005a) mutants was used to transform *C. jejuni* NCTC 11168 using the standard biphasic method for natural transformation and construction of *cmeB, cmeF* and *cmeR* mutants, as described by Klančnik *et al.* (2012). The cultures were stored at −80°C in brain–heart infusion broth (Oxoid, Hampsire, UK) supplemented with 5% horse blood (Oxoid) and glycerol (Kemika, Zagreb, Croatia). The isolates were subcultured on Columbia agar (Oxoid), supplemented with horse blood at 42°C under micro-aerophilic conditions in gas-tight containers (5% O_2, 10% CO_2, 85% N_2).

Antimicrobial susceptibility testing

The *Alpinia* extracts were dissolved in DMSO to prepare stock solutions and further diluted in the appropriate media to the working concentrations. Twofold serial dilutions were used at concentrations from 0·016 to 5 mg ml^{-1}. Control wells were prepared with culture medium (sterility control), plant extract in 50 μl of Müller Hinton broth (negative control), bacterial suspension in 50 μl of Müller Hinton broth (positive control) and DMSO in amounts corresponding to the highest quantity present (from 2·5%) (positive control to prove that there was no bacterial growth inhibition by DMSO). The lyophilized rosemary (*Rosmarinus officinalis* L.) V40 ethanolic extract (with 40% carnosic acid) (supplied by Vitiva d.d., Markovci, Slovenia), (-)-epigallocatechin gallate (EGCG; Sigma-Aldrich GmbH, Steinheim, Germany) and vine-leaf (VL; *Vitis vinifera* L.) ethanolic extract (provided by Višnja Katalinić, University of Split, Split, Croatia) were dissolved in absolute ethanol to provide the stock solutions. They were further diluted in the appropriate media to the working concentrations. Twofold serial dilutions of V40 and EGCG were used at concentrations from 0·00061 to 1·25 mg ml^{-1} and of VL at concentrations from 0·0078 to 16 mg ml^{-1}. Controls were tested as described above. The DMSO and ethanol were used at concentrations previously shown to be noninhibitory for bacterial cells.

The broth microdilution method was used for measuring of minimal inhibitory concentrations (MICs), as previously described (Klančnik *et al.* 2009). The MICs,

Table 1 Susceptibilities of *Campylobacter* spp. isolates from various sources to the different *Alpinia katsumadai* seed extracts

Campylobacter strain					
Alpinia extracts	Distribution of MIC (%)				
AlpE (mg ml^{-1})	4·103	2·051	1·025	0·512	0·256
Erythromycin-resistant *C. coli*	0	44·4	44·4	11·1	0
Erythromycin-resistant*C. jejuni*	0	0	42·8	57·1	0
Erythromycin-resistant *C. coli*	0	0	100	0	0
Erythromycin-sensitive *C. jejuni*	0	0	60	40	0
AlpM (mg ml^{-1})	4·103	2·051	1·025	0·512	0·256
Erythromycin-resistant *C. coli*	11·1	55·5	33·3	0	0
Erythromycin-resistant *C. jejuni*	0	0	71·3	28·6	0
Erythromycin-resistant *C. coli*	0	100	0	0	0
Erythromycin-sensitive *C. jejuni*	0	20	60	20	0
AlpD (mg ml^{-1})	4·103	2·051	1·025	0·512	0·256
Erythromycin-resistant *C. coli*	11·1	33·3	11·1	33·3	11·1
Erythromycin-resistant *C. jejuni*	0	0	0	71·3	28·6
Erythromycin-resistant *C. coli*	0	100	0	0	0
Erythromycin-sensitive *C. jejuni*	0	20	80	0	0
AlpH (mg ml^{-1})	4·103	2·051	1·025	0·512	0·256
Erythromycin-resistant *C. coli*	0	55·5	44·4	0	0
Erythromycin-resistant *C. jejuni*	0	28·6	71·3	0	0
Erythromycin-resistant *C. coli*	0	0	100	0	0
Erythromycin-sensitive *C. jejuni*	0	20	60	20	0

AlpE, ethanol extract; AlpM, methanol extract; AlpD, dichloromethane extract; AlpH, hexane extract.

defined as the lowest concentrations of natural antimicrobials where no metabolic activity was seen after 24 h, were determined on the basis of the bioluminescence signals measured using a microplate reader (Tecan, Mannedorf/Zurich, Switzerland) after adding CellTiter-Glo reagent (Promega Corporation, Madison, WI, USA) to the culture media (Klančnik *et al.* 2009). All of the measurements of MIC values were repeated in triplicate. Control wells were prepared with culture medium, bacterial suspension only, alternative antimicrobial only and ethanol, in amounts corresponding to the highest quantity present.

Efflux pump inhibitors

Chemical EPIs

To investigate the contributions of the CmeABC and CmeDEF efflux pumps in AlpE resistance, the isolates and the reference and mutant strains were tested both in the absence and presence of five EPIs in Müller Hinton broth: PAβN (20 μg ml^{-1}), NMP (100 μg ml^{-1}), verapamil (100 μg ml^{-1}), reserpine (100 μg ml^{-1}) and CCCP (0·25 μg ml^{-1}). Microdilution tests were also performed in preliminary independent experiments to determine the MICs of the EPIs used for all of the tested strains. The selected concentrations had no inhibitory effects on bacterial growth for any of the tested strains.

Putative EPIs

To investigate the activities of putative EPIs and resistance-modifying agents, the MICs of erythromycin (Sigma-Aldrich, St Louis, MO, USA), ciprofloxacin (Fluka, Biochemika), bile salts (Sigma-Aldrich), sodium deoxycholate (Sigma-Aldrich) and ethidium bromide (EtBr, Sigma-Aldrich) were determined in the absence and presence of the AlpE, V40 and VL extracts in Müller Hinton broth at the subinhibitory concentrations of 0·25 MICs, in ten Campylobacter isolates and the three efflux pump mutant strains. Three independent experiments were carried out to confirm the reproducibility of all of the MIC data.

Statistical analysis

The MICs of antimicrobial assays were compared with the independent-samples *t*-tests to define the significance of the differences in resistances between *C. jejuni* and *C. coli* and between erythromycin-sensitive and erythromycin-resistant strains. The correlations of antimicrobial MIC distributions and the effects of EPI were compared by Pearson χ^2-test. A Pearson coefficient was calculated for the correlation matrix between antimicrobial MICs and the effects of EPI. Results were considered significant when $P \leq 0{\cdot}05$ and $r_{xy} \leq 0{\cdot}9$. Statistical analyses were performed with IBM PASW Statistic software, ver. 18.0.

Results

Chemical compositions of the *A. katsumadai* extracts

Chemical screening of the ethanolic extract of the *A. katsumadai* seeds (AlpE) by LC-PDA-ESI-MS analysis revealed the flavonoids alpinetin (**1**), pinocembrin (**2**) and cardamomin (**3**), the diarylheptanoids and (5*R*)-trans-1,7-diphenyl-5-hydroxy-6-hepten-3-one (**4**) and *trans, trans*-1,7-diphenyl-4,6-heptadien-3-one (**5**) as the major components (Fig. 1). As this extract was studied in detail microbiologically, the quantities in the extract according to HPLC-UV analysis were established as 5·69 ± 0·17% (**1**), 9·47 ± 0·32% (**2**), 1·85 ± 0·04% (**3**), 3·28 ± 0·06% (**4**) and 12·89 ± 0·20% (**5**).

In the hexane extract of the *A. katsumadai* seeds (AlpH), **4** and **5** dominated, whereas compound **1** was found in traces only. The dichloromethane extract of the *A. katsumadai* seeds (AlpD) showed a composition similar to AlpE, with the exception of a lower content of **5.** Finally, in the methanolic extract of the *A. katsumadai* seeds (AlpM), the flavonoids **1** and **2** were present at much higher levels than **5**.

Antimicrobial activity of the *A. katsumadai* extracts

In total, 22 food, animal, water and human *Campylobacter coli* or *C. jejuni* isolates were tested for the MICs of the four *A. katsumadai* seed extracts that differed according to their solvent extraction procedures: with ethanol (AlpE), methanol (AlpM), dichloromethane (AlpD) and hexane (AlpH) (Table 1). All the four of these extracts showed antimicrobial activities against all *Campylobacter* isolates, with MICs of 0·512 to 2·051 mg ml^{-1} for AlpE and AlpH, and of 0·256 to 4·103 mg ml^{-1} for AlpD, with no statistically significant differences between the erythromycin-sensitive and erythromycin-resistant *Campylobacter* isolates or between *C. jejuni* or *C. coli* isolates.

For MDR, eight isolates of multi-resistant *C. coli* (137, 140, 171, FC8, FC10, VC7114, VC10076 and 809) (Kurinčič *et al.* 2012) were also tested, and their MIC values showed antimicrobial activities similar to those detected for the other isolates (Table 1).

Although the quantitative composition of the major compounds differed in the extracts from the different extraction procedures, their anti-*Campylobacter* activities were not significantly different. AlpE was slightly more effective (Table 1), so it was chosen for the further investigations.

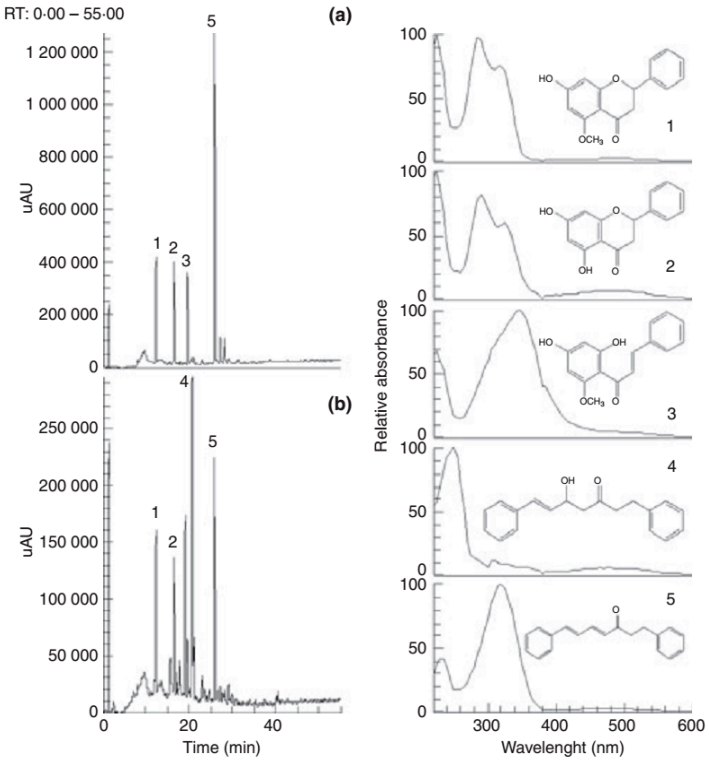

Figure 1 Analysis of *Alpinia katsumadai* EtOH extract (AlpE) by HPLC-PDA. Left: chromatogram at 320 nm (a) and 260 nm (b). Right: UV spectra of major components; 1 = alpinetin, 2 = pinocembrin, 3 = cardamomin, 4 = (5*R*)-trans-1,7-diphenyl-5-hydroxy-6-hepten-3-one; 5 = *trans, trans*-1, 7-diphenyl-4,6-heptadien-3-one.

The role of CmeABC and CmeDEF in resistance to the *A. katsumadai* extracts

We used insertional inactivation mutations as the effective tools for studying the CmeABC and CmeDEF resistance mechanisms in *Campylobacter* spp. The mutants of the *cmeB* (encoding a transporter protein), *cmeF* (encoding an inner membrane transport protein) and *cmeR* (encoding a transcriptional repressor that directly interacts with the cmeABC promoter and modulates cmeABC expression) efflux pump genes were compared with wild-type *C. jejuni* 11168 using antimicrobial susceptibility microdilution analysis (Table 2). The insertional inactivation of the *cmeB* gene increased the susceptibility of the *C. jejuni* NCTC 11168 reference strain to all of the tested *A. katsumadai* extracts, by 4- to 16-fold (Table 2). The MICs for the AlpM and AlpH extracts decreased by fourfold. More significant decreases were observed in the *cmeB* mutant strain to AlpE (eightfold) and AlpD (16-fold) (Table 2). In contrast, inactivation of the *cmeF* and *cmeR* genes had smaller effects on the MICs of *A. katsumadai* extracts. Inactivation of the *cmeF* and *cmeR* genes had no influence on the AlpE and AlpD activities. Compared with the wild-type strain, in both of these mutants, the MICs for AlpM and AlpH increased by twofold and fourfold, respectively (Table 2).

Effects of EPIs on the resistance to AlpE

Furthermore, we studied the role of efflux in the resistance to AlpE, in the absence and presence of each of the EPIs (PAβN, NMP, verapamil, reserpine, CCCP). We tested nine *Campylobacter* spp. isolates, wild-type *C. jejuni* NCTC 11168 and its efflux pump mutants (*cmeB, cmeF, cmeR*). The MIC values are given in Table 3. A statistically significant correlation between the effects of PAβN and NMP and AlpE resistance was observed. PAβN had the greatest effects in all of the sensitive and resistant isolates, as eightfold to >32-fold AlpE MIC reductions. NMP reduced the MICs in most of the tested strains by fourfold to >32-fold. Additionally, CCCP reduced the MICs by up to eightfold, although not for all

Table 2 Susceptibilities of *Campylobacter* jejuni reference strain and its mutants to the different *Alpinia katsumadai* seed extracts

Campylobacter strain	AlpE MIC (mg ml^{-1})	AlpM MIC (mg ml^{-1})	AlpD MIC (mg ml^{-1})	AlpH MIC (mg ml^{-1})
Reference strain				
C. jejuni 11168	0·512	0·512	0·512	0·512
Mutant strains				
cmeB	0·064	0·128	0·032	0·128
cmeF	0·512	1·025	0·512	2·051
cmeR	0·512	1·025	0·512	2·051

AlpE, ethanol extract; AlpM, methanol extract; AlpD, dichloromethane extract; AlpH, hexane extract.

of the tested isolates. On the other hand, verapamil and reserpine had no significant influence on the MICs of AlpE (Table 3). No statistically significant differences were observed in the effects of different EPIs to AlpE resistance between *C. jejuni* and *C. coli* as well as between erythromycin-resistant and sensitive strains (Table 3).

The results obtained for the mutant strains were similar. The MICs are presented in Table 2. PAβN provoked >fourfold MIC reduction in the *cmeB* mutant, and interestingly, had even greater effects, in both of the other mutants (*cmeF* and *cmeR*) with at least 16-fold reductions. Both of the other possible EPIs in *Campylobacter* spp., NMP and CCCP, also reduced the AlpE MICs in all three of the tested efflux pump mutants. However, there were no effects of verapamil and reserpine on any of tested mutant constructs (Table 3).

AlpE, V40 and VL extracts as modulators of *Campylobacter* resistance to antibiotics, EtBr, biocides, plant phenolic extract and plant phenolic compound

AlpE, V40 (rosemary) and VL (vine-leaf) extracts were further tested for modulation of *Campylobacter* resistance to antibiotics (erythromycin, ciprofloxacin), EtBr, biocides (bile salts, sodium deoxycholate), plant phenolic extract (V40) and plant phenolic compound (EGCG). The concentrations of the natural putative EPIs selected for this investigation were always subinhibitory, at 0·25 MIC for each *Campylobacter* strain tested. Table 4 gives the effects of the putative natural EPIs on the resistance of *C. jejuni* and *C. coli* isolates and efflux pump mutants to erythromycin, ciprofloxacin, EtBr, bile salts, sodium deoxycholate, V40 and EGCG. Additionally, Table 4 includes the activities of the known EPIs, PAβN and NMP, respectively.

AlpE decreases the resistance to erythromycin by 4- to 16-fold in above 80% of the tested strains, and for ciprofloxacin in 60%. AlpE also produces a large decrease in resistance to the V40 plant extract, where it reduces the MICs by fourfold in 80% of both the sensitive and resistant *Campylobacter* spp. isolates. The effects of AlpE on the MIC of EGCG in these selected *Campylobacter* spp. was much smaller (Table 4). AlpE showed modulatory activity also in combination with EtBr, where in 90% of these strains it reduced the MICs by 4- to 16-fold. Moreover, significant effects were seen for AlpE on the bile salts and sodium deoxycholate MICs, for 75% of the tested strains. In this case, AlpE reduces the resistance of both by at least 4- to 16-fold (Table 4). However, a Pearson correlation matrix showed also statistically significant correlation between the resistance to AlpE and the modulatory activity of AlpE to resistance to ciprofloxacin, EtBr, bile salts and sodium deoxycholate (Table 4).

The effects of V40 and VL, as putative EPIs in resistance to the tested antibiotics, EtBr, biocides, plant phenolic extract and phenolic compound were minimal in comparison with the EPIs PAβN and NMP, and also with AlpE (Table 4).

The MICs of the selected antibiotics, biocides, EtBr, plant phenolic extract and phenolic compound were tested also in the presence of PAβN and NMP. The greatest effects of PAβN were seen in the reduction in the MICs of V40 and EGCG by fourfold to >64-fold in 80% of these strains and of the MICs of erythromycin and sodium deoxycholate by fourfold to >256-fold in all of the strains tested (100%) (Table 4). Interestingly, significantly higher effect of PAβN to erythromycin resistance was found in erythromycin-resistant isolates. Conversely, the significant effect of PAβN to resistance to V40 was higher in erythromycin-sensitive isolates (Table 4). Additionally, as an EPI, NMP restored erythromycin and sodium deoxycholate resistance by 4- to 512-fold in all of the strains tested (100%) (Table 4). Statistically significant differences in the effect of NMP to resistance to sodium deoxycholate and V40 were also found between erythromycin-resistant and sensitive isolates (Table 4). A Pearson correlation matrix was calculated also for the activity of PAβN and NMP. Statistically significant correlations between the effect of these EPIs to resistance to ciprofloxacin and V40 were observed (Table 4).

The putative EPIs were further evaluated in the mutant strains (Table 4). AlpE reduced the erythromycin, ciprofloxacin, sodium deoxycholate, V40 and EGCG MICs by fourfold or eightfold in the *cmeR* mutants, and it had a fourfold effect in the reduction in the erythromycin, bile salts and sodium deoxycholate MIC in the *cmeF* mutant. In comparison with this activity, PAβN and NMP increased the susceptibility for erythromycin by 16-fold in the *cmeF* mutant, and NMP reduced the erythromycin MIC in the *cmeR* mutant. In contrast, AlpE produced generally smaller MIC reductions in the *cmeB* mutant. PAβN and NMP

Table 3 Susceptibilities of the *Campylobacter* spp. isolates and the reference strain and its mutants to the AlpE *Alpinia katsumadai* extract in the absence and presence of PA*β*N, NMP, verapamil, reserpine and CCCP

Campylobacter strain	AlpE FMIC (mg ml^{-1})	+PA*β*N (20 *μ*g ml^{-1})		+NMP (100 *μ*g ml^{-1})		+Verapamil (100 *μ*g ml^{-1})		+Reserpine (100 *μ*g ml^{-1})		+CCCP (0·25 *μ*g ml^{-1})	
		MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.
Erythromycin resistant											
C. coli 137	2·051	0·128	**16**	0·256	**8**	1·025	2	2·051	2	0·512	**4**
C. coli 140	1·025	<0·032	**>32**	<0·128	**>8**	1·025	1	1·025	1	0·256	**4**
C. coli 171	2·051	0·128	**16**	0·256	**4**	1·025	2	2·051	1	0·512	**4**
C. coli FC8	1·025	0·064	**16**	1·025	1	1·025	1	1·025	1	1·025	1
C. coli VC7114	2·051	0·128	**16**	2·051	1	2·051	1	2·051	1	2·051	1
C. jejuni 375-06	0·512	<0·032	**>16**	<0·032	**>16**	0·256	2	0·512	1	0·256	2
Erythromycin sensitive											
C. jejuni K49/4	0·512	<0·016	**>32**	<0·016	**>32**	0·512	1	0·512	1	0·256	2
C. jejuni V1 - 846	1·025	0·128	**8**	0·128	**8**	0·512	2	1·025	1	0·512	2
C. jejuni 573/03	1·025	0·128	**8**	0·128	**8**	0·512	2	1·025	1	0·256	**4**
C. jejuni 11168	0·512	<0·032	**>16**	0·064	**8**	0·128	**4**	1·020	0·5	0·512	1
Mutant strains											
cmeB	0·064	<0·016	**>4**	0·016	**4**	0·064	1	0·064	1	<0·032	>2
cmeF	0·512	<0·032	**>16**	0·128	**4**	0·512	1	0·512	1	0·128	**4**
cmeR	0·512	<0·032	**>16**	0·128	**4**	0·512	1	0·512	1	0·064	**8**

'Fold diff.' indicates fold difference, which is calculated using the formula: MIC without an EPI/MIC with an EPI. ≥Fourfold changes are indicated in bold.

mostly increased the susceptibilities for both phenolic extract and compound in all of the mutants tested, but the AlpE produced MIC reduction only in the *cmeR* mutant. Unlike AlpE, V40 and VL did not reduce the MICs in any of the mutants (Table 4).

Discussion

Although it is known that components of *A. katsumadai* have antioxidant activities (Lee *et al.* 2003), few studies have already investigated their biological activities. Molecular insights into the neuraminidase inhibitory activities of diarylheptanoids isolated from *A. katsumadai* seeds were shown by Grienke *et al.* (2010). Recently, a series of new diarylheptanoids isolated from *A. katsumadai* seeds were described as heat-shock-factor-1 inducers (Nam *et al.* 2011). Previous investigations of *A. katsumadai* have reported a variety of diarylheptanoids, flavonoids, monoterpenes, sesquiterpenoids and stilbenes in the seeds (Saiki *et al.* 1978; Kuroyanagi *et al.* 1983; Brown and Rice-Evans 1998). Diarylheptanoid monomers in *A. katsdumadai* usually have either unsubstituted or monosubstituted phenyl rings at the end of the heptyl chain (Kuroyanagi *et al.* 1983; Nam *et al.* 2011). In addition, diarylheptanoid – chalcone dimers and kavalactones – appear to be characteristic compounds in *A. katsumadai* (Li *et al.* 2010; Nam *et al.* 2011). These latter constituents could only be identified in minor amounts in the extracts, whereas major compounds were the flavanones, chalcones and simple diarylheptanoids, whereby their proportions depending on the solvent used for extraction. Therefore, in this study, we initially evaluated four different extraction procedures for the seeds of *A. katsumadai* in biological inhibition assays. However, the potential for antimicrobial activities of extracts of *A. katsumadai* seeds has hardly been investigated, with only one study reporting antibacterial activities of compounds **1** and **5** against *Helicobacter pylori* (Huang *et al.* 2006). Strikingly, in the same study, pinocembrin (**2**), which is closely related to alpinetin **1**, was much less active (**1**, MIC = 0·001 mg ml^{-1}; **2**, MIC = 0·32 mg ml^{-1}), with moderate-to-low antimicrobial activities also detected when compounds **1–3** and **5** were tested against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli*. Moderate antibacterial effects of **1** and **2** were also reported in the studies of *Helichrysum forskahlii* (Al-Rehaily *et al.* 2008) and knotwood extracts (Välimaa *et al.* 2007), respectively.

With specific reference to the campylobacters, no antimicrobial activities have been previously reported for *A. katsumadai* seed extracts. According to the MIC values in this study, these *A. katsumadai* extracts were moderately effective in the inhibition of food-borne *Campylobacter* spp. pathogens. These strains of *C. coli* and *C. jejuni* revealed similar susceptibilities to each of

Table 4 Susceptibilities of the *Campylobacter* spp. isolates and the reference strain and its mutants to erythromycin, ciprofloxacin, EtBr, bile salts, sodium deoxycholate, VL and EGCG in the absence and presence of PAβN, NMP, AlpE, V40 and VL

Isolate	Erythromycin		Ciprofloxacin		EtBr		Bile salts		Sodium deoxycholate		V40		EGCG	
Antimicrobial ± inhibitor	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.
Erythromycin resistant														
C. coli 137	512		8		2		256		32		0·313		0·313	
+PAβN	32	**16**	4	2	1	2	32	**8**	1	**32**	0·078	**4**	0·156	2
+NMP	128	**4**	4	2	0·5	**4**	16	**16**	1	**32**	0·078	**4**	0·156	2
+AlpE	32	**16**	4	2	0·5	**4**	32	**8**	2	**16**	0·078	**4**	0·156	2
+V40	256	2	8	1	2	1	128	2	16	2	–	–	0·313	1
+VL	256	2	8	1	1	2	128	2	16	2	0·156	2	0·313	1
C. coli 140	512		32		0·5		64		4		0·625		0·313	
+PAβN	16	**32**	32	1	0·125	**4**	64	1	1	**4**	0·312	2	0·078	**4**
+NMP	64	**8**	8	**4**	0·125	**4**	32	2	2	2	0·312	2	0·156	2
+AlpE	64	**8**	16	2	0·063	**8**	32	2	2	2	0·312	2	0·156	2
+V40	256	2	32	1	0·5	1	64	1	4	1	–	–	0·313	1
+VL	512	1	32	1	0·25	2	64	1	4	1	0·625	1	0·313	1
C. coli 171	512		16		1		16		16		0·313		0·313	
+PAβN	32	**16**	16	1	1	1	4	**4**	1	**16**	0·078	**4**	0·078	**4**
+NMP	128	**4**	8	2	0·5	2	2	**8**	1	**16**	0·156	2	0·156	2
+AlpE	128	**4**	4	**4**	ND	ND	2	**8**	2	**8**	0·156	2	0·156	2
+V40	512	1	8	2	ND	ND	8	2	16	1	–	–	0·313	1
+VL	512	1	8	2	ND	ND	16	1	8	2	0·156	2	0·313	1
C. coli FC8	4		64		2		64		64		0·313		0·625	
+PAβN	0·125	**32**	32	2	0·5	**4**	16	**4**	8	**8**	0·078	1	0·156	**4**
+NMP	0·5	**8**	16	**4**	0·5	**4**	1	**64**	4	**16**	0·156	1	0·156	**4**
+AlpE	1	**4**	8	**8**	0·031	**16**	16	**4**	16	**4**	0·156	4	0·625	1
+V40	4	1	16	**4**	2	1	64	1	32	2	–	–	0·312	2
+VL	4	1	16	**4**	4	0·5	32	2	64	1	0·156	1	0·312	2
C. coli VC7114	512		8		0·25		16		2		0·313		0·313	
+PAβN	32	**16**	8	1	0·031	**4**	8	2	0·25	**8**	0·078	**4**	0·078	**4**
+NMP	<4	**>128**	4	2	0·125	2	4	**4**	0·25	**8**	0·156	2	0·156	2
+AlpE	256	2	1	**8**	0·016	**8**	8	2	0·25	**8**	0·078	**4**	0·626	0·5
+V40	256	2	8	1	0·25	1	16	1	2	1	–	–	0·313	1
+VL	512	1	8	1	0·25	1	16	1	2	1	0·313	1	0·156	2
C. coli 375-06	64		128		0·5		16		16		1·25		0·078	
+PAβN	2	**32**	2	**64**	0·125	**4**	8	2	2	**8**	0·078	**16**	0·002	**>32**
+NMP	4	**16**	2	**64**	0·125	**4**	4	**4**	0·5	**32**	0·312	**4**	0·002	**>32**
+AlpE	32	2	64	2	0·125	**4**	4	**4**	8	2	0·312	**4**	1·25	**0·06**

(*Continued*)

Table 4 *(Continued)*

Isolate Antimicrobial ± inhibitor	Erythromycin		Ciprofloxacin		EtBr		Bile salts		Sodium deoxycholate		V40		EGCG	
	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.
+V40	64	1	64	2	1	0·5	16	1	16	1	–	–	0·039	2
+VL	64	1	64	2	0·5	1	16	1	16	1	0·625	2	0·078	1
Erythromycin sensitive														
C. jejuni K49/4	0·5		1		2		64		64		1·25		0·078	
+PAβN	0·031	**16**	0·5	2	2	1	16	**4**	32	2	0·156	**8**	<0·001	**>64**
+NMP	0·125	**4**	0·5	2	0·5	**4**	32	2	32	2	0·313	**4**	0·020	**4**
+AlpE	0·125	**4**	0·125	**8**	0·25	**8**	8	**8**	4	**16**	0·313	**4**	0·156	0·5
+V40	1	0·5	0·5	2	2	1	64	1	64	1	–	–	0·078	1
+VL	0·5	1	0·5	2	2	1	64	1	64	1	1·25	1	0·078	1
*C. jejuni*V1-846	1		16		1		8		16		0·625		0·078	
+PAβN	0·125	**8**	4	**4**	1	1	2	**4**	8	2	0·078	**8**	0·002	**32**
+NMP	0·25	**4**	8	2	1	1	4	2	8	2	0·156	**4**	0·010	**8**
+AlpE	0·25	**4**	4	**4**	0·25	**4**	4	2	8	2	0·156	**4**	0·039	2
+V40	1	1	ND	ND	0·5	2	8	1	16	1	–	–	ND	ND
+VL	1	1	ND	ND	0·5	2	ND	ND	ND	ND	0·313	2	ND	ND
C. jejuni 573/03	1		32		2		8		16		1·25		0·313	
+PAβN	0·125	**8**	32	1	2	1	4	2	0·5	**32**	0·156	**8**	0·019	**16**
+NMP	0·125	**8**	8	**4**	0·5	**4**	1	**8**	8	2	0·313	**4**	0·078	**4**
+AlpE	0·25	**4**	16	2	0·25	**4**	4	2	4	**4**	0·313	**4**	0·156	2
+V40	1	1	32	1	1	2	8	1	8	2	–	–	0·156	2
+VL	1	1	32	1	1	2	8	1	16	1	1·25	1	0·156	2
C. jejuni 11168	0·25		0·25		2		32		64		0·078		0·078	
+PAβN	0·063	**4**	0·125	2	0·5	**4**	16	2	8	**8**	0·005	**16**	0·008	**8**
+NMP	0·063	**4**	0·25	1	1	2	8	**4**	16	**4**	0·019	**4**	0·078	1
+AlpE	0·031	**8**	0·063	**4**	0·5	**4**	8	**4**	8	**8**	0·019	**4**	0·019	**4**
+V40	0·5	0·5	0·25	1	0·5	**4**	32	1	32	2	–	–	0·039	2
+VL	0·5	0·5	0·5	0·5	0·5	**4**	32	1	32	2	0·078	1	0·078	1
Mutant strains														
cmeB	0·016		0·063		0·063		8		32		0·010		0·078	
+PAβN	0·016	1	0·015	**4**	0·063	1	4	2	8	**4**	<0·0003	**>32**	0·019	**4**
+NMP	0·016	1	0·015	**4**	0·063	1	4	2	16	2	<0·005	**>2**	0·019	**4**
+AlpE	0·008	2	0·031	2	0·015	**4**	4	2	8	**4**	0·005	2	0·039	2
+V40	0·008	2	0·063	1	0·063	1	8	1	32	1	–	–	0·039	2
+VL	0·016	1	0·063	1	0·063	1	ND	ND	ND	ND	0·010	1	0·078	1
cmeF	0·5		0·156		0·5		16		128		0·156		0·078	

(Continued)

the tested *A. katsumadai* extracts. Furthermore, we also examined their anti-*Campylobacter* activities on isolates that have previously shown resistance to erythro-

Table 4 *(Continued)*

Isolate Antimicrobial ± inhibitor	Erythromycin		Ciprofloxacin		EtBr		Bile salts		Sodium deoxycholate		V40		EGCG	
	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.	MIC (mg ml^{-1})	Fold diff.
+PAβN	0·031	**16**	0·078	2	1	0·5	4	**4**	64	2	0·010	**16**	0·019	**4**
+NMP	0·031	**16**	0·078	2	0·5	1	8	2	64	2	0·078	2	0·019	**4**
+AlpE	0·125	**4**	0·078	2	0·125	2	4	**4**	32	**4**	0·078	2	0·039	2
+V40	0·5	1	0·078	2	0·5	1	16	1	128	1	–	–	0·039	2
+VL	0·5	1	0·156	1	0·5	1	ND	ND	ND	ND	0·078	2	0·078	1
cmeR	0·5		0·156		0·25		16		128		0·156		0·313	
+PAβN	0·063	**8**	0·039	**4**	0·25	1	16	1	64	2	0·010	**16**	0·010	**32**
+NMP	0·063	**8**	0·039	**4**	0·25	1	8	2	64	2	0·078	2	0·010	**32**
+AlpE	0·125	**4**	0·039	**4**	0·125	2	8	2	32	**4**	0·039	**4**	0·039	**8**
+V40	0·25	2	0·156	1	0·25	1	16	1	128	1	–	–	0·313	1
+VL	0·5	1	0·156	1	0·25	1	ND	ND	ND	ND	0·156	1	0·156	2

ND, not defined.
'Fold diff.' indicates fold difference, which is calculated using the formula: MIC without an EPI/MIC with an EPI. ≥Fourfold changes are indicated in bold. PAβN at 20 μg ml^{-1}; NMP at 100 μg ml^{-1}; AlpE at 0·25 MIC for each strain tested; V40 at 0·25 MIC for each strain tested; VL at 0·25 MIC for each strain tested.

mycin, ciprofloxacin and tetracycline (Kurinčič *et al.* 2012). The challenge for the development of new antibacterial products for the treatment of MDR bac-

teria arises as these pathogens are uniquely suited to survival in toxic environments (Simões *et al.* 2009). We examined here whether *Campylobacter* spp. have resistance mechanisms that are in some cases not specific to the antibacterial product to which they are exposed, but instead represent more general mechanisms. *Alpinia katsumadai* extracts showed similar MICs against the sensitive and the multi-resistant *C. coli*, thus confirming the antimicrobial activities of the *A. katsumadai* extracts also against multi-resistant *Campylobacter* spp. isolates. As can be seen, the *Campylobacter* spp. susceptibilities to *A. katsumadai* extracts do not correlate with their susceptibilities or resistance to different antibiotics, indicating that the modes of action of *A. katsumadai* extracts are different from those of the antibiotics tested. This has also been reported for several natural antimicrobials (Gibbons *et al.* 2004; Simões *et al.* 2009). Overall, these data indicate that *A. katsumadai* is a promising plant, with antibacterial activities seen for all of the extracts tested against sensitive and resistant *Campylobacter* spp. isolates. However, the ethanolic (AlpE) and dichloromethane (AlpD) extracts were slightly more effective.

We further investigated whether the efflux pumps can extrude the active components of these *A. katsumadai* seed extracts across the outer membrane and thus reduce their effectiveness. The significant decrease in the MICs in the gene-specific knockout mutant that lacks the *cmeB* efflux pump gene indicates that these extracts contained substrates for CmeABC in *Campylobacter* spp., whereby the inactivation of this efflux pump can recover the susceptibilities for these extracts. Interestingly, inactivation of the CmeF efflux pump protein had no effect on the MIC when AlpE and AlpD were tested, but decreased the susceptibilities for AlpM and AlpH. These data indicate that the CmeDEF efflux pump also has a modest role in the resistance mechanisms to *A. katsumadai* in *Campylobacter* spp. As previously reported, *cmeR* encodes a transcriptional repressor that directly interacts with the *cmeABC* promoter and modulates the expression of *cmeABC* (Lin *et al.* 2005a). In this study, the MICs of these different *A. katsumadai* extracts varied in the *cmeR* mutant. The inactivation of the transcriptional repressor CmeR, and hence overexpression of CmeABC, slightly increased (up to fourfold MIC increase) the resistance to AlpM and AlpH. These unexpected MIC changes indicate that the exclusion of one of these efflux systems can lead to increased activity of another efflux system, possible recently described CmeG efflux pump (Jeon *et al.* 2011), or even the activation of other resistance mechanisms. This coincides with a previous finding on the interactive involvement of the CmeABC and CmeDEF efflux pumps in the extrusion of toxic compounds in *C. jejuni,* where the inactivation of CmeDEF led to increased expression of CmeABC, whereby CmeDEF might be primarily responsive to certain conditions (Akiba *et al.* 2006).

