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Anti-adhesion activity of phytochemicals to prevent *Campylobacter jejuni* biofilm formation on abiotic surfaces

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Abstract Biofilms provide a protective environment for pathogens such as *Campylobacter jejuni*, the most prevalent foodborne pathogen, and biofilm formation can enhance bacterial survival in hostile environments. Adhesion of bacteria to the different materials of industrial surfaces is the first step in biofilm formation. Modulation of bacterial adhesion and biofilm formation thus represent important targets in alternative control strategies for reduction of pathogens in food-processing environments. With the high prevalence of *C. jejuni* and the lack of effective control measures, new control strategies are needed to block adhesion and biofilm formation on food contact surfaces in the food industry, with a focus here on natural antimicrobial phytochemicals. Plants remain a poorly recognized yet vast source of such antimicrobials. Valuable phytochemicals can be obtained directly from plant materials but also from agro-food by-products and waste materials. These materials represent a source of important plant bioactive phytochemicals that are effective for prevention of bacterial adhesion. In this review, we will focus on the anti-adhesion activities of phytochemicals targeted against *C. jejuni*, on the appropriate methodologies to determine anti-adhesion effects of phytochemicals, on the mechanisms of *C. jejuni* adhesion, and thus possible targets for reduction and control of this foodborne pathogen in food processing environments.

Keywords Bioactive phytochemicals · Anti-adhesion · Target mechanism · *Campylobacter jejuni* · Abiotic surface

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Introduction

Many bacterial species have developed the unique survival strategy of biofilm formation, which provides selective advantages over their planktonic and free-existing cells. They can attach to a surface through electrostatic and van der Waals forces, and thence start to communicate, to establish a biofilm community (Dunne 2002; Flemming et al. 2016). Further cell–cell interactions, cell signalling and bacterial replication lead to a more organised structure, with the establishment of a dense population that is encapsulated in a mainly self-produced matrix of extracellular polymeric substances (EPS). This matrix is composed of polysaccharides, proteins, nucleic acids and lipids. In biofilms, the EPS holds the cells close together, which

results in higher concentrations of cell-produced signalling molecules, and at sufficient quantities these can promote changes in cellular behaviour and the activation of various genes. At this stage, intercellular communication leads to synergy within the community, with the diffusion of oxygen and nutrients inwards, and waste and signals outwards (Limoli et al. 2015). Further formation of the mature biofilm in its three-dimensional (3D) architecture increases the adhesion between the bacteria and with the surface, and protects the bacteria from radiation damage, dehydration, pH fluctuations and antimicrobial compounds (Flemming et al. 2016).

Further problems can arise from multispecies situations compared to monocultures, whereby the growth and biofilm formation of pathogenic bacteria can also be influenced by other bacterial species. For example, spoilage bacteria, such as *Pseudomonas*, can decompose meat through their extracellular hydrolases and penetrate deeper into food (Shirai et al. 2017). This will provide further nutrients and create potential pathways for *Escherichia coli* to grow and form biofilms, and consequently to penetrate and persist in the food. Thus, such mixed bacterial populations can enhance growth and biofilm formation, and consequently bacterial persistence (Sterniša et al. 2019).

Although the regulatory mechanisms of many aspects of biofilm formation still remain unclear, it is known that this involves a succession of several steps that begins with the initial and crucial step of attachment. However, the exact mechanisms of the adhesion process and the different molecules that are involved in different organisms remain to be defined. It is known that adhesion of bacteria to abiotic surfaces is mediated via non-specific (e.g., hydrophobic) interactions (Sulaeman et al. 2012), whereas their adhesion to other living cells is through specific molecular mechanisms, such as those that involve lectins, adhesin and other specific ligands (Backert and Hofreuter 2013; Svensson et al. 2014; Teh et al. 2014).

Many bacterial species have become resistant to antibiotics, such that many of the available antibiotics are no longer effective against some infections that confront patients and physicians in hospitals. Bacterial resistance mechanisms are pandemic, and they can create clinical and financial burdens on healthcare systems (O'Neill 2016). The Review on Antimicrobial Resistance (AMR) indicate that bacterial infections are the major cause of morbidity and mortality worldwide (EFSA and ECDC 2019a). As stated by the World Health Organisation (WHO), the rate of antibiotic resistance has been accelerated by over-use and misuse of antibiotics, and by poor infection prevention and control practices (World Health Organization 2015). Consequently, new effective and practical measures are needed for the control of contamination by the major foodborne pathogens in these resistant forms.

In this review, we will discuss the current understanding of these initial biofilm-forming processes that are based on the adhesion properties of bacteria to abiotic surfaces, with a focus on *C. jejuni*. We highlight that elimination of adhesion as the first important step in biofilm formation can reduce the potential for cell adaptation to the different materials of industrial surfaces, and thus prevent bacterial survival and spread through the food-production chain. Natural extracts and isolated pure phytochemicals will be the focus as anti-adhesion antimicrobials. Furthermore, we will provide a literature overview of methods used to determine anti-adhesion effects of phytochemicals on *Campylobacter* spp. adhering to abiotic surfaces. Additionally, we will evaluate and compare the efficacy of some of these methods to detect the anti-adhesion effect of thymol and carvacrol in vitro. Finally, we will provide an overview of important targets for anti-adhesion activity of phytochemicals against *C. jejuni* and thus expose the importance of the study of mechanisms of antimicrobial action of phytochemicals.

Campylobacter as a food safety problem and a model to study anti-adhesion strategies

According to the regulatory agencies, including the US Food and Drug Administration and the ECDC, alternative strategies are urgently needed for the control of *Campylobacter* spp. (Kaakoush et al. 2015). Thermophilic *Campylobacter* spp., mainly *C. jejuni*, are Gram-negative spiral, rod-shaped or curved, and bipolar flagellated motile bacteria which grow under microaerobic conditions (85% N_2, 3–5% CO_2, and 5–10% O_2) optimally at 42 °C, but always above 30 °C (Rovira et al. 2006). As human pathogenic intestinal bacteria they can be transmitted into the human food chain from the environment, through infected animals or contaminated food. *C.*

jejuni is a common gut commensal of several animal species, especially birds. The route from environmental contaminant to chicken caeca, to poultry carcass contaminant, and finally, to human disease agent contains many hurdles. However, *C. jejuni* can surmount these hurdles, to represent a major public health problem in the food industry, as the cause of campylobacteriosis, the most common bacterial gastrointestinal infection throughout the world (EFSA and ECDC 2018; Kaakoush et al. 2015). In the latest EFSA report for 2018, beside *Salmonella* species, *Campylobacter* is the second most frequently reported pathogenic agent which accounted for 524 outbreaks or 10.2% of total (5146) food-borne and waterborne outbreaks reported in the EU in 2018. Large *Campylobacter* outbreaks (> 100 cases of illness) were reported in Hungary, France and Sweden (EFSA and ECDC 2019b).

Campylobacter spp. persist and survive outside of the host in environments that are detrimental to them, which represents an enormous problem in the food industry. The main contributions are due to the spread of *Campylobacter* resistance through intensive use of antimicrobial agents, combined with the international trade in raw materials and food products. The European Food Safety Authority and the European Centre for Disease Control have reported increased frequencies of these antibiotic-resistant pathogenic bacteria, which show high resistance to the more widely used antibiotics. For example, the recently reported prevalence of ciprofloxacin resistant *C. jejuni* isolates from humans was 57.7%, and for *Campylobacter coli*, 63.5%, as also for tetracycline resistant *C. jejuni* of 45.4% and *C. coli* of 68.3%. The prevalence of resistant strains is even higher in broiler chickens, with *C. jejuni* strains showing 66.9% ciprofloxacin resistance and 50.7% tetracycline resistance, with still increasing rates and worse situations in southern European countries (EFSA and ECDC 2019a, b).

This growing resistance to antimicrobials has been mainly attributed to mutations in specific genes, or to be conferred by efflux pumps. These efflux pumps can limit the access of antibiotics to their targets by actively pumping them out of the cell, and thereby preventing the intracellular concentrations that are needed for their lethality (Smole Možina et al. 2011). In biofilms, *Campylobacter* spp. are also protected against antimicrobial substances, as these can only penetrate slowly through the biofilm layers. Furthermore, while the sublethal antibiotic concentrations that such biofilm bacteria are exposed to can result in slower growth, this can also promote the expression of resistance genes.

Campylobacter appear to be unusual food-borne pathogens, as they lack many of the adaptive responses to environmental stresses, although they can still survive under the conditions used during food processing and preservation. Several mechanisms appear to be involved in the *Campylobacter* survival strategy, including dynamic adaptation (McDougald et al. 1998) and transformation into a viable-but-not-culturable (VBNC) state (Rollins and Colwell 1986), both of which are promoted by high genetic heterogeneity. *Campylobacter* spp. can also form monospecies biofilms (Joshua et al. 2006) and colonise pre-existing biofilms. They can form different biofilm structures attached to a surface, as a pellicle formed at the surface of a liquid, and as aggregates that float in liquid cultures (Joshua et al. 2006). Attachment of *Campylobacter* spp. and formation of biofilms on food-contact surfaces has been shown, particularly for food processing environments, such as stainless steel, glass and plastic (Joshua et al. 2006; Teh et al. 2014). Also, adhesion of *Campylobacter* spp. to the surface of protozoa can increase the transmission of infections, while their adhesion to the surfaces of human and animal intestinal cells promotes their pathogenicity, with the consequent effects on human and animal health (Indikova et al. 2015; Bolton 2015).

Prevention of *Campylobacter* spp. adhesion in the first place will thus reduce these sources of further contamination that biofilms represent (Indikova et al. 2015). Furthermore, this will limit *Campylobacter* spp. infections in the early stages of their development, and hence avoid or reduce the exposure of their biofilms to antibiotics, as this represents a further risk for the development of bacterial resistance. This will also serve to avoid potential adverse effects of antimicrobials on the host microbiome (Yin et al. 2019).

Thus, it is important to discuss the use of antimicrobial agents through alternative approaches to limit the increasing *Campylobacter* spp. resistance to antibiotics. This might be achieved by using phytochemicals as antimicrobial agents that can prevent *Campylobacter* spp. adhesion, and thus combat bacterial infections without providing selection pressure for the emergence of further antibiotic-resistant

bacteria. Additionally, there is the need to sufficiently investigate the mechanisms of adhesion of *Campylobacter* to abiotic surfaces, to provide insight into different targets in this novel anti-*Campylobacter* anti-adhesion strategy (Fig. 1).

Phytochemicals for the prevention of biofilm formation

The use of phytochemicals in infection medicine gradually diminished as they were replaced by antibiotics. This situation has, however, become reversed more recently as a result of increased antibiotic resistance, and as phytochemicals often show fewer side effects compared to synthetic drugs (Petrovska 2012). Indeed, plant extracts with anti-adhesive activities offer advantages over other antimicrobials as they show no deleterious effects on the host microbiota (Klemm et al. 2010).

