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Natural Antimicrobial Agents

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Preface

Plant Kingdom and Biodiversity: An Endless Source of Antimicrobials in Human and Plant Health

Use of plants for various ailments is as old as human civilization. Continuous efforts are being made not only to improve the knowledge on the phytochemical composition and the biological activities of medicinal plants, but also to produce their bioactive secondary metabolites in high amounts through various high technologies. About 200,000 natural products of plant origin are known, and many more are being identified from higher plants and microorganisms. Some plant-based drugs are used since centuries and still remain at present time essential medicines. Morphine, obtained from poppy straw of *Papaver somniferum*, is on the WHO Model List of Essential Medicines and is widely used as antalgic, primarily to treat both acute and chronic severe pain. Drug discovery from medicinal plants or marine microorganisms continues to provide an important source of new drug leads.

Research on new antibacterial and new antifungal agents represents a real and timely challenge of this century, in particular with the current contexts on the control of pathogenic agents in biomedicine, agriculture, and food industry. One of the main problems to fight human infections is the widespread of multidrug-resistant bacteria for which common antibiotics become less efficient. Among the most problematic Gram-positive bacteria are methicillin-resistant *Streptococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and multidrug-resistant *Mycobacterium tuberculosis* (XDR-TB) strains. Infections caused by resistant Gram-negative bacteria, in particular by extended-spectrum β-lactamase-producing Enterobacteriaceae, carbapenem-resistant Enterobacteriaceae, multidrug-resistant strains of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, also constitute a serious threat to public health worldwide. In agriculture, the management of crop diseases is submitted to two main constraints: (i) occurrence and widespread of fungicide resistance in most plant pathogenic fungi and (ii) societal and politic pressures aiming at reducing the use of conventional fungicides in crop protection because of their potential impacts on both the environment and human health. At last, in food

industry, the use of conventional chemical preservatives to control food pathogens is also controversial.

Thus, new strategies to control bacterial infections in human health are highly sought. A number of compounds found in plants are being cited as antimicrobials and resistance-modifying agents. The natural products can either have direct antibacterial action on resistant strains alone, or as synergists of potentiators of other antibacterial agents, or act as bacterial resistance-modifying agents (RMAs). In plant health, different types of biocontrol products are being developed since they have lower risks on the environment and human health than synthetic pesticides.

The search for new antiparasitic and antiviral natural products is far from devoid of interest. According to the WHO report in 2013, malaria still represents some 207 million cases worldwide and more than 3 billion of people are still exposed to this risk. Similarly, about 350 million people are considered at risk of contracting leishmaniosis. The fight against some viruses also requires that the research on natural products continues. Hepatitis C virus (HCV) infection is a leading cause of chronic liver diseases. According to the World Health Organization, more than 150 million people are chronic carriers of HCV. The development of highly effective treatment regimens, including direct-acting antiviral drugs, has revolutionized the treatment of chronic hepatitis C infection. This new generation of antiviral drugs currently available on the market has a superior efficacy and a better safety profile than previous therapies. However, the cost of these treatments is very high and not easily accessible to an exposed population in developing countries. In addition, the appearance of treatment-resistant viral variants has been noted in some patients.

Documenting the latest research in the field of different pathogenic organisms, this book compiles the recent information about natural sources of antimicrobials and their sustainable utilization in the following areas: (I) plants as a source of antibacterials (Human Health); (II) natural occurring antifungal natural products (Plant Health); (III) antiparasitic natural products (Human Health); (IV) antiviral natural products (Human Health). This book will be useful to researchers and students in microbiology, biotechnology, pharmacology, chemistry, and biology as well as medical professionals.

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Part I
Plants as a Source of Antibacterials (Human Health)

Chapter 1
Antimicrobial Natural Products Against *Campylobacter*

Sonja Smole Možina, Anja Klančnik, Jasna Kovac, Barbara Jeršek and Franz Bucar

Abstract Campylobacteriosis is the world's leading bacterial foodborne illness and the most frequently reported zoonosis in humans. The present review aims to present an overview of the alternative strategies to limit *Campylobacter* contamination and to prevent *Campylobacter* infections using natural products from various sources. Additionally, natural products may improve the sensory characteristics of foods and extend their shelf life. The most effective intervention is inhibiting *Campylobacter* growth and thus reducing their prevalence and levels in vitro and in vivo along the food supply chain and on food products. Further, development of innovative growth and virulence control strategies using natural products in subinhibitory concentrations that do not pose selective pressure, may be beneficial. At such low concentrations, natural products can act as resistance modulators (e.g., efflux pump inhibitors) and thus enhance anti-*Campylobacter* activity of antibiotics. Low doses of natural compounds that are not cytotoxic can prevent adhesion of *Campylobacter* to abiotic surfaces, hence preventing biofilm formation, or to biotic surfaces, hence preventing attachment to animal or human epithelial cells.

Keywords Natural products · Antimicrobial · Resistance mechanism
Efflux pump inhibitors · Anti-adhesion · *Campylobacter*

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1.1 Introduction

Thermotolerant *Campylobacter* spp., mainly *C. jejuni* and *C. coli*, are the most common gastrointestinal bacterial foodborne pathogens in the European Union, and they can even surpass other infections due to *Salmonella*, *Shigella* and *Escherichia coli* (EFSA 2013). *Campylobacter* is generally regarded as being sensitive to environmental conditions outside of the animal and human host. However, it can survive the adverse conditions during food processing and storage (Humphrey et al. 2007; Klančnik et al. 2009, 2014), and can cause bacterial gastroenteritis in humans.

1.1.1 Campylobacter Contamination and Infection

Infections with *Campylobacter* are often contracted by eating and/or handling undercooked poultry meat or other cross-contaminated foods that are not thermally treated prior to ingestion (EFSA 2012). Outbreaks of *Campylobacter* are mostly associated with the consumption of contaminated water and poultry (Halberg Larsen et al. 2014). Broiler flocks are commonly colonized with *Campylobacter* through exposure to farm environmental factors. Once the pathogen is introduced to the farm, it can rapidly spread among birds in the flock via faecal-oral route (Sahin et al. 2015). Furthermore, when infected birds are slaughtered, the release of the intestinal content can lead to cross-contamination of the slaughterhouse environment and consequently of poultry meat from non-infected flocks (Reuter et al. 2010). *Campylobacter* can also be transmitted to humans through unpasteurized milk and dairy products from colonized dairy cattle, or by cross-contamination with manure. Additionally, people can be infected through direct contact with the faeces of infected farm or companion animals (Halberg Larsen et al. 2014). In addition to diarrhoeal disease, chronic complications associated with *Campylobacter* infection include reactive arthritis and Guillain–Barré syndrome. Severe autoimmune responses and axonal damage have been observed in *Campylobacter*-associated Guillain–Barré syndrome, which is mostly problematic in the poorer countries, where patients have limited access to health care and treatment (Nyati and Nyati 2013).