However, the MICs of the *A. katsumadai* extracts were affected by *cmeB* inactivation, and for two of the four extracts tested, also by *cmeF* inactivation, which indicate that they are substrates of CmeABC and possibly of the CmeDEF efflux pump. Significant MIC reductions in the *cmeB* mutant and visible susceptibility changes in the *cmeR* mutant for AlpM and AlpH indicate that the CmeABC efflux pump has an important and broad role in the resistance to *A. katsumadai* extracts in *Campylobacter* spp.

Intrinsic and acquired resistance in bacteria is mostly demonstrated by the use of EPIs, which can enhance drug accumulation inside the bacterial cell, thereby increasing bacterial susceptibility to antimicrobials (Garvey and Piddock 2008; Hannula and Hänninen 2008). The ethanolic extract, AlpE, was specifically selected for these experiments because of its easier preparation, less toxic solvent and its similar composition to the equally active dichloromethane extract (AlpD). The resistance to AlpE of all of the sensitive and MDR isolates tested were statistically significantly reduced by PAβN and NMP. These data support our results that active RND efflux via CmeABC is an important resistance mechanism against *A. katsumadai* extracts in *Campylobacter* spp. On the basis of the restored sensitivity in the presence of CCCP, we suggest that the less-known CCCP can be used as an EPI also in *Campylobacter* spp. CCCP has previously been reported as an EPI in *Bacillus subtilis* and *Streptococcus pneumoniae*, where it blocks RND-type efflux pumps, as is known for PAβN and NMP (Lomovskaya *et al.* 2001; Garvey and Piddock 2008).

A significant difference in the efficiencies of the EPIs verapamil and reserpine in comparison with the other tested EPIs was observed, with relatively little or no effects on the resistances to AlpE. This indicates that they have different modes of action. However, verapamil and reserpine had no effects on the resistance to the *A. katsumadai* extract, and we confirmed that these are not appropriate as EPIs in *Campylobacter* spp.

Similar to what was observed with the wild-type *C. jejuni* 11168, PAβN, NMP and CCCP showed greater potentiating effects in the efflux pump mutants than the other EPIs. The changes in susceptibilities after EPIs addition in the *cmeB* mutant resulted in increased activities of another efflux system, indicating that the CmeABC efflux pump is not the only active efflux system involved to *Campylobacter* resistance to AlpE. Changes in susceptibility in *cmeF* after efflux inhibition with EPIs

indicate the involvement of other non-CmeDEF efflux pumps in the extrusion of these plant substrates. Of particular interest, the EPIs PAβN, NMP and CCCP also had effects in the *cmeR* mutant, showing the possible contribution of another, third, active efflux system in the mediation of the resistance to such *A. katsumadai* antimicrobials, or even the activation of other resistance mechanisms.

We confirm that PAβN, NMP and CCCP can potentiate the anti-*Campylobacter* spp. activities of AlpE, but there is the need for new efflux inhibitors and/or resistance-modifying agents with inhibitory efficacy and no toxic effects on mammalian cells. As is seen in the testing of the activities of putative natural EPIs in sensitive and resistant isolates of *Campylobacter* spp., AlpE inhibited the efflux of all of these antimicrobial agents, except for EGCG. Thus, we confirm resistance-modifying activity of AlpE from *A. katsumadai* seeds in *Campylobacter* spp. In comparison with PAβN and NMP, the effects of AlpE on selected antimicrobial resistances were smaller, although they were still remarkable. Additionally, the efficiency of AlpE as an EPI with both biocides tested was comparable with NMP and PAβN.

No effects of the rosemary V40 and vine-leaf extracts on the resistances to the antibiotics, biocides and plant extracts were observed in the *Campylobacter* strains tested.

Collectively, the results of this study show that these extracts from *A. katsumadai* seeds are important sources of antimicrobial compounds against sensitive and MDR *Campylobacter* spp. isolates. This is the first report to show that at least two of the efflux systems are involved in the resistance to *A. katsumadai* antimicrobial seed extracts. The mechanism of active efflux from the cell is mostly through the CmeABC pump, but there is also the involvement of the CmeDEF efflux pump, with a modest role in this resistance indicated. The additional effects of the EPIs also demonstrate possible contributions of other efflux mechanisms to *Campylobacter* spp. resistance to AlpE.

For the inhibitory effects, we can confirm that the EPIs PAβN, NMP and CCCP potentiate the anti-*Campylobacter* spp. activities of *A. katsumadai*. Verapamil and reserpine had no effects on the resistances to the *A. katsumadai* extract. Significant effects of AlpE on the antibiotics, the biocides, EtBr and also the V40 plant extract in resistant and sensitive *Campylobacter* strains were also shown, with inhibition of the efflux of all of these antimicrobial agents. This efficacy of the AlpE extract represents the first evidence of a plant-derived resistance-modifying agent in *Campylobacter* spp. On the other hand, rosemary V40 and the VL extracts did not show resistance-modifying activities.

Our findings provide an evaluation of the anti-*Campylobacter* spp. and resistance-modifying activities of *A. katsumadai* extracts and demonstrate the potential use of AlpE for the control of *Campylobacter* and its antimicrobial resistance in the food industry.

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2.1.3 Antimicrobial and resistance modulatory activity of *Alpinia katsumadai* seed extract, essential oil and post-distillation extract

Protimikrobno in odpornostno modulatorno delovanje semenskega izvlečka, eteričnega olja in podestilacijskega izvlečka rastline *Alpinia katsumadai*

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Antimicrobial resistance of food-related bacterial pathogens is becoming a serious problem, especially after the emergence of multidrug-resistant strains. To overcome this problem, new and effective antimicrobials or resistance modulators are highly needed and plant kingdom represents a valuable source of these compounds. We investigated antimicrobial and resistance modulatory activity of the phenolic extract, essential oil and post-distillation extract of *Alpinia katsumadai* seeds against *Campylobacter jejuni* and *Staphylococcus aureus*. Among the tested plant formulations, *A. katsumadai* seed extract and post-distillation extract showed moderate antimicrobial activity against *C. jejuni*, while *S. aureus* was more resistant. When evaluating resistance modulatory potential of *A. katsumadai* phenolicextract, essential oil and post-distillation extract in *C. jejuni* against ciprofloxacin, erythromycin, triclosan, bile salts and ethidium bromide, plant formulations exhibited modulatory activity in combination with all antimicrobials. Modulation of resistance was more strain- and antimicrobial-specific in *S. aureus*, but very efficient in the case of reduced resistance to bile salts. Essential oil from *A. katsumadai* seeds efficiently increased intracellular ethidium bromide accumulation and was thus confirmed as potential inhibitor of efflux pumps in *C. jejuni* and *S. aureus*.

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Antimicrobial and Resistance Modulatory Activity of *Alpinia katsumadai* Seed Extract, Essential Oil and Post-Distillation Extract

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Summary

Antimicrobial resistance of food-related bacterial pathogens is becoming a serious problem, especially after the emergence of multidrug-resistant strains. To overcome this problem, new and effective antimicrobials or resistance modulators are highly needed and plant kingdom represents a valuable source of these compounds. We investigated antimicrobial and resistance modulatory activity of the phenolic extract, essential oil and post-distillation extract of *Alpinia katsumadai* seeds against *Campylobacter jejuni* and *Staphylococcus aureus.* Among the tested plant formulations, *A. katsumadai* seed extract and post-distillation extract showed moderate antimicrobial activity against *C. jejuni*, while *S. aureus* was more resistant. When evaluating resistance modulatory potential of *A. katsumadai* phenolic extract, essential oil and post-distillation extract in *C. jejuni* against ciprofloxacin, erythromycin, triclosan, bile salts and ethidium bromide, plant formulations exhibited modulatory activity in combination with all antimicrobials. Modulation of resistance was more strain- and antimicrobial-specific in *S. aureus*, but very efficient in the case of reduced resistance to bile salts. Essential oil from *A. katsumadai* seeds efficiently increased intracellular ethidium bromide accumulation and was thus confirmed as potential inhibitor of efflux pumps in *C. jejuni* and *S. aureus.*

Key words: antimicrobial activity, antimicrobial resistance, resistance modulation, plant antimicrobials, plant resistance modulators, efflux inhibition

Introduction

Despite many recent technological advances in the food industry, which have contributed to increased safety in the food supply chain, we are still facing high incidence of foodborne illnesses. Only in 2011, there were 69 553 human cases originating from foodborne zoonotic outbreaks in the EU (*1*). Ninety-three of them were fatal. In the same year, *Campylobacter*, as the most prevalent foodborne zoonotic agent, caused 220 209 registered, mostly sporadic human illnesses and it is assumed that the real burden is even much higher (*1*). Besides emerging and reemerging food-related zoonotic agents, the non-zoonotic pathogens are also transmitted by foods. Together with their high incidence and the increasing resistance against antibiotics and other antimicrobials used in the food chain (*2*,*3*), microbial pathogens compromise food safety, especially with the emergence of multidrug-resistant strains (*4*).

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Search for alternative antimicrobials derived from plants seems to be a viable solution for mitigation of resistance (*5*). In addition to searching for new antimicrobially active plant formulations, the strategies for combating antibiotic resistance are focused also on resistance modulators. These are not necessarily antimicrobially active, but can decrease the resistance of pathogens when administered together with other antimicrobials. The microbial sensitization by resistance modulators is mostly due to efflux inhibition, increased membrane permeability, increased porin production or change in porin profile (*6*).

Plant extracts rich in phenolic compounds (phenolic extracts), and essential oils have long been shown to possess antimicrobial activity and were frequently studied and reviewed (*7–10*). They are especially interesting as they are generally recognized as safe and have a potential to be used as preservatives in food products. *Alpinia katsumadai* Hayata (syn. *A. katsumadae* Hayata; Zingiberaceae) is widely used in traditional Chinese medicine as an anti-emetic remedy and to increase the appetite, but also in animal feed, to facilitate rapid growth of domestic animals (*11,12*). In our study we have focused on investigating the bioactivity of *A. katsumadai* extract, essential oil and also waste material that remains after the essential oil production. Such residual materials are often disposed of and may present an environmental problem. Their high phenolic content and the potential to provide an economically feasible source of natural antioxidants and antimicrobials is unused (*13*).

In our study ethanolic extract, essential oil and post-distillation extract of *A. katsumadai* seeds were tested against drug-sensitive and resistant strains of Gram-negative *Campylobacter jejuni* and Gram-positive *Staphylococcus aureus*, and their potential to modulate the resistance of these pathogens against different antimicrobials was evaluated. Additionally, intracellular accumulation of ethidium bromide was tested to confirm efflux inhibition with the plant formulation, which was most efficient in resistance modulation.

Materials and Methods

Bacterial strains, growth conditions and antibiotic resistance

All bacterial strains were stored at –80 °C. *Campylobacter* was grown on Columbia blood agar (Oxoid, Basingstoke, UK) at 42 °C for 24 h under microaerobic conditions. *Staphylococcus* was grown on Mueller-Hinton agar (MHA, Oxoid) at 37 °C for 24 h. For the antimicrobial activity assays, cultures were suspended in Mueller-Hinton broth (MHB, Oxoid) to 10^5–10^6 CFU/mL. Antibiotic-sensitive and -resistant strains of both species were included in testing. Their resistance against five or more medically important antibiotics was determined by broth microdilution method (Sensititre® TREK Diagnostic Systems Inc., Thermo Scientific, Independence, OH, USA) or CellTiter-Blue® reagent (Promega, Madison, WI, USA) and automated fluorescence signal detection (*14*).

Preparation of plant formulations

Alpinia katsumadai seeds were bought from a commercial source (Cat. no. 680381, Plantasia, Oberndorf/Salzburg, Austria) and extracted with 96 % ethanol for 24 h at room temperature to obtain an extract rich in phenolic compounds which was dried by gradual pressure decrease at 45 °C using rotavapor. Part of the extract was suspended in water and hydrodistilled for 2 h using Clevenger-type apparatus to obtain the essential oil. The remaining material after hydrodistillation was freeze-dried and dissolved in ethanol for further testing as post-distillation extract.

Total phenolic content and DPPH radical-scavenging assay

The total content of phenolic compounds (TPC) of tested formulations was determined spectrophotometrically using Folin-Ciocalteu reagent (*15*). The reaction mixture contained 100 μL of 0.1 % phenolic extract or post-distillation extract diluted in methanol, 500 μL of 0.2 M Folin-Ciocalteu reagent (Fluka Chemicals, AG, Buchs, Switzerland), and 400 μL of 10 % aqueous Na_2CO_3 solution (Lach-Ner, Brno, Czech Republic). The absorbance was measured spectrophotometrically (Agilent Technologies, Santa Clara, CA, USA) at 760 nm after a 30-minute incubation in the dark. The results are expressed as mg of gallic acid equivalents (GAE) per g of tested dried material.

The radical-scavenging potential of the tested plant formulations was evaluated using the DPPH assay (*16*), with some modifications. Briefly, the samples were dissolved in methanol and tested at four concentrations ranging from 3.75 to 150 μg/mL. The reaction mixtures contained 50 μL of the sample dilution and 150 μL of 50 μM DPPH dissolved in methanol. Spectrophotometric absorbance at 535 nm was measured after 30 min, against methanol as a blank on a Wallac 1420 Victor2 multilabel counter (PerkinElmer, Waltham, MA, USA). The RSC was calculated using the following equation:

$$RSC=[(A_c-A_s)/A_c]\cdot 100 \qquad /1/$$

where A_s is the absorbance of the sample, and A_c is the absorbance of the control.

Antimicrobial activity assay

Antimicrobial activity was evaluated using broth microdilution assay in microtiter plates as previously described using CellTiterBlue® kit (Promega) (*14*). After 24-hour incubation under species-specific growth conditions, viability was measured based on the intensity of the fluorescence signal with a microplate reader (Tecan, Mannedorf/Zurich, Switzerland). Minimal inhibitory concentration (MIC) was determined as the lowest concentration of the tested antimicrobial at which no fluorescence signal was detected. Negative control, growth control and dimethyl sulphoxide (DMSO) control were included and each assay was repeated in triplicate.

Resistance modulatory activity assay

The resistance modulatory assay was performed by the same principle as the antimicrobial activity assay with the addition of the tested modulators in concentrations of half MIC value into the medium.

Ethidium bromide accumulation assay

Ethidium bromide (EtBr) accumulation assay (*17*) was carried out to evaluate the potential efflux inhibitory activity of essential oil at 0.5 and 0.25 MICs of essential oil on *C. jejuni* NCTC 11168 and *S. aureus* 5.3. Additionally, 100 μg/mL of verapamil were tested as a positive efflux inhibitor reference. The overnight cultures were resuspended in MHB to achieve $A_{600\ nm}$=0.2 and incubated for 4 h at 37 °C (*S. aureus*) or 42 °C (*C. jejuni*). Further on, the cells were washed and resuspended in phosphate buffered saline (PBS) to achieve $A_{600\ nm}$=0.2. Plant formulations and EtBr at final concentration of 0.5 μg/mL were added to the culture in black microtitre plates (100 μL per well). Intracellular EtBr accumulation kinetics was measured at 500 nm excitation and 608 nm emission wavelength with a microplate reader (Tecan) for 1 h. Culture with EtBr, but without inhibitor was used as a baseline accumulation control and EtBr alone as blank. Three independent experiments with three replicates were carried out.

Statistical analysis

The results were statistically analyzed using the SPSS® software, v. 21 (IBM Corp., Armonk, NY, USA). Comparisons of the group mean values and the significances of the differences between the groups were verified by one-way ANOVA. Pearson coefficients were calculated for the correlations between different variables. The results were considered significant when $p \leq 0.05$.

Results and Discussion

Phenolic extract, essential oil and the post-distillation extract of *A. katsumadai* seeds were tested for their antimicrobial activity against five *C. jejuni* and five *S. aureus* isolates. Among each tested bacterial species, antibiotic-sensitive and resistant strains, including multidrug-resistant ones, were included (Table 1; *18*).

Statistically significant differences between the antimicrobial activities of different plant formulations were observed ($p<0.0001$). According to the average MICs for *Campylobacter* (275 μg/mL), *A. katsumadai* phenolic extract was the most efficient antimicrobial plant formulation, followed by *A. katsumadai* post-distillation extract. Generally, plant formulations were more effective against *C. jejuni* than against *S. aureus* (Table 1). We could not confirm any difference between the antimicrobial activities of the tested plant formulations against antibiotic-sensitive or resistant strains. They were active also against multidrug-resistant strains (Table 1).

Besides direct antimicrobial activity, we also tested and confirmed remarkable resistance modulatory activity of *A. katsumadai* extract, essential oil and post-distillation extract in *C. jejuni*, as well as in *S. aureus* (Table 2). *A. katsumadai* seed ethanol extract was confirmed for the first time to modulate antibiotic resistance in *Campylobacter* in our recent work (*12*). Here we comparatively investigated the resistance modulatory potential of additional *A. katsumadai* formulations, essential oil and post-distillation extract, and the activity of all three formulations in *S. aureus*, including MRSA strains (Tables 1 and 2). They were tested as modulators in concentrations half of their MIC on each individual strain of *C. jejuni* and *S. aureus* and in combination with antimicrobials, *i.e.* two antibiotics (ciprofloxacin and erythromycin), triclosan, bile salts and ethidium bromide. The cut-off value of signifi-

Table 1. Antimicrobial activity of *Alpinia katsumadai* plant formulations against antibiotic-sensitive and -resistant *Campylobacter jejuni* and *Staphylococcus aureus* strains, including multidrug-resistant ones

	MIC/(μg/mL)								
C. jejuni									
	PE	PDE	EO	ERY	CIP	TET	GEN	STR	CHL
NCTC 11168	250	250	1000	0.25 (S)	0.25 (S)	0.25 (S)	0.25 (S)	2 (S)	<2 (S)
ATCC 33560	125	250	1000	1 (S)	0.25 (S)	0.5 (S)	1 (S)	4 (R)	4 (S)
375/06	500	500	2000	128 (R)	32 (R)	0.25 (S)	0.5 (S)	<1 (S)	<2 (S)
573/03	250	250	4000	1 (S)	32 (R)	2 (S)	0.25 (S)	<1 (S)	<2 (S)
K49/4	250	1000	2000	0.5 (S)	1 (R)	0.25 (S)	0.25 (S)	2 (S)	<2 (S)
S. aureus									
	PE	PDE	EO	ERY	CIP	TET	GEN	PEN	MET
5.1	1000	1000	250	0.25 (S)	2 (R)	32 (R)	>16 (R)	>16	(R)
5.2	1000	1000	2000	>1024 (R)	0.25 (S)	<0.5 (S)	<0.25 (S)	>16	(S)
5.3	1000	1000	4000	0.5 (S)	0.5 (S)	<0.5 (S)	<0.25 (S)	<0.06	(S)
5.5	250	500	2000	0.5 (S)	64 (R)	>32 (R)	<0.25 (S)	0.008	(R)
5.6	1000	500	4000	0.25 (S)	0.5 (S)	8 (R)	<0.25 (S)	0.5	(S)

MIC=minimal inhibitory concentration; PE=*A. katsumadai* phenolic extract, PDE=*A. katsumadai* post-distillation extract, EO=*A. katsumadai* essential oil; ERY=erythromycin, CIP=ciprofloxacin, TET=tetracycline, GEN=gentamicin, STR=streptomycin, CHL=chloramphenicol, PEN=penicillin, MET=methicillin; R=resistant, S=sensitive. Resistant phenotypes were determined according to EUCAST breakpoints (*18*), where these are defined

cant resistance modulation was set at more than twofold decrease in the MIC of the tested antimicrobial and was referred to as modulation factor (MF, Table 2). When testing the post-distillation extract and essential oil as modulators at half of the MIC on *C. jejuni*, we found that the mean MF values were 34 and 78, respectively, considering combinations with all antimicrobials. Testing the extract, essential oil and post-distillation extract in *S. aureus* resulted in mean MFs of 63, 40 and 22, respectively. According to this, the essential oil was the best modulator in *C. jejuni*, while in *S. aureus* the most effective formulation was the phenolic extract, followed by essential

Table 2. Resistance modulatory activity of *Alpinia katsumadai* plant formulations in *Campylobacter jejuni* and *Staphylococcus aureus*

	Ciprofloxacin		Erythromycin		Triclosan		Bile salts		EtBr	
	MIC/(μg/mL)	MF	MIC/(μg/mL)	MF	MIC/(μg/mL)	MF	MIC/(μg/mL)	MF	MIC/(μg/mL)	MF
C. jejuni										
NCTC 11168	0.25		0.25		32		32		2	
+PE*	0.063	**4**	0.03	**8**	n.d.	n.d.	8	**4**	0.5	**4**
+PDE	0.25	1	0.063	**4**	8	**4**	0.5	**>64**	2	2
+EO	<0.125	**>2**	0.004	**>64**	<0.5	**>64**	<0.125	**>256**	<0.078	**>256**
375/06	32		128		8		>2		1	
+PE*	16	2	64	2	n.d.	n.d.	4	**4**	0.25	**4**
+PDE	16	2	n.d.	n.d.	4	2	0.5	**>4**	0.5	2
+EO	<0.5	**>64**	n.d.	n.d.	<0.25	**>32**	<0.016	**>128**	<0.03	**>32**
K49/4	1		0.5		32		128		1	
+PE*	0.125	**8**	0.125	**4**	n.d.	n.d.	16	**8**	0.125	**8**
+PDE	<0.016	**>64**	<0.125	**>4**	<0.5	**>64**	<2	**>64**	<0.02	**>64**
+EO	<0.016	**>64**	<0.125	**>4**	<0.5	**>64**	<2	**>64**	<0.02	**>64**
573/03	32		1		16		8		0.5	
+PE*	16	2	0.25	**4**	n.d.	n.d.	4	2	0.125	**4**
+PDE	<2	**>16**	<0.5	**>2**	<0.25	**>256**	<0.25	**>32**	0.5	1
+EO	<2	**>16**	<0.5	**>2**	<0.25	**>256**	<0.25	**>32**	<0.02	**>32**
S. aureus										
5.1	2		0.25		0.12		4		64	
+PE	<0.03	**>64**	<0.03	**>8**	<0.001	**>128**	<0.06	**>128**	16	**4**
+PDE	2	1	2	1	0.12	1	0. 26	**16**	32	2
+EO	1	2	1	2	>0.12	<1	1	**4**	8	**8**
5.2	0.25		>1024		0.03		>4		8	
+PE	0.25	1	>1024	n.d.	0.12	0.25	<0.03	**>256**	8	1
+PDE	0.06	**4**	>1024	n.d.	<0.001	**>32**	0.51	**>16**	4	2
+EO	1	0.25	1024	>1	<0.001	**>32**	0.26	**>32**	8	1
5.3	0.5		0.5		0.002		>4		2	
+PE	0.25	2	0.06	**8**	<0.0001	**>32**	<0.03	**>256**	<0.03	**>64**
+PDE	0.25	2	<0.008	**>64**	0.0001	**16**	<0.03	**>256**	<0.03	**>64**
+EO	0.25	2	0.125	**4**	0.0001	**16**	<0.03	**>256**	<0.03	**>64**
5.5	64		0.5		0.06		4		4	
+PE	32	2	0.25	2	0.06	1	2	2	2	2
+PDE	32	2	1	0.5	>0.06	<1	0.51	**8**	2	2
+EO	128	0.5	1	0.5	>0.06	<1	1	**4**	4	1
5.6	0.5		0.25		>1		>8		8	
+PE	0.125	**4**	0.125	2	<0.008	**>256**	<0.06	**>256**	2	**4**
+PDE	0.25	2	0.125	2	>1	n.d.	1	**>16**	4	2
+EO	0.008	**32**	<0.004	**>128**	<0.008	>256	1	**>16**	<0.06	**>128**

*The results were previously published with 0.25 MIC of modulators (*12*). 0.5 MIC=half of minimal inhibitory concentration, 0.25 MIC=quarter of minimal inhibitory concentration; n.d.=not determined; MF=modulation factor; PE=*A. katsumadai* phenolic extract, PDE=*A. katsumadai* post-distillation extract, EO=*A. katsumadai* essential oil; EtBr=ethidium bromide. Numbers in bold represent significant resistance modulatory activity (MF>2)

oil and the post-distillation extract. However, these differences could not be confirmed as statistically significant in *C. jejuni* (p=0.071) nor in *S. aureus* (p=0.202). The mean modulation factors of all formulations in combination with individual antimicrobials ranged from 13 to 93, confirming the high modulation potential of *A. katsumadai* seed formulations in *C. jejuni*. The comparative analysis of modulatory activity of *A. katsumadai* formulations in *C. jejuni* confirmed them as equally efficient in combination with different antimicrobials, including bile salts (MF_{mean}>55, Table 2). This is important since the intestinal tract with the presence of bile salts is a natural environment and reservoir of *Campylobacter* sp., so resistance to bile salts is essential for *C. jejuni* survival and virulence potential. It was also shown that active efflux is one of the resistance mechanisms used by *C. jejuni* to resist the bactericidal effects of bile salts (*19,20*). With this work we confirmed *Alpinia* formulations to be very efficient in restoring *C. jejuni* sensitivity to bile salts (Table 2) and thus potentially influence its survival and infection capacity. On the other hand, in *S. aureus*, there were significant differences in modulation effects when formulations were combined with different antimicrobials (p=0.005). The strongest resistance modulation was achieved in combination with bile salts (MF_{mean}=101), followed by triclosan (MF_{mean}=57) and ethidium bromide (MF_{mean}=23). The weakest, yet still exceptional increase in sensitivity was obtained when plant formulations were used together with antibiotics, where mean modulation factor was 18 in the case of erythromycin and 10 in the case of ciprofloxacin. Phenolic extract and essential oil were able to restore resistance of *S. aureus* 5.1 against ciprofloxacin, while all of the tested plant formulations were able to reverse ciprofloxacin resistance in *C. jejuni* strain K49/4. Compared to other studies of resistance modulatory activity of rosemary extract, vine leaf extract and epigallocatechin gallate in *Campylobacter*, our plant formulations performed equally good or better, according to the modulation factors (*12,14*). This might also be due to the differences in concentrations of modulators used. In these previous studies the strain specificity of the resistance modulation had been observed and this phenomenon was to some degree observed also in our study, especially in *S. aureus*. Catechins and gallates had previously been investigated as resistance modulators in methicillin-resistant *S. aureus*. Among them, (–)-epicatechin gallates could significantly decrease the resistance to flucloxacillin, imipenem and meropenem (*21*).

There are several possible mechanisms of increasing susceptibility of bacteria to antibiotics and other antimicrobials. The most promising are focusing on increasing the antibiotic influx by destabilizing lipopolysaccharides in Gram-negative bacteria and increasing the membrane permeability, and blocking the efflux using efflux pump inhibitors (*6*).

In order to elucidate the mechanism of modulatory activity of *A. katsumadai* essential oil in *C. jejuni* and *S. aureus*, we have evaluated its potential to increase the accumulation of the common efflux pump substrate EtBr, which is an indicator of the efflux inhibition. The extract and post-distillation extract were excluded from the experiments because of the high autofluorescence. We compared the levels of EtBr accumulation in cultures treated with half and quarter MIC values of essential oil, relative to the untreated culture, to evaluate whether it can potentiate the intracellular EtBr accumulation. The known efflux pump inhibitor verapamil was included in the study as a positive reference. The results show significant (p<0.0001) increase in the EtBr accumulation in the presence of *A. katsumadai* essential oil, compared to untreated culture of *C. jejuni*, as well as *S. aureus* (Figs. 1 and 2). The accumulation of EtBr was 1.7-fold better in the presence of *A. katsumadai* essential oil in half of its MIC than in the presence of positive control, verapamil (Table 3). The accumulation of EtBr in the presence of essential oil

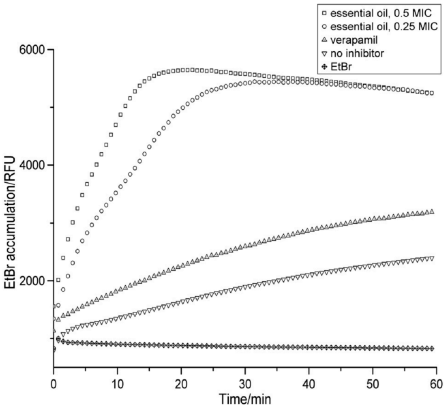

Fig 1. Ethidium bromide accumulation in *Campylobacter jejuni* NCTC 11168 in the presence of *Alpinia katsumadai* essential oil and verapamil. RFU=relative fluorescence units; 0.5 MIC=half of minimal inhibitory concentration, 0.25 MIC=quarter of minimal inhibitory concentration; EtBr=ethidium bromide

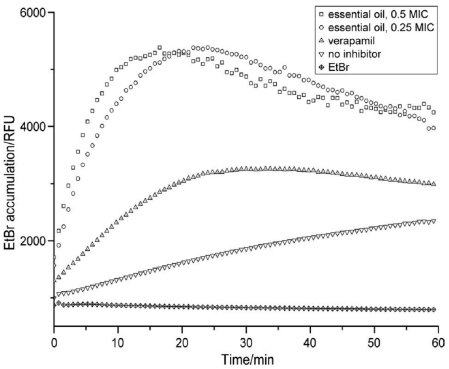

Fig. 2. Ethidium bromide accumulation in *Staphylococcus aureus* 5.3 in the presence of *Alpinia katsumadai* essential oil and verapamil. RFU=relative fluorescence units; 0.5 MIC=half of minimal inhibitory concentration, 0.25 MIC=quarter of minimal inhibitory concentration; EtBr=ethidium bromide

Table 3. Ethidium bromide accumulation in *Campylobacter jejuni* and *Staphylococcus aureus*

Plant formulation	EtBr accumulation*/RFU	
	C. jejuni	*S. aureus*
essential oil, 0.5 MIC	5303±39	4300±52
essential oil, 0.25 MIC	5298±31	4211±141
verapamil	3128±41	3045±35
no inhibitor	2335±39	2291±8
EtBr control	832±3	797±2
	N=9	*N*=9

*Ethidium bromide accumulation was calculated from the measurements during the last 10 minutes of each assay. The results are expressed as mean values±standard deviations. The differences between accumulation when essential oil or verapamil were added are statistically significant ($p<0.0001$), compared to the culture without added inhibitor. RFU=relative fluorescence units; 0.5 MIC=half of minimal inhibitory concentration, 0.25 MIC=quarter of minimal inhibitory concentration; EtBr=ethidium bromide

in half of MIC started to slowly decrease in *C. jejuni* after reaching the maximum accumulation after 15 min, whereas the accumulation in the presence of verapamil increased linearly and did not reach the plateau after 60 min (Fig. 1). Similar mean accumulation of EtBr in the presence of verapamil was achieved in *S. aureus*, but it reached the plateau after 25 min and then the accumulation started to decrease slowly. The same trend of EtBr accumulation as in *C. jejuni* in the presence of essential oil was observed in *S. aureus*, but the mean accumulation in the last 10 min was by 19 % lower than in *C. jejuni*. The mean EtBr accumulation values in the presence of essential oil in quarter of MIC were almost the same as in the presence of half of MIC (Table 3), only the time needed to reach the maximum accumulation was different (Figs. 1 and 2). Gradual decrease in EtBr accumulation after reaching the plateau might be due to the facilitation of EtBr influx by the essential oil and insufficient capacity to block the efflux.

Compounds from *A. katsumadai* had previously been confirmed to increase the EtBr accumulation in *Mycobacterium smegmatis* (*22*). Recently, several triterpenoids isolated from *Momordica balsamina* and coumarins from *Mesua ferrea* exhibited efflux-inhibitory activity in *S. aureus* (*23*,*24*); however, the activity was not directly comparable, due to the different experiment settings.

Since the phenolic compounds are often identified as main active biomolecules in plant formulations (*25*), we have determined the total phenolic content (TPC) of the phenolic extract and post-distillation extract (Table 3) in order to see whether the TPC correlates with their bioactivity. Essential oil was not expected to be rich in phenols and therefore it was not tested. The TPC of *A. katsumadai* seed extract was 24.7 μg/mL, which is lower than in its post-distillation extract (39.9 μg/mL). However, the antimicrobial activity of the extract was better than of the post-distillation extract in all bacterial targets (Table 1), which indicates that some valuable antimicrobially active compounds have been lost or deactivated during hydrodistillation. A similar result was observed in the analysis of resistance modulation. The extract had the strongest potential to decrease the resistance in *S. aureus*, followed by the essential oil and the post-distillation extract. In *C. jejuni*, *A. katsumadai* essential oil was the best modulator. Post-distillation extract was compared to the phenolic extract and the essential oil and it was concluded that it is the least effective modulator, although still showing very good resistance modulation in both, *S. aureus* as well as in *C. jejuni*. This indicates the loss of some bioactive compounds during the hydrodistillation of the extract. Since both, phenolic extract and essential oil, had valuable resistance modulatory activities, it is possible that different compounds, other than phenolics, are responsible for the activity.

Compared to other plant extracts, also from medicinal plants, formulations in our study had comparable or higher phenolic content (*25*–*27*). Plant extracts rich in phenolic compounds often possess good radical scavenging potential (*25*); therefore, the DPPH radical scavenging of the tested plant formulations was determined. As seen in Table 4, the post-distillation extract, which had a higher TPC, was also a more effective radical scavenger (IC_{50}=14.5 μg/mL), compared to the phenolic extract (IC_{50}=64.7 μg/mL).

Table 4. Total phenolic content (TPC) and DPPH radical scavenging potential of *Alpinia katsumadai* plant formulations

Plant formulation	TPC	DPPH
	mg of GAE per g of d.m.	IC_{50}/(μg/mL)
phenolic extract	24.7±0.2	64.7±6.5
post-distillation extract	39.9±0.03	14.5±1.5
essential oil	n.d.	DPPH inhibition <50 %

GAE=gallic acid equivalents; d.m.=dried material; n.d.=not determined

All of the tested formulations originate from a plant that is used traditionally as an anti-emetic remedy, culinary spice and to increase the appetite, as well as in animal feed to increase growth. They also contain compounds that are generally recognized as safe or are approved as food additives by the FDA (*8*), which is why they have a good potential to be exploited as natural antimicrobial and antioxidative preservatives in food products. However, more research needs to be done to evaluate their sensorial impacts, activity in the real food model (*28*), and to prove that they do not cause any negative side-effects, like allergic reactions and irritations (*8*).

Conclusions

Alpinia katsumadai seed phenolic extract, essential oil and post-distillation extract showed moderate antimicrobial activity against *Campylobacter jejuni*, while they were less efficient against *Staphylococcus aureus*. However, all tested plant formulations were confirmed as good modulators of *C. jejuni* and *S. aureus* resistance against vari-

ous antimicrobials, including antibiotics and bile salts. Modulatory activity of *A. katsumadai* essential oil was confirmed to be at least partly due to the efflux pump inhibition, when tested with ethidium bromide accumulation assay. All tested plant formulations have a potential to be used as natural preservatives and functional additives in food products.