Plants produce numerous highly diverse secondary metabolites, many of which have been optimised through evolution for specific biological functions, and which are still far from being exhaustively investigated (Atanasov et al. 2016). These phytochemicals are often referred to as phytonutrients, and they are natural bioactive compounds that can be found in foods like vegetables, fruit, whole grain products, nuts and seeds, legumes, tea and dark chocolate. Although there are tens of thousands of phytochemicals in plants, only a small number have been purified and studied (Cao et al. 2017; Singh and Chaudhuri 2018). Due to their potential benefits, scientific evaluation of various pharmacological effects of phytochemicals has increased throughout the world more recently. Indeed, as reported by the

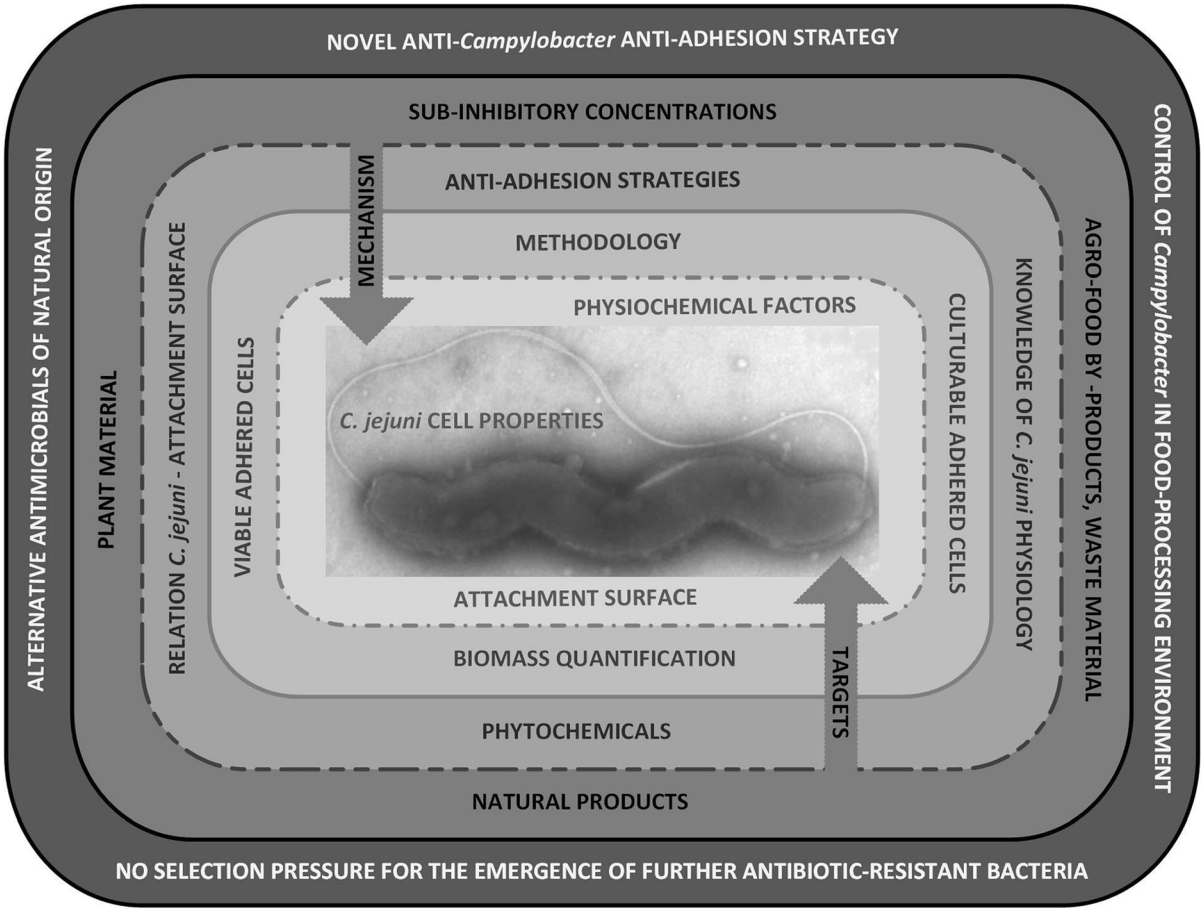

Fig. 1 The scheme of factors involved in adhesion of *Campylobacter* to abiotic surface, to provide insight into different targets and to present novel anti-*Campylobacter* anti-adhesion strategies by phytochemicals

World Health Organisation, more than 80% of populations from developing countries still depend on traditional medicines for their primary health care, and these are mainly plant based (World Health Organisation 2019). Among small molecule anti-infectives, natural products still play a major role. In a report of Newman and Cragg only 33% of small molecule infectives approved between 1981 and 2014 are of pure synthetic origin (Newman and Cragg 2016).

Plants synthesise phytochemicals as part of their defence against pathogens, and many of these have effective antimicrobial activities. The biodiversity of the sources of phytochemicals provides unique and renewable resources for the discovery of novel biological activities (Bacanlı et al. 2017; Chen et al. 2018; Curti et al. 2017; Vinayagam et al. 2017; Zhao et al. 2017). In this review we will focus on the anti-adhesion and anti-biofilm activities of some of the known bioactive phytochemicals.

Plants can produce a large number of diverse bioactive compounds, with > 8000 different phenolic compounds characterised to date. Fruit and vegetables have been the primary sources of these natural antioxidants and antimicrobials (Altemimi et al. 2017). Even less investigated in terms of bioactive chemicals are edible mushrooms (Kalač 2009; Wang et al. 2014) which have also been covered by this review.

Here, we present the first report on the methodologies used for evaluation of *Campylobacter* spp. adhesion to abiotic surfaces, and of the anti-adhesion activities of phytochemicals (Fig. 1). Recent progress in alternative antimicrobials has focused on the identification and evaluation of the properties of bioactive phytochemicals as agriculture by-products, or as by-products of other raw materials that are rich in these bioactive compounds. Alternative approaches are needed to control *Campylobacter* spp. using phytochemicals, probiotic bacteria, or bacteriophages, with investigations of their potential mechanism(s) of actions (Upadhyay et al. 2019).

Collectively, *C. jejuni,* the most prevalent cause of campylobacteriosis, is used as the model system to define novel strategies to combat contamination and infection. Currently, there is no effective control strategy, as the *Campylobacter* spp. 'lifestyle' enables them to withstand stress in the environment, both outside and within their hosts. We will review the possibility to target the adhesion of *Campylobacter* spp. to different materials of industrial surfaces, and their formation of biofilms. Here, *C. jejuni* can be used as a model to study the mechanisms that these bacteria use to adhere to surfaces, which is crucial for the application of novel control strategies. These anti-adhesion strategies will thus allow modulation of *Campylobacter* spp. virulence and elimination of infections at the early stages of bacterial adhesion. Only a few studies have presented such alternative strategies using low doses of bioactive phytochemicals, pure compounds or phytochemicals obtained as agro-food by-products. Additionally, we investigate the adhesion mechanism involved, an understanding of which is crucial for the application of such novel antimicrobial strategies. Finally, we will provide an overview of modern methods to determine the adhesion properties of *Campylobacter* to abiotic surfaces.

Determination of *Campylobacter* adhesion and biofilm formation on abiotic surfaces

Biofilm formation is a multifactorial process that comprises different stages that are governed by different physical and chemical processes, and these all begin with the initial attachment. Adhesion of *C. jejuni* to abiotic surfaces is mediated by the following (Sulaeman et al. 2012; Indikova et al. 2015; Bohinc et al. 2014; Šikić Pogačar et al. 2016; Trošt et al. 2016; Klančnik et al. 2017a, b, 2018a, b):

1. The properties of the microbial cells, which include: their cell surface hydrophobicity and charge; their cell morphology, membranes, membrane proteins, aggregation, flagellation and motility; the host cell proteins, fatty-acid metabolism, intracellular transport and chaperones; their quorum-sensing (QS) and signalling molecules; their chemotaxis, stress responses and extracellular polymeric matrix formation.
2. The surface properties, such as hydrophobicity, electric charge and surface roughness.
3. The physicochemical factors, fluid properties and environmental conditions, including polarity, flow velocity, pH, ionic strength, temperature, atmosphere, presence of salts, antimicrobials, nutrient availability and different media.

Collectively, the internal microbial properties provide possible detection targets in the methods used

to evaluate anti-adhesion and anti-biofilm activities of phytochemicals. However, *Campylobacter* spp. can adhere to inert surfaces of different materials used in different industries, such as stainless steel, glass fibre, glass beads and coverslips, nitrocellulose membranes, and various plastics (Sulaeman et al. 2012; Teh et al. 2014; Šikić Pogačar et al. 2015; Klančnik et al. 2017b). Indeed, the most commonly used model for determination of anti-adhesion activities of phytochemicals is polystyrene microtiter plates, followed by stainless steel coupons of diverse surface roughness, and glass surfaces (Bezek et al. 2016; Klančnik et al. 2017b, 2018b; Wagle et al. 2019). It is important to include abiotic surfaces with different degrees of roughness, to thus present different in vivo conditions of surface irregularities, which have been shown to provide the cell shelter that is relevant for biofilm formation in food processing facilities (Bohinc et al. 2014).

The anti-adhesion and anti-biofilm activities of plant-based compounds also depend on the origin of the *Campylobacter* strain, its growth phase, the addition of glucose to the growth medium, and the incubation time (Reeser et al. 2007). The only strains used so far to determine such anti-adhesion activities of plant compounds have been the reference strains of *C. jejuni* NCTC 11168 (Parkhill et al. 2000) and *C. jejuni* ATCC33560 (Hyytiäinen and Hänninen 2012), and the food isolates *C. jejuni K49/4, C. jejuni* 225421, and two mutants of *cmeB* and *luxS* of *C. jejuni* NCTC 11168.

Furthermore, bacterial cell concentrations need to be taken into account, as these affect bacterial growth and the rate of cell adherence, and can influence the anti-adhesion or anti-biofilm activities of phytochemicals. For research purposes, standardised concentrations of *C. jejuni* of 10^5 to 10^6 CFU/mL have been most commonly used (Bezek et al. 2016; Castillo et al. 2014; Klančnik et al. 2017b, 2018b), and the followed parameters have been used: (1) media: Mueller–Hinton broth, *Campylobacter* enrichment (Bolton) broth or *Brucella* broth; (2) incubation conditions: microaerophilic (5% O_2, 10% CO_2, 85% N_2) or aerobic atmospheres, and temperatures of 20, 37 and 42 °C (Bezek et al. 2016; Castillo et al. 2014; Klančnik et al. 2017b, 2018b; Wagle et al. 2019).

It is very important to select concentrations of phytochemicals that do not influence the bacterial growth (sub-inhibitory), to limit the potential *Campylobacter* resistance. Thus, minimal inhibitory concentrations (MICs) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical Laboratory Standards Institute (CLSI) guidelines have to be determined for each phytochemical tested, and for each *C. jejuni* strain tested. Additionally, the influence on bacterial growth kinetics must be determined for the MICs, and also for selected sub-inhibitory concentrations. According to the literature, sub-inhibitory concentrations for growth effects have been used, mostly as 0.1 × MIC to 0.5 × MIC. Furthermore, whatever methods are used for evaluation of the anti-adhesion and anti-biofilm activities of phytochemicals, care is needed in terms of the concentration of solvent used in the preparation of the phytochemicals, and the relevant controls need to be included. We have found that solutions with 1% ethanol or DMSO do not influence *C. jejuni* adhesion to abiotic surfaces (results not shown, Šimunović et al. 2020), although higher concentrations have not been tested.

Collectively, bacteria inoculated in broth are incubated with the investigational phytochemicals. After the addition of the phytochemicals at sublethal concentrations, short incubation times of up to 24 h are used to study anti-adhesion processes, and with over 24 h used to study anti-biofilm activities. After the incubation and the interactions of the bacteria in terms of their contact with the investigational surface, there is the need to first remove the unattached bacteria, with a wash with phosphate-buffered saline (PBS). Further different direct and indirect methods can be used to provide insights into the various parameters investigated, and the further specific detection steps will depend on the methodology selected. The most critical point for the anti-adhesion or anti-biofilm determination of these phytochemicals is to adopt a relevant method that is reliable and robust enough to be carried out under various environmental conditions (Šikić Pogačar et al. 2016; Trošt et al. 2016; Klančnik et al. 2017b, 2018a).