1.1.2 Campylobacter Control

The European Union recently banned the use of antibiotic growth promoters in animal feed. The awareness of increasing *Campylobacter* resistance has encouraged the search for new effective strategies to reduce the incidence of *Campylobacter jejuni* infection (Smole Možina et al. 2011). In the food industry, the problem arises

due to modern food production facilities and the emergence and spread of resistance through intensive use of antimicrobial agents and the international trade in raw materials and food products. *Campylobacter* spp. has developed resistance to many of the available antibiotics, and as several infections are now not controllable with the available antibiotics, the regulatory bodies have restricted the use of antibiotics in the food supply chain (Cars et al. 2011). *C. jejuni* can also form mono- and multispecies biofilms which enhance survival in hostile environments, as their physiology and behaviour are significantly different from their planktonic counterparts (Reeser et al. 2007; Teh et al. 2014). It is known that *C. jejuni* can form biofilms on different abiotic surfaces including polystyrene or glass and on biotic surfaces, and adhere to animal and human intestinal cell lines, such as PSI, Caco2, H4 and HT29 cells (Šikić Pogačar et al. 2009, 2010, 2016; Kurinčič et al. 2016). The surfaces of equipment used for food handling, storage and processing thus represent major sources of *Campylobacter* cross-contamination (Dunne 2002; Simões et al. 2010; Nguyen et al. 2010). This can also lead to transmission of *Campylobacter* to food and to the next host.

Due to the public health significance of *C. jejuni* infection, it is important to understand its survival in the environment and in the food supply chain (Joshua et al. 2006) and to control *Campylobacter* contamination, growth and transmission in the food supply chain. For example, in broiler meat production chain, the control is needed especially on preharvest, harvest and postharvest levels and is possible in several steps, such as (i) in primary production; (ii) during transportation and before slaughter; (iii) at slaughter, dressing and processing (EFSA 2011; Halberg Larsen et al. 2014). Conventional cleaning and sanitation regimens can contribute to inefficient biofilm control. In order to prevent *Campylobacter* persistence and cross-contamination at post-harvest level, effective cleaning and sanitation procedures must be in place in food processing environment.

To improve the current situation, approaches for the prevention of *Campylobacter* contamination are focused on alternative antimicrobials of natural origin and on the early stage, such as safety of feed and drinking water for food animals. In addition, consumers are increasingly demanding fresh or minimally processed foods without added chemical additives with the longest possible shelf life. Natural antimicrobial substances are an alternative to replace synthetic chemical additives. The main purpose of many studies of natural antimicrobial substances is to obtain a substance with antimicrobial activity in the food and thus contribute to its stability and safety by growth inhibition or inactivation of spoilage and pathogenic microorganisms (Burt 2004; Lucera et al. 2012). Furthermore, new strategies using subinhibitory concentrations and targeting more specific mechanisms like quorum sensing, adhesion and biofilm formation and/or efflux pumps or other cell membrane functions could reduce *Campylobacter* growth and attenuate virulence. Such treatment is not bactericidal, so resistance is less likely to occur (Bensch et al. 2011; Kovač et al. 2015; Šikić Pogačar et al. 2016). However, public scientific evidence on resistance-modifying mechanisms, anti-adhesion and antiquorum-sensing activity of natural antimicrobials to reduce *Campylobacter* persistence is very limited.

1.2 Natural Products Inhibiting Growth of *Campylobacter*

Control of *Campylobacter* growth by natural products has been subject to numerous studies. Usually, cultivation of *Campylobacter* strains in vitro under microaerophilic conditions and application of extracts or pure compounds in increasing concentrations are applied. Natural antimicrobial products are isolated from different sources (Davidson et al. 2015):

(i) Animal sources such as lysozyme (egg, milk), lactoperoxidase (milk), lactoferrin (milk), chitosan;
(ii) Plant sources—species and their essential oils and extracts (i.e. cloves, cinnamon, oregano, thyme), berry fruits (i.e. cranberry, cloudberry, raspberry, strawberry), hop, olives, brassica, onion, garlic, grape;
(iii) Microbial sources such as natamycin, nisin, other bacteriocins, fermentation metabolites, protective cultures, bacteriophages.

For the application and potential use of natural products in controlling *Campylobacter* contamination and infection, it is important to study the target activity of natural products inside the *Campylobacter* cell. Only few studies have demonstrated mechanisms of antimicrobial activity. The activity of organic acids is known to be based on the ability of their undissociated form to penetrate through the cell membrane and to dissociate inside the cell, decreasing the intracellular pH value, thus disrupting homeostasis which is essential for the control of ATP synthesis, RNA and protein synthesis, DNA replication and cell growth. Besides the decrease in intracellular pH, the perturbation of membrane functions by organic acid molecules may be also responsible for the microbial inactivation. High concentration of anions (due to dissociation) inside the cells might result in an increased osmolarity and consequently the metabolic disruption (Hirshfield et al. 2003). To be able to react to potential development of resistance against natural products, it is essential to study the mechanisms of bacterial resistance to these products.