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2.1.4 Antibiotic resistance modulation and modes of action of (-)-α-pinene in *Campylobacter jejuni*

Modulacija antibiotske odpornosti in načini delovanja (-)-α-pinena pri bakterijah *Campylobacter jejuni*

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The aim of the study was to investigate the mode of action of (-)-α-pinene in terms of its modulation of antibiotic resistance in *Campylobacter jejuni.* Broth microdilution and ethidium bromide accumulation assays were used to evaluate the (-)-α-pinene antimicrobial activity, modulation of antimicrobial resistance, and inhibition of antimicrobial efflux. The target antimicrobial efflux systems were identified using an insertion mutagenesis approach, and *C. jejuni* adaptation to (-)-α-pinene was evaluated using DNA microarrays. Knock-out mutants of the key up-regulated transcriptional regulators *hspR* and *hrcA* were constructed to investigate their roles in *C. jejuni* adaptation to several stress factors, including osmolytes, and pH, using Biolog phenotypical microarrays. Our data demonstrate that (-)-α-pinene efficiently modulates antibiotic resistance in *C. jejuni* by decreasing the minimum inhibitory concentrations of ciprofloxacin, erythromycin and triclosan by up to 512-fold. Furthermore, (-)-α-pinene promotes increased expression of *cmeABC* and another putative antimicrobial efflux gene, *Cj1687*. The ethidium bromide accumulation was greater in the wild-type strain than in the antimicrobial efflux mutant strains, which indicates that these antimicrobial efflux systems are a target of action of (-)-α-pinene. Additionally, (-)-α-pinene decreases membrane integrity, which suggests that enhanced microbial influx is a secondary mode of action of (-)-α-pinene. Transcriptomic analysis indicated that (-)-α-pinene disrupts multiple metabolic pathways, and particularly those involved in heat-shock responses. Thus, (-)-α-pinene has significant activity in the modulation of antibiotic resistance in *C. jejuni*, which appears to be mediated by multiple mechanisms that include inhibition of microbial efflux, decreased membrane integrity, and metabolic disruption. These data warrant further studies on (-)-α-pinene to develop its use in the control of antibiotic resistance in *Campylobacter*.

Antibiotic resistance modulation and modes of action of (-)-α-pinene in *Campylobacter jejuni*

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Abstract

The aim of the study was to investigate the mode of action of (-)-α-pinene in terms of its modulation of antibiotic resistance in *Campylobacter jejuni*. Broth microdilution and ethidium bromide accumulation assays were used to evaluate the (-)-α-pinene antimicrobial activity, modulation of antimicrobial resistance, and inhibition of antimicrobial efflux. The target antimicrobial efflux systems were identified using an insertion mutagenesis approach, and *C. jejuni* adaptation to (-)-α-pinene was evaluated using DNA microarrays. Knock-out mutants of the key up-regulated transcriptional regulators *hspR* and *hrcA* were constructed to investigate their roles in *C. jejuni* adaptation to several stress factors, including osmolytes, and pH, using Biolog phenotypical microarrays. Our data demonstrate that (-)-α-pinene efficiently modulates antibiotic resistance in *C. jejuni* by decreasing the minimum inhibitory concentrations of ciprofloxacin, erythromycin and triclosan by up to 512-fold. Furthermore, (-)-α-pinene promotes increased expression of *cmeABC* and another putative antimicrobial efflux gene, *Cj1687*. The ethidium bromide accumulation was greater in the wild-type strain than in the antimicrobial efflux mutant strains, which indicates that these antimicrobial efflux systems are a target of action of (-)-α-pinene. Additionally, (-)-α-pinene decreases membrane integrity, which suggests that enhanced microbial influx is a secondary mode of action of (-)-α-pinene. Transcriptomic analysis indicated that (-)-α-pinene disrupts multiple metabolic pathways, and particularly those involved in heat-shock responses. Thus, (-)-α-pinene has significant activity in the modulation of antibiotic resistance in *C. jejuni*, which appears to be mediated by multiple mechanisms that include inhibition of microbial efflux, decreased membrane integrity, and metabolic disruption. These data warrant further studies on (-)-α-pinene to develop its use in the control of antibiotic resistance in *Campylobacter*.

Keywords: *Campylobacter jejuni*, microbial efflux inhibitor, adaptation, microbial resistance

Introduction

The increasing incidence of bacterial pathogens that show antibiotic resistance has led to impaired efficacy of clinical treatments, prolonged illness and greater mortality. This rising antimicrobial resistance has been accompanied by a decline in new antibiotic discovery over the last few decades, which now poses a serious threat to public health. In Europe it has been estimated that 25,000 patients die annually due to incurable infections with multidrug-resistant (MDR) bacteria, and that the healthcare system is burdened with at least an additional 1.5 billion euros each year because of the prolonged treatments that are needed for infections with MDR bacteria [1]. *Campylobacter jejuni* is the most prevalent bacterial cause of gastroenteritis, and it shows high rates of ciprofloxacin resistance; as such, *C. jejuni* represents an important part of this healthcare burden [2, 3]. Drug resistant *Campylobacter* are on the CDC list of serious threats in the U.S., which further indicates the importance of *Campylobacter* in public health [4]. *Campylobacter* resistance to quinolone antibiotics is especially problematic, because quinolone resistance has increased globally and tends to spread clonally [5].

One of the major mechanisms that contributes to the resistance of MDR bacteria is enhanced antimicrobial efflux, which extrudes antimicrobials of out of bacterial cells with broad specificity. The most important antimicrobial efflux pump in *C. jejuni* is CmeABC, while CmeDEF and CmeG have secondary roles [6-8]. Alone or in combination with specific point mutations and antibiotic resistance genes, these antimicrobial efflux pumps provide increased resistance to clinically important classes of antibiotics.

To restore the activity of antibiotics that are already available on the market, research has been devoted to finding new compounds with activities that can be used to inhibit antimicrobial efflux in bacteria [9-12]. However, to date, no inhibitors of these antimicrobial

efflux pumps have been licensed for clinical use, although some drugs, such as the calcium ion influx inhibitor verapamil that is licensed for arrhythmia treatment, also show inhibitory activity against antimicrobial efflux. Also, some new natural products have recently been identified for potential use as antimicrobial efflux pump inhibitors, based on findings from *in-vitro* studies [13-17].

One of these natural compounds that has been shown to have antimicrobial activity against various microorganisms is the monoterpene α-pinene, which is naturally present in various essential oils [18]. α-Pinene is also one of the constituents of an essential oil from *Alpinia katsumadai* seeds (J. Kovač, unpublished data), which we have shown to have modulatory activity towards antimicrobial resistance in *C. jejuni* and *Staphylococcus aureus* [10, 13]. α-Pinene exists naturally as both (+)-α-pinene and (-)-α-pinene [11]. (-)-α-Pinene was investigated here, in terms of its activity as an antimicrobial, its modulation of antimicrobial resistance, and its inhibition of antimicrobial efflux, using antibiotic-susceptible and antibiotic-resistant *C. jejuni* isolates from different sources. Furthermore, the *C. jejuni* responses to treatment with (-)-α-pinene were studied using transcriptomic and phenotypic microarray approaches.

Materials and Methods

Chemicals

Erythromycin, ciprofloxacin, ethidium bromide (EtBr), carbonyl cyanide-m-chlorophenylhydrazone (CCCP), reserpine, (-)-α-pinene, resazurin sodium salt, and menadion were from Sigma-Aldrich Chemie (Steinheim, Germany), triclosan and chloramphenicol were from Calbiochem (Merck KGaA, Darmstadt, Germany), ampicillin was from Roche Diagnostics (Mannheim, Germany), and kanamycin was from Merck (Darmstadt, Germany).

96

97 Bacterial strains and growth conditions

98 Frozen stocks (from -80 °C storage) of the *C. jejuni* strains listed in Supporting Information
99 S1 Table were cultured on selective Karmali agar and Mueller-Hinton agar (Oxoid,
100 Hampshire, UK), or in Mueller-Hinton broth (Oxoid), and incubated at 42 °C under
101 microaerobic conditions (5% O_2, 10% CO_2, in N_2). *Escherichia coli* were cultured on Luria-
102 Bertani agar (Oxoid) at 37 °C. When needed, the Mueller-Hinton agar was supplemented with
103 kanamycin (30 mg/L) or chloramphenicol (4 mg/L), and the Luria-Bertani agar was
104 supplemented with ampicillin (50 mg/L).

105

106 Antimicrobial and resistance-modulation assays

107 The antimicrobial activity of (-)-α-pinene was determined on 17 *C. jejuni* strains and two *C.*
108 *jejuni* mutants with knocked-out antimicrobial efflux genes (Δ*cmeB* and Δ*Cj1687*), using the
109 broth microdilution method. Briefly, the tested compounds were dissolved in
110 dimethylsulphoxide (final concentration, 2.5%) and serially diluted in Mueller-Hinton broth
111 in black microtitre plates, and the *C. jejuni* cultures were added at a concentration of 5×10^5
112 CFU/mL, to the final volume of 0.1 mL/well. After 24 h incubation at 42 °C under
113 microaerobic conditions, 10 µL resazurin reagent was added to each well, which consisted of
114 10 mM tetrazolium salts and 0.8 mM menadion. Following a 2-h incubation at 42 °C, the
115 fluorescence intensity was measured at 550 nm and 959 nm, using a microplate reader (Tecan,
116 Mannedorf/Zurich, Switzerland) [19]. The minimum inhibitory concentrations (MICs) were
117 defined as the minimal concentrations at which the fluorescence signal declined to the level of
118 the blank. Modulation of antimicrobial resistance was evaluated using the same method, for
119 nine *C. jejuni* strains and two knocked-out antimicrobial efflux mutants (Δ*cmeB* and

120 Δ*Cj1687*), except that the medium was supplemented with subinhibitory concentrations of (-)-
121 α-pinene (62.5 mg/L or 125 mg/L) for the determination of the MICs of ciprofloxacin,
122 erythromycin, triclosan, and ethidium bromide. The (-)-α-pinene modulation factors were
123 defined as the ratios of the MICs for the antimicrobial alone and for the antimicrobial in the
124 presence of (-)-α-pinene. A modulation factor >2 was set as the cut-off for biologically
125 significant resistance modulation [14].
126

127 **Membrane integrity**

128 The influence of 62.5 mg/L and 125 mg/L (-)-α-pinene on membrane integrity of *C. jejuni*
129 NCTC 11168 was followed using LIVE/DEAD BacLight Bacterial Viability kits (L-7012;
130 Molecular Probes, Eugene, Oregon, USA) [20]. A mixture of the green fluorescent dye SYTO
131 9 and propidium iodide was prepared and used according to the manufacturer instructions
132 (Molecular Probes). This dye mixture was added to 100 µL *C. jejuni* cultures (OD_{600}, 0.2; 1:1,
133 v/v) that were untreated or treated with (-)-α-pinene. The kinetics of propidium iodide
134 intracellular penetration were followed by measuring the relative fluorescence units (RFUs) in
135 60-s intervals over 1 h, in terms of the SYTO 9 fluorescence at 481 nm and 510 nm in a
136 microplate reader (Tecan). Two independent experiments were carried out in triplicate in
137 black microtitre plates. The membrane disruption (%) was calculated from the kinetics
138 measurements of the treated relative to the untreated cultures over the last 10 min of the
139 assay.
140

141 **Ethidium bromide accumulation**

142 The influence of 62.5 mg/L and 125 mg/L (-)-α-pinene on EtBr accumulation in *C. jejuni*
143 NCTC 11168, Δ*cmeB* and Δ*Cj1687* was determined. Further screening was carried out with

62.5 mg/L (-)-α-pinene on 16 *C. jejuni* isolates (Supporting Information S1 Table), as previously described [13]. Briefly, exponential phase cultures were washed and resuspended in phosphate-buffered saline (OD_{600}, 0.2). After a 10-min incubation at 37 °C, the efflux pump inhibitors were added to 100 µL cultures in black microtitre plates, and incubated for 10 min at 37 °C before adding EtBr to a final the concentration of 0.5 mg/L. The kinetics of intracellular EtBr accumulation were measured at 500 nm and 608 nm using a Tecan microplate reader, at 45-s intervals for 1 h. In parallel to the (-)-α-pinene treatments, the reference efflux pump inhibitors CCCP (10 mg/L) and reserpine (100 mg/L) were tested. The selected concentrations of the efflux pump inhibitors had no inhibitory effects on bacterial growth. Other EPIs, such as verapamil, NMP, and PaβN were also tested in the preliminary experiments, but they were excluded from further studies due to their low activity (data not shown). Measurements were carried out in triplicate and the means of the last 10 measurements were used in the statistical analysis.

Transcriptional response to (-)-α-pinene

The influence of (-)-α-pinene on gene expression in *C. jejuni* NCTC 11168 was determined using DNA microarrays and qRT-PCR. Exponential phase cultures were adjusted to OD_{600} 0.2 in Mueller-Hinton broth. Five millilitres of culture were treated with 62.5 mg/L (-)-α-pinene dissolved in dimethylsulphoxide. Only dimethylsulphoxide (0.048%) was added to the untreated samples. The cultures were incubated for 2 h, microaerobically and with shaking (160 rpm), at 42 °C. Experiments were carried out as four biological replicates. RNA Protect Bacteria reagent (Qiagen, Maryland, USA) was added to the cultures, and the total RNA was isolated using RNeasy mini kits (Qiagen), and treated with Ambion Turbo DNA-*free* kits (Invitrogen, USA).

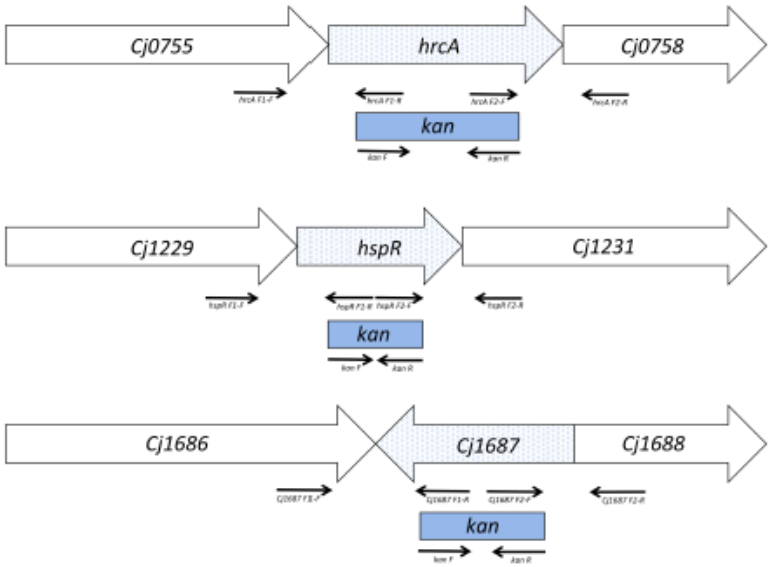
168 Microarrays with 4,751 probes that targeted 1,756 genes that are specific to *C. jejuni*
169 NCTC 11168 (Mycroarray, Biodiscovery-LLC, MI, USA) were used for the gene-expression
170 analysis, as described previously [21]. The cDNA was synthesised with random hexamers,
171 SuperScript III Reverse Transcriptase, and aminoallyl dUTP (all supplied by Invitrogen,
172 USA) and labelled with a monofunctional NHS-ester dye, as Cy3 or Cy5 (GE Healthcare,
173 Buckinghamshire, UK). The DNA concentration and the labelling efficiency were determined
174 spectrophotometrically (NanoDrop 1000; Thermo Scientific, Waltham, MA, USA). Four
175 biological replicates were hybridised to four microarrays, according to the manufacturer
176 protocol, and incubated for 24 h at 42 °C, and scanned at 550 nm for Cy3 and 650 nm for Cy
177 5, using a microarray scanner (GenePix 4100A; Molecular Devices, Sunnyvale, CA, USA), to
178 5 µm resolution. The fluorescence intensities were converted to digital signals using the
179 GenepixPro 7.0 programme (MolecularDevices, Sunnyvale, CA, USA). The microarray data
180 was deposited with the NCBI Gene Expression Omnibus, under the accession number
181 GSE59879, GPL19011.
182 The expression of four genes was confirmed by quantitative real-time PCR using a
183 real-time PCR detection system (ABI 7500; Applied Biosystems, Thermo Scientific,
184 Waltham, MA, USA) with Universal KAPA SYBR One-Step qRT-PCR kits (Kapa
185 Biosystems, Boston, USA) and the same RNA template as in the microarray experiments.
186 Quantitative RT-PCR primers (Supplementary Information S2 Table) were designed using the
187 online tool Primer3 (http://frodo.wi.mit.edu/). Standard quantification curves were generated
188 for each of the RNA templates, using 10-fold dilution series between 25 ng/µL and 0.0025
189 ng/µL. Quantitation was performed in 15 µL reactions with three technical replicates of three
190 biological replicates, according to the following programme: 10 min at 50 °C, 5 min at 60 °C,
191 3 min at 95 °C and 40 cycles of 10 s at 95 °C and 30 s at 58 °C, including the melt curve
192 analysis performed after amplification. Here, 16S rRNA was used for the internal

193 normalisation standard for the calculation of the relative fold-change in gene expression, by
194 the comparative threshold cycle method (ΔΔCt) [22].
195

196 **Construction of insertional mutants**

197 Three *C. jejuni* NCTC 11168 knock-out mutants with disrupted open reading frames of the
198 *hspR, hrcA* and *Cj1687* genes were constructed (Supporting Information S2 Table) using an
199 insertion mutagenesis approach. The internal fragments of the genes were replaced with the
200 kanamycin resistance cassette *aphA-3* (Fig. 1). The PCR products used for construction of
201 these mutants were amplified with GoTaq polymerase (Promega Corporation, Madison, WI,
202 USA) using the primers listed in Supporting Information S2 Table. Following amplification,
203 these were purified using a Wizard SV gel and PCR clean-up system (Promega), and digested
204 with HindIII or PstI (Thermo Scientific, Waltham, MA, USA), or both. The restricted PCR
205 products were incubated at 80 °C, to deactivate the restriction enzymes, ligated using Quick
206 ligation kits (New England Biolabs, Ipswich, MA, USA), and cloned into pGEM-T Easy
207 Vectors using T4 DNA ligase (Promega). The plasmid was amplified in competent *E. coli*
208 DH5α, isolated using GenElute Plasmid Miniprep kits (Sigma, St. Luis, MO, USA), and
209 transformed into *C. jejuni* NCTC 11168 by natural transformation. The transformants with the
210 insert were selected on Mueller-Hinton agar supplemented with kanamycin (30 mg/L), and
211 confirmed with PCR (Supporting Information S2 Table).

10



212

213 **Fig. 1. Scheme of the insertional mutagenesis strategies (not to scale).** Box arrows, target
214 open reading frames; solid arrows, primers used for DNA fragment amplification (from left to
215 right: F1-F, F1-R, F2-F, F2-R, specific for each individual mutant, as listed in Supporting
216 Information S2 Table); blue dots, target gene; blue rectangles, deleted regions substituted with
217 a kanamycin cassette (*kan*).

218

219 Metabolic response to inactivated genes

220 *C. jejuni* NCTC 11168 and the Δ*hspR* and Δ*hrcA* mutants were prepared in IF-0a inoculating
221 fluid (Biolog, Hayward, CA, USA), mixed with redox indicator Dye D, according to the
222 manufacturer instructions, and dispensed (100 µL/well) using carbon utilisation, osmolality,
223 and pH phenotypic plates PM1, PM2A, PM9 and PM10 (Biolog). The PM plates were placed
224 in plastic bags together with microaerophilic atmosphere generating sachets (Oxoid), sealed

and fixated in the automatic plate reader (Omnilog; Biolog) trays using adhesive tape. Plates were incubated in automatic plate reader at 42 °C and the metabolism of different carbon sources, growth on different osmolytes and at different pHs was followed spectrophotometrically every 15 min for 48 h. The endpoint values were used in the comparative statistical analysis.

Statistical analysis

The statistical significances of the data for the antimicrobial actions, modulation of antimicrobial resistance, and EtBr accumulation were determined using one-way ANOVA with appropriate *post-hoc* tests, using the SPSS software, version 21 **(IBM Corp., Armonk, NY, USA)**. Analysis of the Biolog phenotypic microarray data was carried out using MS Excel and Student's t-tests, and analysis of the DNA microarray data was performed with the R software using the LIMMA package, as previously described [21, 23]. Statistical significance was set at $p < 0.05$, and the cut-off for significance of the relative fold-changes in the modulation of antimicrobial resistance set at >2, the gene expression between untreated and treated cultures set at $\geqslant 2$, and in the growth on phenotypic plates set at $\geqslant 1.5$.

Results

Antimicrobial activity and modulation of antimicrobial resistance of (-)-α-pinene

The antimicrobial activity of (-)-α-pinene was determined on 16 strains of *C. jejuni* (Supporting Information S1 Table). The MICs of (-)-α-pinene were 1000 mg/L or higher in all

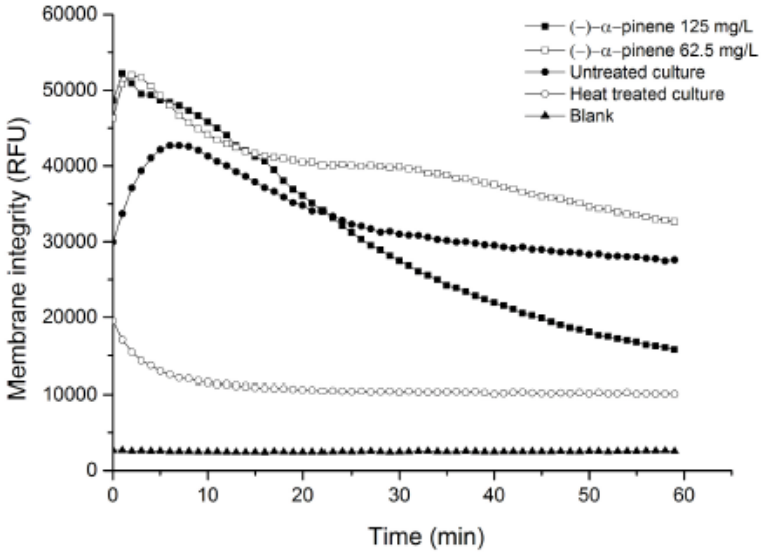
of the tested strains, including those tested in the antimicrobial-resistance-modulation assay (Table 1). These high MICs confirmed the insignificant antimicrobial activity of (-)-α-pinene. As a potential modulator of antimicrobial resistance, (-)-α-pinene was tested at 62.5 mg/L and 125 mg/L in combination with the antibiotics ciprofloxacin and erythromycin, the disinfectant triclosan, and the antimicrobial efflux pump substrate EtBr, on the *C. jejuni* NCTC 11168 reference strain. At 62.5 mg/L, (-)-α-pinene decreased the MICs of these antimicrobials by up to 2-fold. However, at 125 mg/L, (-)-α-pinene reduced the MICs of the various antimicrobials from 32-fold to over 512-fold (Table 1).

Due to the significantly stronger modulation of antimicrobial resistance of (-)-α-pinene at 125 mg/L, this higher concentration was further tested on eight other *C. jejuni* isolates and two mutants with inactivated antimicrobial efflux genes, Δ*cmeB* and Δ*Cj1687* (Table 1). These data indicated pronounced modulation of antimicrobial resistance by (-)-α-pinene with all of these strains for most of the antimicrobials tested. Significant MIC reductions were produced also in the Δ*cmeB* and Δ*Cj1687* mutants derived from the wild type NCTC 11168, which suggest that this modulatory activity of 125 mg/L (-)-α-pinene is not dependent on its inhibition of antimicrobial efflux.

Influence of (-)-α-pinene on membrane integrity

As for the modulation of antimicrobial resistance, both 62.5 mg/L and 125 mg/L (-)-α-pinene were tested for their influence on membrane integrity in *C. jejuni*, to determine whether membrane permeability is the main mechanism of its modulation of antimicrobial resistance. The membrane integrity of cultures treated with 62.5 mg/L (-)-α-pinene was 20% higher than that of the untreated cultures after 1-h treatment, while at 125 mg/L (-)-α-pinene, the membrane integrity was decreased by 39% (Fig. 2). Hence, at this higher concentration of 125 mg/L (-)-α-pinene, the consequent disruption of the membranes is likely to have contributed

272 to its modulation of antimicrobial resistance. Cultures incubated at 80 °C for 15 min were
273 used as a positive control for disrupted membranes, and these showed 64% decreased
274 membrane integrity, compared to the untreated control cultures. These differences were
275 calculated based on the kinetics measurements over the last 10 min of the 1-h assays, and they
276 were statistically significant ($p < 0.0001$). The disruptive impact of (-)-α-pinene on membrane
277 integrity is not surprising, as it is known that monoterpenes can cause alterations in membrane
278 permeability [24].



279

280 **Fig. 2. Influence of (-)-α-pinene on membrane integrity of *C. jejuni* NCTC 11168.**
281 Membrane integrity of wild-type *C. jejuni* NCTC 11168 cultures, untreated culture, culture
282 treated with 125 mg/L (-)-α-pinene or 62.5 mg/L (-)-α-pinene, heat treated culture and blank.

283

Inhibition of antimicrobial efflux by (-)-α-pinene

Again, both 62.5 mg/L and 125 mg/L (-)-α-pinene were tested for their impact on intracellular EtBr accumulation in *C. jejuni* NCTC 11168 and the two mutants (Δ*cmeB* and Δ*Cj1687*). Compared to the non-treated control, both of these concentrations of (-)-α-pinene increased the EtBr accumulation; however, 62.5 mg/L (-)-α-pinene resulted in greater EtBr accumulation (Fig. 3). As 62.5 mg/L (-)-α-pinene did not have a negative impact on membrane integrity, this was used in the further screening on a larger set of 16 isolates (Supporting Information S1 Table). This enabled the investigation of only the inhibitory effects of (-)-α-pinene on antimicrobial efflux. Here, there was increased EtBr accumulation in all of these 16 tested strains when 62.5 mg/L (-)-α-pinene was added (Fig. 4). This (-)-α-pinene concentration increased the EtBr accumulation significantly more than seen for CCCP and reserpine in all of these tested strains, which confirms its inhibitory activity against antimicrobial efflux. Here it is important to note that (-)-α-pinene was tested at a lower concentration (62.5 mg/L) than reserpine (100 mg/L), and at a higher concentration than CCCP (10 mg/L), to avoid the toxic effects of CCCP.

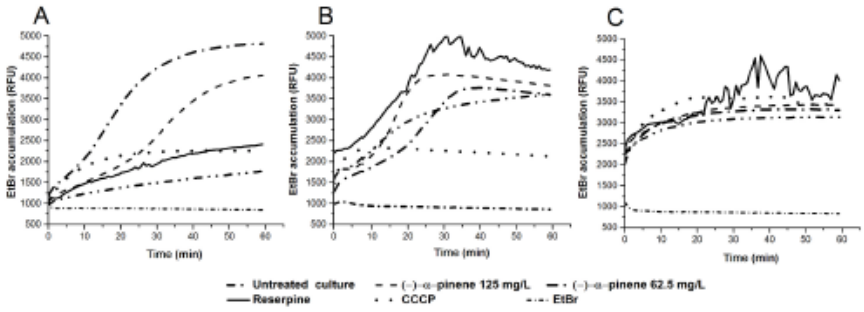
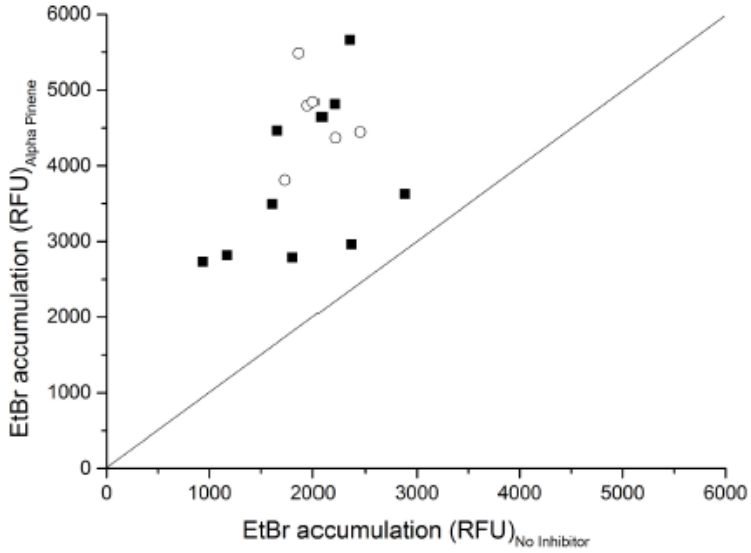


Fig. 3. Influence of (-)-α-pinene, CCCP and reserpine on ethidium bromide accumulation. (A) Reference strain *C. jejuni* NCTC 11168, (B) *C. jejuni* NCTC 11168 ΔcmeB knock-out mutant, (C) *C. jejuni* NCTC 11168 Δ*Cj1687* knock-out mutant.



303

304 **Fig. 4. Influence of (-)-α-pinene on EtBr accumulation in the 16 *C. jejuni* strains.**

305 Relationship between EtBr accumulation in untreated cultures and in cultures treated with
306 62.5 mg/L (-)-α-pinene. Each symbol represents a single isolate. Solid squares, antibiotic
307 resistant strains (resistant to three or more unrelated antibiotics); open circles, antibiotic
308 sensitive strains.

309

310 To identify the target efflux system of (-)-α-pinene, EtBr accumulation in the two
311 mutants in the antimicrobial efflux genes was investigated. In comparison with the control
312 wild-type *C. jejuni* NCTC 11168, the increase in EtBr accumulation with 62.5 mg/L (-)-α-
313 pinene was significantly lower in the Δ*cmeB* and Δ*Cj1687* mutants (Fig. 3), although this
314 remained above the EtBr accumulation in the wild-type. This finding suggests that (-)-α-
315 pinene inhibits both of these antimicrobial efflux systems.

316

Transcriptional response to (-)-α-pinene

After a 2-h treatment with 62.5 mg/L (-)-α-pinene, 128 genes of *C. jejuni* NCTC 11168 were differentially expressed (with the cut-off set at ≥2-fold difference; Table 2 and Supporting Information S3 Table). Of those, 108 were up-regulated, and 20 were down-regulated. The Clusters of Orthologous Groups functional classification of these differentially expressed genes revealed that the up-regulated genes were mainly involved in amino-acid transport and metabolism (12 genes), translation (12 genes), inorganic ion transport and metabolism (8 genes), posttranslational modification and chaperones (8 genes), and unknown functions (36 genes, which included 'poorly characterised', 'function unknown', 'general function prediction only'). The down-regulated genes were scattered into 12 of the functional categories (Table 2). Interestingly, however, the most strongly up-regulated genes (>10-fold) fell into the groups of posttranslational modification and chaperones, and transcription, which included the chaperone-encoding genes *grpE*, *dnaK*, and *clpB*, and the heat-shock regulator *hrcA*. In these two functional categories, another two heat-shock-response genes that encode the chaperones *groES* and *cbpA* and the transcriptional repressor *hspR* were also up-regulated, although to lower levels (2-fold to 4-fold). As well as these heat-shock-response genes, the antimicrobial efflux pump genes *cmeABC* were up-regulated by 2.7-fold to 2.2-fold, respectively. Another, as yet uncharacterised gene that encodes a putative antimicrobial efflux protein, *Cj1687*, was 2.5-fold up-regulated after the treatment with (-)-α-pinene. The *omp50* gene that was previously identified as an outer membrane protein and a phosphotyrosine kinase [25] was 4.9-fold up-regulated here. Several ribosomal genes (*rps, rpl*) and genes involved in purine biosynthesis (*purM, apt, purD*), glutamate metabolism (*murI, metE, metF, glnA, gltBD*), and iron–sulphur homeostasis (*chuAD, tonB3, tonB2*) were expressed at higher levels (2.1-fold to 8.1-fold up-regulated) after the treatment with (-)-α-pinene. On the other hand, aspartate-ammonia lyase (*aspA*) was the most strongly down-regulated (4.6-fold)

342 metabolic gene, together with the genes that encode succinate dehydrogenase (*sdhA, sdhC*),
343 fumarate hydratase (*fumC*), and anaerobic C4-dicarboxylate antiporter *dcuA,* which were up
344 to 3.2-fold down-regulated following the treatment with (-)-α-pinene.
345

346 ## Metabolic response to inactivated genes

347 The *hspR, hrcA* mutants and wild-type *C. jejuni* NCTC 11168 were grown on Biolog
348 phenotype MicroArrays with different carbon sources and in presence of different osmolytes
349 and pH, to investigate their roles in adaptation to these stresses. The growth of the mutants
350 was compared to the growth of the wild-type strain (Supporting Information S4 Table). The
351 *hspR* mutant grew better on some of the osmolytes, such as 6% NaCl with KCl, sodium
352 sulphate, sodium formate, sodium lactate, and sodium phosphate, while growth of the *hrcA*
353 mutant, on the other hand, was inhibited by these and several of the other osmolytes. The
354 *hrcA* mutant did not use a wide range of the carbon sources as efficiently as the wild-type.
355

356 # Discussion

357 (-)-α-pinene has negligible anti-*Campylobacter* activity, with MICs of 1000 mg/L or higher
358 against all of the tested strains. Unlike (+)-α-pinene, this (-)-α-pinene enantiomer was also
359 previously reported to have no inhibitory effects in fungi and with *S. aureus* [11]. Although (-
360)-α-pinene did not inactivate *C. jejuni* growth, it showed remarkable modulatory activity of
361 antimicrobial resistance when applied in combination with ciprofloxacin, erythromycin,
362 triclosan and ethidium bromide. We observed concentration-dependent activity of (-)-α-
363 pinene here, and investigated the mechanisms of its action at 62.5 mg/L and 125 mg/L.

364 Although the modulation of antimicrobial resistance was substantially more pronounced at
365 125 mg/L (-)-α-pinene, the EtBr accumulation remained at similarly high levels.

366 These data suggest the involvement of at least two different modes of action. Indeed,
367 investigation of the membrane integrity revealed increased membrane permeability with 125
368 mg/L (-)-α-pinene. At this concentration of (-)-α-pinene, the synergistic effects of increased
369 membrane permeability and intracellular accumulation due to the inhibition of antimicrobial
370 efflux provided the best modulation of antimicrobial resistance.

371 Previous reports have demonstrated the modulation of antimicrobial resistance and
372 efflux of the diarylheptanoid and flavonoid constituents of *A. katsumadai* seeds in
373 *Mycobacterium smegmatis* at comparable concentrations, although its terpene compounds
374 were studied only as a mixture, with the essential oil at significantly higher concentrations
375 [13, 14]. Inhibition of antimicrobial efflux was also previously achieved in *M. smegmatis* and
376 *S. aureus*, with paradol-related and gingerol-related compounds isolated from *Aframomum*
377 *melegueta*, and with a novel antibacterial natural product that was isolated from *Hypericum*
378 *olympicum* L. cf. *uniflorum*, respectively [15, 17].

379 To determine the antimicrobial efflux system that (-)-α-pinene targets, we carried out
380 comparative accumulation assays in the wild-type and with the mutant strains with inactivated
381 antimicrobial efflux genes, Δ*cmeB* and Δ*cj1687*. In the presence of (-)-α-pinene, the decrease
382 in EtBr accumulation almost to the level of the untreated culture in Δ*cmeB* and Δ*Cj1687*
383 suggests that these two efflux proteins are the likely the targets of (-)-α-pinene.

384 To date, (-)-α-pinene is the first plant-derived compound that shown inhibition of
385 antimicrobial efflux in *Campylobacter* to be described and characterized. Cytotoxicity of α-
386 pinene was previously investigated with the HeLa and Cos7 cell lines, where IC_{50} values of
387 357.9 mg/L and 337.5 mg/L were reported, respectively [26]. As these IC_{50} values for α-
388 pinene are more than 2-fold greater than those used in the present modulation assays, and

more than 5-fold greater than those used in the present EtBr accumulation assays, (-)-α-pinene has the potential to be further investigated as a means for the control of antibiotic resistant *C. jejuni*.