The quantification of *Campylobacter* adhesion is based on various indirect approaches, such as biomass determination, cultivability, viability, metabolic activity, or other cell properties, with most of these determined in microtiter plates. According to the target parameters, the different methods will provide different data. To evaluate and quantify the anti-

adhesion and anti-biofilm activities of the phytochemicals, it is advisable to use different methods to measure several relevant parameters. At the same time, not all methods are always feasible, as unexpected reactions of phytochemicals can occur with the reagents used. Once the data have been obtained, they need to be taken through different data processing formats. The methods generally used to evaluate adhesion and biofilm formation of *C. jejuni*, and the inhibitory effects of phytochemicals, are collected in Table 1.

One of the most commonly used methods to determine anti-adhesion and anti-biofilm activities is based on biomass reduction. These studies used different dyes, such as crystal violet (Klančnik et al. 2017b) and safranin (Castillo et al. 2014), which bind non-specifically to negatively charged surface molecules, and thus bind to both the bacteria and the extracellular biofilm components. In this indirect method, the absorbance of the dye used is then determined, to evaluate the adherence of the bacteria.

At the same time, these dyes can also bind to the phytochemicals themselves. For this reason, when using such non-specific dyes, there is also the need to determine whether the phytochemicals can form a conditioning layer, and the phytochemical interaction with the dye must be included. When the read-out data of absorbance are obtained, the negative control absorbance must also be determined. The mean absorbance as a measure of bacterial adhesion is thus calculated according to Eq. (1) (Harvey et al. 2007):

$$\Delta\bar{A} = \sum_{i=1}^{n}\left(\frac{A - \bar{A}_o}{n}\right) \quad (1)$$

where $\Delta\bar{A}$ is the mean bacterial absorbance, $\bar{A}_o$ is the arithmetic mean of the absorbance of the wells with the negative control, and n is the number of wells with the inoculated bacterial strain. Thus, the reduction of bacterial adhesion in the presence of the natural compounds can be calculated as the inhibition of each parameter (expressed as a percentage) using Eq. (2) (Jadhav et al. 2013):

$$Inhibition(\%) = \left(1 - \left[\frac{T}{C}\right]\right) \times 100 \quad (2)$$

where C is the mean for the control samples that contain bacteria in medium without the plant phytochemical, and T is the mean for the treated samples that contained the bacteria in medium with the phytochemicals.

Recently, Wagle et al. (2019) used 2,3,5-triphenyltetrazolium chloride (generally known as TTC) for adhesion and biofilm assays, with staining of live adhered or biofilm cells. These were then air-dried, dissolved in 20% acetone in ethanol, and measured for absorbence at 500 nm (A_{500}).

The number of adhered or biofilm cells can be evaluated indirectly by the viability of the cells, in terms of the cell cultivability. Cell cultivability is determined on agar plates using the colony counting plate technique, and is expressed as colony forming units (CFU) (Vesterlund et al. 2005; Gamble and Muriana 2007; Klančnik et al. 2017b). The bacterial cell growth in broth to a certain turbidity can also be measured (i.e., optical density [OD]) (Bezek et al. 2016; Klančnik et al. 2017b, 2018a; Šikić Pogačar et al. 2016). To determine the cultivability of adhered or biofilm cells, after rinsing off the unattached cells with PBS, the adhered or biofilm cells need to be detached using sonication, glass beads or scraping (Bezek et al. 2016; Šikić Pogačar et al. 2016; Klančnik et al. 2017b, 2018a; Wagle et al. 2019), and then diluted in PBS. Again, the bacterial cells are then calculated using the colony counting plate technique, and expressed as CFU/mL (ISO 4833). These data expressed as CFU/mL, or as CFU/surface, are compared to the control. When turbidity is measured, the surface is washed/rinsed with PBS, fresh medium is added, and the cells are detached and allowed to grow, followed by optical density measurements compared to the control.

The number of adhered or biofilm cells can be evaluated indirectly by the viability of the cells according to the cell metabolism. However, this approach also detects cells that are in a state of very low metabolic activity. These cells cannot therefore divide, although they are alive and might not be considered by plate counting methods (and hence are VBNC). *Campylobacter* enter the VBNC state as a response to stress, due to adverse nutrient, temperature, osmotic and oxygen conditions, and also due to addition of antimicrobial compounds. Interestingly, there have only been two methods used in the literature

Table 1 Methods generally used to evaluate the adhesion and biofilm formation of *Campylobacter jejuni*, and the inhibitory effects of phytochemicals

Parameter evaluated	Method	Conditions/work	Processing	Surface	Assay	References
Biomass determination	Crystal violet assay	Microaerobic atmosphere, 42 °C for 24 h	Wash, dry, 1% Crystal violet (wt/vol), A_{584}	Polystyrene microtiter plates	Adhesion	Trošt et al. (2016), Klančnik et al. (2017b)
	Crystal violet assay	Microaerobic atmosphere, 37 °C for 48 h	Wash, dry, 1% Crystal violet (wt/vol), A_{570}	Polystyrene microtiter plates	Biofilm formation	Duarte et al. (2015, 2016)
	Safranin assay	Microaerobic atmosphere, 42 °C for 72 h	Wash, dry, 1% Safranin (wt/vol), A_{492}	Polystyrene microtiter plates	Biofilm formation	Castillo et al. (2014, 2015)
Attached, biofilm cells	2,3,5-Triphenyltetrazolium chloride	Aerobic atmosphere, 20/37 °C for 24/48 h; medium/chicken meat juice	Wash, dry, 0.05% Triphenyltetrazolium chloride (wt/vol), A_{500}	Polystyrene microtiter plates, stainless steel coupons	Adhesion, biofilm formation	Wagle et al. (2019)
Cultivability of adhered cells	Plating (CFU/mL)	Microaerobic atmosphere, 42 °C for 1/24/48/72 h	Wash, detach cells (vortex, glass beads), plate	Stainless steel coupons	Adhesion	Bezek et al. (2016)
	Plating (CFU/mL)	Microaerobic atmosphere, 42 °C for 24 h	Wash, detach cells (sonication), plate	Polystyrene microtiter plates	Adhesion	Šikić Pogačar et al. (2016), Trošt et al. (2016), Klančnik et al. (2017b)
	Plating (CFU/mL)	Aerobic atmosphere, 20/37 °C for 24/48 h; medium/chicken meat juice	Wash, detach cells (glass beads), plate	Polystyrene microtiter plates, stainless steel coupons	Adhesion, biofilm formation	Wagle et al. 2019
Viability of adhered cells	BacTiterGlo kit	Microaerobic atmosphere, 42 °C for 24 h	Wash, detach cells (sonication), add reagent, measure bioluminescence	Polystyrene microtiter plates	Adhesion	Trošt et al. (2016), Klančnik et al. (2017b)
	5-Cyano-2,3-ditolyl tetrazolium chloride (CTC)	Microaerobic atmosphere, 37 °C for 48 h	Wash, detach cells, CTC (5 mM)	Polystyrene microtiter plates	Biofilm formation	Duarte et al. (2015)
DNA quantification	PCR-based (qPCR/dPCR)	Microaerobic atmosphere, 42 °C for 24 h; influence of glucose levels, monoculture/co-culture with *Listeria monocytogenes*	Wash, DNA isolation from adhered cells	Polystyrene microtiter plates, stainless steel coupons (polished, etched)	*ccoN* sequence	Klančnik et al. (2018b)

Table 1 continued

Parameter evaluated	Method	Conditions/work	Processing	Surface	Assay	References
Microscopy techniques	Confocal laser scanning microscopy	Aerobic/ microaerobic atmosphere, 37/42 °C for 24/48 h	FilmTracer Live/Dead Biofilm Viability kits (SYTO-9, propidium iodide); cyanine dye (BOBO3); calcofluor white dye	Stainless steel coupons, Lab-Tek two-chamber (no. 1) borosilicate coverglass system, polystyrene	Biofilm formation	Oh et al. (2018), Wagle et al. (2019)
	Environmental scanning electron microscopy	Aerobic atmosphere, 37 °C for 48 h		Stainless steel coupons Lab-Tek two-chamber (no. 1) borosilicate cover-glass system, polystyrene	Biofilm formation	Wagle et al. (2019)

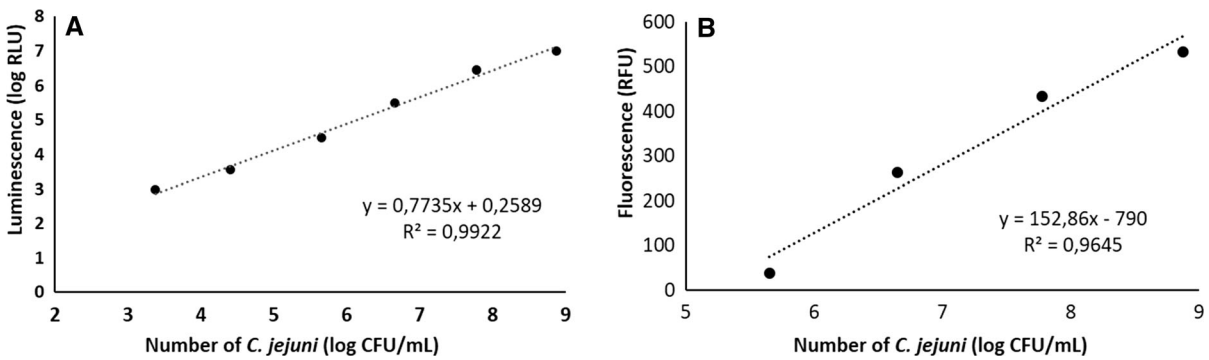

Fig. 2 Calibration curves for *C. jejuni* 11168 numbers with the addition of the BacTiterGlo reagent (**a**) and the resazurin reagent (**b**). RLU/RFU, relative luminescence/fluorescent units

to determine viable adhered cells after treatments with natural compounds: (1) spectrophotometric measurements for bioluminescence detection of ATP, using BacTiter-Glo assays (Klančnik et al. 2017b); and (2) absorbance measurements after using tetrazolium salts, as methyl tetrazolium chloride (Duarte et al. 2015).

For accurate determination of the anti-adhesion activity of a phytochemical, very low numbers of adhered viable cells have to be detected. Thus, for this review, we prepared some examples of calibration curves from overnight *C. jejuni* 11168 cultures for BacTiterGlo (Fig. 2a) and the resazurin reagent (Fig. 2b). These data clearly demonstrate that with BacTiterGlo the lower limit of detection is $\sim$ 3 log CFU/mL, and this assay is thus better suited for detection of anti-adhesion, compared to the resazurin reagent, where the lower limit of detection is $>$ 5 log CFU. Thus, when the expected number of adhered cells was $<$ 5 log CFU, they were not detected with the resazurin reagent, but they were detected with BacTiterGlo. The main reason for this might be that the detection of cell viability with resazurin is based on the reduction of resazurin to fluorescent resorufin by the metabolically active cells, while with BacTiterGlo, the ATP in the cells is detected. As the metabolic activity of adhered cells can differ from that of their planktonic counterparts, the determination of adhered cells with resazurin and other such reagents

based on the metabolic activity of the cells might be problematic and not accurate.