1.2.1 Methods for Identification of Antimicrobial resistance Activity

Several methods for measurement of minimal inhibitory concentration (MIC) values of natural products are available, but there is no standard procedure established. Since results from numerous reports of antimicrobial activity against *Campylobacter* need to be equivalent, it is important to evaluate different methods for antimicrobial activity assessment. The assay needs to be customized to suit individual bacterial species growth requirements by selecting the appropriate growth medium, incubation temperature, time and atmosphere and finally, the method for detection of bacterial growth inhibition. Thus, the comparison of

reported activities of plant extracts and compounds should be made with caution, and the methods used for MIC determination (such as cultivation; bacterial growth evaluation; kinetics of inhibition via survival curves) have to be taken into account. An evaluation of different test methods like disc diffusion, agar dilution, broth macrodilution and microdilution assays revealed that broth microdilution assays are most suitable for fast screening of growth inhibitory potential of plant extracts and compounds (Klančnik et al. 2010). While for Gram-positive bacteria results from agar dilution and broth dilution were in good agreement, for Gram-negative bacteria like *Campylobacter* spp. lower MIC values were determined by broth dilution compared to agar dilution methods (Klančnik et al. 2010). A comparison of growth kinetics for 24 h by a macrodilution assay at certain MIC values could then be used to confirm the antibacterial effects. In case of *Campylobacter* spp., low MIC values seen in broth microdilution assays clearly correlate with the actual growth inhibitory effects of the test compounds (Klančnik et al. 2010).

Critical point in the assessment of antibacterial activity in broth microdilution assays performed in 96-well microtiter plates is the way bacterial growth is examined. Since natural products, especially plant extracts, are rarely colourless, the determination of MIC by broth microdilution assays based upon measurement of bacterial optical density (OD) may be hindered. Therefore, growth indicators such as tetrazolium salts (Klančnik et al. 2010), resazurin (Kovač et al. 2014, 2015) or luminescent ATP detectors (Klančnik et al. 2009) associated with bacterial metabolic activity were found to be more appropriate to use for MIC determination of natural products. Viability determination of aerobic bacteria is possible by observing visible growth utilizing tetrazolium salts like XTT (2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide), INT (p-iodonitrotetrazolium violet) or resazurin (Klančnik et al. 2010). Tetrazolium salts are reduced by bacterial oxidative enzymes by acting as electron acceptors and are therefore not suitable for detection of metabolic activity in microaerophilic bacteria, such as *Campylobacter* (Klančnik et al. 2010). Thus, in case of *Campylobacter* growing under microaerophilic conditions with lower reduction kinetics, activity measurement of ATP or resazurin proved to be most suitable. ATP activity can be measured via bioluminescence after adding BacTiterGlo Reagent (Promega, USA) and incubation in the dark (Klančnik et al. 2009). Resazurin is converted by viable cells to the fluorescent resorufin product which can be detected by fluorescence signals measured with a microplate reader (excitation/emission wavelengths) after adding BacTiterBlue Reagent (Promega, USA) (Kovač et al. 2015).

Apart from adjusting the growth detection methodology to suit the testing organism, also the potential interference of testing material with the growth detector needs to be ruled-out, in order to reliably determine the MICs. Natural products themselves, especially plant extracts rich in phenolic compounds, may namely reduce the growth detector resulting in false-positive growth results. From this aspect, the use of ATP detection systems for MIC determination of natural products seems to be the safest choice, although use of significantly more cost-effective resazurin is often appropriate, as well (Klančnik et al. 2012a, b; Kurinčič et al. 2012a; Kovač et al. 2014, 2015; Klančnik et al. 2012b).

1.2.2 Anti-Campylobacter Activity of Isolated Plant Compounds

Among substances of plant origin, the most active ones were found within isothiocyanates, diallysulphides and coumarins (see Table 1.1). In a study of Dufour et al. (2012), isothiocyanates (allyl-, benzyl-, ethyl-, 3-(methylthio)propyl-isothiocyanates) were tested against 24 *C. jejuni* isolates from chicken faeces, human infections and contaminated food together with two reference strains, NCTC 11168 and 81-176. They tested also the *ggt* mutant of 81-176 with deleted gene for γ-glutamyl transpeptidase (GGT enzyme). Remarkable differences in anti-*Campylobacter* activity could be recorded with respect to the growth medium. Particularly, allyl isothiocyanate showed antibacterial effects with MIC of 50 to 200 mg/L measured by agar dilution assay and a higher effect with MIC of 5 to 10 mg/L measured by broth dilution assay. Also for benzyl isothiocyanate, a significant decline in MIC of 1.25 to 5 mg/L versus 0.625 to 1.25 mg/L measured by broth dilution assay could be observed. Both isothiocyanates were found to be bactericidal rather than bacteriostatic. In the preliminary screening (agar dilution assay and reference strain NCTC 11168), ethyl isothiocyanate and allyl isothiocyanate showed no or moderate activity (MIC $\geq$ 200 mg/L), whereas benzyl isothiocyanate and 3-(methylthio) propyl-isothiocyanate resulted in MIC values of 5 mg/L indicating the pronounced influence of the alkyl residue on the antibacterial activity of isothiocyanates and opening the window for exploring synthetic derivatives. Comparing wild-type 81-176 strain and its *ggt* mutant, let the authors conclude that the GGT enzyme might be involved in detoxification of isothiocyanates also in *Campylobacter*. However, resistance to isothiocyanate depended on additional, so far not unveiled other factors. Sulforaphane, an isothiocyanate originating from glucoraphanin which is abundant in brokkoli and other vegetables of the Brassicaceae family, revealed a similar potency as benzyl isothiocyanate with an MIC value of 15 mg/L measured by agar dilution but was not investigated further (Dufour et al. 2012). A series of diallyl sulphides abundant in essential oils of chives and garlic were tested against a number of food pathogens including *C. jejuni* ATCC 49349. A clear structure–activity relationship could be observed with increasing potency correlated to increasing number of sulphide groups, diallyl tetrasulphide being the most active (Rattanachaikunsopon and Phumkhachorn 2008). Alkaloids have been rarely investigated; however, the bisbenzylisoquinoline alkaloid cocsoline, isolated from the root bark of *Epinetrum villosum* (Menispermaceae), showed remarkably good activity on *C. jejuni* and *C. coli* (MIC of 15.62 to 31.25 mg/L; MBC 62.5 mg/L) (Otshudi et al. 2005).

Among many phenolic compounds evaluated as growth inhibitors of *Campylobacter* spp., the prenylated 4-phenylcoumarin mammea A/AA isolated from the stem bark of *Mammea africana* (Calophyllaceae) showed remarkable high activity on *C. jejuni* ATCC 33291 in vitro with an MIC value of 0.25 mg/L measured by broth dilution assay. Other Gram-negative bacteria like *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Salmonella* typhimurium were much less sensitive to this plant compound (MIC $\geq$ 16 mg/L).