To explore the *C. jejuni* responses to (-)-α-pinene, we analysed the changes in the *C. jejuni* transcriptome before and after the 2-h (-)-α-pinene treatment. Here, we observed increases in the expression of the *cmeABC* and *Cj1687* antimicrobial efflux genes that were identified as potential targets for antimicrobial efflux inhibition, which confirms the increased need for CmeABC and Cj1687 activity after inhibition by (-)-α-pinene. The involvement of (-)-α-pinene in alterations to membrane permeability, as a complementary mechanism of its action, was also indicated by the increased expression of the outer membrane protein product of the *omp50* gene. This protein was previously shown to modulate phosphorylation of proteins involved in capsule production [25]. Up-regulation of *omp50* appears to be a strategy for bacterial adaptation, as *opm50* is likely to enhance the production of surface polysaccharides, and thereby to contribute to decreased membrane permeability and decreased antimicrobial influx [25].

Similar responses of *C. jejuni* were also observed previously after exposure to erythromycin, which like (-)-α-pinene, is a hydrophobic compound [27]. Furthermore, treatment of *C. jejuni* with (-)-α-pinene evoked increased expression of ribosomal and purine biosynthesis genes, which indicated that there were metabolic changes, and thereby the associated need for increased protein synthesis. Previous reports have indicated similar transcriptional changes in *C. jejuni* in response to stomach acid, which evoked up-regulation of *purB* [28]. The product of the up-regulated glutamate synthetase gene (*gltBD*) catalyses the condensation of glutamate, which is involved in osmoadaptation and can be further converted to aspartate. Then, as well as coding for heat-shock proteins, up-regulation of the *gltD* and *glnA* genes was previously reported in *C. jejuni* in response to salt stress [29]. Aspartate-

414 ammonia lyase (*aspA*), which is the most strongly down-regulated gene associated with
415 energy metabolism, cleaves carbon–nitrogen bonds and catalyses the conversion of L-
416 aspartate into fumarate and ammonia. Additionally, down-regulation of succinate
417 dehydrogenase genes (*sdhA, sdh*) contributes to inhibition of fumarate biosynthesis,
418 obstruction of oxidative energy metabolism, and conversion of fumarate to malate by
419 fumarate hydratase (*fumC*). Metabolic changes and an imbalance in Krebs cycle intermediates
420 were also reflected in the down-regulation of *fumC* and the anaerobic C4-dicarboxylate
421 antiporter gene *dcuA*. Up-regulation of iron–sulphur homeostasis genes, such as *chuAD*,
422 *tonB3* and *tonB2*, suggested an increase in the transport of the metal ions and siderophores
423 that are involved in respiration and DNA synthesis.

424 The changes in gene expression caused by (-)-α-pinene are strikingly similar to those
425 that have previously been observed in a mutant with a defective transcriptional regulator
426 *hspR*, which showed highly up-regulated heat-shock-related genes: *grpE, dnaK, clpB* and
427 *hrcA* [30]. Similar responses were also observed after treatment with the natural antimicrobial
428 compound benzyl isothiocyanate, which promotes protein aggregation [31]. The simple heat-
429 shock-response model from a study by Holmes et al. (2010) suggested repression of *groES*
430 and *groEL* by *hrcA*, and repression of *dnaK*, *cbpA* and the *clpB* operon by *hspR*, which
431 become derepressed in the event of heat shock, allowing the expression of *hrcA* [30]. HrcA,
432 however, needs the assistance of *groES* and *groEL* for posttranscriptional modifications that
433 enable its activity [30].

434 Characterisation of mutant strains using phenotypic microarrays revealed increased
435 adaptation of Δ*hspR* to increased concentrations of several sodium salts. On the other hand,
436 the growth of the mutant Δ*hrcA* was attenuated in the presence of a wide variety of osmolytes
437 and high pH. The Δ*hrcA* mutant also did not use a wide range of carbon sources, compared to

the wild-type. These data demonstrate that the heat-shock transcriptional regulators are also important for efficient responses to stresses other than high temperatures.

In conclusion, (-)-α-pinene is here confirmed as an efficient modulator of antimicrobial resistance in *C. jejuni*, with at least two different mechanisms that contribute synergistically to this activity. Lower concentrations of (-)-α-pinene show pronounced inhibition of antimicrobial efflux through the targeting of the main efflux system CmeABC and another, as yet uncharacterised, efflux protein, Cj1687. The higher concentration of (-)-α-pinene additionally targeted the membrane, with increased permeability, thereby promoting the influx of antimicrobials. The low antimicrobial activity that we observed appears to be derived from the effective bacterial adaptation to (-)-α-pinene treatment provided by the heat-shock response, and efficient changes in protein synthesis and energy metabolism. Due to the promising modulation of antimicrobial resistance and its previously reported low cytotoxicity, (-)-α-pinene has the potential to be further investigated for the control of antimicrobial resistant in *C. jejuni*.

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542
543

544 **Table 1.** Antimicrobial activity and modulation of antimicrobial resistance of (-)-α-pinene for
545 the different strains of *Campylobacter jejuni*.

C. jejuni strain	MIC (mg/L)												
	AP	CIP			ERY			TC			EtBr		
		-AP	+AP	MF	-AP	+AP	MF	-AP	+AP	MF	-AP	+AP	MF
NCTC 11168*	1000	0.063	0.031	2	0.5	0.25	2	8	4	2	1	1	1
NCTC 11168	1000	0.063	<0.002	>32	0.5	<0.002	>256	8	<0.125	>64	1	<0.008	>128
K49/4	2000	0.063	0.002	32	0.25	<0.002	>128	4	0.25	16	2	0.016	128
53124	1000	4	2	2	0.25	0,063	4	4	4	1	0.5	0.5	1
375/06	2000	4	1	4	0.125	<0.008	>16	4	<0.125	>32	0.25	<0.008	>32
57360	1000	4	<0.008	>512	0.25	<0.002	>128	8	<0.063	>128	0.25	<0.008	>32
58429	1000	4	<0.031	>128	0.25	<0.125	>2	4	<0.125	>32	0.25	<0.063	>4
60089	>2000	8	2	4	0.125	0.063	2	32	32	1	8	8	1
1518/08	2000	8	<0.031	>256	0.125	<0.002	>64	16	<0.031	>512	0.5	<0.008	>64
573/03	2000	16	4	4	0.125	0.063	2	16	<0.063	>256	0.5	<0.008	>64
ΔcmeB	1000	0.016	<0.001	>16	0.063	<0.002	>32	8	<0.063	>128	0.063	<0.008	>8
Δ*Cj1687*	2000	0.063	<0.001	>63	0.125	<0.002	>64	8	<0.063	>128	0.5	<0.008	>64

546
547 MIC, minimal inhibitory concentration; AP, (-)-α-pinene; CIP, ciprofloxacin; ERY:
548 erythromycin; TC, triclosan; EtBr, ethidium bromide; -AP, in the absence of (-)-α-pinene;
549 +AP, with 125 mg/L (-)-α-pinene, MF, modulation factor.
550 * In this case +AP was applied in concentration 62.5 mg/L.
551
552

27

553 **Table 2.** Functional categories of the differentially expressed genes in *Campylobacter jejuni*.

Clusters of orthologous groups category	Up-regulated genes	Down-regulated gens	Differentially expressed genes
Energy production and conversion	2	3	5
Amino acid transport and metabolism	12	1	13
Nucleotide transport and metabolism	5	0	5
Carbohydrate transport and metabolism	1	2	3
Coenzyme transport and metabolism	4	1	5
Lipid transport and metabolism	2	0	2
Translation	12	0	12
Transcription	3	2	5
Replication, recombination and repair	5	0	5
Cell wall/ membrane biogenesis	5	2	7
Cell motility	1	0	1
Posttranslational modification, chaperones	8	3	11
Inorganic ion transport and metabolism	8	1	9
General function prediction only	9	2	11
Function unknown	1	1	2
Signal transduction mechanisms	1	1	2
Defense mechanisms	3	1	4
Poorly characterized	26	0	26
Total	**108**	**20**	**128**

554

555 **S1 Table**. Bacterial strains and plasmids used in the study.

Bacterial strain/ plasmid	Designation	Description	Reference
Bacterial strains	NCTC 11168	*Campylobacter jejuni*, human isolate	NCTC
	ATCC 33560	*Campylobacter jejuni*, animal isolate	ATCC
	K49/4	*Campylobacter jejuni*, meat isolate	5
	58429	*Campylobacter jejuni*, meat isolate	5
	53124	*Campylobacter jejuni*, meat isolate	5
	57360	*Campylobacter jejuni*, meat isolate	5
	60089	*Campylobacter jejuni*, meat isolate	5
	9581	*Campylobacter jejuni*, human isolate	5
	9090	*Campylobacter jejuni*, human isolate	5
	9711	*Campylobacter jejuni*, human isolate	5
	1190/09	*Campylobacter jejuni*, animal isolate	5
	375/06	*Campylobacter jejuni*, human isolate	5
	573/03	*Campylobacter jejuni*, meat isolate	5
	1518/08	*Campylobacter jejuni*, animal isolate	5
	C2	*Campylobacter jejuni*, meat isolate	5
	C33	*Campylobacter jejuni*, meat isolate	5
	816	*Campylobacter jejuni*, water isolate	5
	660/08	*Campylobacter jejuni*, animal isolate	5
	11168 Δ*cmeB*	*NCTC 11168* Δ*cmeB* knock-out mutant	9
	11168 Δ*Cj1687*	*NCTC 11168* Δ*Cj1687* knock-out mutant	This study
	11168 Δ*hspR*	*NCTC 11168* Δ*hspR* knock-out mutant	This study
	11168 Δ*hrcA*	*NCTC 11168* Δ*hrcA* knock-out mutant	This study
	DH5α	*Escherichia coli*, competent strain	Promega
Plasmids	pGEM®-T Easy Vector	Cloning vector, ampiciline resistance	Promega
	pGEMΔ*Cj1687*	Δ*Cj1687* cloned into pGEM®-T Easy Vector	This study
	pGEMΔ*hspR*	Δ*hspR*cloned into pGEM®-T Easy Vector	This study
	pGEMΔ*hrcA*	Δ*hrcA* cloned into pGEM®-T Easy Vector	This study

556

557

558 **S2 Table.** Primers used for mutant construction and qRT-PCR.

Purpose	Primer code	Sequence (5' → 3')	Reference
Species confirmation	CJF	ACTTCTTTATTGCTTGCTGC	9
	CJR	GCCACAACAAGTAAAGAAGC	9
	CCF	GTAAAACCAAAGCTTATCGTG	9
	CCR	TCCAGCAATGTGTGCAATG	9
	CLF	TAGAGAGATAGCAAAAGAGA	9
	CLR	TACACATAATAATCCCACCC	9
	CUF	AATTGAAACTCTTGCTATCC	9
	CUR	TCATACATTTTACCCGAGCT	9
	CFF	GCAAATATAAATGTAAGCGGAGAG	9
	CFR	TGCAGCGGCCCCACCTAT	9
	23SF	TATACCGGTAAGGAGTGCTGGAG	9
	23SR	ATCAATTAACCTTCGAGCACCG	9
Mutant construction	*hrcA* F1-F	ATGCAAGTGGGAGTGGTAGC	This study
	hrcA F1-R	CGCGGATCCCCGAAGCAGGTATGCAAAGGT	This study
	hrcA F2-F	GAACTGCAGTATGGGGCTTAAGGTGGATG	This study
	hrcA F2-R	GCACTTGAACCACTTCACCA	This study
	hspR F1-F	TGGAGGATTTGGAGGTTTTG	This study
	hspR F1-R	CGCGGATCCTTGCCTTAAGGTTTGTGGATG	This study
	hspR F2-F	GAACTGCAGCGGCTAGTAAAGCCGTTGTT	This study
	hspR F2-R	AAAAGCAAAATTCCCAATGC	This study
	Cj1687 F1-F	TCTTTGGCATCTTTGGCTTT	This study
	Cj1687 F1-R	CGCGGATCCTAGCAGGCAGAGCAGATGAA	This study
	Cj1687 F2-F	GAACTGCAGAATCGCCTTAGCTTTGCTTG	This study
	Cj1687 F2-R	TTATCCCTGGAATTCGTCCA	This study
	kan F	CGCGGATCCCGCTTATCAATATATCTATAGAATGG	This study
	kan R	GAACTGCAGGATAATGCTAAGACAATCACTAAAG	This study
qRT-PCR	*dnaK*-F	TGGCGCATCAAGTAGAAAAA	This study
	dnaK-R	TCACGCAAATCATCAAGAGC	This study
	grpE-F	TGAAAAACATGGAGTGGCTCT	This study
	grpE-R	GCACTTGAACCACTTCACCA	This study
	aspA-F	TGGGGAATTGGAAATCTCTG	This study
	aspA-R	CCCTAACAAAGCGAGGAAAA	This study
	dcuA-F	CAACAAGCAGGTGGACTTGA	This study
	dcuA-R	TTGTAAGCAACCACCCACAA	This study

559

560

561 **S3 Table.** Differentially expressed genes in (-)-α-pinene treated *C. jejuni* NCTC 11168.

Gene ID		Functional category	Fold change	
			Microarray	qRT-PCR
Cj0757	*hrcA*	Putative heat shock regulator	25.8	
Cj0509c	*clpB*	ATP-dependent Clp protease ATP-binding subunit	18.3	
Cj0759	*dnaK*	Heat-shock protein	14.7	6.11
Cj0758	*grpE*	Heat-shock protein	11.9	6.95
Cj1373		Putative ntegral membrane protein	8.2	
Cj0699c	*glnA*	Glutamine synthetase	8.1	
Cj0567		Hypothetical protein	8	
Cj0908		Putative periplasmic protein	7.8	
Cj0970		Hypothetical protein	7	
Cj0561c		Putative periplasmic protein	6.8	
Cj1622	*ribD*	Riboflavin-specific deaminase/reductase	6.3	
Cj0790	*purU*	Formyltetrahydrofolate deformylase	6	
Cj0030		Hypothetical protein	5.9	
Cj0037c		Putative cytochrome C	5.5	
Cj0013	*ilvD*	Dihydroxy-acid dehydratase	5.3	
Cj0121		Conserved hypothetical protein	5.3	
Cj1410c		Putative membrane protein	5.2	
Cj0022c		Putative ribosomal pseudouridine synthase	5.1	
Cj0007	*gltB*	Glutamate synthase (NADPH) large subunit	5	
Cj0945c		Putative helicase	4.9	
Cj1170c	*omp50*	50-kDa outer membrane protein precursor	4.9	
Cj0321	*dxs*	L-deoxy-D-xylulose-5-phosphate synthase	4.8	
Cj1001	*rpoD*	RNA polymerase sigma factor (sigma-70)	4.8	
Cj1229	*cbpA*	Putative curved-DNA binding protein	4.7	
Cj1652c	*murI*	Glutamate racemase	4.7	
Cj1103	*csrA*	Carbon storage regulator homolog	4.5	
Cj1230	*hspR*	Heat-shock transcriptional regulator	4.1	
Cj1169c		Putative periplasmic protein	3.9	
Cj0816		Hypothetical protein	3.7	
Cj1250	*purD*	Phosphoribosylamine-glycine ligase	3.7	
Cj0692c		Putative membrane protein	3.4	
Cj1713		Putative radical SAM domain protein	3.4	
Cj0456c		Hypothetical protein	3.2	
Cj0163c		Hypothetical protein	3	
Cj0262c		Putative methyl-accepting chemotaxis signal	3	

Cj0849c		Conserved hypothetical protein	3
Cj1303	*fabH2*	Putative 3-oxoacyl-[acyl-carrier-protein]	3
Cj1584c		Putative peptide ABC-transport system	3
Cj0198c		Helicase-like protein	2.9
Cj0733		Putative HAD-superfamily hydrolase	2.9
Cj1101		ATP-dependent DNA helicase	2.9
Cj1201	*metE*	5-Methyltetrahydropteroyltriglutamate--	2.9
Cj0008		Conserved hypothetical protein	2.8
Cj0009	*gltD*	Glutamate synthase (NADPH) small subunit	2.8
Cj0293	*surE*	Multifunctional protein SurE homolog	2.8
Cj0485		Putative oxidoreductase	2.8
Cj0957c		Hypothetical protein	2.8
Cj0348	*trpB*	Tryptophan synthase beta chain	2.7
Cj0367c	*cmeA*	Periplasmic fusion protein CmeA	2.7
Cj0772c		Putative NLPA family lipoprotein	2.7
Cj1082c	*thiD*	Phosphomethylpyrimidine kinase	2.7
Cj1553c	*hsdM*	Putative type I restriction enzyme M protein	2.7
Cj1614	*chuA*	Haemin uptake system outer membrane receptor	2.7
Cj0927	*apt*	Adenine phosphoribosyltransferase	2.6
Cj1199		Putative iron/ascorbate-dependent	2.6
Cj1242		Hypothetical protein	2.6
Cj0366c	*cmeB*	Inner membrane efflux transporter CmeB	2.5
Cj0494		Putative exporting protein	2.5
Cj1472c		Putative membrane protein	2.5
Cj1630	*tonB2*	Putative TonB transport protein	2.5
Cj1687		Putative efflux protein	2.5
Cj0017c	*dsbI*	Disulphide bond formation protein	2.4
Cj0018c	*dba*	Disulphide bond formation protein	2.4
Cj0391c		Hypothetical protein	2.4
Cj0706		Conserved hypothetical protein	2.4
Cj0989		Putative membrane protein	2.4
Cj1202	*metF*	5.10-Methylenetetrahydrofolate reductase	2.4
Cj1231	*kefB*	Putative glutathione-regulated potassium-efflux	2.4
Cj1255		Putative isomerase	2.4
Cj1479c	*rpsI*	30S ribosomal protein S9	2.4
Cj1529c	*purM*	Phosphoribosylformylglycinamidine cyclo-ligase	2.4
Cj0419		Putative histidine triad (HIT) family protein	2.3
Cj0667		Putative S4 domain protein	2.3
Cj0727		Putative periplasmic solute-binding protein	2.3
Cj0859c		Hypothetical protein	2.3

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Cj0963		Hypothetical protein	2.3
Cj1200		Putative NLPA family lipoprotein	2.3
Cj1298		Putative N-acetyltransferase	2.3
Cj1388		Putative endoribonuclease L-PSP	2.3
Cj1480c	*rplM*	50S ribosomal protein L13	2.3
Cj1659	*p19*	Periplasmic protein p19	2.3
Cj1718c	*leuB*	3-Isopropylmalate dehydrogenase	2.3
Cj0145		Putative TAT (Twin-Arginine Translocation)	2.2
Cj0365c	*cmeC*	Outer membrane channel protein CmeC	2.2
Cj0415		Putative GMC oxidoreductase subunit	2.2
Cj0453	*thiC*	Thiamin biosynthesis protein	2.2
Cj0753c	*tonB3*	TonB transport protein	2.2
Cj1583c		Putative peptide ABC-transport system permease	2.2
Cj1707c	*rplC*	50S ribosomal protein L3	2.2
Cj1708c	*rpsJ*	30S ribosomal protein S10	2.2
Cj1717c	*leuC*	3-Isopropylmalate dehydratase large subunit	2.2
Cj0080		Putative membrane protein. Functional	2.1
Cj0414		Putative oxidoreductase subunit	2.1
Cj0518	*htpG*	Hsp90 family heat shock protein	2.1
Cj0568		Hypothetical protein	2.1
Cj0736		Hypothetical protein	2.1
Cj1070	*rpsF*	30S ribosomal protein S6	2.1
Cj1072	*rpsR*	30S ribosomal protein S18	2.1
Cj1181c	*tsf*	Elongation factor TS	2.1
Cj1617	*chuD*	Putative haemin uptake system periplasmic	2.1
Cj1704c	*rplB*	50S ribosomal protein L2	2.1
Cj1706c	*rplD*	50S ribosomal protein L4	2.1
Cj0146c	*trxB*	Thioredoxin reductase	2
Cj0295		Putative acetyltransferase	2
Cj1071	*ssb*	Single-strand DNA binding protein	2
Cj1220	*groES*	10 kDa chaperonin (cpn10)	2
Cj1658		Putative iron permease	2
Cj1721c		Putative outer membrane protein	2
Cj0360	*glmM*	Phosphoglucosamine mutase	-2
Cj0069		Hypothetical protein	-2
Cj0361	*lspA*	Lipoprotein signal peptidase	-2.1
Cj0362		Putative integral membrane protein	-2.1
Cj1226c		Putative two-component sensor (histidine	-2.1
Cj1227c		Putative two-component regulator	-2.1
Cj1279c		Putative fibronectin domain-containing	-2.1

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Cj1364c	*fumC*	Fumarate hydratase	-2.1	
Cj1131c	*gne*	UDP-GlcNAc/Glc 4-epimerase	-2.2	
Cj0440c		Putative transcriptional regulator	-2.3	
Cj0596	*peb4-cbf2*	Major antigenic peptide PEB-cell binding factor	-2.4	
Cj0597	*fba*	Fructose-bisphosphate aldolase	-2.4	
Cj0950c		Putative lipoprotein	-2.4	
Cj1380		Putative periplasmic protein	-2.5	
Cj1130c	*pglK*	Flippase	-2.6	
Cj0437	*sdhA*	Succinate dehydrogenase flavoprotein subunit	-3.2	
Cj0439	*sdhC*	Putative succinate dehydrogenase subunit C	-3.3	
Cj0088	*dcuA*	Anaerobic C4-dicarboxylate transporter	-3.6	-7.5
Cj0358		Putative cytochrome C551 peroxidase	-3.6	
Cj0087	*aspA*	Aspartate ammonia-lyase	-4.6	-7.1

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564 **S4 Table.** Influence of deactivation of *hspR* and *hrcA* on *Campylobacter jejuni* use of
565 different carbon sources (Biolog PM1 and PM2) and growth in presence of different
566 osmolytes and pH (Biolog PM9 and PM10).

Growth on substrate relative to wild type CJ NCTC 11168	
CJ NCTC 111168Δ*hspR*	**CJ NCTC 111168Δ*hrcA***
↑ Glycerol	↑ L-Mallic acid
↑ Pyruvic acid	↑ 3-0-β-D-Galactopyranosyl-D-arabinose
↑ 1% NaCl	↓ L-arabinose
↑ 6.5% NaCl	↓ Succinic acid
↑ 6% NaCl + creatinine	↓ D-Galactose
↑ 6% NaCl + KCl	↓ L-Aspartic acid
↑ 6% NaCl + glycerol	↓ L-Proline
↑ 6% NaCl + octopine	↓ D-Mannose
↑ 4% Potassium chloride	↓ D-Serine
↑ 2% Sodium sulphate	↓ L-Fucose
↑ 3% Sodium sulphate	↓ D-Glucoronic acid
↑ 1% Sodium formate	↓ D-Xylose
↑ 4% Sodium formate	↓ Formic acid
↑ 5% Sodium formate	↓ L-Glutamic acid
↑ 1% Sodium lactate	↓ D-Glucose-6-phosphate
↑ 9% Sodium lactate	↓ D-Galactonic acid-γ-lactone
↑ 100 mM Sodium phosphate pH 7	↓ D-Robose
↓ Glycolic acid	↓ L-Rhamnose
↓ Glycyl-L-proline	↓ D-Fructose
↓ Tyramine	↓ Acetic acid
↓ D-Lactic acid methyl ester	↓ D-Glucose
↓ 3-0-β-Galactopyranosyl-D-arabinose	↓ L-Asparagine
↓ pH 8	↓ D-Glucosaminic acid
↓ pH 9.5 + cadaverine	↓ Tween 40
	↓ α-Keto-glutaric acid
	↓ α-Keto-butyric acid
	↓ α-Methyl-D-galactoside
	↓ L-Glutamine
	↓ m-Tartaric acid
	↓ D-Fructose-6-phosphate
	↓ Tween 80
	↓ α-Hydroxy butyric acid
	↓ Glycyl-L-aspartic acid
	↓ Citric acid
	↓ D-Threonine
	↓ Glycolic acid
	↓ Glyoxylic Acid
	↓ Glycyl-L-aspartic acid

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↓	Citric acid
↓	D-Threonine
↓	Glycolic acid
↓	Glyoxylic acid
↓	Glycyl-L-glutamic acid
↓	Tricarballylic acid
↓	L-Serine
↓	Acetoacetic acid
↓	Monomethyl succinate
↓	D-Psicose
↓	L-Lyxose
↓	Glucuronamide
↓	D-Galacturonic acid
↓	D-Lactic acid methyl ester
↓	L-Pyroglutamic acid
↓	6% NaCl + MOPS
↓	6% NaCl +KCl
↓	6% NaCl + N-acetyl L-glutamine
↓	3% Sodium sulphate
↓	4% Sodium sulphate
↓	10% Ethylene glycol
↓	15% Ethylene glycol
↓	3% Sodium formate
↓	2% Urea
↓	3% Urea
↓	5% Urea
↓	6% Urea
↓	7% Urea
↓	8% Sodium lactate
↓	100 mM Sodium phosphate pH 7
↓	200 mM Sodium benzoate pH 5.2
↓	10 mM Ammonium sulphate pH 8
↓	20 mM Ammonium sulphate pH 8
↓	50 mM Ammonium sulphate pH 8
↓	10 mM Sodium nitrate
↓	20 mM Sodium nitrate
↓	40 mM Sodium nitrate
↓	60 mM Sodium nitrate
↓	80 mM Sodium nitrate
↓	100 mM Sodium nitrate
↓	pH 9.5 + L-Glutamine
↓	pH 9.5 + L-Tyrosine
↓	pH 9.5 + L-Norvaline
↓	X-α-D-Glucoside
↓	X-β-D-Glucoside
↓	X-α-D-Galactoside

567 ↑, increased growth of mutant relative to wild-type *C. jejuni* NCTC 11168;

568 ↓, decreased growth of mutant relative to wild type *C. jejuni* NCTC 11168;

569 Cut-off for significant change in growth: ≥1.5-fold difference between wild-type and mutant

570 for $p < 0.05$; only statistically significant changes are included.

2.2 UNPUBLISHED WORK

2.2.1 Modelling the prevalence of foodborne pathogens in EU countries

2.2.1.1 Introduction

As the prevalence of foodborne diseases is thought to be significantly underestimated, there is a need to evaluate the effectiveness of the reporting process in order to appreciate the real burden of disease. The prevalence of infectious intestinal disease is underestimated due to underreporting and underdiagnoses associated with passive surveillance, incorrect diagnosis and inefficient communication, as well as with the large number of cases with mild symptoms that do not seek medical help.

Some (longitudinal) studies have attempted to reconstruct the surveillance pyramid for foodborne diseases by evaluating a few vital fractions (rates), which quantify the underdiagnoses and underreporting of the diseases. These fractions include the probability for a patient to visit a GP, the probability for a GP to submit a stool, the probability for a positive result and the probability for a positive result to be reported etc. The probability for diarrhoea case to seek medical help and for a GP to submit the patient's stool sample for microbiological analysis are the most important components contributing to the overall notification fraction according to the data from previous studies (Haagsma et al., 2012; Scallan et al., 2011; Hall et al., 2008; Scallan et al., 2006; Michel et al., 2000). Patients seeking medical help, as well as GP decision making, is influenced by the severity of symptoms and, in particular the appearance of bloody diarrhoea. Rates from cases with bloody diarrhoea or otherwise are often analysed separately (Haagsma et al., 2012; Scallan et al., 2011; Scallan et al., 2006). Although some studies report similar proportions of diarrhoea cases seeking medical attention despite the differences in structure and access to health care system, e.g. in Australia, Canada, Ireland and United States (Scallan et al., 2005), others reveal significant differences in medical help seeking behaviour among countries within EU (Haagsma et al., 2012).

2.2.1.2 Modelling the prevalence for foodborne pathogens

Prevalence is normative, characterizing the rate of cases in a population at a specific time and it is therefore easy to visualize and to compare different values. Prevalence in a population can be estimated from finite samples. Reported cases of food borne illness occur according to a binomial process with prevalence taking to role of the binomial probability it is therefore convenient to represent uncertainty in prevalence by beta distribution. Because of the systematic relationship between the binomial process and a beta distribution (conjugacy) existing belief about the prevalence can be systematically updated given data

from successive finite samples. Bayes' theorem can be used to express how to change a prior belief by taking evidence into account. Parameters affecting the prevalence for reporting of food borne illness by general practitioners (GPs) can be visualized by construction of a graphical representation. Since different parameters affecting the process of reporting are involved in cases of hospitalization, these have to be acknowledged with a separate branch in the graphical model. Because of the differences in symptoms and severity of the disease caused by different pathogens, as well as the differences in medical and reporting practice among European Union Member States (EU MSs), some of the parameters are dependent of the country and others of the pathogen.

2.2.1.3 The MedVetNet model

Haagsma et al. (2012) have built a probabilistic model for prediction of the reporting of prevalence for seven foodborne pathogens in seven EU MSs, including Denmark, Germany, Italy, Netherlands, Poland, Sweden, and United Kingdom. They included *Campylobacter* spp., *Salmonella* spp., *Yersinia enterocolitica*, *Shigella* spp., Shiga-toxin producing *Escherichia coli* O157(STEC), enteropathogenic *Escherichia coli* (EPEC), and *Cryptosporidium* spp. in their model. The Haagsma model acknowledges that general practitioner (GP) cases occur in a separate branch from hospitalized cases and consists of a series of country-specific and pathogen-specific parameters. The model also distinguishes the cases involving bloody diarrhoea from those with non-bloody diarrhea and addresses them independent of one another. The country-specific parameters used in the model were obtained from a variety of sources including expert opinions and harmonized longitudinal surveys of acute gastroenteritis incidence in the community.

The MedVetNet model concerns the probability for reporting of a case of gastroenteritis. The total probability is constructed from the probability for general practitioner cases (P_{GP}) and the probability for hospitalized cases (P_H). Each probability is the product of several parameters

$$P_{GP} = a^* (1\text{-}p_H)\, c^* f\, j\, h = a^* (1 - p_H) \quad \ldots(1)$$

$$P_H = a^* p_H\, e\, g\, j\, i = a^*\ p_H\, o \quad \ldots(2)$$

$$a^* = k\, a + (1 - k)\, b \quad \ldots(3)$$

$$c^* = k\, c + (1 - k)\, d \quad \ldots(4)$$

Where a^* is the probability for a gastroenteritis case to consult a general practitioner and c^* and e are probabilities for a stool sample to be submitted for the laboratory analysis, j is the sensitivity of laboratory analysis and i and h the probabilities for a positive result to get in the records.

The probability that a practitioner refers a case for hospitalization, p_H, can be related to the fraction of reports that are associated with a hospital setting (f_H) according to

$$f_H = op_H / (n(1-p_H) + o\,p_H)) \quad \ldots(5)$$

Then the total probability for a case to become a report is

$$P_{TOT} = 1 / M = (a^* on) / (nf_H + o(1 - f_H)) \quad \ldots(6)$$

As we can observe from the above described dependency, the multiplier M depends on the fractional, rather than absolute rate of hospital cases.

Dependencies between the important parameters are summarized by the arrows in Figure 1, where country-specific parameters are marked by green colour and pathogen-specific parameters by pink colour. Each arrow in the model means one variable depends on its parents. The model builds up from the number of cases within a population to the number of cases that are included in the records – and hence gives the representation for the multiplier.

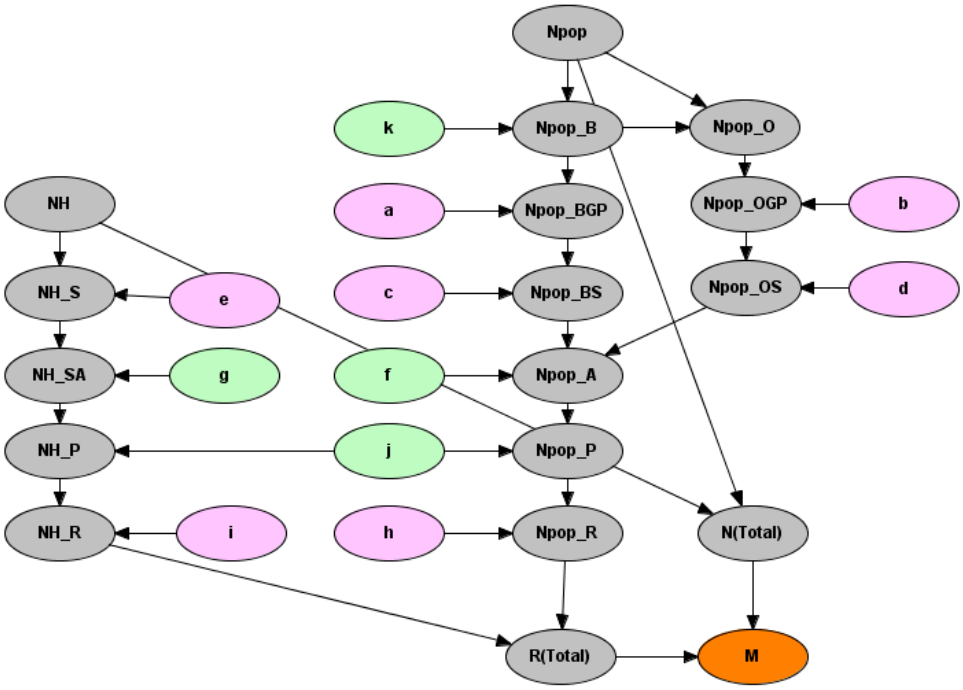


Figure 1: Probabilistic graphical model for reporting foodborne disease prevalence
Slika 1: Verjetnostni grafični model za poročanje prevalence bolezni, povezanih s hrano

We start from a total number of GP cases in the population Npop and partition by taking into account the parameter *k*, which stands for the fraction of cases with bloody diarrhoea. In this case the dependency is multiplication. After this step, the model splits in two branches; one

describing the behaviour related to bloody diarrhoea cases on the left (Npop_B) and the one describing the non-bloody cases (Npop_O) on the right side of the graphical model. The next step adds in the parameters describing the probability for a diarrhoea case to visit a general practitioner, which is labelled as *a* in case of bloody diarrhoea and as *b* in case of the non-bloody diarrhoea. Following step is multiplication by parameters *c* or *d*, which stands for probability of submitting a stool sample for analysis for a patient with bloody or non-bloody diarrhoea, respectively. At this point a new, separate branch acknowledging the hospital flow, which starts with the population NH, is introduced. The equivalent parameter for probability of stool submission is introduced to the model as parameter *e* for the hospitalized patients. After this point the two branches of GP cases reconnect (add) and the resulting distribution is multiplied by parameter *f*, which represents the probability of analysing for a particular pathogen. The equivalent parameter introduced to the hospital branch of the model is labelled as *g*. The next parameter, *j*, describes the sensitivity of laboratory analysis for each individual pathogen and is common to both branches of the model. The terminal parameters *h* and *i* describe the probability for reporting the positive laboratory result to the authorities in case of GP consultation or hospitalization, respectively. The final distributions Npop_R and NH_R combine to give the total number of reports and therefore describe the number of cases of foodborne disease that get in the official records. In turn Npop and NH combined with the total number of reports can be transformed into a multiplier (M) that quantifies the efficiency of the reporting process. The multiplier can be used to calculate the prevalence of a particular disease (associated with a particular pathogen in a MS) based on the number of official reports. In the MedVetNet model each of 37 different scenarios is quantified.

2.2.1.4 The parameter uncertainty

In order to evaluate the uncertainty associated with the multiplier each parameter of the model includes its own uncertainty (as a distribution) and the multiplications are carried out throughout the model by using parameter probability density functions, rather than point values. In that way it is possible to follow the contribution of uncertainty for each new parameter introduced to the model. The probability density distributions are informative of any unusual situations occurring in the model, which could not be observed from a simple median value of parameters.

We have evaluated the role of parameter uncertainty by comparing the multiplier values (M_{Med}) that are obtained as point values with information on parameter location alone, and the median of the multiplier distributions ($\%50(M_{MC})$) when associated uncertainty is included by using Monte Carlo simulations (Table 1).