The quantification of adhesion and biofilm formation has thus relied on different approaches and techniques, which will lead to different evaluations for the adhered cells. Thus, for this review, we carried out an experiment using four different methods for determination of the anti-adhesion activities of some phytochemicals. The aim was to determine the anti-adhesion activities of carvacrol and thymol using polystyrene microtiter plates according to four different methods. *C. jejuni* 11168 were prepared to an OD_{600} of 0.1 in Mueller–Hinton broth. The conditions were then without (control; in 1% DMSO) and with addition of carvacrol and thymol at 0.25 × MIC (in 1% DMSO), in 96-well polystyrene microtiter plates (100 µl/well). After a 24-h incubation at 42 °C under microaerobic conditions, the plates were washed three times with PBS, and where appropriate, treated for 5 min in an ultrasonic bath (2, 3, and 4). The levels of adhesion were then determined, as follows:

1. Quantification of the adhered cell biomass using crystal violet staining, as described by Klančnik et al. (2017b);
2. Direct quantification of the adhered cells using serial dilutions and plating on Mueller–Hinton agar, as CFU/well (Klančnik et al. 2017b);
3. Quantification of adhesion using BacTiterGlo and measurement of the luminescence signal, as described by Klančnik et al. (2017b);
4. Quantification of adhered cells using 100 µl resazurin reagent per well with measurement of excitation and emission fluorescence at 560 nm and 590 nm, adapted method which was previously described by Kovač et al. (2015) for MIC determination.

These data of the anti-adhesion activities of carvacrol and thymol are presented in Fig. 3. As the four assays provide different types of information, the ratio of crystal violet will indicate the biomass of the adhered cells. These data show no significant differences between the biomass of the adhered *C. jejuni* cells of untreated control and those treated with carvacrol and thymol (Fig. 3a). The reason for this might be that in the first 24 h, *C. jejuni* have less possibility to form strong biofilms. As previously reported, adhesion of *C. jejuni* to polystyrene is strain dependent (Bronnec et al. 2016), and more than 24 h is needed to form adequate biomass (Melo et al. 2017; Klančnik et al. 2017b). However, the ability of *C. jejuni* to adhere strongly is increased under conditions similar to those in the avian industry (e.g., in chicken juice). The nutritive particles available in chicken juice can form a conditioning layer on polystyrene and glass surfaces, which can facilitate bacterial adhesion. Thus, only 4 h of incubation was sufficient for the initial establishment of the biofilm structure, and this is why *Camplyobacter* spp. act as a constant source of contamination in the food industry (Schluter et al. 2015; Melo et al. 2017).

Furthermore, the data in Fig. 3b show that using the direct method of colony counting, there appeared to be significantly fewer adhered cells on the polystyrene when treated with both carvacrol and thymol. The adhesion of the untreated control *C. jejuni* to the polystyrene surface of microtiter plates was 6.77 ± 0.40 log CFU/well. The greater reduction was seen for adhesion of *C. jejuni* in the presence of thymol, to 4.19 ± 0.76 log CFU/well, which represented 38% inhibition of *C. jejuni* adhesion. Significant anti-adhesion effects were also seen for carvacrol, as 7.70 ± 0.70 log CFU/well, which represented 31% inhibition of *C. jejuni* adhesion.

Interestingly, these findings were not confirmed with the methods here that measured the presence of ATP, as representative of the metabolic activity of the adhered cells. With BacTiterGlo, the anti-adhesion effect of thymol was again significant, although carvacrol showed no anti-adhesion effect (Fig. 3c). This difference in these data for the direct counting and the BacTiterGlo analysis for carvacrol can be explained by the presence of *C. jejuni* as VBNC, which cannot be detected by the cultivability assay, but are detectable using BacTiterGlo. Additionally, for this review, we used the calibration curve from Fig. 2a to quantify the numbers of metabolically active cells, to define them as log CFU/mL. These data for the adhesion of the untreated control *C. jejuni* to the polystyrene surface of the microtiter plates was 6.93 ± 1.56 log CFU/mL. Again, here the only significant reduction seen for adhesion of *C. jejuni* was with thymol, down to 5.23 ± 1.31 log CFU/mL, which represented 25% inhibition of *C. jejuni* adhesion. The differences obtained for the anti-adhesion effects of carvacrol potentially confirm the presence of the VBNC form of *C. jejuni* as a response to this stress condition. On the other hand, for the anti-adhesion

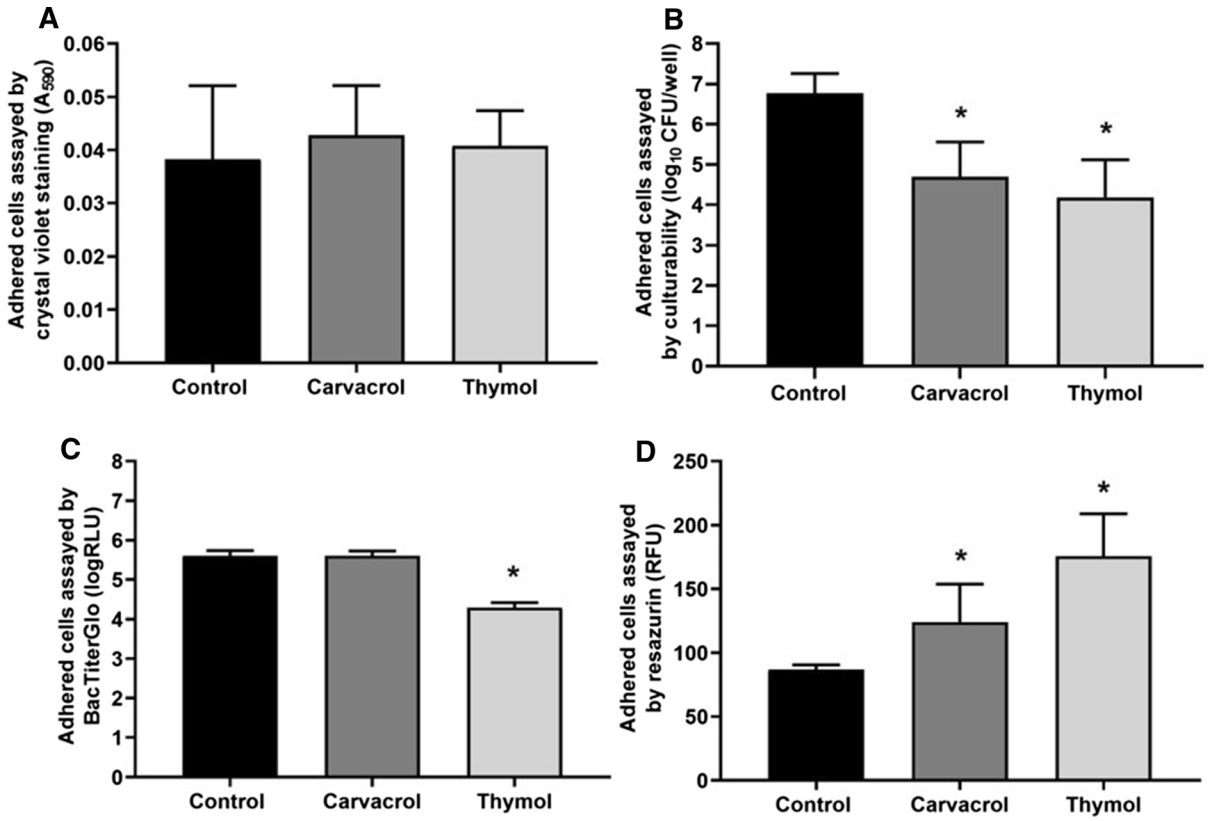

Fig. 3 Anti-adhesion activities against *C. jejuni* NCTC 11168 (on polystyrene microtiter plates) for carvacrol (0.25 × MIC) and thymol (0.25 × MIC), as compared to the untreated controls. **a** Quantification with crystal violet staining and A_{590} measures. **b** Direct quantification of cultivable cells, as $\log_{10}$ CFU/well. **c** Quantification with BacTiterGlo, as relative luminescent units (log RLU). **d** Quantification using the resazurin agent, as relative fluorescent units (RFU). *, $p < 0.05$, versus relevant control (Student's t-tests)

data obtained with the methods that used the resazurin reagent, which is reduced to the highly fluorescent resorufin in proportion to the metabolic activity of a cell population, these were not comparable with the methods using BacTiterGlo (Fig. 3c, d). Additionally, it should be noted that all of these data, even for the control, were around the limit of detection for the resazurin reagent (5 log units; see also above), which means that the resazurin quantification will be less accurate.

On the basis that it is known that *Campylobacter* spp. can attach to food contact surfaces, to represent an important hazard for safe food production, and that their clinical importance is based on their formation of biofilms, it is surprising that there is no standard method already defined to determine the numbers of adhered cells. Besides the commonly used method to quantify biofilm mass according to culturable cell numbers, there is no simple method to determine the viability of the adhered cells, which is needed for effective control of biofilm systems. These data provided here suggest that anti-adhesion research should include at least two methods to analyse adhered cells, one being the culturable counts by the plate method, and the alternative measurement being the viability or metabolic activity of the bacteria, such as used with the dyes in BacTiterGlo.

Recently, PCR-based methods have been used as a novel approach for adhesion assays and in biofilm research (Winkelströter et al. 2014; Klančnik et al. 2018b). Quantitative real-time PCR (qPCR) has been introduced to detect low levels of DNA, as a rapid, accurate, sensitive and specific method for detection and quantification of adhered *C. jejuni* cells directly from the microtiter plates. Additionally, qPCR takes into account *C. jejuni* that are VBNC, which might not

be considered using the plate counting methods. We can highlight that these studies have used the newer technology to optimise these analyses, such as digital PCR and qPCR, with the construction of standard curves for quantification. Briefly, Klančnik et al. (2018b) used a *C. jejuni* DNA mix from 14 *C. jejuni* strains for digital PCR quantification, with the standard curve created with serial dilutions using qPCR detection of specific sequences of *ccoN* (Toplak et al. 2012). The standard curve was calculated from the C_t values and the corresponding DNA copy numbers (Klančnik et al. 2018b). This is the only study that has used this novel approach of PCR-based methods for quantification of adherent *C. jejuni* in monospecies biofilms, and also as multispecies biofilms, with the anti-adhesion determinations using polystyrene and sterile polished and etched stainless-steel coupons (Klančnik et al. 2018b).

Different microscopy techniques have also been used for direct determination of numbers of cells attached to an abiotic surface or formed into a biofilm (Asakura et al. 2007; Wagle et al. 2019). The formation of biofilms can also be assessing according to EPS production, using fluorescence microscopy, where the biofilms can be stained as follows (Oh et al. 2018):

1. *SYTO 9* This dye has a high affinity for DNA, and is used to detect total DNA (both intracellular and extracellular);
2. *BOBO-3* This is an intercalative DNA-binding red fluorescent dye, and as it cannot penetrate through membranes, it is used to detect extracellular DNA;
3. *Calcofluor white* This fluorescent dye binds to β1–3 and β1–4 carbohydrate linkages of polysaccharides, and it has been used to detect extracellular polysaccharides in *C. jejuni* biofilms.

Electron microscopy is also an important method to examine bacterial adhesion patterns and biofilm formation (Chae and Schraft 2000; Hazelton and Gelderblom 2003; Golding et al. 2016). Scanning electron microscopy provides micrographs of surfaces, and has often been the method of choice to study bacterial adhesion and biofilm characteristics (Gomes and Mergulhão 2017). Wagle et al. (2019) used environmental scanning electron microscopy to validate the influence of phytochemicals on mature biofilms of *C. jejuni* formed on stainless-steel coupons and borosilicate covers. They followed the biofilm architecture and the modulation of the biofilm structure or loss of EPS after treatments with trans-cinnamaldehyde, eugenol and carvacrol.

Confocal laser scanning microscopy is useful to study the viability of adhered *C. jejuni* cells, as well as to study the architecture and viability of *C. jejuni* biofilms (Asakura et al. 2007; Wagle et al. 2019). The viability of *C. jejuni* cells in a biofilm is often determined using Live/Dead Viability kits (Molecular Probes, Eugene, OR, USA), where two fluorescent dyes used allows differentiation of live from dead cells: SYTO 9 and propidium iodide (Asakura et al. 2007; Wagle et al. 2019).