Table 1.1 Anti-*Campylobacter* activity of selected compounds of plant origin

Compound	Species	Assay; MIC (mg/L)	Viability	Reference
Allyl isothiocyanate (AITC)	*C. jejuni*	AD; MIC 50–200	VIS	Dufour et al. (2012)
		BD; MIC 5–10		
Benzyl isothiocyanate (BITC)	*C. jejuni*	AD; MIC 1.25–5	VIS	Dufour et al. (2012)
		BD; MIC 0.625–1.25		
α-Bisabolol	*C. jejuni*	BMD; MIC 0.125–0.5	OD 600 nm; INT	Kurekci et al. (2013)
	C. coli			
Carnosic acid	*C. jejuni*	BMD; MIC 19.5–78	ATP activity	Klančnik et al. (2012b)
	C. coli			
Chlorogenic acid	*C. jejuni*	BMD; MIC 156–313	ATP activity	Klančnik et al. (2012b)
	C. coli			
Cineole	*C. jejuni*	BMD; MIC 0.25	OD 600 nm INT	Kurekci et al. (2013)
	C. coli			
Cocsoline	*C. jejuni*	BMD; MIC 15.62–31.25 MD; MBC 62.5	n.i.	Otshudi et al. (2005)
	C. coli			
Diallyl mono-, di-, tri, tetrasulphide	*C. jejuni*	BMD; MIC 56; 12; 2; 1	n.i.	Rattanachaikunsupon and Phumkhachorn (2008)
Elemicin	*C. jejuni*	BMD; MIC 250	n.i.	Rossi et al. (2007)
Epigallocatechin gallate	*C. jejuni*	BD; MIC 600	OD	Engels et al. (2011)
		BD; MBC 700		
Ferulic acid	*C. jejuni*	BMD; MIC 78–313	ATP activity	Klančnik et al. (2012b)
	C. coli			
Gallic acid	*C. jejuni*	BMD; MIC 313	ATP activity	Klančnik et al. (2012b)
Hepta-O-galloylglucose	*C jejuni*	BD; MIC < 100	OD	Engels et al. (2011)
		BD; MBC < 100		
Kaempferol-, myricetin- and quercetin glycosides	*C. jejuni*	BMD; MIC 130–250	INT	Madikizela et al. (2013)
Linalool	*C. jejuni*	BMD; MIC 0.5–1	VIS	Duarte et al. (2016)
	C. coli	BMD; MBC 0.5–1		
Mammea A/AA	*C. jejuni*	BMD; MIC 0.25	VIS	Canning et al. (2013)
			OD 590 nm	
Methyl gallate	*C. jejuni*	BMD, MIC 60	INT	Madikizela et al. (2013)

(continued)

Table 1.1 (continued)

Compound	Species	Assay; MIC (mg/L)	Viability	Reference
Methyl isoeugenol	*C. jejuni*	BMD; MIC 150	n.i.	Rossi et al. (2007)
Nerolidol	*C. jejuni*	BMD; MIC 0.5–1	OD 600 nm	Kurekci et al. (2013)
	C. coli		INT	
Pterostilbene (Pts), Pinosylvin (Ps); cyclodextrin inclusion complexes (Pts-CIC; Ps-CIC)	*C. jejuni*	BMD; MIC	OD	Silva et al. (2015)
	C. coli	Pts 50→400		
		Ps 25–50		
		Pts-CIC 128→1024		
		Ps-CIC 16–64		
α-Pinene	*C. jejuni*	BMD; MIC 1000→2000	Resazurin FL 550/959 nm	Kovač et al. (2015)
Resveratrol (Rv); cyclodextrin inclusion complexes (Rv-CIC)	*C. jejuni*	BMD; MIC	OD	Duarte et al. (2015)
	C. coli	Rv 50–100		
		Rv-CIC 64–256		
Rosmarinic acid	*C. jejuni*	BMD; MIC 78–156	ATP activity	Klančnik et al. (2012b)
	C. coli			
Sinapic acid	*C. jejuni*	BMD; MIC 313	ATP activity	Klančnik et al. (2012b)
Syringic acid	*C. jejuni*	BMD; MIC 156–313	ATP activity	Klančnik et al. (2012b)
	C. coli			
α-Terpinene	*C. jejuni*	BMD; MIC 0.125–0.25	OD 600 nm	Kurekci et al. (2013)
	C. coli		INT	
Terpinen-4-ol	*C. jejuni*	BMD; MIC 0.06	OD 600 nm INT	Kurekci et al. (2013)
	C. coli			
Thymol (Ty); thymol-β-D-glucopyranoside (t-gluc)	*C. jejuni*	BMD	Viable cell count	Epps et al. (2015)
	C. coli	Ty (1 mM): decrease in log CFU/ml for 1.88 to 4.80; t-gluc (1 mM): no effect		
Vanillic acid	*C. jejuni*	BMD; MIC 313	ATP activity	Klančnik et al. 2012b

'AD' agar dilution; 'BD' broth dilution; 'BMD' broth microdilution method; 'MIC' minimal inhibitory concentration; 'MBC' minimal bactericidal concentration; 'n.i.' not indicated, 'OD' optical density, 'VIS' visually observation

However, when tested on normal and cancer cell lines, it became obvious that mammea A/AA displays a general cytotoxic effect which decreases its potential as anti-*Campylobacter* drug (Canning et al. 2013). Stilbenes like pinosylvin (25 to 50 mg/L) and resveratrol (50 to 100 mg/L) can be regarded as another promising group of plant phenolics with growth-inhibiting effects on *C. jejuni* and *C. coli.* Preparing cyclodextrin inclusion complexes might be an option to improve the

solubility of this type of compounds (Silva et al. 2015; Duarte et al. 2015). Other compounds with phenolic structural features with antibacterial effects were flavonols like kaempferol, quercetin and myricetin glycosides (Madikizela et al. 2013), epigallocatechin gallate (Engels et al. 2011), hepta-O-galloyl glucose (Engels et al. 2011), methyl gallate (Madikizela et al. 2013), phenolic acids such as chlorogenic acid, rosmarinic acid, ferulic acid, gallic acid and syringic acid (Klančnik et al. 2012b), the phenolic terpenoids thymol (Epps et al. 2015) and carnosic acid (Klančnik et al. 2012b), or the phenylpropanoids elemicin and methyl isoeugenol (Rossi et al. 2007). Their anti-*Campylobacter* potency varied in a wide range between MIC values of 19.5 mg/L (*C. jejuni/C. coli*, carnosic acid) (Klančnik et al. 2012b) and 60 mg/L (*C. jejuni*, methyl gallate) (Madikizela et al. 2013) to 600 mg/L (*C. jejuni,* epigallocatechin gallate) (Engels et al. 2011; Klančnik et al. 2012b). Mono- and sesquiterpenes isolated from essential oils generally can be regarded as moderately active, and promising structures within this class of compounds were terpinen-4-ol (*C. jejuni*, *C. coli*; MIC 0.6 ml/L) and α-bisabolol (*C. jejuni*; MIC 1.25 ml/L) (Kurecki et al. 2013).