As observed from the ratio between $\%50(M_{MC})$ and M_{Med}, the values obtained from median parameter values turn out to be very good approximations of multipliers in most cases. When the parameter uncertainty increases, the median value is no longer useful, as observed in the case of STEC. The difference between the multiplier distribution and the multiplier median value in this case is substantially different, which indicates an anomalous situation.

Table 1: Median multiplier values for prevalence of foodborne disease established from Monte Carlo simulations and point values obtained from median values of parameters
Preglednica 1: Mediane množiteljev prevalence patogenov povezanih s hrano, pridobljene z Monte Carlo simulacijami in točkovne vrednosti median posameznih parametrov

Median Multiplier		$\%50(M_{MC})$	M_{Med}	$M_{Med}/\%50(M_{MC})$
UK	*Campylobacter*	52.15	52.37	1.00
UK	*Salmonella*	39.71	38.67	0.97
UK	*Yersinia*	2185.99	2279.13	1.04
UK	*Shigella*	61.04	59.75	0.98
UK	STEC	1602.41	1504.42	0.94
UK	*Cryptosporidium*	92.91	94.68	1.02
NL	*Campylobacter*	49.41	49.08	0.99
NL	*Salmonella*	19.80	18.69	0.94
NL	*Yersinia*	45.74	43.59	0.95
NL	*Shigella*	52.33	49.96	0.95
NL	STEC	88.97	68.86	0.77
NL	*Cryptosporidium*	2085.10	2078.35	1.00
DE	*Campylobacter*	9.29	9.32	1.00
DE	*Salmonella*	6.69	6.52	0.97
DE	*Yersinia*	13.03	12.57	0.97
DE	*Shigella*	10.56	10.42	0.99
DE	STEC	23.02	19.33	0.84
DE	EPEC	34.09	31.06	0.91
DE	*Cryptosporidium*	101.43	92.49	0.91
DK	*Campylobacter*	29.36	29.23	1.00
DK	*Salmonella*	16.63	14.77	0.89
DK	*Yersinia*	20.12	19.48	0.97
DK	*Shigella*	29.63	27.90	0.94
DK	STEC	32.75	23.40	0.71
DK	EPEC	102.71	87.17	0.85
IT	*Campylobacter*	90.66	86.49	0.95
IT	*Salmonella*	16.73	14.66	0.88
PL	*Campylobacter*	295.91	247.09	0.84
PL	*Salmonella*	35.01	32.52	0.93
PL	*Yersinia*	227.78	199.87	0.88
PL	*Shigella*	58.03	53.05	0.91
SE	*Campylobacter*	17.14	16.33	0.95
SE	*Salmonella*	10.08	8.96	0.89
SE	*Yersinia*	13.66	12.40	0.91
SE	*Shigella*	18.06	16.08	0.89
SE	STEC	13.35	9.85	0.74

$\%50(M_{MC})$ median multiplier values established from Monte Carlo simulations, M_{Med} multiplier values established from median values of model parameters, $M_{Med}/\%50(M_{MC})$ the ratio of multiplier values established from median values and Monte Carlo simulations

The analysis of the data shows that the differences arise from a considerably higher proportion of bloody diarrhea within the population (*k*) for STEC, in comparison to other pathogens. Consequently the importance of parameters *a* and *c* is increased. Since those are associated with notably higher uncertainty than parameters *b* and *d*, the median value no longer serves as a good approximation for the multiplier. An example of a well-defined distribution of the multiplier for *Campylobacter* in UK and a highly uncertain distribution for the multiplier for STEC in UK is presented in Figure 2.

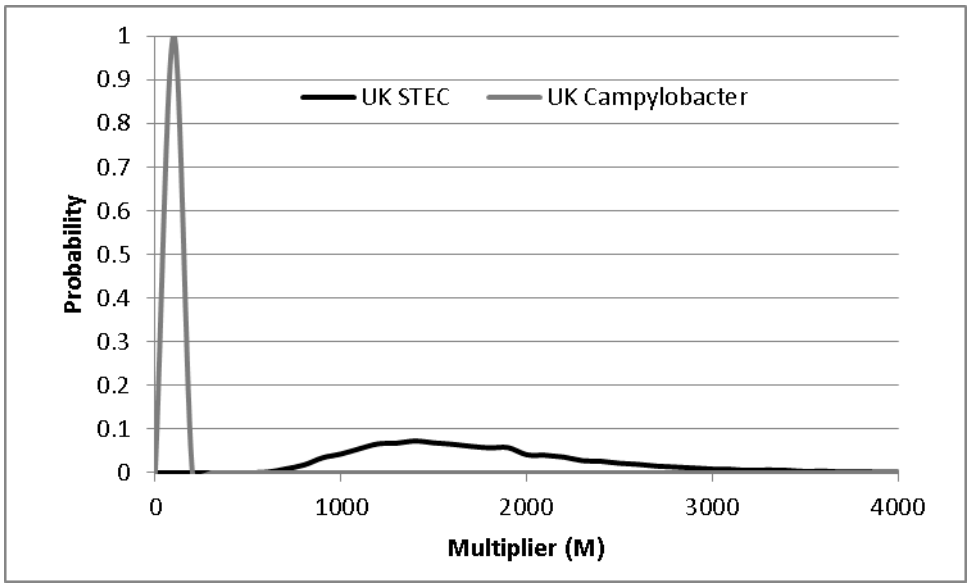


Figure 2: The distribution of prevalence multipliers for *Campylobacter* and STEC in UK
Slika 2: Porazdelitev množiteljev prevalence za *Campylobacter* in STEC v Velki Britaniji

2.2.1.5 Sensitivity of the model

In order to evaluate the robustness of the model, it is important to identify the parameters that have the greatest impact on the model output by performing a sensitivity analysis. Since testing the sensitivity of the parameter distributions is highly complex, we have simplified the approach by using the model that is based on median values. When operating with numbers, such as median values, the sensitivity of multiplier (*M*) is a derivative *dM/dp* with respect to parameter value (*p*), for each individual parameter. A large derivative indicates strong sensitivity of a model with respect to the tested parameter, meaning that a minor change in the parameter value will substantially increase the multiplier. When the derivative is negative, the model output will decline when parameter value is increased.

For easier comparison of the parameter sensitivity values, we have analysed the fractional rather than absolute increase in multiplier values by using (*1/M*) *dM/dp*. Furthermore, in order to easily compare all the parameters on a single scale, we have followed the variation of fractional changes in M with fractional changes of each individual parameter *(p/M)*

dM/dp, which are presented in Table 2. When normalized sensitivity of the parameter is -1 a 10% increase in the parameter value will result in 10% decrease in the multiplier value etc. Similarly, the sensitivity value -0.5 means that the 10% increase in the parameter value will result in a 5% decline of the multiplier value.

Table 2: Normalized sensitivity values *(1/M) p dM/dp* for parameters $p = a - k$ of the prevalence model
Preglednica 2: Normalizirane vrednosti občutljivosti *(1/M) p dM/dp* za parameter $p = a - k$ modela prevalence

		a	b	c	d	e	f	g	h	i	j	k
UK	CAMP	-0.22	-0.78	-0.24	-0.74	-0.03	-0.97	-0.03	-0.97	-0.03	-1.00	-0.15
UK	SALM	-0.45	-0.55	-0.47	-0.51	-0.02	-0.98	-0.02	-0.98	-0.02	-1.00	-0.30
UK	YERS	-0.40	-0.60	-0.44	-0.56	0.00	-1.00	0.00	-1.00	0.00	-1.00	-0.27
UK	SHIG	-0.31	-0.69	-0.34	-0.65	-0.01	-0.99	-0.01	-0.99	-0.01	-1.00	-0.21
UK	STEC	-0.89	-0.11	-0.63	-0.07	-0.30	-0.70	-0.30	-0.70	-0.30	-1.00	-0.47
UK	CRYP	-0.01	-0.99	-0.01	-0.99	0.00	-1.00	0.00	-1.00	0.00	-1.00	-0.01
NL	CAMP	-0.44	-0.56	-0.60	-0.36	-0.05	-0.95	-0.05	-0.95	-0.05	-1.00	-0.85
NL	SALM	-0.69	-0.31	-0.74	-0.15	-0.11	-0.89	-0.11	-0.89	-0.11	-1.00	-1.17
NL	YERS	-0.66	-0.34	-0.79	-0.20	-0.01	-0.99	-0.01	-0.99	-0.01	-1.00	-1.18
NL	SHIG	-0.56	-0.44	-0.72	-0.26	-0.02	-0.98	-0.02	-0.98	-0.02	-1.00	-1.05
NL	STEC	-0.96	-0.04	-0.96	-0.02	-0.02	-0.98	-0.02	-0.98	-0.02	-1.00	-1.56
NL	CRYP	-0.02	-0.98	-0.05	-0.95	0.00	-1.00	0.00	-1.00	0.00	-1.00	-0.06
DE	CAMP	-0.24	-0.76	-0.22	-0.72	-0.06	-0.94	-0.06	-0.94	-0.06	-1.00	-0.15
DE	SALM	-0.47	-0.53	-0.41	-0.47	-0.12	-0.88	-0.12	-0.88	-0.12	-1.00	-0.29
DE	YERS	-0.42	-0.58	-0.38	-0.53	-0.09	-0.91	-0.09	-0.91	-0.09	-1.00	-0.26
DE	SHIG	-0.33	-0.67	-0.31	-0.62	-0.07	-0.93	-0.07	-0.93	-0.07	-1.00	-0.21
DE	STEC	-0.89	-0.11	-0.74	-0.09	-0.17	-0.83	-0.17	-0.83	-0.17	-1.00	-0.53
DE	EPEC	-0.33	-0.67	-0.30	-0.61	-0.09	-0.91	-0.09	-0.91	-0.09	-1.00	-0.21
DE	CRYP	-0.01	-0.99	-0.01	-0.96	-0.04	-0.96	-0.04	-0.96	-0.04	-1.00	-0.01
DK	CAMP	-0.49	-0.51	-0.24	-0.73	-0.03	-0.97	-0.03	-0.97	-0.03	-1.00	-0.48
DK	SALM	-0.73	-0.27	-0.46	-0.49	-0.05	-0.95	-0.05	-0.95	-0.05	-1.00	-0.75
DK	YERS	-0.70	-0.30	-0.43	-0.54	-0.02	-0.98	-0.02	-0.98	-0.02	-1.00	-0.71
DK	SHIG	-0.61	-0.39	-0.34	-0.63	-0.03	-0.97	-0.03	-0.97	-0.03	-1.00	-0.61
DK	STEC	-0.96	-0.04	-0.88	-0.10	-0.02	-0.98	-0.02	-0.98	-0.02	-1.00	-1.09
DK	EPEC	-0.61	-0.39	-0.34	-0.63	-0.02	-0.98	-0.02	-0.98	-0.02	-1.00	-0.61
IT	CAMP	-0.22	-0.78	-0.90	-0.09	-0.02	-0.98	-0.02	-0.98	-0.02	-1.00	-0.94
IT	SALM	-0.45	-0.55	-0.84	-0.03	-0.13	-0.87	-0.13	-0.87	-0.13	-1.00	-0.95
PL	CAMP	-0.32	-0.68	-0.02	-0.88	-0.09	-0.91	-0.09	-0.91	-0.09	-1.00	-0.02
PL	SALM	-0.57	-0.43	-0.06	-0.77	-0.17	-0.83	-0.17	-0.83	-0.17	-1.00	0.08
PL	YERS	-0.53	-0.47	-0.06	-0.87	-0.08	-0.92	-0.08	-0.92	-0.08	-1.00	0.08
PL	SHIG	-0.43	-0.57	-0.04	-0.82	-0.15	-0.85	-0.15	-0.85	-0.15	-1.00	0.00
PL	STEC	-0.93	-0.07	0.00	0.00	-1.00	0.00	-1.00	0.00	-1.00	-1.00	-0.52
PL	EPEC	-0.43	-0.57	-0.03	-0.72	-0.25	-0.75	-0.25	-0.75	-0.25	-1.00	-0.03
SE	CAMP	-0.61	-0.39	-0.14	-0.81	-0.05	-0.95	-0.05	-0.95	-0.05	-1.00	-0.50
SE	SALM	-0.82	-0.18	-0.30	-0.64	-0.06	-0.94	-0.06	-0.94	-0.06	-1.00	-0.64
SE	YERS	-0.79	-0.21	-0.27	-0.68	-0.05	-0.95	-0.05	-0.95	-0.05	-1.00	-0.62
SE	SHIG	-0.72	-0.28	-0.20	-0.75	-0.04	-0.96	-0.04	-0.96	-0.04	-1.00	-0.58
SE	STEC	-0.98	-0.02	-0.63	-0.14	-0.23	-0.77	-0.23	-0.77	-0.23	-1.00	-0.70

UK United Kindgdom, NL Netherlands, DE Germany, DK Denmark, IT Italy, PL Poland, SE Sweden, NR number of reported cases, NH number of hospitalized cases

As observed from the values from Table 2, the most sensitive parameter is the one for the laboratory analysis for each individual pathogen (*j*), followed by *f* and *h* - the probabilities for analysing a pathogen and reporting the positive result, respectively. The model is also highly sensitive for changes in probability for a non-bloody case of diarrhoea to visit a GP (*b*) and probability for submitting a stool sample for analysis for a patient with non-bloody diarrhoea (*d*). Table 2 also shows that, for STEC, the parameters a and c, are sources of sensitivity corresponding with the higher rate for bloody diarrhoea (see above). Table 2 also indicates some national differences; for Germany and the UK the multiplier is significantly more sensitive to parameters b and d (corresponding with non-bloody diarrhoea) but for The Netherlands, Denmark and Sweden the results are equally sensitive to parameters a and c.

2.2.1.6 Additional models for country-specific parameters

The data needed to define the key parameters contributing to the estimation of the real prevalence (i.e. a community case rate) is usually collected in the healthcare system by evaluating special cohort studies, such as the longitudinal study of infectious intestinal disease in the UK (Wheeler et al., 1999; Tam et al., 2011) or Sensor in Netherlands (Wit et al., 2001). The cohort studies as these are broad undertakings over a long period of time that result in large quantities of collected data, however they are laborious and expensive and only wealthy countries can afford to carry out studies with this type of design. For this reason the data needed for modelling the community prevalence of foodborne pathogens in less wealthy European Union Member States (EU MSs) is sparse to non-existing.

In order to implement the Haagsma model in other EU MSs, we have searched for the required data without success (by direct approach to food safety authorities in some cases). Since the data is not available, we have explored the possibility to use the published data from countries within the MedVetNet model to predict the country-specific model parameters for EU MSs which cannot afford comparable studies. In practice we aimed to predict the reporting efficiency, and hence the community prevalence, for *Campylobacter* spp. in Slovenia. To do this, we modelled the crucial country-specific parameters *a*, *b*, *c*, *d* and *e* using linear regression based on the existing data for seven foodborne pathogens in seven EU countries. Only common statistical data freely available for EU countries through Eurostat (Table 3) was used to construct a model for the parameter uncertainty distributions.

The model parameter for the probability of a case of bloody diarrhea to visit a GP (*a*) was fitted to data on average traveling distance to a GP and the number of GPs per 100,000 people in the country. The medical help seeking behavior in case of experiencing a severe form of gastroenteritis with blood in the stool was associated with the availability of the medical help, which is related to the number of general practitioners per member of the population and to the accessibility of the healthcare.

Probability for a non-bloody case to visit a GP (*b*) was modelled using the data on total population in the country and the fraction of middle aged people (age 25 to 64), which was believed to influence the medical seeking behaviour in case of gastroenteritis of low severity.

Table 3: Statistical data extracted from Eurostat (2014) that was used for linear regression fitting of the additional models for prediction of prevalence of foodborne diseases
Preglednica 3: Statistični podatki, izvlečeni iz Eurostata (2014) in uporabljeni za prileganje dodatnih modelov za napoved prevalence bolezni, povezanih s hrano z linearno regresijo

	D/GP	GPs	Pop	fMA (%)	GNI pC	*RL	fCE (%)	PuF (%)	THE/ capita	PrF (%)
PL	4.2	46.2	38564000	58.6	20450	16	28.6	71.6	1423	28.4
SE	8.6	63.1	9591000	51.2	42350	21	35.6	78	3870	22
DK	11.2	60.9	5612000	52.2	42330	14	35	84.3	4564	15.7
UK	2.2	79.9	64231000	52.8	36970	8	34.2	96.9	3322	3.1
IT	2.3	95.1	59626000	55.7	32350	20	34.3	57.1	3130	42.9
NL	1.4	125	16798000	54.1	43770	30	33.8	70.7	5122	29.3
DE	1.7	155.4	82141000	55.2	40170	16	33.9	77.5	4371	22.5
SI	4.4	51	2062000	58.9	26040	9	30.5	80.8	2551	19.2

PL Poland, SE Sweden, DK Denmark, UK United Kingdom, IT Italy, NL Netherlands, DE Germany, D/GP distance to GP, GPs number of GPs/100,000, Pop population, fMA fraction of middle aged people, GNI pC Gross national income per capita, RL number of regional laboratories, fCE fraction of children and elderly, PuF public funding of healthcare system, THE/capita total health expenditure per capita, PrF private funding of healthcare system. *Data obtained from the national public health institutes web pages.

The parameter *c*, which describes the probability for a stool sample, from a case with bloody diarrhoea, to be submitted for laboratory analysis by a GP, was modelled using data on gross national income (GNI) per capita and the number of regional labs. The wealth of the country was believed to affect a GP's decision making when considering sending the bloody stool sample for analysis. The availability and geographical remoteness and capacities of public health laboratories were also believed to play an important role in assessing the value of this parameter.

The probability for a GP to submit a sample, from a non-bloody case, for laboratory analysis (*d*) was modelled based on the data for the fraction of children (0-14 year old) and elderly (>65 year old), which represent the most vulnerable groups, and the share of public funding of the healthcare system.

The probability for a stool sample of a hospitalized patient being submitted (*e*) was modelled using data on total health expenditure per capita and on the share of private funding in the healthcare system. Most of the evidence indicates that the value of the multiplier is quite insensitive to the cases that are treated in hospital (see the final column in table 2).

The parameters were modelled using the following expression:

$$z = (\alpha\, x/\delta\, x) + (\beta\, y/\delta\, y) - ((\alpha\langle x\rangle/\delta\, x) + (\beta\langle y\rangle/\delta\, y)) + \gamma \quad \ldots(7)$$

where z is the median value of the parameter being estimated, x and y are the variables listed in the Table 2, and α, β and γ are fitting coefficients established by least squares fitting to the existing data from seven EU MSs published by Haagsma et al., 2012 (Table 3). The fitting procedure was completed using the SOLVER add in for Microsoft Excel. Model fits are presented in Figure 3a-e.

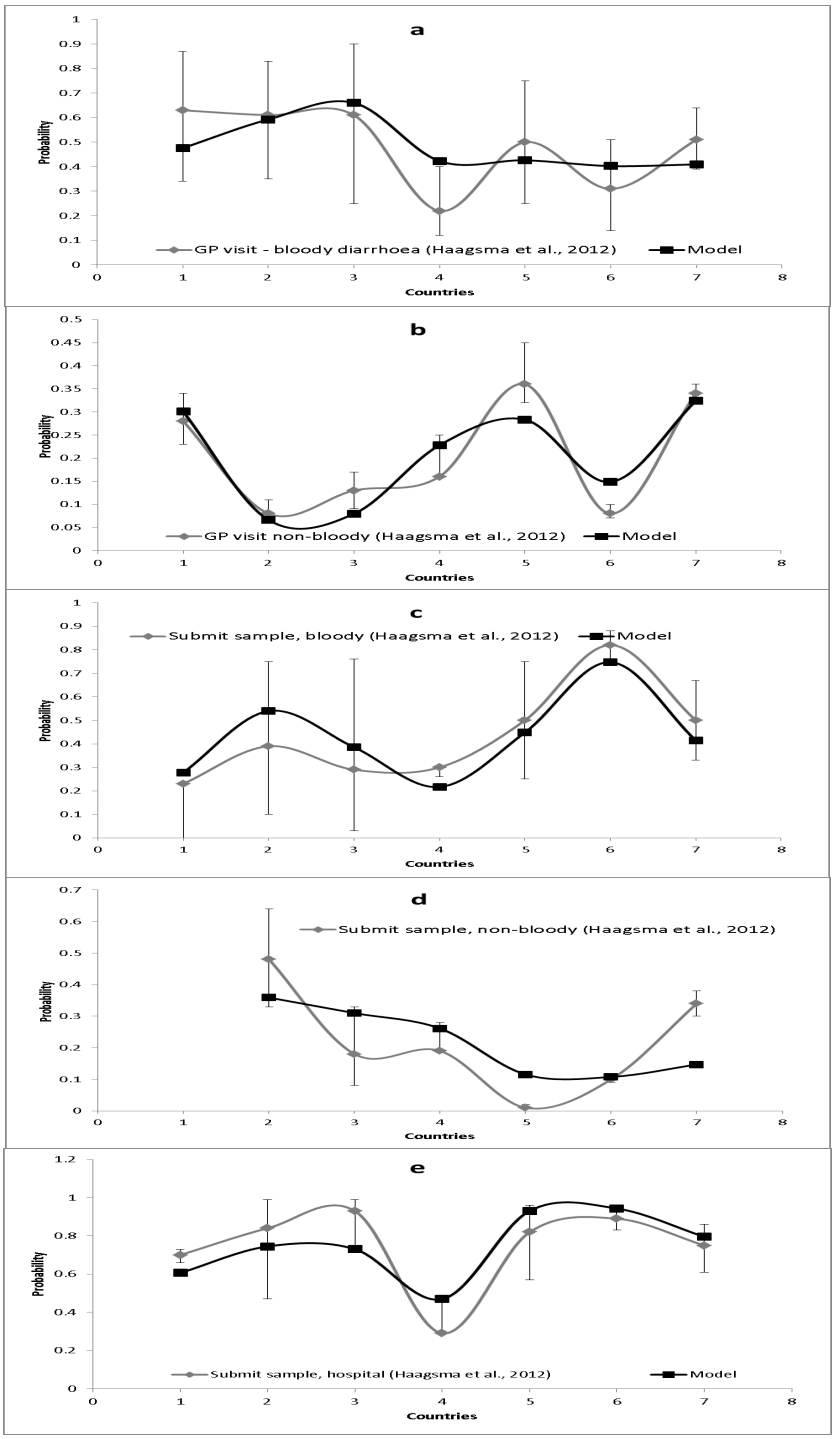


Figure 3: Additional models for parameters *a* (probability for a case of bloody diarrhoea to visit general practitioner), *b* (probability for a case of non-bloody diarrhoea to visit general practitioner), *c* (probability for submitting a stool for a case of bloody diarrhoea), *d* (probability for submitting a stool for a case of non-bloody

diarrhoea) and *e* (probability for submitting a stool for hospitalized case). The probabilities from Haagsma model were fitted to common population and healthcare statistic data using linear regression. The error bars indicate the maximal uncertainty associated with the Haagsma model values.
Slika 3: Dodatni modeli parametrov *a* (verjetnost obiska zdravnika v primeru krvave diareje), *b* (verjetnost obiska zdravnika v primeru nekrvave diareje), *c* (verjetnost odvzema vzorca v primeru krvave diareje), *d* (verjetnost odvzema vzorca v primeru nekrvave diareje) in *e* (verjetnost odvzema vzorca pri hospitaliziranem primeru). Verjetnosti iz modela Haagsma so bile prilegane na splošno dostopne prebivalstvene in javno-zdravstvene podatke z uporabo linearne regresije. Napaka prikazuje maksimalno negotovost povezano z vrednostmi v modelu Haagsma.

The values in the chart represent the each individual parameter's values for seven EU countries – Poland, Sweden, Denmark, United Kingdom, Italy, Netherlands, and Germany - presented as numbers 1-7 on the x axis, respectively. The lines connecting the values serve only for visualization.

Based on the median values of parameters *a* – *e* obtained using the above described additional models (built from data for 7 EU MSs), we have calculated the beta distribution parameters α_1 and α_2 for each of the additional models that correspond with Slovenia in order to attribute the associated uncertainty using Monte Carlo simulations. For each model parameter the beta distribution exponents were calculated according to the following equations

$$\alpha_1 = (\mu^2 / \delta^2)\ (1 - \mu) \quad \ldots(8)$$

$$\alpha_2 = (\alpha_1\ (1-\mu)) / \mu \text{ or } ((\mu\ (1- \mu^2)/ \delta^2 \quad \ldots(9)$$

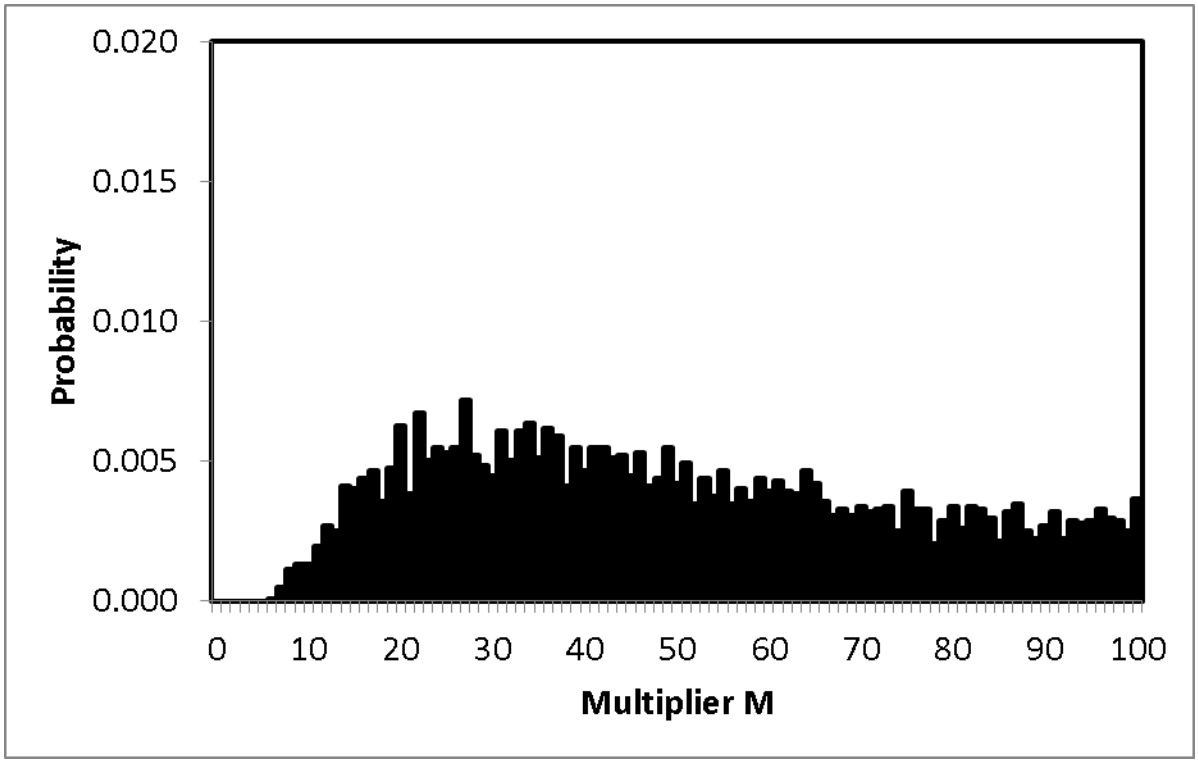
where α_1 and α_2 are parameters defining the beta distribution, μ is median and δ is standard deviation (Table 3). The standard deviations used in the calculations were maximal standard deviations for each of the parameters from the Haagsma study.

Table 4: Parameters of additional models for prediction of prevalence of foodborne diseases
Preglednica 4: Parametri dodatnih modelov za napoved realne prevalence bolezni povezanih s hrano

	a	b	c	d	e
α	0.2602	0.0532	0.0573	0.0836	0.5842
β	0.1688	0.0468	0.1504	0.0546	0.1485
γ	0.4843	0.2043	0.4329	0.1886	0.7457
M	0.3038	0.2248	0.1458	0.1047	0.5842
α1	2.86	13.54	1.07	2	8.4
α2	6.54	150.11	6.29	17.13	5.98

α, β, γare fitting coefficients , a – e are parameters of the prevalence model, M is median value (for Slovenia), α_1 and α_2 are parameters defining beta distributions of additional models (for Slovenia)

Monte Carlo simulations of modelled parameters were used to estimate beliefs about prevalence of *Campylobacter* in Slovenia, but these could be obtained for prediction of any of the seven foodborne pathogens in any of the EU MSs.



4: The probability distribution of prevalence multiplier for *Campylobacter* spp. cases in Slovenia.
Slika 4: Verjetnostna porazdelitev množiteljev prevalence za primere *Campylobacter* spp. v Sloveniji.

The obtained median multiplier for *Campylobacter* GP cases in Slovenia when acknowledging modelled parameters a – e was 181.3. As observed from the figures 4, there is a high uncertainty attached to the estimation.

A more complete analysis might use models fitted to only part of the MedVetNet data and use the remainder as a validation but it is clear from this analysis that there may be opportunities to establish beliefs, concerning community prevalence of infectious intestinal disease, without performing expensive community wide studies.

2.2.2 Clonal expansion of quinolone resistance among clinical Campylobacter isolates from Oxfordshire, United Kingdom

2.2.2.1 Introduction

Campylobacter spp. as the most prevalent bacterial cause of foodborne gastroenteritis represents a serious public health burden in UK, as well as in other EU countries, with *C. jejuni* being responsible for a vast majority of the cases (EFSA, 2014a). The disease itself is usually self-limiting, but the need for antibiotic treatment arises in more severe cases. Currently, macrolide and quinolone antibiotics are recognized as the drugs of choice for clinical treatment of campylobacteriosis. While the resistance to macrolides is successfully controlled, the prevalence of quinolone resistance was reported to rapidly increase over the last decade (Cody et al., 2010b; EFSA, 2014b). Although the use of quinolones as growth promoters of food production animals in EU was banned in 2006, they are still allowed to be used as therapeutics in poultry, which is recognized as the main source of human *Campylobacter* infections (Wilson et al., 2008; Mullner et al.., 2009; Sheppard et al., 2009; WHO, 2011). Since the quinolone resistant *C. jejuni* mutants have better colonization fitness in chicken host, compared to the quinolone susceptible strains (Luo et al., 2005), the inappropriate use of quinolones in veterinary medicine can result in rapid selection of resistant isolates in animal reservoir (van Boven et al., 2003). The introduction of quinolone antibiotic to the farm environment would expect to substantially decrease the genetic variability of *Campylobacter* population within the farm by selecting for only the handful of the fittest resistant genotypes. Transmission of those from animals to humans through the food production chain is thereafter essentially unavoidable and leads to decreased efficacy of quinolones in human clinical treatment.

There are several different mechanisms contributing to the quinolone resistant phenotype in *Campylobacter*. The most widely recognized is the mutation Thr86Ile in the quinolone resistance determining region (QRDR) of the DNA gyrase subunit A gene (*gyrA*), although several other resistance-conferring mutations on positions 70, 85, 86, and 104 within this region have been reported to date (Wieczorek and Osek, 2013). The mutations in the *gyrA* gene were observed to occur at frequency as high as 10^{-6}, however the rate was shown to reach 100-fold reduction in mutants lacking the *mfd* gene (Han et al., 2008). Apart from point mutations, resistance is conferred also through antibiotic efflux provided by the activity of *Campylobacter* principal efflux pump CmeABC (Jeon and Zhang, 2009).

Recently, several reports have demonstrated the clustering of quinolone resistant *Campylobacter* isolates within certain lineages (Kittl et al., 2013; Wimalarathna in sod., 2013), indicating the local clonal expansion of quinolone resistant isolates based on the MLST typing. Moreover, the genetic relatedness of QRDR among those clustering within

the specific clonal complex provided further evidence for clonal nature of the quinolone resistance spreading (Kovač et al., 2014). The sequencing of QRDR region was found to be also a useful genotyping approach informative of the isolate's ecological niche. When used together with MLST, it increased the discriminatory power and improved the source attribution (Ragimbeau et al., 2014).

In present study we exploited the existing whole-genome sequence data of 1727 *Campylobacter* spp. isolated from humans in Oxfordshire, UK from June 2011 to June 2013 to characterize the quinolone resistance spreading using the typing and phylogenetic data. We have investigated the prevalence of genotypic quinolone resistance, as determined based on the presence of previously reported resistance conferring mutations within the QRDR region. The inferred genotypic resistance information was used to assign the resistance phenotype to *gyrA* alleles deposited in the PubMLST database and thereby predict the quinolone resistance phenotype of investigated isolates. The comparative analyses based on the variability within the *gyrA, mfd* and *cmeB* genes were carried out and clonal expansion of resistant genotypes was investigated by analysing the clustering of *gyrA* types among the established core genome phylogenies within the individually analysed groups of isolates.

2.2.2.2 Materials and methods

Isolates and whole genome sequence data

The publicly available whole-genome sequence data for *Campylobacter jejuni* and *Campylobacter coli* strains isolated from human stool samples submitted to the microbiology laboratory of John Radcliffe Hospital in Oxford between June 2011 and June 2013 were included in the study. The data was produced by the research groups from Department of Zoology from University of Oxford (Maiden group) and John Radcliffe Hospital, and was deposited to the online database PubMLST (http://pubmlst.org/campylobacter; Jolley and Maiden, 2010).

Variability of *gyr*A gene and genotypic quinolone resistance

The variability of gyrase subunit A gene (*gyrA*) among the investigated isolates was evaluated by extracting the arbitrary integer identifier of CAMP0950 (*gyrA*) locus automatically assigned to each isolate deposited in the PubMLST database. To the date of analysis (2nd October 2014), there were 554 unique *gyrA* allele variants defined in the *Campylobacter* locus/sequence definition database pubMLST (Jolley and Maiden, 2010).

Literature search was carried out to identify 9 mutations on 5 positions (A70T, D85Y, T86I, T86K, T86A, T86V, D90N, D90Y and P104S) in quinolone resistance determining region of the *gyrA* gene, which were previously associated with phenotypic resistance to quinolone

antibiotics (Wieczorek and Osek, 2013). Resistant or susceptible phenotypes were assigned to the 554 unique *gyrA* allele types defined in *Campylobacter* locus/sequence definition database based on the presence of any single or a combination of listed mutations within the QRDR. The defined resistant *gyrA* genotypes were used to predict the phenotypic resistance of the investigated isolates.

Comparative analysis of quinolone resistance associations

The distribution of resistant and susceptible *gyrA* alleles among investigated group of isolates was analysed, and the associations of specific *mfd* and *cmeB* alleles with genotypic resistance was explored. Furthermore, the association of quinolone resistance with specific clonal complexes (CCs) and sequence types (STs) within the highly resistant clonal complexes were evaluated.

Genome Comparator analyses

In order to investigate the clonal expansion of the quinolone resistance among isolates from highly resistant clonal complexes, we have inferred their phylogenetic relationships based on the core genome using the Genome Comparator module of the BIGSdb (Jolley and Maiden, 2010). The pairwise allelic differences were computed for a core genome defined as all the shared loci within the analysed group of isolates. The established distance matrices were visualized in SplitsTree version 4.12.8 (Huson and Bryant, 2006) by employing the Neighbor-net algorithm (Bryant in Moulton, 2004), which accounts for the recombination and horizontal gene transfer and does not assume the tree-like hierarchy. This makes it more appropriate for visualisation of frequently ambiguous phylogenetic relationships among highly recombinant species.

The *gyrA* alleles were mapped over the phylogenetic trees comprising the isolates from each individual analysed clonal complex, in order to identify the clustering of specific resistance-associated *gyrA* alleles indicative of clonal expansion of the resistance.