Although microscopy is an important method to examine the adhesion patterns of *C. jejuni* to abiotic surfaces and the effects of phytochemicals, standard methods for the use of scanning electron microscopy and confocal laser scanning microscopy have not yet been described. Wagle et al. (2019) showed that three plant secondary metabolites, trans-cinnamaldehyde, carvacrol and eugenol, were effective for reduction of *C. jejuni* mature biofilm formation and inactivation of mature biofilms on polystyrene and stainless-steel surfaces. They used confocal laser scanning microscopy in combination with differential staining of live and dead cells by Live/Dead Biofilm Viability Kit in which SYTO-9 and propidium iodide stains were used. They show that after 10 min of treatment, the treated samples were red indicating inactive cells and the cells in the control sample were green indicating active cells. This pointed towards strong anti-biofilm effects of these plant secondary metabolites.

Anti-adhesion and anti-biofilm compounds of natural origin

The activities of extracts of plants, mushrooms, essential oils and isolated compounds, for inhibition of adhesion and biofilm formation by *C. jejuni* on abiotic surfaces has become of increasing interest over the last few years. A summary of the data that have been reported for plant and mushroom extracts, and for extracts of waste materials from essential oil and plant food production, is presented in Table 2, along with the data for various fractions from plant extracts. Also, similar studies with pure compounds are summarised in Table 3. The structures of the most commonly identified compounds that show anti-adhesive and

Table 2 Anti-adhesive and anti-biofilm activities of extracts of plants and mushrooms against *Campylobacter jejuni* on abiotic surfaces

Plant	Preparation	Strain	Test concentration	Assay	Activity	References
Achillea millefolium L.	Herb; 96% ethanol macerate, pre-distillation (a), post-distillation (b)	*C. jejuni* NCTC11168	0.25 × MIC = 250 μg mL^{-1}	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h; CFU counts on Mueller–Hinton agar	Inhibition 57% (a), 62% (b)	Šimunović et al. (2020)
Alpinia katsumadai Hayata	Seeds, 96% ethanolic macerate	*C. jejuni* NCTC11168 as mono culture, *C. jejuni* in mixed culture (1:1:1) with *E. coli* ATCC11229 and *L. monocytogenes* 4b	50 μg mL^{-1}	Adhesion of bacteria to polystyrene plates at 37 °C for 24 h	Inhibition of adhesion to polystyrene by 10.6% (*C. jejuni* mono culture), 46.3% (mixed culture). Combination with epigallocatechin gallate (50 μg ml^{-1}): 51.5% (mono culture), 45.5% (mixed culture)	Klančnik et al. (2018a)
Armillaria borealis Marxm. & Korhonen	Fruiting bodies: cold phosphate-buffered saline; dialysis (3 kDa cut-off)	*C. jejuni* NCTC11168	1 μg mL^{-1} (a), 10 μg mL^{-1} (b)	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, biomass determination using crystal violet	Inhibition 16.9% (a), 16.6% (b)	Klančnik et al. (2017a)
Clitocybe nebularis (Batsch) P. Kumm.	Fruiting bodies: cold phosphate-buffered saline; dialysis (3 kDa cut-off)	*C. jejuni* NCTC11168	1 μg mL^{-1} (a), 10 μg mL^{-1} (b)	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, biomass determination using crystal violet	Inhibition 20.9% (a), 22.0% (b)	Klančnik et al. (2017a)
Coriandrum sativum L.	Essential oil	*C. jejuni* ATCC33560 *C. jejuni* 225421	0.5–4 × MIC = 0.25–2 μl mL^{-1} (ATCC33560); 0.5–4 μl mL^{-1} (225421)	1. Biofilm formation on polystyrene microtiter plates at 37 °C for 48 h; 2. Mature biofilms on polystyrene microtiter plates at 37 °C for 48 h	1. Inhibition of biofilm formation: ~ 35% (0.5 × MIC) to 75% (ATCC33560), 85% (22521) at 4 × MIC; 2. Degradation of mature biofilms: ~ 85% (ATCC33560), 87% (22521) at 4 × MIC	Duarte et al. (2016)
Euodia ruticarpa (A. Juss.) Benth.	Fruit; ethanol (96%) macerate (EREE), quinolone fraction (Q)	*C. jeuni* NCTC11168; wild-type and *cmeB* and *luxS* mutants of *C. jeuni* NCTC11168	256 μg mL^{-1} (EREE, Q) (11168); 16 μg mL^{-1} (EREE), 64 μg mL^{-1} (Q) (cmeB); 128 μg mL^{-1} (EREE), 256 μg mL^{-1}(Q) (luxS)	Adhesion and biofilm formation on stainless steel coupons at 42 °C for 1, 24, 48, 72 h	Highest inhibition of biofilm formation (~ 50%) by Q (48, 72 h) followed by EREE (~ 30%) after 24 h (11168); inhibition of biofilm formation also with cmeB mutant (Q, EREE; 24, 48 h).	Bezek et al. (2016)

Table 2 continued

Plant	Preparation	Strain	Test concentration	Assay	Activity	References
Juniperus communis L.	Fruit (JFE) and post-distillation waste material, 96% ethanol; essential oil (JFEO); flavonoid fractions of JFE	*C. jejuni* K49/4 as mono culture, *C. jejuni* in mixed culture (1:1) with *L. monocytogenes* ŽM58 4b	1 mg mL^{-1}	Adhesion to polystyrene microtiter plates and stainless steel coupons,mono and mixed cultures with *L. monocytogenes*. DNA copy numbers of adhered *C. jejuni* by qPCR	Inhibition of adhesion 65.0–99.7% in mono cultures and 99.3% in mixed cultures. Highest activity for JFEO. Significant antiadhesive effects under food-related conditions (JFE; JFEO).	Klančnik et al. (2018b)
	Essential oil	*C. jejuni* NCTC11168	0.25 × MIC = 62.5 μg mL^{-1}	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, CFU counts on Mueller–Hinton agar	Inhibition 99%	Šimunović et al. (2020)
Lavandula hybrida E.Rev. ex Briq.	Essential oil	*C. jejuni* NCTC11168	0.25 × MIC = 62.5 μg mL^{-1}	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, CFU counts on Mueller–Hinton agar	Inhibition 96%	Šimunović et al. (2020)
Lycoperdon pyriforme Schaeff.	Fruiting bodies: cold phosphate-buffered saline; dialysis (3 kDa cut-off)	*C. jejuni* NCTC11168	1 μg mL^{-1} (a), 10 μg mL^{-1} (b)	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, biomass determination using crystal violet	Inhibition 18.7% (a), 8.9% (b)	Klančnik et al. (2017a)
Olea europaea L.	Leaves; 70% ethanol macerate	*C. jejuni* K49/4	0.2–200 μg mL^{-1}	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, biomass determination using crystal violet	Inhibition of adhesion to polystyrene plates by up to 20% at 3.2 μg mL^{-1}	Šikić Pogačar et al. (2016)
Origanum vulgare L.	Leaves (a), flowers (b); 96% ethanol macerate	*C. jejuni* NCTC11168	0.25 × MIC = 62.5 μg mL^{-1}	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, CFU counts on Mueller–Hinton agar	Inhibition 54% (a), 36% (b)	Šimunović et al. (2020)
Pleurotus ostreatus (Jacq.) P. Kumm.	Fruiting bodies: cold phosphate-buffered saline; dialysis (3 kDa cut-off)	*C. jejuni* NCTC11168	1 μg mL^{-1} (a), 10 μg mL^{-1} (b)	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, biomass determination using crystal violet	Inhibition 27.3% (a), 26.6% (b)	Klančnik et al. (2017a)

Table 2 continued

Plant	Preparation	Strain	Test concentration	Assay	Activity	References
Salvia rosmarinus Spenn. (syn. *Rosmarinus officinalis* L.)	Essential oil	*C. jejuni* NCTC11168	0.25 × MIC = 31.25 μg mL^{-1}	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, CFU counts on Mueller–Hinton agar	Inhibition 91%	Šimunović et al. (2020)
Sparassis crispa (Wulfen) Fr.	Fruiting bodies: cold phosphate-buffered saline; dialysis (3 kDa cut-off)	*C. jejuni* NCTC11168	1 μg mL^{-1} (a), 10 μg mL^{-1} (b)	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, biomass determination using crystal violet	Inhibition 29.6% (a), 16.7% (b)	Klančnik et al. (2017a)
Syzygium aromaticum (L.) Merr. & L.M.Perry	Essential oil	*C. jejuni* NCTC11168	0.25 × MIC = 62.5 μg mL^{-1}	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, CFU counts on Mueller–Hinton agar	Inhibition 79%	Šimunović et al. (2020)
Thymus vulgaris L.	Herb (TE) and post-distillation waste material (TE-R); 45% ethanolic macerate	*C. jejuni* K49/4	0.2–200 μg mL^{-1}	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, biomass determination using crystal violet	Inhibition of adhesion to polystyrene plates by up to 35% (TE) and 40% (TE-R) at 0.2–200 μg mL^{-1} (TE, TE-R)	Šikić Pogačar et al. (2016)
	Essential oil	*C. jejuni* NCTC11168	0.25 × MIC = 15.62 μg mL^{-1}	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, CFU counts on Mueller–Hinton agar	Inhibition 56%	Šimunović et al. (2020)
Tricholoma sulphureum (Bull.) P. Kumm.	Fruiting bodies: cold phosphate-buffered saline; dialysis (3 kDa cut-off)	*C. jejuni* NCTC11168	1 μg mL^{-1} (a), 10 μg mL^{-1} (b)	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, biomass determination using crystal violet	Inhibition 0.8% (a), 23.4% (b)	Klančnik et al. (2017a)

Table 2 continued

Plant	Preparation	Strain	Test concentration	Assay	Activity	References
Vitis vinifera subsp. *vinifera* L.	'Pinot noir' grape skin and seed waste (GSS); 50% ethanol, ultrasonication	*C. jejuni* K49/4	12.5–200 µg mL^{-1}, 1.25–6.25 mg mL^{-1} (MIC–5 × MIC);	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for24 h.	Inhibition of adhesion to polystyrene up to ~ 75% (5 × MIC), comparable effect when extract pre-incubated with bacteria (~ 85%)	Klančnik et al. (2017b)
			12.5–200 µg mL^{-1}	Adhesion of bacteria to stainless steel coupons (two roughnesses)	Inhibition of adhesion to polished and brushed stainless steel coupons ~ 50–60% (no dose dependency)	Trošt et al. (2016)

anti-biofilm effects against *C. jejuni* when exposed to abiotic surfaces are presented in Fig. 4.

Extracts prepared from the fruiting bodies of 21 wild basidiomycete mushrooms and cultivated oyster mushroom (*Pleurotus ostreatus*) have shown different anti-adhesion effects against *C. jejuni* on polystyrene surfaces (Klančnik et al. 2017a). The highest activities were seen for *Sparassis crispa* and *P. ostreatus* aqueous extracts, with ~ 30% and 27% inhibition, respectively, although no dose dependency was seen for the comparison of 1 µg/mL and 10 µg/mL extract concentrations. The only extract that showed increased activity at higher concentrations and inhibition of > 20% was prepared from *Tricholoma sulphureum*. However, this type of material needs to be further investigated for anti-adhesive activities, considering also the low concentrations at which some extracts have shown remarkable potential.