Campylobacters became resistant to the point where none of our available antibiotics work for some of the infections that confront patients and physicians in hospitals. However, the mechanisms of action of bioactive phytophenols must be investigated (Smole Možina et al. 2011). Resistance to phenolic compounds and plant extracts involves the CmeABC efflux system. This could be evidenced by comparing growth inhibitory effects on wild-type and efflux mutant strains in genes with crucial role in response and/or defence against antimicrobials (*cmeB, cmeF, cmeR*). In particular, gene knockout mutants of *cmeB* encoding the transport protein CmeB were highly susceptible to rosmarinic, chlorogenic or gallic acid compared to the wild-type strains with modulation factors of 64 to 128 mg/L, i.e. decreasing MIC values in mutant strains by 64 to 128-fold (Klančnik et al. 2012b). Similarly, increased susceptibilities could be shown for a number of herbal extracts like those of rosemary, sage, lemon balm, bearberry or grape leaves (Klančnik et al. 2012b).

Finally, the influence of the gut microbiota on metabolisms of administered plant compounds and its influence on antimicrobial activity should not be neglected as shown by Epps et al. (2015). Thymol-O-β-D-glucoside exerted after coincubation with the gut bacterium *Parabacteroides distasonis* possessing β-glucosidase activity increased activity against *C. coli* and *C. jejuni*, and comparable results were observed after coincubation with porcine or bovine faecal microbes. These results shed light on the important role of metabolic capacities of gut microbiota to convert ingested natural products from inactive prodrugs to potentially antibiotic entities (Epps et al. 2015). This was also illustrated by urolithins, originating from ellagitannins by metabolic action of colon microbiota, which have been shown to inhibit quorum sensing of the enteropathogenic *Yersinia enterocolitica* (Gimenez-Bastida et al. 2012).

1.3 Resistance Modulators and *Campylobacter* Efflux Pump Inhibitors from Natural Sources

Potentiation of antimicrobial activity can be achieved by a combination of different antimicrobials with synergistic antimicrobial effects or by antimicrobials together with resistance modulators. Resistance modulators are compounds or a mixture of compounds (e.g. plant extracts) that are able to enhance the activity of antibiotics without necessarily exhibiting direct antimicrobial activity themselves. By acting so, they can increase bacterial susceptibility to antibiotics and in some cases fully reverse bacterial resistance against antibiotics. Resistance-modifying effect can be achieved through interference of a modulator with specific or general mechanisms of antibiotic resistance existing in bacterial organisms. The modification of antibiotic target and inactivation of the antibiotic itself are here considered as specific mechanisms of resistance, in oppose to the insufficient influx of antimicrobial into a bacterium and efficient extrusion of antibiotic out of a bacterium through enhanced efflux, which are considered to be general, i.e. less specific, resistance mechanisms.

The use of a β-lactam antibiotic amoxicillin together with a β-lactamase inhibitor clavulanic acid (Neu and Fu 1978) is a proof of concept of such resistance-modifying strategy in fight against antibiotic resistance. Its success demonstrates feasibility of applying narrow-spectrum modulators that target specific antibiotic-modifying enzymes, to protect the structural and functional integrity of an antibiotic. To date, the clavulanic acid, naturally produced by *Streptomyces clavuligerus,* which has the genes responsible for the biosynthesis of clavulanic acid and the β-lactam antibiotics clustered together in shared regulation network (Pradkar 2013), is a unique example of a drug licensed for use in conjunction with an antibiotic to enhance its activity, i.e. resistance modulator. It has been developed and patented for use in pharmaceutical formulation with antimicrobial agents in 1994, and the drug is still widely used in a variety of generic forms (FDA Orange Book 2015). Other clinically used drugs, such as synthetic verapamil and naturally occurring alkaloid reserpine, which have been licensed as calcium ion influx inhibitor and hypertensive, respectively (PubChem—Verapamil 2015a; PubChem—Reserpine 2015b), were demonstrated to have multidrug efflux-inhibitory activity in Gram-negative bacteria as well (Klančnik et al. 2012b; Kovač et al. 2014; 2015). Efforts have been placed into search for novel resistance modulators that could counteract accelerated evolution of antibiotic resistance observed under man-made selective pressure created since the beginning of antibiotic era in the past century (Baquero et al. 2009). We here reviewed natural products from plant sources possessing resistance-modifying or efflux-inhibitory activity in *Campylobacter* identified over the past years and the methodology used in this quest.

1.3.1 Methods for Screening of Resistance Modulators and Efflux Inhibitors

Modulation of antibiotic resistance is determined using a modulation assay based on the principle of broth microdilution assay which is described in detail in Sect. 1.2.1. The modulation assay is a modified MIC assay, in which MICs of antibiotic alone and antibiotic in the presence of subinhibitory concentration of potential resistance modulator are determined and expressed as respective ratio called modulation factor (MF) (Lechner et al. 2008). Figure 1.1 presents the growth in modulation assay of *C. jejuni* detected using resazurin metabolic activity indicator. The modulation factor is calculated as in Eq. (1.1):

$$\mathrm{MF} = \mathrm{MIC}_{\mathrm{AB}} / \mathrm{MIC}_{\mathrm{AB+M}} \tag{1.1}$$

where $\mathrm{MIC}_{\mathrm{AB}}$ is the MIC of antibiotic and $\mathrm{MIC}_{\mathrm{AB+M}}$ is the MIC of antibiotic in the presence of a resistance modulator. In this case, $\mathrm{MIC}_{\mathrm{AB}}$ is 0.25 mg/L and $\mathrm{MIC}_{\mathrm{AB+M\ is}}$ 0.031 mg/L resulting in MF 8. The modulation factors above two are considered to be good indicators of resistance-modifying activity, and compounds possessing such activity are often further investigated by exploring their efflux-inhibitory potential.