Statistical analyses

Association of quinolone resistance with specific MLST clonal complexes and sequence types, as well as with *gyrA, mfd*, and *cmeB* allele types was evaluated using Pearson's chi-square or Fischer's exact, when appropriate, in StataIC 10 (StataCorpLP, Texas). The cut-off for statistical significance was set at $p<0.5$.

2.2.2.3 Results and Discussion

Isolates

In total 1727 *Campylobacter jejuni* and *Campylobacter coli* strains isolated from human stool samples submitted to the microbiology laboratory of John Radcliffe Hospital in Oxford between June 2011 and June 2013 were included in the study. The Oxfordshire area comprises the population of approximately 630,000 and is a representative sample (1%) for the entire United Kingdom.

The 880 of strains were isolated in year 1 (June 2011 to June 2012) and 847 in year 2 (June 2012 to June 2013). The whole genome sequencing was performed and resistance to ciprofloxacin determined for each of the isolates. Ten isolates from year 1 and 6 isolates from year 2 were excluded from the analysis due to the incomplete data (missing MLST ST, MLST rST and/or any of the investigated alleles), resulting in a final set of 1711 isolates being analysed.

Variability of *gyrA* and genotypic quinolone resistance

In total 328 unique *gyrA* variants were identified in 1711 isolates included in the analysis, 176 in year 1 and 152 in year 2, indicating a slightly higher diversity among isolates from year 1.

Genotypic quinolone resistance was determined based on the presence of any single or a combination of different quinolone resistance determining mutations within the QRDR, as identified in the literature search. The mutation T86I was identified as the principal mutation responsible for high levels or quinolone resistance, while eight other mutations (A70T, D85Y, R86K, T86A, T86V, D90N, D90Y and P104S) were previously reported to be associated with quinolone resistance, as well. In this study we have identified another mutation, P104T, which was previously not reported elsewhere. This particular mutation was found in two isolates of *C. jejuni* with MLST sequence type 50, and in both cases co-existed with the predominant resistance conferring mutation T86I, which disabled us to evaluate its sole contribution to the resistant phenotype.

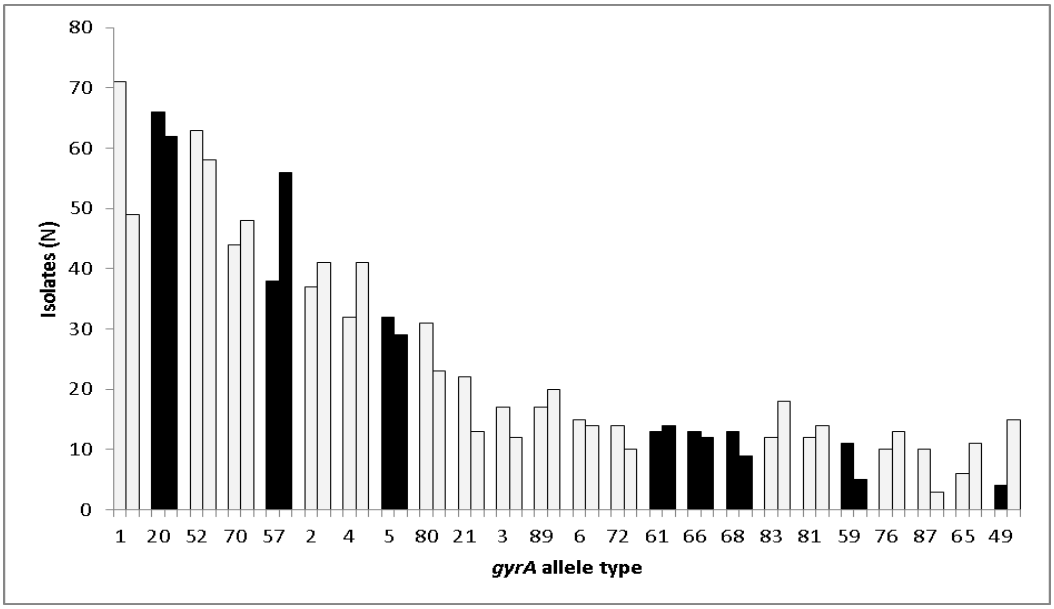


Figure 5: The *gyrA* allele distribution among *Campylobacter jejuni* and *Campylobacter coli* human isolates from John Radcliffe Hospital catchment area, Oxfordshire, Oxford, between June 2011 and June 2013.
Slika 5: Porazdelitev alelov *gyrA* med humanimi izolati *Campylobacter jejuni* in *Campylobacter coli* iz bolnišnice John Radcliff v Oxfordshiru v Oxfordu med junijem 2011 in junijem 2013.

First and second column of each allele represents the number of isolates carrying this allele in year 1 and 2, respectively. Grey columns represent quinolone susceptible genotypes, black columns quinolone resistant genotypes. Only the *gyrA* alleles with 10 or more representative isolates per year are presented.

In order to assign the resistant genotype to the analysed isolates, we first defined it for 554 existing *gyrA* allele types in the *Campylobacter* locus/sequence definition database at pubMLST.org. The 253 of those were determined as resistant and 301 as susceptible. As presented in the Figure 1, the most prevalent *gyrA* alleles associated with resistance were 20, 57 and 5, while others were present in fewer than 15 isolates per year.

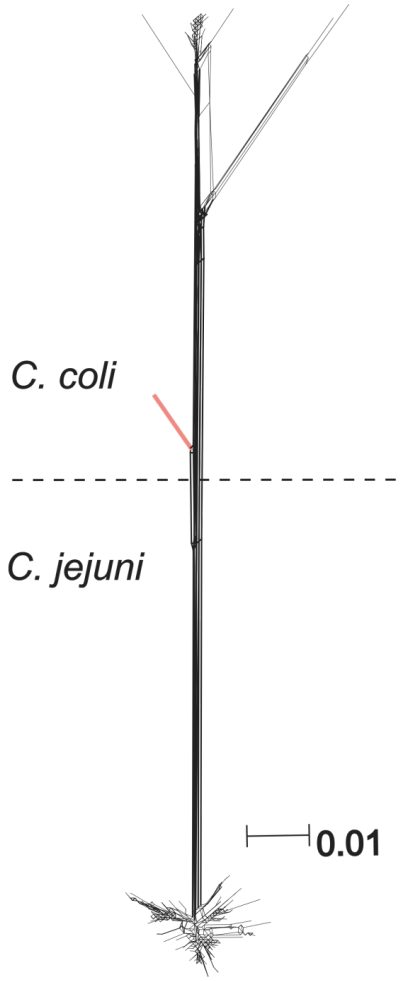


Figure 6: SplitsTree phylogenetic network build using Neighbor-net algorithm based on the unique *gyrA* allele sequences identified in human isolates from John Radcliffe Hospital catchment area, Oxfordshire, Oxford, between June 2011 and June 2013.
Slika 6: Filogenetska mreža zgrajena 2013 v programu SplitsTree z algoritmom Neighbor-net na podlagi unikatnih alelnih variant gena *gyrA* med humanimi izolati iz bolnišnice John Radcliff v Oxfordshiru v Oxfordu med junijem 2011 in junijem.

The phylogenetic network demonstrates the clear discrimination between the *Campylobacter jejuni* and *Campylobacter coli* isolates based on the *gyrA* sequence variability, with the exception of the hybrid allele type 363 marked with red.

The Neighbor-net analyses of unique *gyrA* sequences in SplitsTree demonstrated the clear split between the two analysed species, *C. jejuni* and *C. coli*, with exception of one *C. coli* strain (ID 25067, ST 827) that carried the hybrid *gyrA* allele 363 associated with quinolone resistance (Figure 2). The hybrid allele appears to be the result of recombination between *C. coli* allele 66 and *C. jejuni* allele 5, both associated with resistance and frequently found among analysed isolates (Figure 1).

Based on the presence of resistant *gyrA* variants among the 1711 analysed isolates, we determined 37.9% (N=330) of isolates from year 1 and 38.6% (N=325) isolates from year 2 as resistant to quinolones. The prevailing mutation in resistant isolates was T86I, and was absent from only two resistant isolates in year 1 and three resistant isolates from year 2. In all cases it was substituted by the mutation T86A. The coexistence of two resistance-conferring mutations was observed in 6 isolates from year 1 and in a single isolate from year 2. The obtained results clearly confirm the mutation T86I as the major quinolone resistance-conferring mutation in *Campylobacter*.

MLST and clonal complexes

The MLST profiles for 1711 isolates were generated from WGS data, identifying 206 and 171 STs in years 1 and 2, respectively. These were assigned to 32 clonal complexes in year 1 and 31 clonal complexes in year 2. In year one 49/870 (5.6%) of the isolates were unassigned to any of the existing clonal complexes and in year two 40/841 (4.8%) were left unassigned.

Comparative analysis of quinolone resistance associations

The prevalence of resistant isolates, as determined based on the *gyrA* allele types, was investigated among clonal complexes. The clonal complexes ST-464 and ST-354 had the highest proportions of quinolone resistant isolates and the correlation with resistance was statistically significant ($p<0.001$) in both years (Figure 3). In year 1 51/54 (94.4%) isolates from CC ST-464 were resistant to quinolones, while in year 2 the proportion decreased (66/75, 88%), however the total prevalence of the clonal complex increased by 39%. The rise in prevalence was observed also for clonal complex ST-354, of which 24 cases were recorded in year 1 and 30 cases in year 2. The prevalence of resistance in this clonal complex was 20/24 (83.3%) and 29/30 (96.7%) in year 1 and 2, respectively. The most common clonal complex, ST-21, was also observed to have high proportions of quinolone resistant isolates, however the correlation was not confirmed as statistically significant. Due to the possibility of a specific ST contributing to the high resistance rate within the clonal complex ST-21, we have further investigated the distribution of resistant isolates within the STs of this complex. Indeed the analysis confirmed the significance ($p<0.05$) of quinolone resistance with ST 50, where 38/57 (66.7%) and 31/60 (51.7%) of isolates were resistant in year 1 and 2, respectively. Breaking down the resistant clonal complexes ST-464 and ST-354 revealed in both years fully resistant ST 5136 within clonal complex ST-464, giving the strong indication of clonal expansion of quinolone resistance with this ST. Furthermore, the ST 354 from clonal complex ST-354 had 16/17 (94%) resistant isolates in year 1 and was fully resistant in year 2.

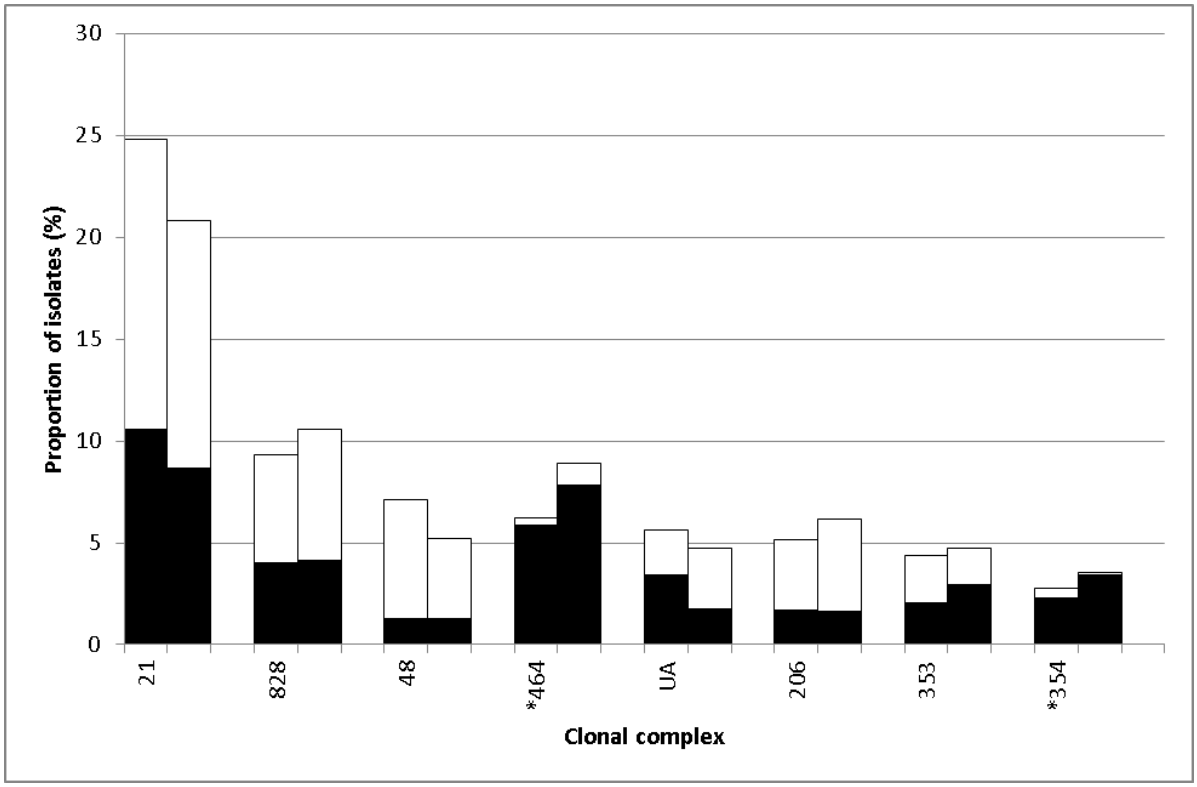


Figure 7: The distribution of quinolone resistant *Campylobacter jejuni* and *Campylobacter coli* human isolates from John Radcliffe Hospital catchment area, Oxfordshire, Oxford, between June 2011 and June 2013 among major clonal complexes. The asterisk indicates the statistically significant correlation of quinolone resistance with particular clonal complex in both years. Only clonal complexes with 10 or more representative isolates per year are presented.
Slika 7: Porazdelitev proti kinolonom odpornih humanih izolatov iz bolnišnice John Radcliffe v Oxfordshiru v Oxfordu med junijem 2011 in junijem 2013 v glavne klonske komplekse. Zvezdica nakazuje statistično značilno povezavo med kinolonsko odpornostjo in klonskim kompleksom v obeh letih. Na sliki so prikazani samo klonski kompleksi z 10 ali več predstavniki.

Clonal expansion was further examined by building the phylogenetic networks based on the core genome of isolates from clonal complex ST-464 (N=129), ST 354 (N=54) and ST-21(N=391) and investigating the distribution of *gyrA* alleles among the established phylogenetic clusters. As observed from the Figure 4, there were clear indications of clonal expansion of quinolone resistant isolates within the clonal complexes ST-464 (A) and ST-354 (B), while clonal structure of resistant isolates within clonal complex ST-21 (C) was less conspicuous.

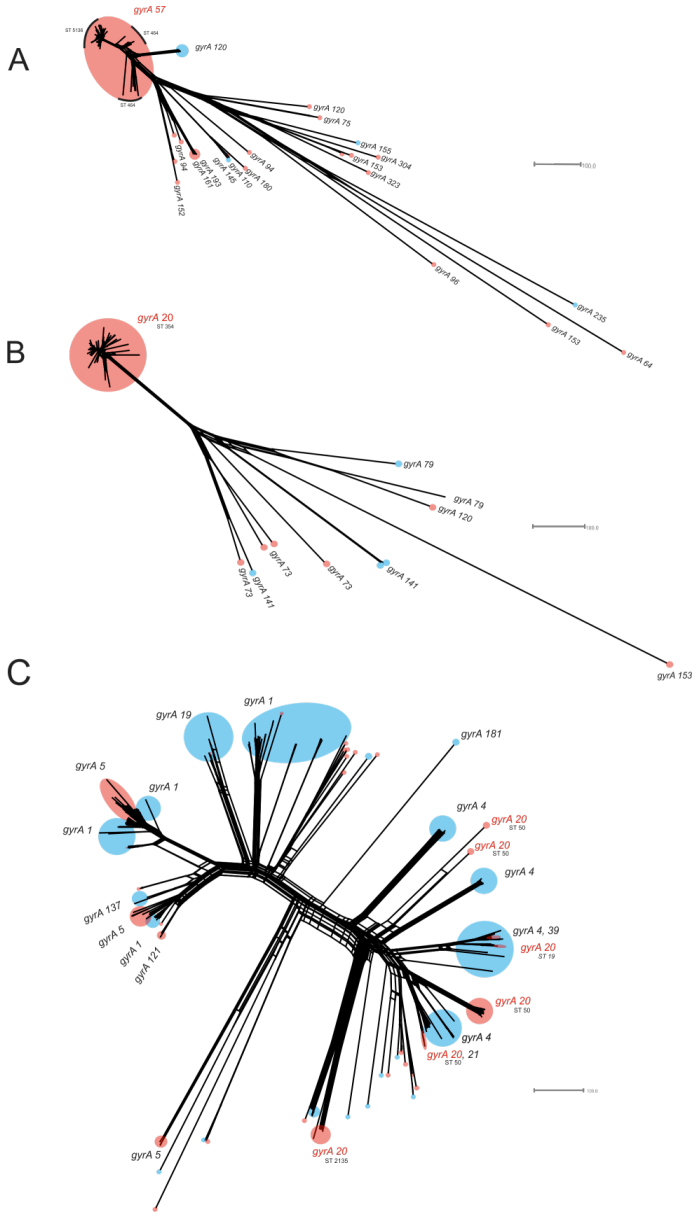


Figure 8: The distribution of *gyrA* alleles over the phylogenetic clusters established from core genomes of isolates from A) clonal complex ST-464, B) clonal complex ST-354 and C) clonal complex ST-21.The blue colour represents quinolone susceptible genotypes and the red colour represents the quinolone resistant genotypes.
Slika 8: Porazdelitev alelnih variant gena *gyrA* med filogenetskimi skupinami, pridobljenimi na podlagi primerjave jedrnih genomov izolatov iz A) klonskega kompleksa ST-464, B) klonskega kompleksa ST-354, C) klonskega kompleksa ST-21. Modra barva predstavlja na kinolone občutljive isolate, rdeča proti kinolonom odporne isolate.

The quinolone resistant isolates assigned to clonal complex ST-464 clearly clustered in three clonal groups, all of them possessing the *gyrA* allele type 57 ($p<0.001$). The *gyrA* allelic structure was uniform in MLST sequence type 5136, unambiguously indicating the clonal development of this group of resistant isolates, while higher level of variability was found in ST 464. There, *gyrA 57* was found in only 14/42 (33.3%) of the isolates, and the resistant isolates from ST 464 carrying the *gyrA* allele 57 formed 2 clusters, indicating the lower level

of clonality. The development of a single major group of resistant isolates in clonal complex ST-354 was associated with *gyrA* allele variant 20 ($p<0.001$) (Figure 4B).

As demonstrated by the consistent clustering of the resistant isolates with ST 354 and *gyrA 20* within a single phylogenetic group, the high quinolone resistance rate in this clonal complex was acquired by clonal growing of the resistant population. In contrast to ST-464 and ST-354, the phylogenetic structure of clonal complex ST-21 was substantially more diverse (Figure 4C). The resistance in ST-21 was associated with two *gyrA* variants – *gyrA 5* 60/165 (36.4%) and *gyrA 20* 85/165 (52.5%) ($p<0.01$). The first variant appeared mostly among isolates of MLST ST 21, while the second was found among MLST ST 19, ST 2135, and ST 50. The ST 50 was the only one of those with statistically significant correlation with quinolone resistance. As presented on the Figure 4C, some level of clonal clustering was observed for resistant isolates from clonal complex ST-21, which carried *gyrA* variant 20, however based on the presented evidence the nature of the resistance spreading within the ST-21 remains disputable.

Due to the previous reports of *mfd* gene contributing to the development of quinolone resistant mutations in *Campylobacter* (Han et al., 2008) we have investigated the link between specific *mfd* allele variants and quinolone resistance within our set of isolates. There were 182 unique *mfd* alleles identified in year 1 and 157 in year 2. The *mfd* alleles with 10 or more quinolone resistant representatives in each year, and with statistically significant association with quinolone resistance, were 1, 106, 39, 19, 31, and 111 (Figure 5).

Of those, the *mfd* allele 1 was associated with quinolone susceptibility in year 1, while *mfd* alleles 106, 39, 19, 31 and 111 were associated with quinolone resistance in both years ($p<0.01$). In each of those, the predominant proportion of resistant isolates carried a single resistance-associated *gyrA* variant. This was *gyrA* 57 in case of *mfd* 106, *gyrA 20* in case of *mfd* 39, 19, and 111, and *gyrA* 59 in case of *mfd* 31 (Figure 4). The combination of *mfd* 19 and *gyrA* 20 was typical for quinolone resistant isolates from CC ST 354, while the combination of *mfd* 39/111 and *gyrA* 20 was mostly found in quinolone resistant isolates from ST 50 ($p<0.0001$). The analysis of representative sequences of *mfd* alleles 1, 19, 39, 106, and 11 revealed 39 variable nucleotides over the total length of 1892 bp. As expected, the nucleotide sequences of *mfd* alleles 39 and 111 (both typical for ST 50) were the most similar to each other, while the *mfd* 106 (common in STs 464 and 5136) was the most distinct from all others.

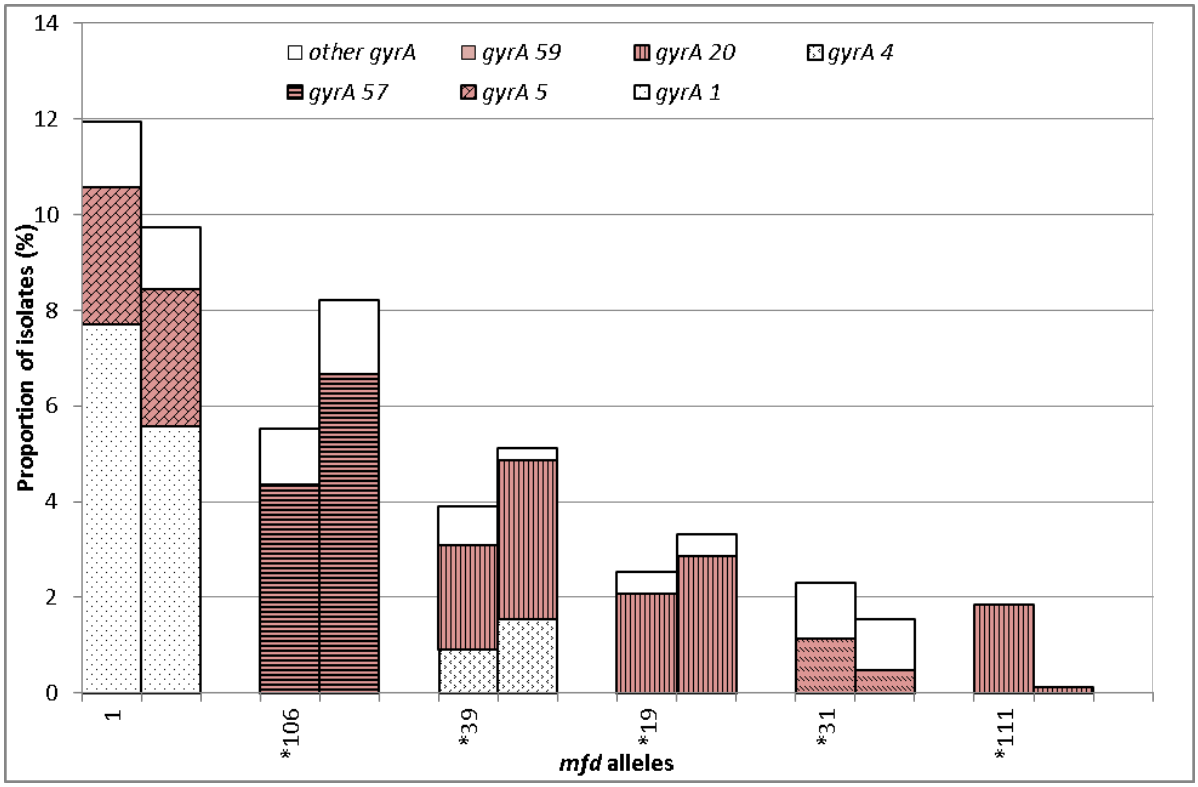


Figure 9: Distribution of *gyrA* alleles within the *mfd* alleles among *Campylobacter jejuni* and *Campylobacter coli* human isolates from John Radcliffe Hospital catchment area, Oxfordshire, Oxford, between June 2011 and June 2013, and their correlation with quinolone resistance.The *mfd* alleles with statistically significant correlation with quinolone resistance in both years are marked with asterisk. Resistant *gyrA* alleles are presented with red colour. Only the *mfd* alleles with 10 or more quinolone resistant representative isolates are presented.
Slika 9: Porazdelitev alelnih variant gena *gyrA* znotraj posameznih alelov gena *mfd* med humanimi izolati *Campylobacter jejuni* in *Campylobacter coli* iz bolnišnice John Radcliffe (junij 2011 - junij 2013). Aleli *mfd* s statistično značilno povezavo s kinolonsko odpornostjo v obeh letih so označeni z zvezdico. Alelne variante *gyrA*, ki so značilne za proti kinolonom odporne seve, so označene z rdečo bravo. Na sliki so predstavljeni samo aleli *mfd*, ki so zastopani z 10 ali več izolati.

Besides the resistance-conferring mutations in QRDR of *gyrA* gene, active efflux was reported to importantly contribute to the phenotypic resistance. We have analysed the variability and the distribution of the inner membrane transporter gene *cmeB* present in our set of isolates and the correlation of specific allele variants with quinolone resistance. The proportion of resistant and susceptible isolates within the seven *cmeB* alleles that had 10 or more resistant representative isolates are presented in Figure 6.

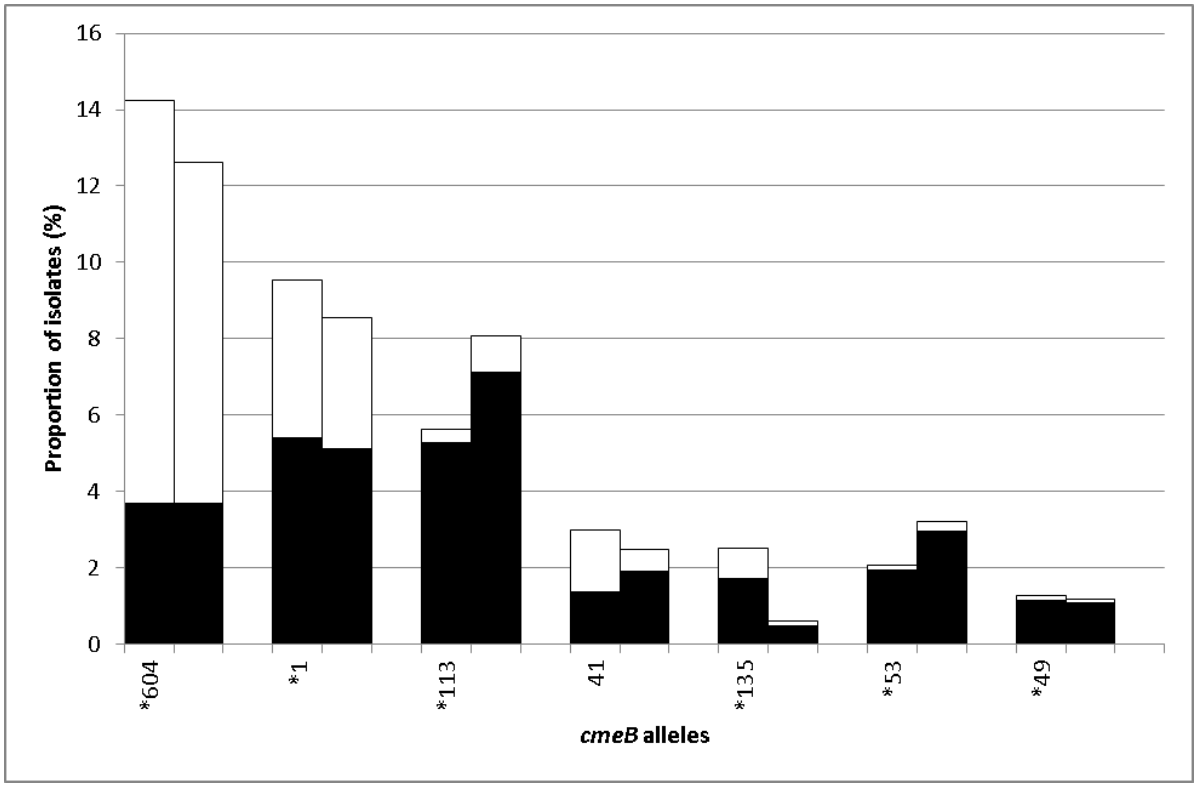


Figure 10: Distribution of *cmeB* alleles among *Campylobacter jejuni* and *Campylobacter coli* human isolates from John Radcliffe Hospital catchment area, Oxfordshire, Oxford, between June 2011 and June 2013, and their correlation with quinolone resistance. The white colour represents quinolone susceptible isolates and black colour quinolone resistant isolates. The *cmeB* alleles with statistically significant correlation with quinolone resistance or susceptibility in both years are marked with asterisk. Only the *cmeB* alleles with 10 or more quinolone resistant representative isolates are presented.
Slika 10: Porazdelitev alelnih variant gena *cmeB* med humanimi izolati *Campylobacter jejuni* in *Campylobacter coli* iz bolnišnice John Radcliffe v Oxfordshiru v Oxfordu (junij 2011 - junij 2013) in njihova povezava s kinolonsko odpornostjo. Bela barva označuje isolate odporne proti kinolonom in črna barva isolate odporne proti kinolonom. Alelne variante gena *cmeB*, ki so statistično značilno povezane s kinolonsko odpornostjo ali občutljivostjo so označene z zvezdico. Na sliki so predstavljeni samo aleli *cmeB*, ki so zastopani z 10 ali več izolati.

Of those, 1, 113, 135, and 53 were significantly linked with quinolone resistance ($p<0.001$). On the other hand, allele 604, along with 94, 51, 5, 14, 24, and 35 was associated with quinolone susceptibility in both years ($p<0.05$). Comparison of variable nucleotides within the two groups of *cmeB* alleles reviled no obvious variable hot spots within the gene, as the variability was observed all over the sequence length. The Neighbor-net phylogeny analysis demonstrated that *cmeB* 35 and 135 cluster in a separate group, and that alleles 1, 14, and 94 form an individual subgroup, suggesting that the resistant genotypes 135 and 1 were developed from susceptible allele types 35 and 14, and 94, respectively (data not shown).

3 GENERAL DISCUSSION AND CONCLUSIONS

3.1 GENERAL DISCUSSION

3.1.1 Underreporting of *Campylobacter* prevalence

Campylobacteriosis is a leading zoonotic food-borne disease in Europe and around the world. Its yearly incidence in 2012 in Europe and Slovenia was 55.49 and 45.4/100.000, respectively (EFSA, 2014a; IVZ, 2012). These numbers are still thought to be strongly underreported, since most campylobacteriosis cases remain unrecognized by the surveillance system due to the mild self-limiting symptoms or undetermined cases of gastroenteritis. In Slovenia, as well as in other European countries, there is a trend of increasing campylobacteriosis incidence (EFSA, 2014a; IVZ, 2012).

In order to evaluate the burden of the disease caused by *Campylobacter* spp. their true incidence needs to be estimated. This is thought to be much higher than reported due to under-diagnosis and underreporting. The under-diagnosis occurs because many cases who do not seek medical help are not acknowledged by the healthcare system, while the underreporting is a consequence of incorrect diagnosis, classification, notification or dissemination to surveillance authorities. All this depends on the pathogen and each individual country, since they have different availability of healthcare use, laboratory practice and surveillance system.

To estimate the true prevalence of campylobacteriosis in Slovenia, we have modelled the EU country-specific and *Campylobacter*-country-specific parameters which can be used in the prevalence estimation of seven food-borne pathogens in any of the EU Member countries. Parameters were modelled based on published data for seven EU Member countries (Haagsma et al., 2012) using common statistical data available through Eurostat.

We have identified the probability for a case of non-bloody diarrhoea to visit a GP to be the most sensitive parameter of the model predicting the real prevalence of campylobacteriosis. This was demonstrated with substantial changes in final multiplier occurring as a consequence of minor changes in this parameter. As finally observed, when calculating the multiplier of campylobacteriosis in Slovenia, the parameter d had strong influence on the uncertainty of the final multiplier, which was estimated to be 181.3. These results demonstrate that the cases of mild diarrhoea are the ones that contribute the most to the underreporting of food borne diseases. The underreporting due to this contributor is especially pronounced in the case of the diseases that evoke only mild diarrhoeal symptoms, such as campylobacteriosis.

3.1.2 Transmission of *C. jejuni* from environment, along the food production chain, to humans

Campylobacter is colonizing the gut of many production animals, in which it rarely causes disease symptoms. The meat of these animals is therefore often contaminated during the slaughtering and serves as a vehicle of transmittance of this pathogen via food chain. It naturally cycles from environmental waters, feedstuff, animals and food to humans (Silva et al., 2011) and is known for its fast adaptation ability and genomic instability (Jayaraman, 2011; Hänninen and Hannula, 2007; Wassenaar et al., 1998). When different reservoirs harbour strains with specific genotypes, it is therefore possible to determine the source of the infection using PFGE, MLST and/or *fla* typing (Sahin et al., 2012; Magnusson et al., 2010; Sheppard et al., 2009). Nevertheless, MLST has become the method of choice for *Campylobacter* genotyping. In combination with sequencing of the short variable region within the flagella-encoding *fla*B gene it allows further strain differentiation within the same MLST sequence types (ST) (Wirz et al., 2010).

We have determined antibiotic resistance profiles, as well as MLST and flaA-SVR types for 53 *C. jejuni* isolated in Slovenia from human, animal, raw and cured chicken meat and water samples. Twenty-nine different sequence types, arranged in eleven clonal complexes (CCs), three new allele types and five new sequence types were identified, indicating the relatively high diversity among a small group of strains. Twenty-three out of 29 STs appeared only once, which further confirms high genetic diversity. Sequence types belonging to clonal complexes ST-21 and ST-45 were previously confirmed to be predominant in European region and typical for poultry (Gripp et al., 2011; Wirz et al., 2010; Korczak et al., 2009) and were highly represented among isolates from poultry meat and faeces (63% and 75%) also in our study. All five isolates with the newly identified *tkt* allele type originated from the same region in Slovenia and were isolated from raw and cured chicken meat and human faeces, indicating chicken as the most probable source of human infection. The comparative analysis revealed some correlation between specific genotypes and their main sources of isolation. Majority of the human isolates (70%) were distributed into CC ST-21 and CC ST-353 and most of the meat isolates (63%) in CC ST-21 and CC ST-45, while animal isolates were mostly (67%) assigned to CC ST-21 and CC ST-354 (Kovač et al., 2014a).

Parallel to MLST typing, *flaA* typing was carried out, which was with 23 different genotypes found to be less discriminatory than MLST. The higher discriminatory power was also confirmed with higher Simpson's diversity index for MLST (0.942), compared to *flaA*-SVR (0.928). The combination of both typing methods increased the discriminatory power to 0.967. The most common *fla*A alleles were 36 (n=9/17%), 278 (n=8/15%) and 256 (n=7/13%). All *fla*A allele types 256 and 36 were distributed in to CC ST-21, while most of the strains with allele type 278 were assigned to CC ST-353 as well as to CC ST-21 and CC ST-48. Cluster analysis of *fla*A SVR sequences revealed six major clusters at similarity level of 95%. Statistical analysis confirmed *fla*A allele type 36 (n=8/89%) as typical for animal isolates and type 278 as typical for meat isolates (n=5/63%) (Kovač et al., 2014a).

The water isolates were genetically the most diverse group and two out of five could not be assigned to any existing clonal complex (Kovač et al., 2014a). With distinct sequence types they present an interesting group with little in common with isolates originating from biotic sources like humans, animals and meat.