Similar anti-adhesive potential at low concentrations (0.2–200 µg/mL) have been reported for extracts of the herb *Thymus vulgaris*, and for the material that remains after hydrodistillation of its essential oils (Šikić Pogačar et al. 2016). Indeed, the hydrodistillation waste material was active at 0.2 µg/mL, although as for the mushroom extracts, no clear dose dependency was seen, except for no anti-adhesive effects at the lowest concentration of the thyme herbal extract. As the hydrodistillation waste material showed an even higher activity than the crude herbal extract, the essential oil produced can be excluded as an effective ingredient in these thyme preparations. However, for carvacrol, which is a phenolic monoterpene that occurs at high proportions in thyme oils, significant inhibition of biofilm formation on polystyrene and on stainless steel coupons has been shown, along with reduction of mature biofilms (Šimunović et al. 2020; Wagle et al. 2019). Quantification of major phenolic compounds in the two thyme extracts recently reported by Šimunović et al. (2020) and Wagle et al. (2019) showed higher levels of 7-O-glucuronides of luteolin and apigenin in the waste material (4.0%, 1.0%, respectively) than in the crude herbal extract (3.7%, 0.8%), but lower levels of rosmarinic acid (5.1%, 5.6%) and 3′-O-(8′′-Z-caffeoyl) rosmarinic acid (3.0%, 3.1%). Here, rosmarinic acid showed no significant anti-adhesion effects (Šimunović et al. 2020), and therefore further investigations are needed to evaluate the role of flavone glycosides as anti-adhesives against *C. jejuni*. This is also supported by a

Table 3 Anti-adhesive and anti-biofilm activities of natural pure compounds against *Campylobacter jejuni* on abiotic surfaces

Compound	Strain	Concentration	Assay	Activity	Reference
Amentoflavone	*C. jejuni* K49/4	1 mg mL^{-1}	Adhesion to polystyrene microtiter plates. DNA copy numbers of adhered *C. jejuni* by qPCR	Inhibition of adhesion 65.0%	Klančnik et al. (2018b)
Carvacrol	*C. jejuni* NCTC 11168	0.26 mM	Biofilm formation without and with 5% chicken meat juice on: 1. Polystyrene microtiter plates; 2. Stainless steel coupons	1. Reduction by ~ 1.5 (20 °C) and 0.75 (37 °C) log CFU, compared to control (48 h); similar results for 5% chicken meat juice 2. Reduction by ~ 0.6 (20 °C) and 0.45 (37 °C) log CFU, compared to control (48 h); similar results for 5% chicken meat juice (48 h)	Wagle et al. (2019)
	C. jejuni NCTC 11168	16.64–66.56 mM	Mature biofilm without and with 5% chicken meat juice at 20 °C, 37 °C; 1–10 min exposure time on: 1. Polystyrene microtiter plates; 2. Stainless steel coupons	1. 10 min exposure time: 20 °C: 7.78 log CFU/mL (control), 4.33 to < 1 log CFU/mL; chicken meat juice: 7.81 (control), 4.80–1.12; 37 °C: 8.39 (control), 6.07–4.46; chicken meat juice: 8.63 (control), 6.46–3.75 2. 10 min exposure time: 20 °C: 6.35 log CFU/mL (control), < 1− < 1 log CFU/mL; chicken meat juice: 6.05 (control), < 1 to < 1; 37 °C: 7.88 (control), 1.95 to < 1; chicken meat juice: 7.75 (control), 2.25 to < 1	Wagle et al. (2019)
	C. jejuni NCTC11168	0.25 × MIC = 7.81 μg mL^{-1}	Adhesion of bacteria to polystyrene plates, 42 °C, 24 h, CFU count on Mueller–Hinton agar	Inhibition 44%	Šimunović et al. (2020)
trans-Cinnamaldehyde	*C. jejuni* NCTC 11168	0.75 mM	Biofilm formation without and with 5% chicken meat juice on: 1. Polystyrene microtiter plates; 2. Stainless steel coupons	1. Reduction by ~ 0.6 (20 °C) and 0.45 (37 °C) log CFU, compared to control (48 h); similar results for 5% chicken meat juice (48 h) 2. Reduction by ~ 0.5 (20 °C) and 0.45 (37 °C) log CFU, compared to control (48 h); similar results for 5% chicken meat juice (48 h)	Wagle et al. (2019)

Table 3 continued

Compound	Strain	Concentration	Assay	Activity	Reference
	C. jejuni NCTC 11168	18.91–75.64 mM	Mature biofilm without and with 5% chicken meat juice at 20 °C, 37 °C; exposure time 1–10 min on: 1. Polystyrene microtiter plates; 2. Stainless steel coupons	1. 10 min exposure time: 20 °C: 7.78 log CFU/mL (control), 4.54− < 1 log CFU/mL; chicken meat juice: 7.81 (control), 5.14–4.59; 37 °C: 8.39 (control), 7.54–5.61; chicken meat juice: 8.63 (control), 7.98–6.99 2. 10 min exposure time: 20 °C: 6.35 log CFU/mL (control), 1.85 to < 1 log CFU/mL; chicken meat juice: 6.05 (control), 3.65to < 1; 37 °C: 7.88 (control), 5.05–3.85; chicken meat juice: 7.75 (control), 5.80–4.94	Wagle et al. (2019)
Diallyl sulphide	*C. jejuni* NCTC 11168 and F38011	0.1 mg l^{-1}	Biofilms on nitrocellulose membrane; incubation of biofilms in Mueller–Hinton broth with diallyl sulphide or antibiotics (CIP, ERY) Alterations in extracellular polymeric substance proteins and polysaccharides assessed by FT-IR and Raman spectroscopy	Total inactivation of bacteria in biofilms after 5 h (both strains), control: CIP and ERY, inactivation > 24 h. Diallyl sulphide showed different and greater alterations of extracellular polymeric substance proteins and polystyrene than CIP and ERY, according to FT-IR and Raman measurements	Lu et al. (2012)
Epigallocatechin gallate	*C. jejuni* NCTC11168; *C. jejuni* NADC5653; *C. jejuni* 238ip	75% minimal bactericidal concentration: 65.25 µg mL^{-1} (11168); 93.75 µg mL^{-1} (5653); 23.25 µg mL^{-1} (238ip)	Biofilm formation in polystyrene microtiter plates at 42 °C for 72 h	Specific biofilm formation: ~ 0.03, control ~ 0.32 (11168); ~ 0.11, control ~ 0.22 (5653); ~ 0.11, control ~ 0.23 (238ip)	Castillo et al. (2015)
	C. jejuni NCTC11168 (as mono culture or mixed culture (1:1:1) with *E. coli* ATCC11229 and *L. monocytogenes* 4b	50 µg mL^{-1}	Adhesion of bacteria to polystyrene microtiter plates at 37 °C for 24 h.	Inhibition of adhesion to polystyrene microtiter plates by 22.8% (*C. jejuni mono* culture), 11.9% (mixed culture). Combination with *Alpinia katsumadai* seed extract (50 µg mL^{-1}): 51.5% (mono culture), 45.5% (mixed culture)	Klančnik et al. (2018a)

Table 3 continued

Compound	Strain	Concentration	Assay	Activity	Reference
Eugenol	*C. jejuni* NCTC 11168	0.61 mM	Biofilm formation without and with 5% chicken meat juice on: 1. Polystyrene microtiter plates; 2. Stainless steel coupons	1. Reduction by ~ 1.0 (20 °C), 0.70 log CFU (37 °C), compared to control (48 h); similar results for 5% chicken meat juice 2. Reduction by ~ 0.6 (20 °C), 0.45 log CFU (37 °C), compared to control (48 h); similar results for 5% chicken meat juice (48 h)	Wagle et al. (2019)
	C. jejuni NCTC 11168	15.22–60.90 mM	Mature biofilm without and with 5% chicken meat juice at 20 °C, 37 °C; exposure time 1–10 min on: 1. Polystyrene microtiter plates; 2. Stainless steel coupons	1. 10 min exposure time: 20 °C: 7.78 log CFU/mL (control), 4.52 to < 1 log CFU/mL; chicken meat juice: 7.81 (control), 5.00–3.93; 37 °C: 8.39 (control), 6.34–4.55; chicken meat juice: 8.63 (control), 6.75–4.52 2. 10 min exposure time: 20 °C: 6.35 log CFU/mL (control), < 1 to < 1 log CFU/mL; chicken meat juice: 6.05 (control), 1.02 to < 1; 37 °C: 7.88 (control), 4.33 to < 1; CMJ: 7.75 (control), 5.13 to < 1	Wagle et al. (2019)
L-Fucose	*C. jejuni* NCTC11168, wild-type and fucP mutant	25 mM	Biofilms in borosilicate test tubes at 37 °C for 5 days	Reduction of biofilm ~ two-fold (NCTC11168); no reduction of biofilm in fucP mutant	Dwivedi et al. (2016)
Linalool	*C. jejuni* ATCC33560, *C. jejuni* 225421	0.25–2 µl mL^{-1} (ATCC33560); 0.5–4 µl mL^{-1} (225421)	1. Biofilm formation in polystyrene microtiter plates at 37 °C for 48 h; 2. Mature biofilms in polystyrene microtiter plates at 37 °C for 48 h	1. Inhibition of biofilm formation: ~ 16% (ATCC33560), ~ 30% (22521) at 0.5 × MIC to ~ 47% (ATCC33560), % (22521) at 4 × MIC; 2. Degradation of mature biofilms: ~ 75% (both strains) at 4 × MIC	Duarte et al. (2016)
Resveratrol	*C. jejuni* K49/4	12.5–200 µg mL^{-1} or 0.313–1.565 mg mL^{-1} (MIC–5 × MIC)	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h	Inhibition of adhesion to polystyrene up to ~ 70% (5 × MIC); comparable effect when extract pre-incubated with bacteria (~ 75%)	Klančnik et al. (2017b)

Table 3 continued

Compound	Strain	Concentration	Assay	Activity	Reference
Resveratrol or resveratrol–cyclodextrin inclusion complex (IC)	*C. jejuni* 225421	Resveratrol: 0.125–2 × MIC (12.5–200 μg mL^{-1}); IC: 0.125–4 × MIC (32–1024 μg mL^{-1})	1. Biofilm formation in polystyrene microtiter plates, at 37 °C for 48 h; 2. Mature biofilms in polystyrene microtitre plates at 37 °C for 48 h	1. Inhibition of biofilm formation: ~ 90% (resveratrol) at 2 × MIC; ~ 60% (IC) at 4 × MIC 2. Degradation of mature biofilms: ~ 87% (resveratrol) at 2 × MIC; ~ 65% (IC) at 4 × MIC	Duarte et al. (2015)
Rutaecarpine	*C. jejuni* NCTC11168; wild-type and cmeB, luxS mutants	256 μg mL^{-1} (11168); 64 μg mL^{-1} (*cmeB*); 256 μg mL^{-1} (*luxS*)	Adhesion and biofilm formation on stainless steel coupons at 42 °C for 1, 24, 48, 72 h	Inhibition of biofilm formation (~ 25%) at 48 h, 72 h (11168), ~ 25% inhibition of cmeB mutant (24 h)	Bezek et al. (2016)
γ-Terpinene	*C. jejuni* NCTC11168	0.25 × MIC = 31.25 μg mL^{-1}	Adhesion of bacteria to polystyrene microtiter plates at 42 °C for 24 h, CFU count on Mueller–Hinton agar	Inhibition 91%	Šimunović et al. (2020)

study of two flavonoid-rich fractions from juniper pseudo-fruits (*Juniper communis*), which showed inhibition of *C. jejuni* attachment to polystyrene of ~ 70%. The flavonoid fractions differed in their compositions for flavone glycosides and biflavones, where the biflavonoid amentoflavone showed the lowest activity, with about 60% inhibition.