Efflux-inhibitory activity of natural products can be evaluated using accumulation (Tegos et al. 2002; Lin et al. 2002; Garvey et al. 2011; Blanchard et al. 2014; Aparna et al. 2014; Kovač et al. 2014, 2015) and efflux assays (Lechner et al. 2008; Gröblacher et al. 2012a, b). There are two common approaches to quantifying the intracellular accumulation and efflux of efflux pump substrates by:

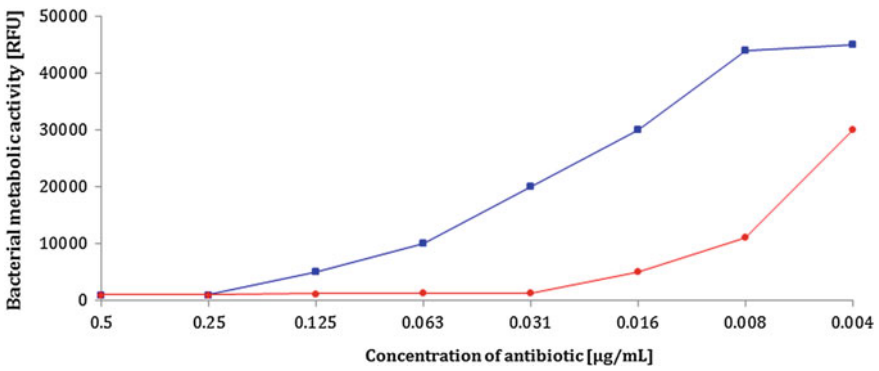

Fig. 1.1 Metabolic activity detection in modulation assay using resazurin. Growth of *C. jejuni* in the presence of antibiotic is represented by the blue curve with full squares and growth in the presence of antibiotic and resistance modulator by the red curve with full circles. The break point in the curve, at which the relative fluorescence signal (RFU) starts to increase, is defined as MIC

(i) Real-time monitoring of intracellular substrate accumulation kinetics;
(ii) Measurement of the accumulated endpoint concentration.

The first approach can be performed by automated spectrofluorometric microplate reader kinetics measurement when using substrates such as ethidium bromide, Nile Red, DiBAC4-(3), berberine or Hoechst 33342 (Lin et al. 2002; Tegos et al. 2002; Garvey et al. 2011), fluorescence of which increases due to their binding with DNA after intracellular accumulation. Measuring the accumulation of antibiotics that do not have the same fluorescence properties becomes more time-consuming and laborious, since it requires manual sampling and processing of the sample, and finally measurement of antibiotic concentration employing chromatographic, spectrofluorometric or radiometric methods (Jeon et al. 2011; Shiu et al. 2013; Blanchard et al. 2014). Assays also differ among each other by the accumulation time, ranging from 10 min (Shiu et al. 2013) up to 60 min (Kovač et al. 2014). The advantage of measuring accumulation of an efflux pump substrate by determining the residual substrate concentration in the medium is that there are no limitations in terms of the substrate fluorescence properties. For example, HPLC and UPLC coupled with MS were successfully used to follow the accumulation of minocycline in *Acinetobacter baumannii*, as well as accumulation of linezolid in *Escherichia coli* and meropenem in *Pseudomonas aeruginosa*, respectively (Blanchard et al. 2014; Zhou et al. 2015). In addition to using a microplate reader, a microfluidic channel device can be used to evaluate the intracellular accumulation of efflux pump substrate using fluorescent microscope, as demonstrated in *Escherichia coli* using fluorescein-di-β-D-galactopyranoside (Matsumoto et al. 2011).

To date, ethidium bromide and ciprofloxacin accumulation assays were performed in *Campylobacter* (Lin et al. 2002; Jeon et al. 2011; Kovač et al. 2014, 2015). Among reference efflux pump inhibitors, cyanide m-chlorophenyl hydrazone (CCCP) and reserpine resulted in best efflux inhibition in *C. jejuni* after 30 min, followed by verapamil (Fig. 1.2).

Somewhat surprisingly, Phe-Arg-β-naphthylamide dihydrochloride (PAβN) and 1-(1-naphthylmethyl)-piperazine (NMP), which were demonstrated to produce substantial resistance modulation in combination with several antimicrobials in *Campylobacter* (Klančnik et al. 2012a, b; Kurinčič et al. 2012b), show poor efflux-inhibitory activity (unpublished data of JK; Fig. 1.2). This may be explained with the synergistic resistance-modifying effects of efflux inhibition and membrane permeabilization, as demonstrated in *Pseudomonas aeruginosa* (Lamers et al. 2013). The increased membrane permeability could negatively influence the intracellular accumulation of small molecules, such as ethidium bromide.

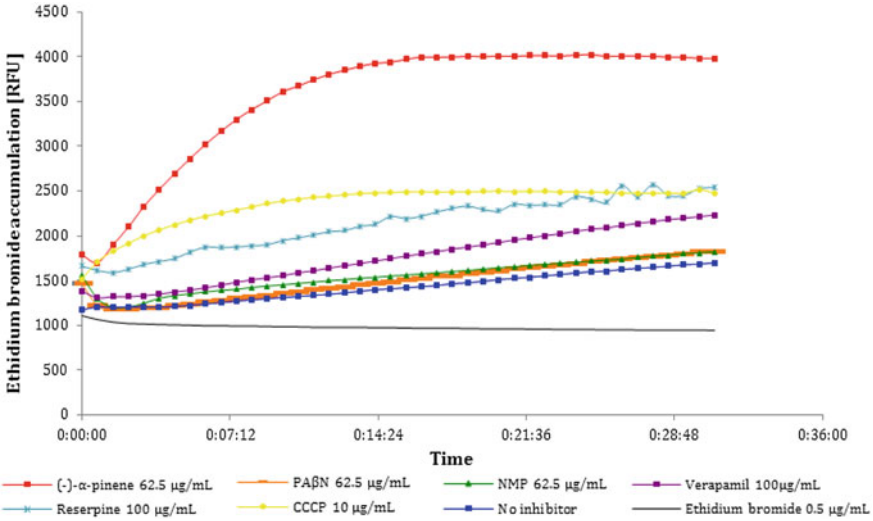

Fig. 1.2 Ethidium bromide accumulation in *Campylobacter jejuni* NCTC 11168 in the presence of natural product (-)-α-pinene, reference inhibitors PAβN, NMP, verapamil, reserpine and CCCP