3.1.3 Prevalence and epidemiological characteristics of *Campylobacter* antibiotic resistance

The prevalence of antibiotic resistance among campylobacters is constantly increasing (EFSA, 2014b). As we have seen in the previous work, the contribution of environment to the resistance prevalence is neglegable (Kovač et al., 2014). On the other hand, the highest incidence of resistance is recorded among the livestock isolates (EFSA, 2014b; Kovač et al., 2014). This is somewhat expected, due to the extensive use of antibiotics in the veterinary medicine (EMA, 2011a; EMA, 2011b). The problem is, that the resistance, together with the pathogen is transmitting along the food chain and, finally, compromising the effective treatment of human campylobacteriosis.

The most problematic antibiotic from the resistance point of view is ciprofloxacin from the class of quinolones. This family of antibiotics is used in veterinary as well as human medicine. In order to elucidate the epidemiology of the quinolone resistance of *C. jejuni*, we have investigated how the antibiotic spreads among isolates from different sources from environment, livestock and human (Kovač et al., 2014a). We have included 52 strains isolated in Slovenia in the study, where we employed the MLST to determine the clonal structure of this group of isolates. Furthermore, we have determined their phenotypical antibiotic resistance against seven antibiotics (gentamicin, streptomycin, ciprofloxacin, tetracycline, erythromycin, nalidixic acid and chloramphenicol). We have recorded that the highest prevalence of resistance to both of the quinolone antibiotics, ciprofloxacin and nalidixic acid, confirming the development of cross-resistance among the antibiotics of the same class. The incidence of resistance to quinolones was the highest in isolates belonging to the MLST clonal complex 21, which was an indication of the clonal spreading of this type of resistance. The resistance of *Campylobacter* spp. to quinolone antibiotics is known to be provided by the point mutation within the quinolone resistance determining region (QRDR) of the *gyrA* gene (Iovine, 2013). Besides this, it was proven that the strains with this point mutation are more fit to survive and colonize the host, compared to the quinolone sensitive strains, even in the absence of the antibiotic selective pressure (Hyytiäinen et al., 2013; Luo et al., 2005). This striking finding has further strengthened our belief about the clonal spread of quinolone antibiotic resistance. We have approached this investigation using the QRDR typing of the phenotypically ciprofloxacin resistant strains. The comparative analysis has revealed that all the quinolone resistant strains from clonal complex 21 were assigned to the separate cluster based on the similarity within the *gyrA* gene. This was the final proof of the quinolone resistance clonality, which rejected the alternative hypothesis about the frequent individual mutation acquirement (Kovač et al., 2014a).

Recently several other high-quality research works studying the similar topic were published and they all observed resistance to quinolones to be related to several clonal complexes, however, none of them provided the molecular evidence in form of genetic similarity in quinolone resistance determining region (Kittl et al., 2013; Wimalarathna et al., 2013; Stone et al., 2013; Wirz et al., 2010)

Due to the fact that the group of analysed strains in our study was relatively small and considered to be local from the total EU aspect, analysis on expanded, international set of strains needed to be carried out in order to confirm that the observed trend is not only local.

For this reason we have exploited the publicly available *Campylobacter jejuni* and *Campylobacter coli* whole genome sequences deposited in PubMLST database for the analysis of the quinolone resistance spreading among human clinical isolates from Oxfordshire, UK. The 1711 isolates included in the final analysis were collected between June 2011 and June 2013. Firstly, the gyrA gene of the analysed isolates was curated and the arbitrary allele number was assigned to each locus variant. The genotypic resistance was then assigned to each unique gyrA variant based on the presence of the resistance-conferring mutations within the QRDR. We have found that the quinolone resistance was the highest within the ST 50 of clonal complex ST-21 (66.7% in year 1 and 51.7% in year 2), within the CC ST-354 (83.3% in year 1 and 96.7% in year 2) and CC ST-464 (94.4%in year 1 and 88% in year 2). In order to explore the nature of the resistance expansion, we have build the core-genome phylogenies of isolates from clonal complexes ST-21, ST-354, and ST-464 and investigated the clustering of gyrA variants within the inferred phylogenetic networks. The distribution of gyrA allele variant 20, which was the main allele type associated with resistance in clonal complex ST-21, was observed in different clusters within the clonal complex, suggesting that it is not expanding clonally. On the other hand, the same *gyrA* variant clearly clustered within a major resistant group of isolates within the CC ST-354. Similarly, we observed the clustering of the isolates carrying the quinolone resistant *gyrA* allele 57 within a single group of CC ST-464, clearly indicating the clonal expansion of quinolone resistance with these two clonal complexes.

The increase in the rate of the resistance-conferring mutations within the *gyrA* gene was previously associated with the presence of the *mfd* (mutation frequency decline) gene (Han et al., 2008), which has lead us to investigate how the variability within the *mfd* might influence the quinolone resistance. We found that the alleles 106, 39, 19, 31 and 111 were associated with quinolone resistance, and that certain *gyrA* variants are significantly associated with certain *mfd* variants, suggesting that these two genes are not evolved independently. Similarly, also certain variants (1, 113, 135, and 53) of inner membrane transporter gene *cmeB* were strongly associated with quinolone resistance, indicating that these variants will result in the efflux pumps with higher activity.

The results of resistance spreading analysis of isolates from two different geographical areas both demonstrate the clonal expansion of the quinolone resistance among *Campylobacter*. Since the clonal spreading was observed with different clonal complexes, which are associated with more than one *gyrA* variant, the further research needs to be done in order to identify the key factors enabling the rapid spread of those resistant clones.

3.1.4 Anti-*Campylobacter*, resistance modulatory and efflux inhibitory activity of *A. katsumadai* seeds formulations

We have demonstrated in a previous study, that antibiotic resistance of *C. jejuni* clearly represents an alarming issue, especially, because it is the most prevalent foodborne bacterial cause of gastroenteritis in EU (EFSA, 2014a; Kovač et al., 2014a). For this reason, it is important to invest in the research of novel antimicrobials, also from plants, which represent an incredibly diverse source of bioactive chemicals. In our two studies we have investigated the anti-*Campylobacter* and resistance modulatory potential of *Alpinia katsumadai* seeds in

form of extracts, essential oil and post-distillation residue extract, which is usually disposed and represents an ecological burden (Kovač et al., 2014b). Plant formulations were tested against antibiotic resistant and antibiotic susceptible strains of *C. jejuni* and *C. coli* (Klančnik et al., 2012a; Kovač et al., 2014b). Additionally, their resistance modulatory and efflux inhibitory activity was investigated.

We have found that ethanol extract of *A. katsumadai* seeds has a moderate antimicrobial activity (125 – 2051 mg/L) and at the same time the best antimicrobial activity, compared to the post-distillation residue extract and the essential oil. The inhibitory effect of the latter was the weakest. There was no difference in antimicrobial potential of extracts prepared using ethanol, methanol, dichloromethane and hexane on the reference *C. jejuni* strain. Also, *C. coli* strains were more resistant to ethanol extract than *C. jejuni* strains (Klančnik et al., 2012a; Kovač et al., 2014b). This suggests that the seeds contain antimicrobial compounds of a wide polarity range.

All three plant formulations (extract, essential oil and the residue extract) were then further investigated for their resistance modulatory activity in quarter (0.25 MIC) or half (0.5 MIC) of their minimal inhibitory concentration in combination with two antibiotics (ciprofloxacin, erythromycin), disinfectant triclosan and common efflux pump substrate ethidium bromide (EtBr). The ethanol extract in a concentration of 0.25 MIC was confirmed as a *Campylobacter* resistance modulator in the first study, where modulation factors (MFs) of up to 64 were confirmed, but the activity was strain-specific (Klančnik et al., 2012a). When testing the post-distillation extract and essential oil as modulators at 0.5 MIC on *C. jejuni*, we found that the mean MF was 34 and 78, respectively, considering combinations with all antimicrobials. Overall the best resistance modulatory effects were observed in case of essential oil (Kovač et al., 2014b). For this reason we have further tested it in an EtBr accumulation assay, which is a good indicator of efflux inhibitory activity. Essential oil was able to significantly increase the accumulation of ethidium bromide in the reference *C. jejuni* strain. The accumulation achieved with *A. katsumadai* essential oil was even better than that with reference efflux pump inhibitor verapamil (Kovač et al., 2014b). To our knowledge, this is the first study of efflux inhibitory activity of plant extracts in *C. jejuni*. The demonstrated activity was exceptionally good, but the concentrations used (500 mg/L) were most likely too high to rationale its applicative use. Therefore we proceeded with screening of the resistance modulatory activity of the main chemical constituents of *A. katsumadai* seed essential oil.

3.1.5 Anti-*Campylobacter*, resistance modulatory and efflux inhibitory activity of (-)-α-pinene

Alpha-pinene was confirmed as one of the chemical constituents of *A. katsumadai* seed essential oil, where it represented 2.18% of total essential oil content (Šikić Pogačar et al., 2015, unpublished work). It was one of the candidate compounds that were likely responsible for antimicrobial and resistance modulatory activity of *A. katsumadai* seed essential oil presented in our previous studies (Klančnik et al., 2012a; Kovač et al., 2014b). It was further tested alone. Because (-)-α-pinene showed better results in preliminary experiments, this enantiomer was tested in further experiments to evaluate its antimicrobial, resistance modulatory and efflux inhibitory activity.

High MICs confirmed insignificant antimicrobial activity of (-)-α-pinene, but it had a high resistance modulatory potential. (-)-α-pinene was tested as a resistance modulator in concentrations 62.5 mg/L and 125 mg/L and it decreased the MICs of the tested antimicrobials by up to 2-fold in lower concentration and from 32 to over 256-fold in higher concentration, which was similar to observed for the essential oil. We have observed when evaluating the impact of (-)-α-pinene on the *C. jejuni* membrane integrity, the higher concentration was able to increase the membrane permeability, which was probably the cause of the better resistance modulatory effect.

Both concentrations of (-)-α-pinene were tested in EtBr accumulation assay to evaluate their efflux inhibitory potential and were equally good in increasing the intracellular EtBr accumulation, therefore all further tests were carried out using the lower concentration, which did not impair the membrane integrity, in order to assess the sole efflux inhibitory impact. It was later found that the same level of activity is remained also when the concentration of the compound is decreased to 8 mg/L. Since we were especially interested in the target of our tested compound, we have tested it also on the mutants with disrupted open reading frames (ORFs) of the *Campylobacter* spp. main efflux system CmeABC and the yet uncharacterized efflux protein Cj1687. In both cases the increase in the EtBr accumulation was significantly lower compared to the wild type, which confirmed these two efflux systems as the targets of (-)-α-pinen. We have therefore confirmed (-)-α-pinene as an efflux inhibitor in *C. jejuni* with potential to be used in medicine, especially because cytotoxicity of α-pinene was already investigated on HeLa and Cos7 cell lines, where IC_{50} concentrations of 357.9 and 337.5 mg/L were determined (Herrmann and Wink, 2011). These are 2 to 5-fold higher than those used in our study.

In a recent study, similarly active *Mycobacterium smegmatis* potential efflux pump inhibitors from *A. katsumadai* seed extracts were identified (Gröblacher et al., 2012a). The activity of those was expressed at concentrations 32 to 64 mg/L and was equal or more pronounced than that of CCCP, but lower than that of verapamil. The active compounds were identified as (5R)-trans-1,7-diphenyl-5-hydroxyhept-6-en-3-one, (3S,5S)-trans-1,7-diphenylhept-1-ene-3,5-diol and the flavanone pinocembrin (dihydrochrysin). Several putative inhibitors of *M. smegmatis* efflux pump were confirmed also in extracts of *Aframomum melegueta* (Gröblacher et al., 2012b). These were active in concentrations 16 to 64 mg/L. Another novel natural product isolated from *Hypericum olympicum* L. cf. uniflorum was recently confirmed to have efflux inhibitory activity also in *Staphylococcus aureus* (Shiu et al., 2013). However, these have not identified the inhibited efflux systems. (-)-α-pinene is the first confirmed *Campylobacter* spp. efflux pump inhibitor from plant sources.

3.1.6 Adaptation of *Campylobacter jejuni* to treatment with (-)-α-pinene

The bacterial response to stress caused by sub-inhibitory concentrations of phytochemicals is not yet well researched and the bacterial adaptation mechanisms are not fully understood. In our study we have investigated the *C. jejuni* response to treatment with sub-inhibitory concentration of efflux pump inhibitor (-)-α-pinen, in order to elucidate its mechanism of action and adaptation mechanism.

After 2-hour treatment of the reference strain with 62.5 mg/L of (-)-α-pinene, 129 genes were differently expressed, with cut-off set at ≥2-fold difference. Of those, 109 were up-regulated and 20 down-regulated. The most strongly up-regulated (over 10-fold) were chaperone encoding genes *grpE, dnaK, clpB* and the heat-shock regulator *hrcA*. Another two heat shock response genes encoding chaperonin *groES, cbpA* and transcriptional repressor *hspR* were also up-regulated, but at lower levels (2 - 4-fold). Besides heat shock-response genes, also efflux pump genes *cmeABC* were 2.7 to 2.2-fold up-regulated, respectively. Another putative efflux protein gene *Cj1687*, which has not yet been characterized in *C. jejuni* was 2.5-fold up-regulated. These results were the first indication of (-)-α-pinene targeting these efflux systems and has lead us to look deeper into it.

The expressional changes caused by treatment with (-)-α-pinene were strikingly similar to those previously observed in a mutant with defected transcriptional regulator *hspR,* i.e. highly up-regulated heat shock related genes *grpE, dnaK, clpB* and *hrcA* (Holmes et al., 2010). The simple heat shock response model from the same study suggests repression of *groES* and *groEL* by *hrcA* and repression of *dnaK*, *cbpA* and *clpB* operon by *hspR*, which become de-repressed in the event of heat shock and consequently *hrcA* is up-regulated. According to our results the same transcriptional avalanche seems to occur after treatment with (-)-α-pinene, suggesting that heat shock response plays an important role in bacterial defence and adaptation to this chemical stress. Similar response was observed also after treatment of *C. jejuni* with natural antimicrobial compound benzyl isothiocyanate (Dufour et al., 2013).

When the growth of mutants with disrupted ORFs *hspR, hrcA* and the wild type were examined in presence and absence of (-)-α-pinene, the wild type was able to grow best at given conditions, followed by *hrcA*, and *hspR*, respectively. The same or reduced growth of Δ*hspR* at normal growth temperatures was previously observed, while growth at high temperatures was increased (Holmes et al., 2010; Szintzi et al., 2005). Furthermore, the exposure to higher concentration of (-)-α-pinene had more pronounced negative effect on bacterial growth, compared to the wild type. The trend of decreasing growth in presence of (-)-α-pinene was observed in both, Δ*hrcA* and Δ*hspR*, indicating that these heat shock transcriptional repressors play an important role in adaptive response to treatment with (-)-α-pinene.

Furthermore, mutants in genes *hspR, hrcA* and wild type *C. jejuni* NCTC 11168 were grown on Biolog phenotype MicroArrays with different carbon sources and in presence of different osmolytes and pH, in order to elucidate their role in adaptation to this type of stresses. Growth of mutants was compared to the growth of wild type strain. The Δ*hspR* was growing better on some of the osmolytes, like 6% NaCl with KCl, sodium sulfate, sodium formate, sodium lactate and sodium phosphate, while growth of *hrcA* mutant, on the other hand, was inhibited by these and several other osmolytes. The *hrcA* was also not able to utilize a wide range of carbon sources as efficiently as the wild type. Besides Δ*hspR* showing increased ability to adapt to high concentrations of selected osmolytes, it was also previously confirmed to tolerate heat shock better than wild type, while the opposite was observed for Δ*hrcA* (Holmes et al., 2010). A former study of Cameron et al. (2012) showed that several heat shock related genes are up-regulated after osmotic shock and demonstrated that pre-

treatment of *C. jejuni* with osmolytes will provide protection against oxidative stress, but not to heat shock stress.

We concluded that the heat shock related genes importantly contribute to *C. jejuni* adaptation to several different stresses, like osmotic and chemical stress, including stress of (-)-α-pinene treatment. However, the mechanism itself is not yet fully understood, especially due to complex posttranslational regulation of heat shock transcriptional repressors HspR and HrcA.

3.2 CONCLUSIONS

In our study we have approached the problematic of campylobacteriosis and antibiotic resistance in *C. jejuni* in a holistic way, investigating the underreporting of the disease, the prevalence and spreading of the antibiotic resistance in the pathogen, as well as finding new solutions to solving the increasing antibiotic resistance problematic by identifying novel antimicrobial, resistance and efflux modulatory natural products. Furthermore the targets of their action and adaptation response of bacteria were investigated to give a clearer picture of their mode of action.

We have employed mathematical modelling using the Bayesian approach and linear regression to model the parameters influencing the underreporting and underdiagnosis (Haagsma et al., 2012). The underdiagnosis is the main reason for prevalence underestimation and the most significant contributions to the underdiagnosis arise, due to the low probability of a patient experiencing diarrhoea to visit a GP. The estimations based on our model show that for every case of reported campylobacteriosis in Slovenia there are 181 unreported cases. Most of the underreporting occurs due to the underdiagnosis of non-bloody diarrhoeal cases.

We have confirmed the reports of high ciprofloxacin resistance, especially among poultry meat and faeces isolates. The resistance is more commonly observed in isolates with genotypes belonging to MLST clonal complex 21. Additionally, we have employed the *gyrA* QRDR typing and confirmed the clonal spreading of ciprofloxacin resistant strains based on the MLST clonality and genetic similarity of QRDR (Kovač et al., 2014a). Clonal expansion of quinolone resistance was additionally confirmed among the larger number of *C. jejuni* and *C. coli* strains from Oxfordshire, UK, based on their whole genome sequences. There however, the clonal complexes ST-354 and ST-464 were the ones with the highest proportions of quinolone resistant clones.

We investigated the antimicrobial, resistance modulatory and efflux inhibitory potential of a Zingiberaceae, *Alpinia katsumadai,* used in traditional Chinese medicine. Its extracts from seeds, post-distillation residue extract, and essential oil had moderate to weak antimicrobial activity and essential oil was found to have high resistance modulatory and efflux inhibitory potential in *C. jejuni* (Klančnik et al, 2012a; Kovač et al, 2014b). Furthermore, the pure terpene compound α-pinene was found as one of the constituents of this essential oil and one of its enantiomers, (-)-α-pinene, was confirmed as a strong modulator of *C. jejuni* resistance against ciprofloxacin, erythromycin, triclosan and ethidium bromide. It has an efflux inhibitory activity that is significantly better than that of the reference efflux inhibitors CCCP and reserpine. The efflux systems CmeABC and Cj1687 were identified as the main targets of its efflux inhibitory activity.

C. jejuni responded to treatment with (-)-α-pinene using the same regulatory set of genes as in heat shock response. This mechanism of adaptation was found also in response to other types of stresses, like osmotic stress and seems to be a type of general stress response system that protects bacteria from protein coagulation and thereby preventing the fatal consequences.

4 SUMMARY

4.1 SUMMARY

Campylobacteriosis is the most prevalent bacterial foodborne disease in EU and its incidence is thought to be significantly underreported, due to the self-limiting nature of the disease, which sometimes is expressed with only mild symptoms (EFSA, 2014a). Even so, the disease poses a tremendous economical and healthcare burden; therefore there have been many attempts of the estimation of its real prevalence. Over the time, many approaches to accomplishing that have been presented, but the most frequently used is the evaluating vital fractions that influence the underdiagnosis and underreporting of the disease. The prevalence is underestimated due to underreporting and under-diagnosis associated with passive surveillance, incorrect diagnosis and inefficient communication with authorities, as well as with the unregistered cases with mild symptoms that do not seek medical help (Haagsma et al., 2012).

All the factors, which we believe are influencing the reporting prevalence, can be systematically updated given the data from successive finite samples using the Bayes' theorem, which rationally changes a subjective belief by taking evidence into account. In order to build a Bayesian probabilistic graphical model, it is necessary to define the model parameters affecting the prevalence reporting, which are then systematically incorporated in the model. Because different parameters affecting the process of reporting are involved in cases of hospitalization, these are incorporated in a separate branch of the model. Also the differences in symptoms and severity of the disease caused by different pathogens, as well as the differences in medical and reporting practice among EU countries are considered, as well as the fact that some of the parameters depend on the country and others depend on the pathogen (Scallan et al., 2006; Scallan et al., 2011; Haagsma et al., 2012).

In our study parameters were modelled using linear regression based on existing data for seven foodborne pathogens in seven EU countries. Only common statistical data available for EU countries was used to model the parameter uncertainty distributions. The Monte Carlo simulations of modelled parameters were used to estimate foodborne pathogen prevalence in additional EU countries. According to the model, the prevalence of campylobacteriosis in Slovenia needs to be corrected for the multiplier 181.

Furthermore, the sensitivity of the model was investigated and the analysis revealed that in the EU countries the crucial factor, contributing the most to the underreporting is the probability of visiting the GP when experiencing the non-bloody diarrhoea. This can be explained with the fact, that the disease is of self-limiting nature and the patients, who experience only mild symptoms do not tend to seek medical help.

Since the real prevalence of the disease is significantly higher than reported, it is important to understand how the pathogen spreads to human, in order to limit the disease rate. Nowadays the poultry meat is identified as the main source of sporadic campylobacteriosis infections, although most of the outbreaks are caused by consumption of contaminated water

or milk. *Campylobacter* spp. are bacteria that naturally cycles from environment to livestock and finally via meat to human, but the role of the environmental isolates in this chain is not fully understood.

In our study we have investigated the transmittance of *Campylobacter jejuni* from environment along the food production chain to humans by employing the multi locus sequence typing (MLST) and *flaA* as the molecular tracing tools. MLST is a convenient typing method which enables to detect the long-term evolutionary genetic changes by examining the sequence variation of multiple loci of conserved housekeeping genes. It is portable, unambiguous and the results can be deposited in the international publicly available PubMLST database. *FlaA* typing was included to additionally increase the discriminatory power. Using MLST we have investigated the clonal structure of the selected *C. jejuni* population, comprising isolates from environmental waters, farm animals, poultry meat and human clinical cases. Forty-eight *C. jejuni* isolates out of total of 52 were assigned to ten pre-defined CCs and 28 STs by MLST. Clonal complexes 21 and 45 predominated and sequence types (ST) 50 and 104 were the most frequent sequence types. Twenty-three STs appeared only once, indicating high genetic diversity within a relatively small group of strain (Kovač et al., 2014a).

The distribution of isolates in the MST analysis indicates some correlation between specific genotypes and their main source of isolation. The majority of human isolates (71%) were distributed in CC 21 and CC 353 and most of the meat isolates (67%) in CC 21 and CC 45, while animal isolates were mostly (67%) assigned to CC 21 and CC 354. Sequence types belonging to clonal complexes 21 and 45 were previously shown to be predominant in Europe and typical for poultry. In our case, 12 of 19 isolates assigned to CC 21 and six of eight assigned to the CC 45 were isolated from poultry meat or faeces. Water isolates were genetically the most diverse group and two out of five could not be assigned to any existing clonal complex. Their distinct sequence types present an interesting group with little in common with isolates from human and animal sources (Kovač et al., 2014a).

Besides understanding the pathogen's epidemiological characteristics, it is of great importance to elucidate also the trends of its antibiotic resistance spreading. Employing the molecular tools has allowed the correlation of specific genotypes with specific antimicrobial resistance profiles and shown that resistance to quinolones and macrolides are mostly associated with point mutations in *gyrA* and 23S rRNA, respectively, acting together with efflux mechanisms. The RND type efflux pumps of some Gram negative bacteria are able to extrude different types of antibiotics and can also be induced by their substrates (Mohamoud et al., 2007). This indicates that environmental selective pressures play an important role in acquiring nonspecific, via active efflux, or specific, via point mutation, resistance against antimicrobial drugs. Specific STs have already been associated with quinolone resistance in strains with point mutations, but it is not yet known whether this is because these genotypes are more prone to the mutations conferring resistance or because they are clonal (Wirz et al, 2010; Kittl et al., 2013; Wimalarathna et al., 2013). In our study we have determined the antibiotic resistance, MLST and analysed the genetic similarity of ciprofloxacin resistant strains based on the quinolone resistance determining region (QRDR) within the gyrase gene *gyrA* in order to elucidate the characteristics of quinolone resistance expansion in *C. jejuni* (Kovač et al., 2014a).

Antibiotic resistance in *C. jejuni* is becoming a significant issue, not only in Slovenia, but also in other European countries with the most problematic being resistance against quinolones, which are the second group of drugs of choice for campylobacteriosis treatment. In Slovenia quinolones are widely used for the nonspecific treatment of bacterial gastroenteritis as well as for enterococcal and *E. coli* infections in poultry flocks. The prevalence of ciprofloxacin resistant animal *Campylobacter* isolates in Europe is reported to range from 37% to 84%, depending on the country which is consistent with our study where 72% of animal and 44% of meat isolates were ciprofloxacin resistant and this increased to 80% for human isolates. Almost all (94%) of ciprofloxacin resistant strains from our study were cross-resistant to nalidixic acid (EFSA, 2014; IVZ, 2014).

Analysis of antibiotic resistance and MLST genotypes revealed that all but one of the 19 strains of *C. jejuni* belonging to the predominant MLST clonal complex (CC) 21 were quinolone resistant. In order to determine whether the association of quinolone resistance with CC 21 was due to individual mutational events or an increased ability to spread clonally, we investigated the most common ciprofloxacin resistance conferring mutation Thr-86-to-Ile and analysed the QRDR by sequence typing. The results show that all phenotypically ciprofloxacin resistant isolates, with the exception of strain 180/08, have the mutation ACA to ATA in the 86th codon. Six additional silent mutations in the QRDR, were identified, which clearly indicate the high degree of genetic relatedness of ciprofloxacin resistant isolates from CC 21, which together with one strain from CC 658 and two strains with unassigned CCs form a separate group, when compared to ciprofloxacin resistant strains from all other clonal complexes. As quinolone resistance is quickly developed under antibiotic selective pressure and can persist long after cessation of treatment, we conclude that the high incidence of such strains within CC 21 found in our study is not due to high genetic plasticity of this particular clonal complex, but rather due to acquired efficiency of clonal spreading (Kovač et al., 2014a).

The clonal expansion of quinolone resistance was further investigated by analysis of the whole genome sequences of 1711 *C. jejuni* and *C. coli* clinical strains isolated in Oxfordshire, UK, between June 2011 and June 2013. The prevalence of quinolone resistance was determined by assigning the resistance to isolates with resistance-conferring mutations in the *gyrA* gene. It was found that the quinolone resistance was the highest within the ST 50 of clonal complex ST-21 (66.7% in year 1 and 51.7% in year 2), within the CC ST-354 (83.3% in year 1 and 96.7% in year 2) and CC ST-464 (94.4%in year 1 and 88% in year 2). In order to explore the nature of the resistance expansion, we have built the core-genome phylogenies of isolates from clonal complexes ST-21, ST-354, and ST-464 and investigated the clustering of *gyrA* variants within the inferred phylogenetic networks. The distribution of *gyrA* allele variant 20, which was the main allele type associated with resistance in clonal complex ST-21, was observed in different clusters within the clonal complex, suggesting that it is not expanding clonally. On the other hand, the same *gyrA* variant clearly clustered within a major resistant group of isolates within the CC ST-354. Similarly, we observed the clustering of the isolates carrying the quinolone resistant *gyrA* allele 57 within a single group of CC ST-464, clearly indicating the clonal expansion of quinolone resistance with these two clonal complexes.

The increase in the rate of the resistance-conferring mutations within the *gyrA* gene was previously associated with the presence of the *mfd* (mutation frequency decline) gene (Han et al., 2008), which has lead us to investigate how the variability within the *mfd* might influence the quinolone resistance. We found that the alleles 106, 39, 19, 31 and 111 were associated with quinolone resistance, and that certain *gyrA* variants are significantly associated with certain *mfd* variants, suggesting that these two genes are not evolved independently. Similarly, also certain variants (1, 113, 135, and 53) of inner membrane transporter gene *cmeB* were strongly associated with quinolone resistance, indicating that these variants will result in the efflux pumps with higher activity.

The results of resistance spreading analysis of isolates from two different geographical areas both demonstrate the clonal expansion of the quinolone resistance among *Campylobacter*. Since the clonal spreading was observed with different clonal complexes, which are associated with more than one *gyrA* variant, the further research needs to be done in order to identify the key factors enabling the rapid spread of those resistant clones.

Increasing antibiotic resistance clearly presents a serious problem, since clinically used antibiotics are becoming less or completely inefficient in campylobacteriosis treatment. Search for alternative antimicrobials derived from plants seems to be a viable solution for mitigation of resistance. Plant extracts rich in phenolic compounds (phenolic extracts), and essential oils have long been shown to possess antimicrobial activity and were frequently studied and reviewed (Cowan, 1999; Burt, 2004; Klančnik et al., 2012c; Negi, 2012). They are especially interesting as they are generally recognized as safe and have potential to be used as preservatives in food products. *Alpinia katsumadai* Hayata (Zingiberaceae) is widely used in traditional Chinese medicine as an anti-emetic remedy and to increase the appetite, but also in animal feed, to facilitate rapid growth of domestic animals. In our study we have focused on investigating bioactivity of the *A. katsumadai* extracts, essential oil and also the extract from the post-distillation material, which remains as a waste product after essential oil production. Such residual materials are often disposed of and may present an environmental problem. Their high phenolic content and the potential to provide an economically feasible source of natural antioxidants and antimicrobials is unused.

Initially *A. katsumadai* seeds, extracted with different solvents were tested for antimicrobial activity against the reference *C. jejuni* strain and there was no difference in the antimicrobial activity (MIC=512 mg/L). Further, ethanol extract, essential oil and the post-distillation extract of *A. katsumadai* seeds were tested for their antimicrobial activity against several *C. jejuni* and/or *C. coli* isolates. Among each bacterial species tested, antibiotic sensitive and resistant strains, including multidrug resistant ones, were included. Statistically significant differences between the antimicrobial activities of different plant formulations were observed. According to the average MICs of each formulation, *A. katsumadai* ethanol extract from seeds was the most efficient antimicrobial plant formulation, with MICs ranging from 125 to 2051 mg/L, followed by its post-distillation extract. We could not confirm any difference between antimicrobial activities of tested plant formulations against antibiotic sensitive or resistant strains; they were active also against multidrug resistant strains. As observed from our results, the antimicrobial activity of tested plant formulations is species- and strain-specific. According to the MICs, which are consistently higher than 100 mg/L, we can conclude, that they have weak to moderate antimicrobial activity in *Campylobacter* (Klančnik et al, 2012a; Kovač et al., 2014b).

In addition to searching for new antimicrobially active plant formulations, the antibiotic resistance combating strategies are focused also on resistance modulators. These are not necessarily antimicrobially active, but can decrease the resistance of pathogens when administered together with other antimicrobials. The microbial sensitization by resistance modulators is mostly due to efflux inhibition, increased membrane permeability, and increased porin production or change in porin profile (Bolla et al., 2011).

Beside direct antimicrobial activity, we tested and confirmed also remarkable resistance-modulatory activity of *A. katsumadai* extract, essential oil and post-distillation extract in *C. jejuni*. *A. katsumadai* seed ethanol extract was confirmed for the first time to modulate antibiotic resistance in *Campylobacter* in our first study, where it was demonstrated to decrease the MICs of tested antimicrobials ciprofloxacin, erythromycin, ethidium bromide, bile salts, sodium deoxycholate, rosemary extract and epigalocatechin gallate for up to 16-fold. In the second study we comparatively investigated the resistance-modulatory potential of additional *A. katsumadai* essential oil and post-distillation extract. They were tested as modulators in concentrations half of their MIC on each individual strain of *C. jejuni* and in combination with antimicrobials ciprofloxacin, erythromycin, triclosan, bile salts and ethidium bromide. When testing the post-distillation extract and essential oil as modulators, we found that the mean MF value was 34 and 78, respectively, considering combinations with all antimicrobials. The essential oil was therefore confirmed as the best modulator. The comparative analysis of modulatory activity of *A. katsumadai* formulations in *Campylobacter* confirmed them as equally efficient in combination with different antimicrobials, including bile salts (mean MF >55). This is important since the intestinal tract with the presence of bile salts is a natural environment and reservoir of *Campylobacter*, so resistance to bile salts is essential for *Campylobacter* survival and virulence potential (Klančnik et al., 2012a; Kovač et al., 2014b).

There are several possible mechanisms of increasing susceptibility of bacteria to antibiotics and other antimicrobials. The most promising are focusing on increasing the antibiotic influx by destabilizing LPS in Gram negative bacteria and increasing the membrane permeability and blocking the efflux using efflux pump inhibitors (Bolla et al., 2011). In order to elucidate the mechanism of modulatory activity of *A. katsumadai* essential oil in *C. jejuni*, we have evaluated its potential to increase the accumulation of the common efflux pump substrate ethidium bromide, which is an indicator of the efflux inhibition. The extract and post-distillation extract were excluded from the experiments because of the high auto fluorescence. We have compared the levels of EtBr accumulation in cultures treated with 0.5 and 0.25 MIC of essential oil, relative to untreated culture, to evaluate whether it can potentiate the intracellular EtBr accumulation. Known efflux pump inhibitor verapamil was included in the study as a positive reference (Kovač et al., 2014b).

The results showed significant increase in EtBr accumulation in presence of *A. katsumadai* essential oil. The accumulation of EtBr was for 1.7-fold better in presence of 0.5 MIC of *A. katsumadai* essential oil than in the presence of positive control, verapamil. The mean accumulation values in presence of 0.25 MIC of essential oil were almost the same as in presence of 0.5 MIC, only the time needed to reach the maximum accumulation was different. The gradual decrease in EtBr accumulation after reaching the plateau might be due

to the facilitation of EtBr influx by essential oil and insufficient capacity to block the efflux (Kovač et al., 2014b).

Because of the efficient resistance modulatory and efflux inhibitory activity of the *A. katsumadai* essential oil, we have aimed to identify the compounds responsible for this activity. We have analysed the chemical composition of the essential oil and identified 95% of the compounds present. The compounds present in amounts over 1% were (in descending order) 2Z,6E-farnesol, 1,8-cineole, α-humulene, cymene, α-phellandrene, carotol, caryophyllene, daucene, α-pinene, δ-cadinene, 2-butanone, 3-phenyl or 2-butanone, 4-phenyl, α-terpineol and β-pinene. Our pre-screening has lead us to analyse more in depth the compound α-pinene, which was present in the essential oil in the amount of 2.2%. Due to the better activity in the initial tests, we have used the (-)-α-pinene in further investigations.

The MICs of (-)-α-pinene were 1000 mg/L or higher in all tested strains of *C. jejuni*, including those tested in resistance modulation assay. High MICs confirmed insignificant antimicrobial activity of (-)-α-pinene. As a potential resistance modulator (-)-α-pinene was tested in concentrations 62.5 mg/L and 125 mg/L in combination with antibiotics ciprofloxacin and erythromycin, disinfectant triclosan and efflux pump substrate EtBr on a reference strain *C. jejuni* NCTC 11168. In concentration 62.5 mg/L it decreased the MICs of these antimicrobials by up to 2-fold and in concentration 125 mg/L from 32 to over 256-fold. Similarly, strong modulatory activity was observed also for seed essential oil.

Before the EtBr accumulation assessment, both concentrations of (-)-α-pinene used in the resistance modulatory assay were tested for their influence on the *C. jejuni* membrane integrity in order to refute the influence on increased membrane permeability as the main mechanism of resistance modulation. The membrane integrity of culture treated with 62.5 mg/L was even by 20% higher relative to untreated culture after 1 h treatment, while higher concentration of 125 mg/L decreased the membrane integrity by 39%.