Consideration of the data summarised in Tables 2 and 3 shows that phenolic compounds other than the flavonoids might also have major roles in these anti-adhesive effects against *C. jejuni.* This is exemplified by olive (*Olea europaea*) leaf extracts, which are rich in oleuropein, a secoiridoid that is esterified with the phenylethanol derivative hydroxytyrosol. Oleuropein, the phenylethanoid verbascoside, and luteolin-7-O-glucoside were reported to be major compounds in an olive leaf extract tested by Šikić Pogačar et al. (2016), at 18.9%, 1.1% and 2.9%, respectively. This olive leaf extract inhibited adhesion of *C. jejuni* to polystyrene even at the low concentration of 3.5 μg/mL. Analysis of the different phenolic fractions in a 50% ethanolic extract of skins and seeds from the 'Pinot noir' grape (*Vitis vinfera*) that inhibited adhesion of *C. jejuni* to polystyrene and stainless steel coupons by 50% to 60% revealed (per kilogram plant material) 0.4 g total flavonoids, 12.4 g total catechins, 1.27 g total anthocyanidins and 0.04 g total hydroxycinnamic acids (Trošt et al. 2016). However, the mode of action of this extract has not been clarified yet, and non-specific binding to the bacteria might be responsible for these activities, as no dose dependency was seen. More detailed studies with distinct fractions, or with the isolated pure compounds, from the various phenolic classes found in grapes is thus still needed.

Also for grapes, the stilbene resveratrol has been tested both as the pure compound (Duarte et al. 2015; Klančnik et al. 2017b) and as an inclusion complex with hydroxypropyl-γ-cyclodextrin, which improves the water solubility of resveratrol (Duarte et al. 2015). Good inhibition was seen here for *C. jejuni* biofilm formation and for detachment of mature *C. jejuni* biofilms from polystyrene surfaces, although at concentrations greater than the MICs. Duarte et al. (2015) concluded that the anti-biofilm effects of resveratrol are associated with regulation of biofilm-related genes, including those involved in QS. On the other hand, morphological changes induced by resveratrol were reported for *C. jejuni* cells by Klančnik et al. (2017b), with the transformation of spiral cells into

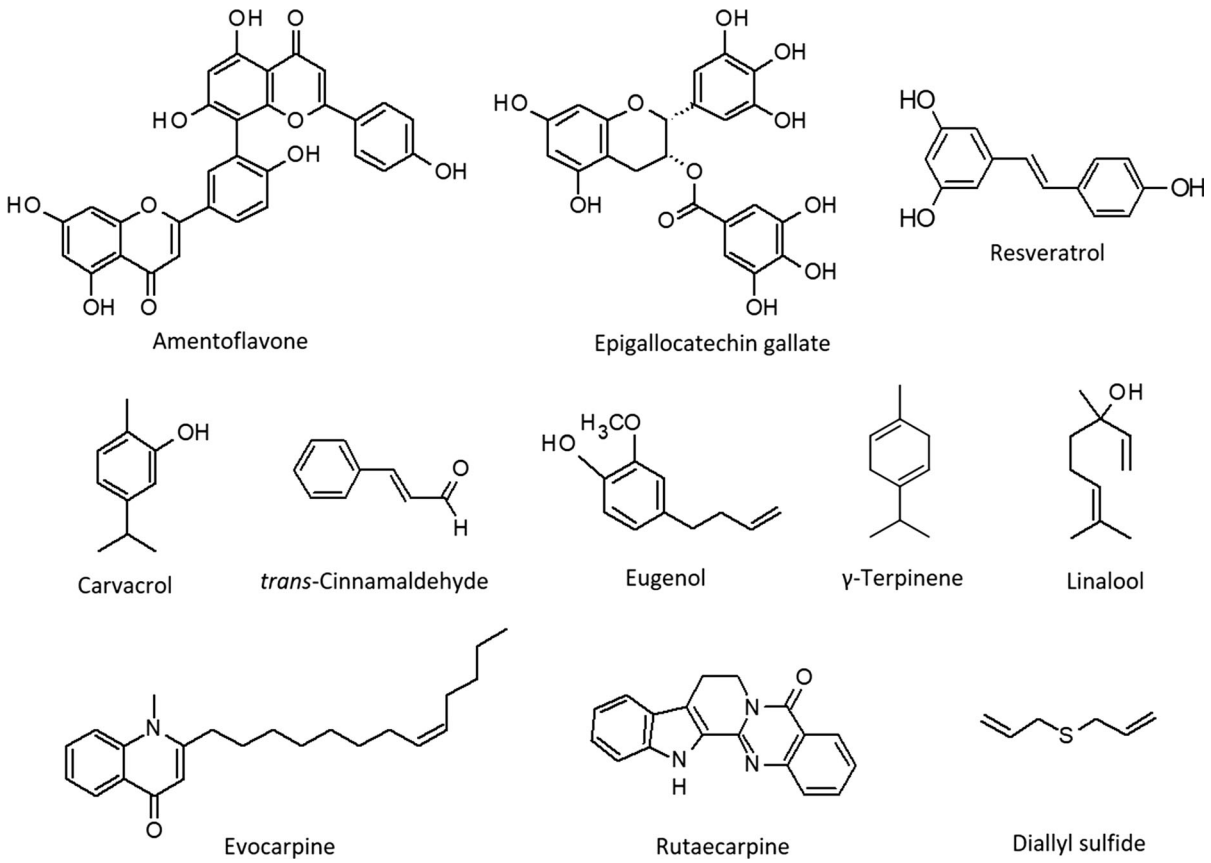

Fig. 4 Structures of compounds that show anti-adhesive or anti-biofilm activities against *C. jejuni* exposed to abiotic surfaces

non-spiral or short bacillary forms, without pits and flagella. The same effects as these seen for resveratrol were also reported for their 'Pinot noir' extract.

As an example of alkaloids with anti-biofilm activities against *C. jejuni*, interesting data have been reported for an extract of *Euodia rutaecarpa* fruit (also known as *Tetradium ruticarpum*), a quinolone-rich fraction from this extract, and its isolated rutaecarpine, an indoloquinazoline alkaloid (Bezek et al. 2016). These compounds have shown promising antimicrobial effects (Pan et al. 2014; Wang et al. 2013). The greatest inhibition of biofilm formation seen here for stainless steel coupons was obtained for a complex mixture of structurally closely related 2-alkyl-4-quinolones, which included evocarpine as its major component (42%). This study by Bezek et al. (2016) went beyond the phenomenological approach to measure reduction of *C. jejuni* biomass on abiotic surfaces, by studying also the influence of these compounds on efflux pumps. They investigated the CmeABC pump in particular, using a *cmeB* knock-out mutant, as well as QS, using the reporter strain *Vibrio harveyi* BB170 to measure autoinducer-2 (AI-2) levels. It has been shown previously that AI-2 has an important role in biofilm development of *C. jejuni* (Reeser et al. 2007). As the LuxS homologous protein is known to be responsible for AI-2 biosynthesis (Elvers and Park 2002), a *luxS* knock-out mutant was also included in these studies of the *Euodia* alkaloids. However, no significant differences were seen for biofilm inhibition between the wild-type and the *luxS* mutant, with no correlation seen between AI-2 levels in the wild-type or the *cmeB* mutant and the CFU on stainless steel coupons (Bezek et al. 2016). Interestingly, in a previous study of the antimycobacterial activities of a hexane extract of *E. rutaecarpa*, evocarpine, and the indoloquinazolines evodiamine and rutaecarpine, antagonistic actions were seen for evodiamine and rutaecarpine on the effects of evocarpine, which were most likely due to their formation

of a complex in solution (Hochfellner et al. 2015). Hence, such interactions cannot be excluded when plant extracts or multicomponent fractions are studied.

Under food-related conditions, *C. jejuni* will not be expected to just form pure biofilms, as mixed cultures of *C. jejuni* with *E. coli* and *Listeria monocytogenes* can occur. On this basis, the data from a study of an ethanolic extract of the seeds ('grains of paradise') of *Alpinia katsumadai*, a plant of the ginger family, are of interest, as this showed greater biofilm inhibition for mixed cultures. This ethanolic extract of *A. katsumadai* seeds was shown to be rich in diarylheptanoids (1,7-diphenyl-4,6-heptadien-3-one, 12.9%) and flavanones (pinocembrin, 9.5%; alpinetin, 5.7%). Combining this extract with the phytochemical epigallogatechin gallate (EGCG) also increased this effect (Klančnik et al. 2018a). EGCG itself has also shown activity at concentrations below its minimal bactericidal concentration under two different conditions (42 °C, 72 h; 37 °C, 24 h), as reported by Castillo et al. (2015) and Klančnik et al. (2018a), respectively.

As adhesion and biofilm formation of *C. jejuni* on abiotic and biotic surfaces involves highly glycosylated structures like flagellins (Sulaeman et al. 2012; Schmidt et al. 2019), it is not surprising that carbohydrates have been shown to interfere with biofilm formation on abiotic surfaces. L-Fucose at 25 mM can reduce biofilm formation on borosilicate surfaces by about 50%, compared to the control (i.e., growth media without L-fucose), whereas biofilm formation of a *fucP* mutant was similar to that of the wild-type control (Dwivedi et al. 2016). The *fucP* gene encodes the fucose permease protein, which is essential for fucose uptake from the extracellular medium (Muraoka and Zhang 2011). These data for the measurements of bacterial biomass using crystal violet staining were in agreement with the analysis by scanning electron microscopy, which showed greatly reduced biofilm formation on borosilicate glass in the samples with L-fucose (Dwivedi et al. 2016).

Overall, the most intensively investigated phytochemicals are those of the essential oils and their individual constituents. Remarkable anti-adhesive effects have been reported for juniper essential oil and an ethanolic extract, with > 90% inhibition at 1 mg/mL (Klančnik et al. 2018b). These materials showed large anti-adhesive effects on *C. jejuni* for stainless-steel coupons with different surfaces, and with *C. jejuni* as individual cultures or as biofilms of mixed cultures with *L. monocytogenes* (Klančnik et al. 2018b). Large anti-adhesive effects of juniper essential oil have also been seen against *C. jejuni* for adhesion to polystyrene plates, at 62.5 µg/mL as 0.25 × MIC (Šimunović et al. 2020), which indicates that this juniper essential oil includes potent anti-adhesives. Essential oils of lavender (*Lavandula hybrida*), rosemary (*Salvia rosmarinus*) and clove (*Syzygium aromaticum*) have also been shown to reduce *C. jejuni* adhesion to polystyrene plates by ~ 80% (clove), and even by > 90% (lavender, rosemary) at concentrations that are clearly below their respective MICs (i.e., 0.25 × MIC). The major compounds contained in clove oil (i.e., eugenol) and lavender oil (i.e., linalool) have also shown inhibitory effects on biofilm formation and on mature biofilms (Duarte et al. 2016; Wagle et al. 2019). Of note, however, although it might be tempting to use the effects of such individual constituents to directly define the activities of the essential oils, the effects of essential oils will rather be the results of the concerted actions of a number of their individual constituents.

Environmental scanning electron microscopy and confocal laser scanning microscopy have also been used to investigate the effects of *trans*-cinnamaldehyde, eugenol and carvacrol on *C. jejuni* biofilm architecture and viability on stainless steel coupons. After treatments for only 10 min, most of the EPS in the biofilms were lost, and the majority of the *C. jejuni* cells were dead. Interestingly, *trans*-cinnamaldehyde significantly down-regulated genes related to cell motility, and eugenol and carvacrol also modulated the expression of genes related to cell motility, surface modifications, stress responses and QS. Alterations were also seen at the proteome level, with up-regulation of the flagellar protein, cytochrome c553, and the putative peptidyl-prolyl *cis*-isomerase, and down-regulation of the periplasmatic nitrate reductase, chaperones and bacterioferritin (Wagle et al. 2019).