1.3.2 Natural Products as Campylobacter Resistance Modulators

Natural products have been demonstrated to be a valuable source of resistance modulators. The resistance-modifying agents from plant sources were comprehensively reviewed by Abreu et al. in 2012 and updated recently (Prasch and Bucar 2015). Among plant extracts, the ethanol extract of *Alpinia katsumadai*, a plant belonging to ginger family (Zingiberaceae), exhibited modulation of ciprofloxacin and erythromycin, ethidium bromide, bile salts and sodium deoxycholate in *C. jejuni* and *C. coli* at subinhibitory concentration 0.25 MIC (Klančnik et al. 2012a). The same study demonstrated similar effects for *Rosmarinus officinalis* L. extract from mint family (Lamiaceae). Beside crude plant extracts, also essential oils have been found to potentiate the antibiotic activity in Gram-negative bacteria. The antimicrobially inactive essential oil from *Alpinia katsumadai* seeds was demonstrated to modulate resistance against antibiotics ciprofloxacin and erythromycin, as well as the disinfectant triclosan, bile salts and ethidium bromide at half MIC concentration for over 256-fold (Kovač et al. 2014).

Recently, two pure compounds responsible for resistance-modifying activity in *Campylobacter* were discovered. First one is catechin (-)-epigallocatechin gallate, a flavanol compound commonly found in green tea. It was confirmed as a modulator of resistance against a number of macrolide antibiotics in *Campylobacter* at concentrations 0.25 MIC (Kurinčič et al. 2012a). The second is (-)-α-pinene, a terpene

compound present among others also in *A. katsumadai* seeds, the extracts of which were previously found to act resistance-modifying (Kovač et al. 2014, 2015).

1.3.3 Natural Products as Campylobacter Efflux Pump Inhibitors

Several synthetic (Blanchard et al. 2014; Vargiu et al. 2014) as well as natural (Aparna et al. 2014; Whalen et al. 2015) inhibitors of Gram-negative bacterial efflux pumps have been discovered in the last years; however, reports on inhibitors of *Campylobacter* efflux pumps are limited. Among plant natural products, the essential oil of *Alpinia katsumadai* (Zingiberaceae) has been identified as a potential inhibitor of *Campylobacter* efflux pumps (Kovač et al. 2014). The terpene compound (-)-α-pinene, abundant in *A. katsumadai*, pine trees, rosemary, lavender and turpentine, has been demonstrated to have inhibitory effects on major resistance–nodulation–division (RND) family efflux pump in *Campylobacter*, the CmeABC efflux pump. Furthermore, its activity extends to another newly discovered putative efflux protein *Cj1687* (Kovač et al. 2015).

1.4 Inhibition of Adhesion and Biofilm Formation of *Campylobacter* by Natural Products

Several studies in recent years have provided evidence that this bacterial adhesion process is a multifactorial event, with the cooperative actions of several factors required mediating the adherence of *C. jejuni* to host cells (Ó Cróinín and Backert 2012; Backert and Hofreuter 2013).

The precise molecular mechanisms involved in the attachment of *C. jejuni* remain largely unknown. Many factors (presented in Table 1.2) have been identified as critical to the adhesion and survival of *Campylobacter* in vivo and in nature, which include those related to survival under stress and to the basic biology of the stress response of the organism (Szymanski and Gaynor 2012; Sulaeman et al. 2010).

1.4.1 Methods for Identification of Adhesion

Given the tremendous clinical importance of adhesion and biofilm formation, it is somewhat surprising that there is no standard method for the investigation of cells during bacterial adhesion. Several methods that are based on different principles are available to detect the adhesion properties of pathogens. For bacteria, a common

Table 1.2 Factors involved in the control of bacterial adhesion

Aspect/factor	Reference
Material surface characteristics	Garrett et al. (2008)
Surface chemistry; composition; topography; roughness; degree of hydrophobicity; hydrogen-bonding capacity	
Surface physicochemical factors	Lemos et al. (2014)
Surface energy; surface charge; functional groups; hydration	
Bacterial properties	Reisner et al. (2006), Harvey et al. (2007), Rode et al. (2007), Dunne (2002), Sulaeman et al. (2010)
Type; surface properties; morphology; cell membrane; host proteins/adhesins; bacterial motility; flagella; hydrophobicity; quorum-sensing inhibition; stress responses; extracellular polymeric matrix	
Environmental conditions	Reuter et al. (2010), Soni et al. (2008), Sulaeman et al. (2010)
pH; temperature; flow conditions; atmosphere and oxygen; nutrient composition	

method is to quantify the mass of the biofilm using crystal violet or safranin staining, followed by extraction of the bound dye with a solvent and measurement of its absorption (Reuter et al. 2010; Kurinčič et al. 2016). However, discussions remain, as the staining of live cells, dead cells and matrix with crystal violet can give misleading information. New methodologies might overcome the limitations of total biomass staining methods that appear when injured bacteria or viable but non-culturable bacteria are present. In some studies, the reduction of adhesion to polystyrene was measured in terms of cell culturability (according to CFU plate counting) and cell viability (according to a metabolic indicator) (Šikič Pogačar et al. 2015).

1.4.2 Natural Products as Campylobacter Anti-adhesives

Different concepts and approaches have been developed to achieve biomaterials that have such anti-infective properties. One of the new methodologies relies on the use of nanosized carriers to transport and controlling the release of an active antimicrobial (Liakos et al. 2014). Recently, there has been increasing interest in the use of phytochemicals for inhibition of adhesion and biofilm formation, particularly through the use of some natural compounds (Table 1.3).