Both concentrations of (-)-α-pinene were tested for their ability to increase the intracellular EtBr accumulation in reference *C. jejuni* strain. The use of lower concentration of 62.5 mg/L resulted in higher EtBr accumulation. At the same time this concentration did not have negative impact on membrane integrity and was therefore used in further screening of a larger set of isolates. This enabled us to investigate solely its efflux inhibitory effects. We observed relative increase in EtBr accumulation when (-)-α-pinene was added in all 16 tested strains. The (-)-α-pinene was able to increase EtBr accumulation significantly better compared to CCCP and reserpine in all tested strains, confirming its remarkable efflux inhibitory activity. It maintained the same level of activity also in concentration 8 mg/L. This suggests that it could be the compound responsible for efflux inhibition in *A. katsumadai* seed essential oil, although the share of (-)-α-pinene in the α-pinene present in the essential oil was not determined. However, the contribution of other constituents to the overall efflux inhibitory activity of the essential oil cannot be excluded.

To determine the target efflux system of (-)-α-pinene, we have compared the EtBr accumulation in wild type and in mutants with interrupted open reading frames of efflux pump genes Δ*cmeB* and Δ*Cj168*. The accumulation of EtBr in the mutants was significantly lower, indicating the absence of (-)-α-pinene target in these two mutants. From these results

we conclude, that efflux systems CmeABC and Cj1687 are the targets of (-)-α-Pinene efflux inhibitory activity.

In order to decipher the bacterial adaptive response to treatment with sub-inhibitory concentrations of (-)-α-pinene used in the EtBr accumulation assay, we have carried out the whole genome expressional analysis using microarray technology and qPCR. After 2-hour treatment with 62.5 mg/L of (-)-α-pinene, 129 genes of *C. jejuni* NCTC 11168 were differently expressed, with cut-off set at ≥2-fold difference. Of those, 109 were up-regulated and 20 down-regulated. The most strongly up-regulated (over 10-fold) were chaperone encoding genes *grpE, dnaK, clpB* and the heat-shock regulator *hrcA*. Another two heat shock response genes encoding chaperonin *groES, cbpA* and transcriptional repressor *hspR* were also up-regulated, but at lower levels (2- 4-fold). Besides heat shock-response genes, also efflux pump genes *cmeABC* were 2.7 to 2.2-fold up-regulated, respectively. Another putative efflux protein gene *Cj1687*, which has not yet been characterized in *C. jejuni* was 2.5-fold up-regulated.

The expressional changes caused by treatment with (-)-α-pinene were strikingly similar to those previously observed in a mutant with defected transcriptional regulator *hspR*. That is highly upregulated heat shock related genes *grpE, dnaK, clpB* and *hrcA*. The simple heat shock response model from the same study suggests repression of *groES* and *groEL* by *hrcA* and repression of *dnaK*, *cbpA* and *clpB* operon by *hspR*, which become derepressed in the event of heat shock and consequently hrcA is upregulated. According to our results the same transcriptional avalanche seems to occur after treatment with (-)-α-pinene, suggesting that heat shock response plays an important role in bacterial defense and adaptation to this chemical stress. Similar response was observed in another study also after treatment of *C. jejuni* with natural antimicrobial compound benzyl isothiocyanate.

To confirm the role of these genes in the adaptive response, we have constructed the mutants in genes *hspR* and *hrcA* and use them in the phenotypical tests. The growth of mutants with disrupted ORFs *hspR, hrcA* and the wild type NCTC 11168 was examined in presence and absence of (-)-α-pinene. The wild type NCTC 11168 was able to grow best at given conditions, followed by *hrcA*, and *hspR*, respectively. Furthermore, the exposure to higher concentration of (-)-α-pinene had more pronounced negative effect on bacterial growth, compared to the untreated cultures. The trend of decreasing growth in presence of (-)-α-pinene was observed in both, Δ*hrcA* and Δ*hspR*, indicating that these heat shock transcriptional repressors play and important role in adaptive response to treatment with (-)-α-pinene.

Furthermore, mutants in genes *hspR, hrcA* and wild type *C. jejuni* NCTC 11168 were grown on Biolog phenotype MicroArrays with different carbon sources and in presence of different osmolytes and pH, in order to elucidate their role in adaptation to this type of stresses. Growth of mutants was compared to the growth of wild type strain. The *hspR* mutant was growing better on some of the osmolytes, like 6% NaCl with KCl, sodium sulphate, sodium formate, sodium lactate and sodium phosphate, while growth of *hrcA* mutant, on the other hand, was inhibited by these and several other osmolytes. The *hrcA* was also not able to utilize a wide range of carbon sources as efficiently as the wild type.

Based on these results we conclude that heat shock related genes importantly contribute to *C. jejuni* adaptation to several different stresses, like osmotic and chemical stress, including stress of (-)-α-pinene treatment. However, the mechanism itself is not yet fully understood, especially due to complex posttranslational regulation of heat shock transcriptional repressors HspR and HrcA.

4.2 POVZETEK

Kampilobakterioza je v državah Evropske Unije najpogostejša bolezen povzročena z okužbami s hrano (EFSA, 2014a). Ker se bolezen pogosto kaže le z blagimi simptomi in se pozdravi tudi brez zdravljenja, se predvideva, da je dejanska prevalenca bolezni znatno višja od poročane. Ker kampilobakterioza predstavlja veliko ekonomsko in javnozdravstveno breme, je ocena realne prevalence bolezni cilj številnih raziskav. Skozi čas so bili predstavljeni različni pristopi k sistematičnemu ocenjevanju stopnje podcenjenosti poročanja, vendar se je najbolj uveljavil način ocenjevanja ključnih dejavnikov, ki prispevajo k podcenjevanju diagnoz in poročanja kampilobakterioz. Prevalenca bolezni je podcenjena zaradi nezadostnega poročanja in diagnosticiranja, ki je posledica pasivnega nadzora, nepravilnih diagnoz in neučinkovitega komuniciranja z oblastmi, kot tudi zaradi neregistriranih primerov z blagimi simptomi, ki se ne odločijo za posvetovanje z zdravnikom (Haagsma in sod., 2012).

Vse dejavnike, za katere verjamemo, da vplivajo na nezadostno poročanje prevalence, lahko sistematično upoštevamo pri oceni realne prevalence s pomočjo Bayesovega teorema, ki racionalno preoblikuje subjektivna prepričanja s tem, da upošteva dokaze v obliki podatkov, pridobljenih iz zaporednih končnih vzorcev. Prvi korak k postavitvi Bayezijskega grafičnega verjetnostnega modela je definicija parametrov modela, za katere verjamemo, da vplivajo na nezadostno poročanje prevalence. Ti parametri so nato sistematično vgrajeni v model. Ker na poročanje prevalence hospitaliziranih primerov vplivajo drugačni parametri, kot na poročanje primerov, ki obiščejo samo osebnega zdravnika, so hospitalizirani primeri v model vključeni kot ločena veja. Naslednja razlika, ki jo je potrebno upoštevati pri grajenju modela, so razlike v simptomih in resnosti bolezni v primeru različnih patogenov, kot tudi razlike v medicinskih in poročevalnih navadah med posameznimi državami Evropske Unije. Tako so nekateri parametri odvisni od patogena, ki povzroča bolezen, spet drugi od države (Scallan in sod., 2006; Scallan in sod., 2011; Haagsma in sod., 2012).

V naši študiji smo parametre modelirali s pomočjo linearne regresije na podlagi obstoječih podatkov za sedem patogenov, povezanih s hrano v sedmih državah Evropske Unije. Pri modeliranju parametrov smo uporabili samo splošne statistične podatke, dostopne na Eurostatu. S pomočjo Monte Carlo simulacij smo upoštevajoč strukturo Bayezijskega modela ocenili prevalence patogenov v dodatnih državah EU. Naša ocena kaže na znatno podcenjenost prevalence kampilobakterioz v Sloveniji, in sicer za faktor 181.

Poleg ocene stopnje nezadostnega poročanja smo analizirali tudi občutljivost modela. Analize so pokazale, da je verjetnost bolnika za obisk zdravnika dejavnik, ki ključno prispeva k nezadostnemu poročanju. Še posebaj majhna je verjetnost za obisk zdravnika pri bolnikih z nekrvavo diarejo. To pojasni dejstvo, da je kampilobakterioza bolezen, ki se pogosto odraža v blagih simptomih in je samoomejujoča. Zaradi tega se veliko bolnikov ne odloči za iskanje medicinske pomoči in njihovi primeri ostanejo spregledani s strain oblasti.

Ker je realna prevalenca znatno višja od poročane, je za njeno omejitev oz. znižanje nujno potrebno razumevanje širjenja patogena do človeka. Dandanes je perutninsko meso identificirano kot glavni vir sporadičnih okužb z bakterijami *Campylobacter* spp., čeprav je večina izbruhov povzročenih zaradi uživanja kontaminirane vode ali mleka. *Campylobacter*

spp. so bakterije, ki naravno krožijo iz okolja do proizvodnih živali in končno preko mesa do ljudi. Kljub temu ostaja vloga okoljskih sevov v tej verigi prenosa slabo pojasnjena.

V našem delu smo raziskovali širjenje bakterij *Campylobacter jejuni* iz okoljskih virov preko živilsko predelovalne verige do ljudi. Pri tem smo uporabili multilokusno sekvenčno tipizacijo (MLST) in tipizacijo na podlagi gena *flaA* kot molekularni sledilni orodji. MLST je priročna tipizacijska metoda, ki omogoča detekcijo dolgoročnih evolucijskih genetskih sprememb. Osredotoča se na analizo sekvenčnih variacij v sedmih ohranjenih hišnih genih. Metoda je prenosljiva, nedvoumna in enostavna. Rezultati so zaradi tega primerljivi med laboratoriji, poleg tega pa se lahko uvozijo v javno dostopno podatkovno bazo PubMLST. Tipizacija na podlagi *flaA* je bila vključena z namenom povišanja diskriminatorne moči. S pomočjo metode MLST smo preučevali klonsko strukturo izbrane populacije *C. jejuni*, v katero smo vključili seve iz okoljskih voda, farmskih živali, piščančjega mesa in humanih kliničnih primerov. Oseminštiridest isolatov *C. jejuni* od skupno dvainpetdesetih je bilo z metodo MLST razporejenih v deset klonskih kompleksov (CC) in 28 sekvenčnih tipov (ST). Prevladovala sta klonska kompleksa 21 in 45 in sekvenčna tipa 50 in 104. Triindvajset sekvenčnih tipov se je pojavilo zgolj enkrat, kar nakazuje na izjemno visoko genetsko diverziteto znotraj relativno majhne skupine sevov (Kovač in sod., 2014a).

Porazdelitev izolatov s pomočjo drevesa minimalnega razpona nakazuje na korelacijo specifičnih genotipov in njihovega vira izolacije. Večina humanih izolatov (71 %) je bila razporejena v CC 21 in CC 353, medtem ko je večina mesnih izolatov bila značilna za CC 21 (67 %) in CC 45. Sedeminšestdeset odstotkov živalskih izolatov je imelo genotipe, značilne za CC 21 in CC 354. Sekvenčni tipi, ki pripadajo CC 21 in 45 so bili že v preteklosti prevladujoči v Evropi in so tipični za perutnino. V našem primeru je bilo 12 od 19 izolatov razvrščenih v CC 21 in šest od osmih razvrščenih v CC 45, iz perutninskega mesa ali fecesa. Vodni izolati so se genetsko najbolj raziskovali od izolatov iz ostalih virov. Dva od petih izolatov nista bila dodeljena nobenemu klonskemu kompleksu. Njihovi različni sekvenčni tipi predstavljajo zanimivo skupino, ki ima malo skupnega z izolati iz živali in ljudi (Kovač in sod., 2014a).

Klonsko širjenje kinolonske odpornosti smo naprej preučevali z analizo sekvenc celotnih genomov 1711 humanih kliničnih izolatov *C. jejuni* in *C. coli*, zbranih v Oxfordširu v obdobju med junijem 2011 in junijem 2013. Prevalenca kinolonske odpornosti je bila določena na podlagi odpornost-zagotavljajočih mutacij v genu *gyrA*. Odpornost proti kinolonom je bila najvišja v v klonskem kompleksu ST-464 (94,4 % in 88 % odpornih v prvem in drugem letu), v klonskem kompleksu ST-354 (83,3 % in 96,7 % odpornih v prvem in drugem letu), ter v sekvenčnem tipu ST 50 iz klonskega kompleksa ST-21 (66,7 % in 51,7 % odpornih v prvem in drugem letu). Z namenom preučiti naravo širjenja kinolonske odpornosti smo na podlagi genomskih sekvenc zgradili filogenetske mreže izolatov iz klonskih kompleksov ST-21, ST-354 in ST-464 in preučili skupinjenje alelnih različic gena *gyrA* znotraj posameznih filogenetskih mrež. Porazdelitev alelne različice 20, ki je bila povezana z odpornostjo v sekvenčnem tipu 50 klonskega kompleksa ST-21, ni potrdila klonskega izvora. Nasprotno se je pa enaka različica gena gyrA v klonskem kompleksu ST-354 jasno porazdelila znotraj glavne skupine odpornih izolatov. Podobno je bilo opaženo tudi za alelno različico 57 v klonskem kompleksu ST-464, kar jasno nakazuje na klonski način širjenja kinolonske odpornosti v teh dveh klonskih kompleksih.

Naraščanje stopnje nastajanja spontanih mutacij v genu *gyrA*, ki zagotavljajo odpornost proti kinolonom je povezana s prisotnostjo gena *mfd* (Han in sod., 2008), zato smo preučili kako je variabilnost v genu *mfd* povezana z odpornostjo proti kinolonom. Ugotovili smo, da so alelni tipi 106, 39, 31 in 111 povezani z odpornostjo in da so določene različice gena *gyrA* znatno povezane z določenimi različicami gena *mfd*, kar nakazuje na to, da se gena nista razvijala neodvisno eden od drugega. Podobno smo opazili, da so določene različice (1, 113, 135 in 53) gena za notranji transmembranski transporter CmeB močno povezane s kinolonsko odpornostjo, kar nakazuje na to, da se te različice izrazijo v proteinih z večjo učinkovitostjo izčrpavanja kinolonskih antibiotikov.

Poleg razumevanja patogenovih epidemioloških značilnosti je pomembno tudi poznavanje trendov širjenja antibiotske odpornosti med bakterijami *C. jejuni*. S pomočjo molekularnih orodij lahko raziskujemo povezave med specifičnimi genotipi in odpornostnimi profili in dokažemo, da je odpornost proti kinolonom in makrolidom povezana s točkovnimi mutacijami v genih *gyrA* in 23S rRNA. Odpornost lahko dodatno okrepi tudi ojačana aktivnost bakterijskih efluksnih sistemov.

Efluksne črpalke tipa RND so v nekaterih Gram negativnih bakterijah sposobne izločanja različnih tipov antibiotikov. Njihova aktivnost je lahko inducirana z njihovimi lastnimi substrati, kar nakazuje na to, da igra selektivni pritisk pomembno vlogo pri pridobitvi nespecifične ali specifične odpornosti proti poritmikrobnim zdravilom (Mahamoud in sod., 2007). Določeni sekvenčni tipi so že bili povezani z odpornostjo proti kinolonom v sevih z omenjenimi točkovnimi mutacijami, vendar do danes še ni bilo pojasnjeno, ali pride do tega zato, ker so ti genotipi bolj nagnjeni k nastanku individualnih mutacij ali gre za klonsko širjenje sevov s pridobljenimi mutacijami, ki zagotavljajo odpornost (Wirz in sod., 2010; Kittl in sod., 2013; Wimalarathna in sod., 2013). V našem delu smo določili antibiotsko odpornost, MLST genotipe in analizirali genetsko sorodnost sevov, odpornih proti ciprofloksacinu, na podlagi regije za zagotavljanje odpornosti proti kinolonom (QRDR) znotraj gena *gyrA*. Namen analize je bil pojasnitev značilnosti širjenja odpornosti bakterij *C. jejuni* proti antibiotikom iz skupine kinolonov (Kovač in sod., 2014a).

Odpornost bakterij *C. jejuni* proti antibiotikom postaja problematično ne le v Sloveniji, temveč tudi v ostalih državah Evropske Unije. Izmed trenutno uporabljanih kliničnih antibiotikov, je nevarno visoka odpornost proti kinolonom. Ta skupina antibiotikov je, poleg makrolidov, prva izbira za zdravljenje kampilobakterioz, zato je njihova učinkovitost velikega pomena. V Sloveniji se kinoloni uporabljajo za zdravljenje nespecifičnih gastroenteritisov bakterijskega izvora, kot tudi za zdravljenje enterokoknih in okužb z *E. coli* pri perutnini. Prevalenca odpornosti proti ciprofloksacinu je med živalskimi izolati v EU od 37 % do 84 % (EFSA, 2014b), kar je v skladu z našimi opažanji, saj smo med 35 mesnimi izolati zabeležili 77 % odpornih proti ciprofloksacinu. Skoraj vsi (94 %) proti ciprofloksacinu odporni sevi iz naše študije so bili navzkrižno odporni tudi proti nalidiksinski kislini (EFSA, 2014b; IVZ, 2014).

Analiza antibiotske odpornosti in MLST genotipov je razkrila, da so vsi, razen enega izmed 19 sevov *C. jejuni*, ki pripadajo klonskemu kompleksu 21, odporni proti kinolonom. V našem delu smo želeli ugotoviti ali povezava med CC 21 in odpornostjo proti ciprofloksacinu obstaja zaradi visoke stopnje individualno nastalih točkovnih mutacij v genu *gyrA*, ali zaradi povečane sposobnosti klonskega širjenja odpornih sevov. Tega smo se lotili

s preučevanjem najbolj značilne mutacije Thr-86-Ile in nukleotidnega zaporedja regije QRDR znotraj gena *gyrA*. Rezultati so pokazali, da so imeli vsi sevi, odporni proti ciprofloksacinu, z izjemo enega, mutacijo ACA v ATA v 86. kodonu. Dodatno so imeli šest tihih mutacij v regiji QRDR, kar jasno nakazuje na visoko stopnjo genetske sorodnosti sevov iz CC 21, odpornih proti ciprofloksacinu. Ker se kinolonska odpornosti hitro razvije pod selektivnim antibiotskim pritiskom in lahko še dolgo vztraja tudi v okolju brez selektivnega pritiska, sklepamo, da je visoka pojavnost takih sevov znotraj klonskega kompleksa 21 posledica pridobljene povečane učinkovitosti klonskega širjenja, in ne zaradi visoke genetske plastičnosti omenjenega klonskega kompleksa (Kovač in sod., 2014a).

Naraščajoča odpornost proti antibiotikom nedvomno predstavlja resen problem, saj klinično uporabljani antibiotiki postajajo manj ali celo popolnoma neučinkoviti pri zdravljenju kampilobakterioz. Iskanje alternativnih protimikrobnih sredstev iz rastlinskih virov tako predstavlja dobro rešitev za premostitev bakterijske odpornosti. Rastlinski izvlečki, bogati s fenolnimi spojinami, in eterična olja so že dolgo znana po tem, da imajo protimikrobne lastnosti in so iz tega razloga pogosti preučevani (Cowan, 1999; Burt, 2004; Klančnik in sod., 2012c; Negi, 2012). Posebej zanimivi so zato, ker so splošno priznani kot varni in imajo potencial za uporabo v vlogi konzervansov v hrani. *Alpinia katsumadai* Hayata (Zingiberaceae) je splošno uporabljana rastlina v tradicionalni kitajski medicini, in sicer kot sredstvo proti želodčnim težavam in slabosti, kot tudi za dvig apetita. Uporablja se tudi kot krma za izboljšanje rasti domačih živali. V našem delu smo se osredotočili na preučevanje bioaktivnosti izvlečka semen *A. katsumadai*, kot tudi eteričnega olja in izvlečka odpadnega materiala po destialciji eteričnega olja. Tovrstni odpadni materiali so pogosto zavrženi in predstavljajo okoljski problem. Njihova visoka vsebnost fenolnih spojin in potencial za izrabo, kot ekonomičen vir naravnih oksidantov in protimikrobnih spojin, pa ostaja neizkoriščen (Klančnik in sod., 2012a; Kovač in sod., 2014b).

Sprva smo izvlečke semen *A. katsumadai*, pripravljene z različnimi topili, testirali za protimikrobno aktivnost na referenčnem sevu *C. jejuni*. Ugotovili smo, da topilo ni pomembno vplivalo na protimikrobni potencial izvlečka, saj je bila pri vseh določena minimalna inhibitorna koncentracija 512 mg/l. Sledilo je testiranje etanolnega izvlečka semena, eteričnega olja in etanolnega izvlečka odpadnega materiala na številčnejši skupini izolatov *C. jejuni* in *C. coli*. Med sevi vsake vrste so bili vključeni tako občutljivi kot proti antibiotikom odporni sevi. Opažene so bile statistično značilne razlike v protimikrobni učinkovitosti testiranih rastlinskih pripravkov. Glede na minimalne inhibitorne koncentracije (MIK) je bil etanolni izvleček protimikrobno najučinkovitejši z MIK-i med 125 in 2051 mg/l, nakar je sledil izvleček odpadnega materiala. Pri protimikrobni učinkovitosti rastlinskih pripravkov nismo ugotovili razlik v učinkovitosti delovanja na proti antibiotikom občutljive in odporne seve. Učinkoviti so bili tudi na mnogokratno odpornih sevih. Ugotovili smo, da je protimikrobna učinkovitost vrstno in sevno specifična. Glede na MIK-e, ki so bili dosledno višji od 100 mg/L, sklepamo, da imajo zmerno do šibko protimikrobno učinkovitost na bakterijah *Campylobacter* (Klančnik in sod., 2012a; Kovač in sod., 2014b).

Poleg iskanja novih protimikrobnih rastlinskih pripravkov se strategije boja proti odpornosti osredotočajo tudi na modulatorje odpornosti. Le-ti niso nujno protimikrobno učinkoviti, vendar lahko znižajo odpornost patogenih bakterij, ko se dodajajo skupaj z obstoječimi protimikrobnimi sredstvi in na ta način povečajo oziroma obnovijo njihovo učinkovitost. Do povečanja občutljivosti protimikrobnih sredstev pride v tem primeru večinoma zaradi

inhibicije efluksa, povečane prepustnosti membrane, povečane sinteze proteinov ali spremembe porinskega profila (Bolla in sod., 2011).

V našem delu smo tako poleg protimikrobne učinkovitosti ugotavljali in potrdili tudi odpornostno mudulatorni potencial rastlinskih pripravkov iz semen *A. katsumadai* na bakterijah *C. jejuni*. Etanolni izvleček je bil potrjen kot modulator odpornosti v *C. jejuni* in *C. coli* v naši prvi študiji, kjer smo dokazali, da v subinhibitorni koncentraciji poveča učinkovitost ciprofloksacina, eritromicina, etidijevega bromida, žolčnih soli, rožmarinovega izvlečka in epigalokatehin galata za do 16-krat. V drugem delu smo primerjalno preučevali odpornostno modulatorno učinkovitost eteričnega olja in izvlečka odpadnega materiala. Kot modulatorja sta bila rastlinska pripravka testirana v polovični minimalni inhibitorni koncentraciji vsakega posameznega seva, in sicer v kombinaciji z antibiotikoma ciprofloksacinom, eritromicinom, razkužilom triklosan, žolčnimi solmi ter splošnim substratom efluksnih črpalk etidijevim bromidom. Eterično olje je v povprečju povečalo učinkovitost testiranih protimikrobnih sredstev za 78-krat, medtem ko je odpadni material povečal prtimikrobno učinkovitost testiranih spojin za 34-krat. Eterično oje je bilo potrjeno kot najučinkovitejši modulator odpornosti pri bakterijah *C. jejuni*. Vsi posamezni testirani rastlinski pripravki so bili enako učinkoviti v kombinaciji z vsemi testiranimi protimikrobnimi sredstvi, vključno z žolčnimi solmi. Slednje je pomembno, ker so žolčne soli naravno prisotne v gastrointestinalnem traktu, ki služi kot rezervoar bakterij *Campylobacter*. Odpornost proti žolčnim solem je torej nujna za preživetje in infektivnost bakterij v tem okolju (Klančnik in sod., 2012a; Kovač in sod, 2014b).

Obstaja več različnih strategij za povečanje občutljivosti bakterij na antibiotike in ostala protimikrobna sredstva. Najbolj obetajoče se osredotočajo na povečanje vnosa antibiotikov v celico z destabilizacijo lipopolisaharidenga sloja Gram negativnih bakterij in povečanje membranske prepustnosti ter blokiranje efluksa z uporabo efluksnih inhibitorjev (Bolla in sod., 2011). Da bi ugotovili kakšen je mehanizem odpornostno modulatornega delovanja eteričnega olja iz semen *A. katsumadai* na baktetrijah *C. jejuni*, smo ocenili njegov potencial za povečanje znotrajcelične akumulacije etidijevega bromida. Povečanje akumulacije tega splošnega substrata efluksnih črpalk je namreč dober indikator za efluks inhibitorno učinkovitost. Etanolni izvleček in izvleček odpadnega materiala sta bila iz tehničnih rezlogov izključena iz analiz zaradi lastne avtofluorescence, ki je onemogočala meritve. V našem delu smo primerjali stopnje akumulacije etidijevega bromida v kulturah, tretiranih s subinhibitorno koncentracijo eteričnega olja (0,25 ali 0,5 MIK) in netretiranih kulturah, da bi ocenili ali eterično olje lahko potencira znotrajcelično akumulacijo etidijevega bromida. Kot pozitivno referenco smo vključili znan efluksni inhibitor verapamil (Kovač in sod., 2014b).

Rezultati so pokazali znatno povečano akumulacijo etidijevega bromida v prisotnosti eteričnega olja semen *A. katsumadai*. Akumulacija etidijevega bromida je bila celo za 1,7-krat boljša v prisotnosti 0,5 MIK eteričnega olja, v primerjavi z verapamilom, kot pozitivno referenco. Povprečna akumulacija v prisotnosti 0,25 MIK eteričnega olja je bila skoraj enaka, kot v prisotnosti 0,5 MIK olja, le čas, potreben za dosego maksimalne akumulacije je bil nekoliko daljši. Postopno upadanje akumulacije etidijevega bromida po tem, ko je dosegel plato, se je najverjetneje zgodilo zaradi olajšanega vstopa etidijevega bromida zaradi vpliva olja na membrano, ali zaradi nezadostne kapacitete olja za blokado efluksa (Kovač in sod., 2014b).

Zaradi učinkovite odpornostno modulatorne in efluks inhibitorne učinkovitosti eteričnega olja semen *A. katsumadai* smo želeli identificirati tarčno spojino, odgovorno za to aktivnost. Analizirali smo kemijsko sestavo eteričnega olja in identificirali 95 % prisotnih spojin. Med spojinami, ki so bila zastopane v koncentraciji, višji od 1 %, so bile farnezol, 1-8-cineol, α-humulen, cimen, α-felandern, 2-butanon, 4-fenil, α-terpineol in β-pinen. Začetni presejalni test nas je vodil do podrobnejše analize učinkovitosti spojine α-pinen, ki je bila v olju zastopana v koncentraciji 2,2 %. Zaradi boljše aktivnosti v začetnih testih smo nadalnje raziskave izvedli z enantiomero (-)-α-pinene.

Minimalna inhibitorna koncentracija (-)-α-pinena je bila 1000 mg/l ali višja pri vseh testiranih sevih *C. jejuni*, vključno s tistimi, vključenimi v teste modulatorne aktivnosti. Potrjeni visoki MIK-i kažejo nesignifikantno protimikrobno učinkovitost (-)-α-pinena. Kot potencialni modulator odpornosti je bil (-)-α-pinen testiran v koncentracijah 62,5 mg/l in 125 mg/l v kombinaciji z antibiotikoma ciprofloksacinom in eritromicinom, razkužilom triclosan in substratom efluksnih črpalk etidijevim bromidom na referenčnem sevu *C. jejuni* NCTC 11168. V nižji testirani koncentraciji je povečal učinkovitost testiranih protimikrobnih sredstev za do dvakrat, v višji koncentraciji pa od 32 do čez 256-krat. Podobno močna učinkovitost je bila opažena tudi pri eteričnem olju.

Pred oceno vpliva (-)-α-pinena na akumulacijo etidijevega bromida smo preverili vpliv obeh koncentracij spojine na integriteto membrane na referenčnem sevu *C. jejuni*. S tem smo želeli ovreči vpliv na prepustnost membrane kot glavni mehanizem modulatornega delovanja (-)-α-pinena. Membranska integriteta kulture, tretirane z 62,5 mg/l je bila po eni uri celo za 20 % višja od netretirane kulture, medtem ko je višja koncentracija (125 mg/l) znižala membransko integriteto za 39 %.

Obe koncentraciji (-)-α-pinena sta bili testirani za sposobnost povečanja intracelularne akumulacije etidijevega bromida v referenčnem sevu *C. jejuni*. Uporaba nižje koncentracije je rezultirala v višji akumulaciji in obenem ni imela negativnega vpliva na membransko integriteto, zato smo jo uporabili v testih na večjem številu sevov. To nam je omogočilo preučevanje samega efluks inhibitornega učinka. Opazili smo relativno povišanje akumulacije etidijevega bromida v prisotnosti (-)-α-pinena pri vseh šestnajstih testiranih sevih. (-)-α-pinene je bi sposoben povečanja akumulacije etidijevega bromida znatno bolje kot referenčna inhibitorja CCCP in reserpine v vseh testiranih sevih, kar potrjuje njegovo izjemno dobro efluks inhibitorno učinkovitost. Spojina je obdržala enak nivo aktivnosti tudi v koncentraciji do 8 mg/l, kar nakazuje na to, da je najverjetneje bil glavna spojina eteričnega olja *A. katsumadai*, ki je inhibirala efluks, vendar tega zaradi nepoznavanja enantiomerne sestave α-pinena v eteričnem olju ne moremo potrditi.

Z namenom določitve tarčnega efluksnega sistema spojine (-)-α-pinene, smo akumulacijo etidijevega bromida testirali na divjem sevu in mutantah s prekinjenim bralnim okvirom efluksnih genov *cmeB* in *Cj1687*. Akumulacija etidijevega bromida je bila v mutantah znatno nižja v primerjavi z divim sevom, kar nakazuje na odsotnost tarče (-)-α-pinena v omenjenih mutantah. Iz teh rezultatov sklepamo, da sta efluksni črpalki CmeABC in Cj1687 glavni tarči efluks inhibitornega delovanja (-)-α-pinena.

Da bi razvozlali bakterijski adaptacijski odziv na tretiranje s subinhibitorno koncentracijo (-)-α-pinena, ki smo jo uporabili v testih akumulacije etidijevega bromida, smo izvedli

analizo izražanja genov na nivoju celotnega genoma referenčnega seva *C. jejuni*. Pri tem smo uporabili tehologijo DNA mikromrež in kvantitativnega PCR v realnem času. Po 2-urnem tretiranju bacterij *C. jejuni* z 62,5 mg/l (-)-α-pinena, je bilo 129 genov različno izražanih, v primerjavi z netretirano kulturo. Pri tem smo upoštevali kot statistično značilne spremembe v izražanju samo tiste, ki so presegle dvakratno spremembo. Od teh je bilo 109 pozitivno raguliranih in 20 negativno reguliranih. Najmočneje pozitivno regulirani (več kot 10-krat) so bili geni, ki kodirajo šaperone (*grpE, dnaK, clpB*) in regulator odziva na toplotni šok, *hrcA*. Dodatna dva pozitivo regulirana gena (2 do 4-krat), vključena v odziv na toplotni šok, sta bila gena, ki kodirata šaperonine *groES* in *cbpA* ter transkripcijski represor *hspR*. Poleg genov, vključenih v odziv na toplotni šok, so bili pozitivno regulirani tudi geni efluksnih črpalk *cmeABC*, in sicer za 2,7 do 2,2-krat.

Spremembe v izražanju genov, ki jih je povzročil (-)-α-pinen, so bile izjemno podobne tistim, ki so jih predhodno opazili v mutanti z okvarjenim transkripcijskim regulatorjem *hspR*. To so močno povečano izražanje genov *grpE, dnaK, clpB* in *hrcA*. Enostaven model odziva na toplotni šok predlaga represijo *groES* in *groEL* s strani regulatorja *hrcA* in represijo operona *dnaK, cbpA* in *clpB* s strani represorja *hspR*, ki v primeru toplotnega šoka postane de-reprimiran. To posledično povzroči pozitivno regulacijo transkripcijskega regulatorja *hrcA*. Glede na naše rezultate se pri tretiranju *C. jejuni* z (-)-α-pinenom sproži podobna kaskada dogodkov, kar kaže na to, da igra mehanizem odziva na toplotni šok pomembno vlogo v bakterijski adaptaciji na ta kemični stres. Podoben odziv so opazili tudi v drugi študiji, in sicer po tretiranju *C. jejuni* z naravnim protimikrobnim sredstvom benzil izotiocianatom.

Da bi potrdili vlogo teh genov v adaptacijskem odzivu *C. jejuni*, smo pripravili mutanti v genih *hspR* in *hrcA* in jih uporabili v fenotipskih testih. Rast mutant s prekinjenimi bralnimi okvirji genov *hspR* in *hrcA* ter divjega tipa *C. jejuni* NCTC 11168 je bila nato testirana v prisotnosti in odsotnosti (-)-α-pinena. Divji sev NCTC 11168 je rastel najboljše v danih pogojih, nakar je sledil *hrcA* ter nazadnje *hspR*. Rast v prisotnosti (-)-α-pinena je bila slabša kot v odsotnosti (-)-α-pinena pri vseh treh sevih. Ker je bil trend padajoče rasti opažen v obeh mutantah, tako *ΔhspR*, kot tudi *ΔhrcA*, sklepamo, da imata oba transkripcijska regulatorja pomembno vlogo v adaptacijskem odzivu na tretiranje z (-)-α-pinenom.

Mutanti *ΔhspR* in *ΔhrcA* in divji tip *C. jejuni* NCTC 11168 so bili dodatno fenotipsko okarakterizirani s pomočjo fenotipskih mikromrež Biolog. Preverjali smo njihovo sposobnost rasti na različnih virih ogljika ter v prisotnosti različnih osmolitov in pH. S tem smo želeli pojasniti vlogo okvarjenih genov v adaptaciji na tovrstne strese. Rast mutant smo primerjali z rastjo divjega tipa. Mutanta *ΔhspR* je rastla bolje na nekaterih osmolitih, kot so na primer 6 % NaCl s KCl, natrijev sulfat, natrijev format, natrijev laktat in natrijev fosfat, medtem ko je bila rast mutante na teh substratih *ΔhrcA* znatno inhibirana. Še več, mutanta *ΔhrcA* ni bila sposobna izrabljati cele vrste virov ogljika tako učinkovito kot divji tip.

Na podlagi teh rezultatov sklepamo, da igra odziv na toplotni šok pomembno vlogo v adaptaciji *C. jejuni* na različne strese, kot so osmotski stres in kemični stres, vključno s stresom tretiranja z efluksnim inhibitorjem (-)-α-pinenom, vendar natančen mehanizem odziva še ni popolnoma pojasnjen. Ena izmed ovir na poti k popolnemu razumevanju adaptacijskega odziva so tudi kompleksne posttranslacijske regulacije transkripcijskih

represorjev HspR in HrcA, ki jih ni mogoče pojasniti z eksperimenti na transkripcijskem nivoju.

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ANNEXES

ANNEX A: Permission of Epidemiology and Infection for publishing the article entiteled 'Anti-*Campylobacter* and resistance-modifying activity of *Alpinia katsumadai* seed extracts' as part of the doctoral thesis.

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