Anti-adhesion or antibiofilm formation activities have been shown for essential oils and for extracts from red wine, grape marc, pine bark and cranberry fruit, and also for specific natural compounds, such as ferulic acid, salicylic acid, phenyl isothiocyanate, *trans*-cinnamaldehyde, carvacrol, thymol, eugenol and others (Sandasi et al. 2010; Abreu et al. 2014; Lemos et al. 2014).

Table 1.3 Treatments for the control of bacterial adhesion

Treatment/compounds	Reference
Phytochemical addition	Simões et al. (2010), Abreu et al. (2014), Borges et al. (2013), Lemos et al. (2014), Soni et al. (2013), Bezek et al. (2016)
Ferulic acid; salicylic acid; phenyl isothiocyanate; *trans*-cinnamaldehyde; carvacrol; thymol; eugenol; gallic acid; thyme extract; olive leaf extract; *Quercus cerris*; cinnamon essential oil; wine extracts; grape marc extracts; pine bark extracts; 7-hydroxycoumarin; indole-3-carbinol; saponin; okra fruit; Euodia ruticarpa; evocarpine and other quinoline fractions	
Biomaterials and biomaterial surfaces	Campoccia et al. (2013)
Cationic peptides; functionalized dendrimers; dendrimers; micro-/nanostructures; intrinsically antimicrobial materials; antimicrobial loading; antibiotic grafting; lysostaphin; phytoactive surfaces; dispersin B; cationic polymer grafting	
In vitro Anti-adhesion of *Campylobacter*	Šikić Pogačar et al. (2016), Bensch et al. (2011), Bezek et al. (2016)
Thyme extract; thyme postdistillation waste; olive waste; cayenne pepper; ginger; okra fruit extract; liquorice, Euodia ruticarpa	

However, recently, we showed for the first time that a thyme extract is effective for inhibition of *C. jejuni* adhesion to a polystyrene surface and thus of biofilm formation, although this extract did not inhibit *C. jejuni* growth or kill *C. jejuni* cells at the effective concentration for the anti-adhesion activity (Šikić Pogačar et al. 2016). Additionally, the antimicrobial activity of this thyme extract was comparable to those of more well-known sources of plant phenolic compounds, like wine (Gañan et al. 2009) and grape skin, as have been tested against different foodborne pathogens (Katalinić et al. 2013).

Adhered biomass is mostly evaluated using crystal violet staining, with measurement of the absorbance at 584 nm, which for the control, untreated *C. jejuni* K9/4 cells, is 0.13 ± 0.01 arbitrary units (a.u.) (Fig. 1.3). The adhered *C. jejuni* biomass to polystyrene can be significantly reduced by rosemary extract and (−)-epigallocatechin gallate (EGCG), by more than 80% of the control adhesion (as shown in Fig. 1.3). The adhesion was significantly reduced also by rosmarinic acid with 50% reduction. Smaller reductions of 15% in the adhered *C. jejuni* biomass were noted for the tested ferulic acid. Interestingly, there was a similar reduction of adhered *C. jejuni* biomass at all tested concentrations which was similar to that seen at subinhibitory 0.25 MIC concentration (Klančnik et al. unpublished) (Fig. 1.3).

Some research has been done for ferulic and gallic acids, which are known to act on the surface properties, particularly the occurrence of local rupture or pore

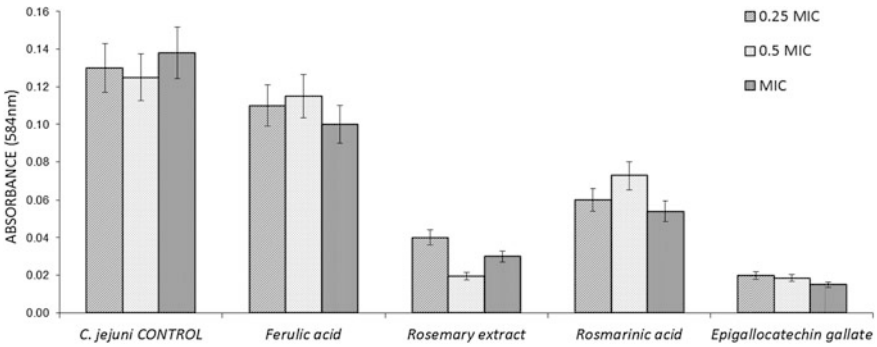

Fig. 1.3 Absorbance determined with microtiter reader for biomass evaluation after dying with crystal violet for *C. jejuni* control cells and when natural compounds were added in MIC (minimal inhibitory concentration), 0.5 and 0.25 MIC concentrations

formation in the cell membranes, although again they can show different modes of action according to the concentrations used (Borges et al. 2013).

Considering the mechanisms of anti-adhesion and the pronounced antimicrobial activities of thyme extract, it is known that more than one specific mechanism is involved, and thus, there are several targets in the cell activity (Šikić Pogačar et al. 2016). Additionally, a thyme postdistillation waste as a by-product of the agro-food industries and an olive (*Olea europaea* L.) leaf extract were tested as waste material. These residues have high economic burdens and can cause environmental problems; however, we showed that they are a source of bioactive phytochemicals that can inhibit *C. jejuni* adhesion at sub-bactericidal concentrations. Indeed, some studies have reported enhanced bacterial growth and induction of biofilm formation by essential oils or their components and also by antibiotics, such as aminoglycosides and β-lactams (Hoffman et al. 2005). The inability to inhibit biofilm growth at higher concentrations of olive leaf extracts confirms that *C. jejuni* cells in a biofilm respond adaptively and develop resistance (Šikić Pogačar et al. 2016).

However, only a few studies have investigated the anti-adhesive activities of such natural substances against *C. jejuni* in different cell lines (Bensch et al. 2011). Low doses of thyme and olive extracts are not cytotoxic and can prevent specific cell adhesion of *Campylobacter* to PSI cells and to H4 human foetal small intestine cells (Šikić Pogačar et al. 2016). Again, these anti-adhesion activities were stable across a range of extract concentrations and occurred at lower concentrations in comparison with those for abiotic surfaces. Thus, these extracts can be used to modulate C. *jejuni* invasion and its intracellular survival, which represent the two most crucial mechanisms of colonization through which *C. jejuni* induces disease (Šikić Pogačar et al. 2016). These plant extracts can thus potentially be used as therapeutic agents, to replace certain bactericidal drugs (Rogers and Paton 2009).