Linalool is also a prominent constituent of coriander oil, which has shown activity against mature biofilms of *C. jejuni* on stainless steel coupons, although only at concentrations well in excess of its MIC (i.e., 4 × MIC). Linalool and coriander essential oil were also studied for their effects on QS using a disc diffusion assay with the *Chromobacterium violaceum* biosensor strain, and on the QS-dependent

production of violacein. At the more relevant lower concentrations of 0.1 µL/mL, the anti-QS effects of linalool were more pronounced than those of coriander oil, with 70% and 43% inhibition, respectively (Duarte et al. 2016).

Alterations to *C. jejuni* membrane permeability have also been reported for α-pinene at concentrations well below its MIC, which is a monoterpene hydrocarbon that has been defined as a constituent of many essential oils (Kovač et al. 2015). Indeed, scanning electron micrographs of *E. coli*, *Staphylococcus aureus* and *Bacillus subtilis* treated with essential oil combinations have clearly shown deformation, permeabilisation and disruption of bacterial cell walls (Lv et al. 2011). Hence, morphological changes to the bacterial cell wall might represent a mechanism for these anti-adhesive effects. Therefore, further investigations of essential oils and their individual constituents on the membrane structures responsible for specific adhesion to abiotic surfaces are needed.

One of the most promising phytochemicals at present appears to be diallyl sulphide, which is a major constituent of processed garlic (*Allium sativum*), including black garlic (Yang et al. 2019). The antimicrobial activities of garlic have been referred to its organosulphur compounds, as shown by changes in the bacterial cell membrane components using Fourier-transform infrared and Raman spectroscopy (Lu et al. 2011). The low concentration of 0.1 µg/mL of a garlic concentrate showed total inactivation of *C. jeuni* adhesion in biofilms on nitrocellulose membranes. Decay of the EPS matrix of these *C. jejuni* biofilms was seen within 1 h of the treatment with diallyl sulphide, followed by the destruction of cell integrity, as also seen for planktonic cells (Lu et al. 2012). Hence, it appears that organosulphur compounds represent a still underexplored source of anti-biofilm compounds of natural origin, and thus these should gain more attention in future studies.

Adhesion mechanisms and identification of targets against adhesion of *C. jejuni*

Campylobacter have developed a multiplicity of adhesion mechanisms. As is known, their attachment takes place in two phases: (1) the initial physical reversible phase that is mediated by van der Waals and electrostatic interactions; and (2) the irreversible phase that is mediated by the bacterial surface structures (e.g., flagella, polysaccharides), which potentially bridge the bacterial cell and the attachment surface, to overcome the energy barrier established by the negative charges of the bacterial cell and attachment surface (Nguyen et al. 2012). A number of further cell properties are also involved, such as cell morphology, membranes, membrane proteins, aggregation and motility, and host proteins, fatty-acid metabolism, intracellular transport, chaperones, hydrophobicity, QS, chemotaxis, stress responses, and EPS matrix formation. Additional external factors are also involved, such as the characteristics and physicochemical factors of the attachment surface, and the environmental conditions (Reuter et al. 2010; Sulaeman et al. 2012; Szymanski and Gaynor 2012; Giaouris et al. 2015; Kurinčič et al. 2016).

Collectively, the properties of the bacterial cell and contact surface represent the main targets for the anti-adhesion activities of phytochemicals. The design of effective anti-adhesion strategies is at present limited by the lack of knowledge of *C. jejuni* cell physiology and their cell structures in relation to different attachment surfaces. Natural extracts and phytochemicals with anti-adhesion activities against *C. jejuni* will use different mechanisms of action against these pathogens. However, extracts and compounds with known anti-adhesion activities against *C. jejuni* have been poorly studied in terms of their mechanisms against bacterial adhesion.

Trans-cinnamaldehyde, carvacrol and eugenol, have been shown to reduce *C. jejuni* adhesion to an abiotic surface through an anti-adhesion mechanism that acts through reduced expression of genes involved in *C. jejuni* motility: *flgA, flaA, flaB* and *motA* (Wagle et al. 2019). In *C. jejuni*, flagella-mediated motility is a crucial factor for adhesion to an abiotic surface. Joshua et al. (2006) reported that aflagellate *C. jejuni* mutants that lack *maf*5 (located in the flagellin glycosylation locus) and *fliS* cannot attach to glass surfaces, which shows the importance of the flagella as a mediator of this adhesion. Furthermore, Moe et al. (2010) tested the adhesion of *C. jejuni* Δ*flaA,* an aflagellate mutant, and *motA*, a flagellate but non-motile mutant, and reported that both of these mutations impaired *C. jejuni* adherence to glass. Citrus extracts reduced *C. jejuni* motility and expression of the *flaA-B* genes (Castillo et al. 2014), while carvacrol modulates genes that encode for energy taxis

(*cetB*), motility (*motA*), binding (*cadF*) and attachment (*jlpA*) (Shrestha et al. 2019). This shows that motility itself, and not only the presence of the flagella, is important for *C. jejuni* attachment to abiotic surfaces, and their subsequent biofilm formation. Both the flagella as a structure and *C. jejuni* motility can be considered as targets for the anti-adhesion activities of phytochemicals.

The phytochemical EGCG has been shown to reduce *C. jejuni* adhesion (Klančnik et al. 2018a) and inhibit *C. jejuni* efflux pumps (Kurinčič et al. 2012), and thus efflux pump inhibition by EGCG can be considered as a mechanism of anti-adhesion. In *C. jejuni*, the efflux pumps have gained attention mainly as facilitators of antibiotic resistance, although their involvement spans other important areas, such as host colonisation, cell viability, bile resistance and oxidative stress (Lin et al. 2003; Akiba et al. 2006; Jeon et al. 2010; Kurinčič et al. 2012), and also adhesion to abiotic surfaces. For this review we used *C. jejuni* NCTC 11168 wild-type and its efflux pump mutants that lack the functional genes for expression of the efflux pump proteins CmeB, CmeR, CmeF (Klančnik et al. 2012), Cj1687 and CmeG, with the aim being able to determine the differences in the adhesion properties to stainless steel. The reference and mutant strains were prepared as previously described (Klančnik et al. 2012; Kovač et al. 2015), and *C. jejuni* adherence to stainless steel was evaluated by cell cultivability (Trošt et al. 2016; Klančnik et al. 2017a), with a 24-h incubation in Mueller–Hinton broth at 42 °C under microaerobic conditions. This was determined after washing and ultrasound treatment of the discs, and the data are presented as the numbers of *C. jejuni* cells (log CFU/mL). The data here are shown in Fig. 5 for the involvement of the efflux pumps CmeDEF, CmeGH and Cj1687 in *C. jejuni* adhesion to this abiotic surface. Interestingly, the mutants that lacked functional *cmeF, cmeG* and *cj1687* genes showed impaired adhesion, compared to the wild-type. According to these data, the efflux pumps CmeDEF, CmeGH and Cj1687 can be considered as targets for the anti-adhesion activities of these phytochemicals.

As well as the efflux pumps in the membrane, the membrane as a whole and the cell surface can be considered as targets for anti-adhesion activities. *C. jejuni* show high abundance of membrane proteins involved in adhesion and biofilm formation (Asakura et al. 2007; Sulaeman et al. 2012), which indicates the

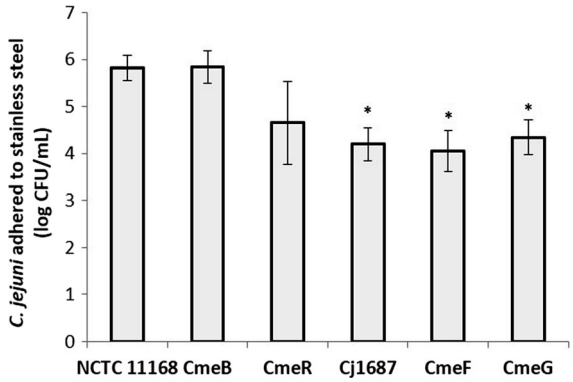

Fig. 5 *Campylobacter jejuni* adhered to stainless steel after 24 h incubation at 42 °C in a microaerobic atmosphere. The samples include wild-type *C. jejuni* NCTC 11168, and its efflux pump mutants that lack functional genes for expression of the efflux pump proteins CmeB, CmeR, Cj1687, CmeF and CmeG (as indicated). *, $p < 0.01$ (Student's t-tests)

importance of membrane proteins in *C. jejuni* adhesion to abiotic surfaces. Furthermore, Sulaeman et al. (2012) showed a link between the expression of the *cadF* gene, which encodes an outer membrane protein, and *C. jejuni* adhesion. Compounds that reduced the expression of *cadF*, such as *trans*-cinnamaldehyde, carvacrol and eugenol, also reduced *C. jejuni* adhesion to abiotic surfaces (Upadhyay et al. 2017; Wagle et al. 2019); thus, alterations to the *C. jejuni* membrane can be considered as one of their mechanisms of anti-adhesion action.

The physiological properties of the cell surface also have an important role in *C. jejuni* adhesion to abiotic surfaces, in terms of its hydrophobicity and surface charge (Nguyen et al. 2012; Joshua et al. 2006). In *C. jejuni*, a link between cell surface hydrophobicity and adhesion to stainless steel and glass has been shown, although no relationship to cell surface charge was evident (Nguyen et al. 2011). This might be explained by the production of polysaccharides by *C. jejuni*, as their presence changes the cell surface physicochemical properties and will influence the attachment of *C. jejuni* to an abiotic surface (Gilbert et al. 2008; Nguyen et al. 2012).

Considering in particular thyme extracts, in which the major constituent is carvacrol, these are known to inhibit *C. jejuni* motility, modify the *C. jejuni* cell membrane, and reduce *C. jejuni* adhesion to polystyrene (Šikić Pogačar et al. 2016). We believe that this motility reduction and the membrane

modifications define the main mechanisms of anti-adhesion of thyme extracts, although this remains to be confirmed more directly. Indeed, the spiral-shape and motility of *Campylobacter* are of great importance for their attachment, and consequently for their pathogenesis (Klančnik et al. 2013, 2014; Frirdich et al. 2019). *Campylobacter* are well known for their morphology changes during exposure to adverse environmental conditions, and especially for transition from spiral to coccoid form (Klančnik et al. 2006), which appears to be a potentially dormant form, and also to the VBNC or degenerative form (Park 2002; Klančnik et al. 2009).

To look at this further here, we used *C. jejuni* K49/4, with the aim to determine the morphology changes to the planktonic cells in the presence of sub-inhibitory concentrations of a 'Pinot noir' grape skin and seed extract, and of resveratrol. The electron micrograph in Fig. 6a shows the spiral morphology of the control, untreated, *C. jejuni* K49/4, with a flagellum at each cell pole, prepared as reported previously and observed under transmission electron microscopy (CM100; Philips Electronics, N.V. Eindhoven, The Netherlands) (Klančnik et al. 2009). When subinhibitory concentrations of the 'Pinot noir' skin and seed extract (200 µg/mL; Fig. 6b) or resveratrol (200 µg/mL, Fig. 6c; 100 µg/mL, Fig. 6d) were added, different *C. jejuni* forms were seen, mainly as short bacillary forms without flagella, and as cells in clusters, with the coccoid forms rarely seen.

Many other phytochemicals have the potential for prevention of *C. jejuni* adhesion to abiotic surfaces. For example, α-pinene (Kovač et al. 2015) is an efflux pump inhibitor and thus has the potential to influence *C. jejuni* adhesion via this mechanism. Two terpenoids, carvone and carveol, result in the separation of *Rhodococcus erythropolis* cells through their decreased hydrophobicity (de Carvalho and de Fonseca 2007), and can be considered as *C. jejuni* anti-adhesion agents. Pinostrobin acts as an inhibitor of the major facilitator superfamily type efflux pump in *S. aureus*, and is also an anti-biofilm agent (Christena et al. 2015). An essential oil from *Juniperus communis* has been shown to damage bacterial cell membranes (Meng et al. 2016), and it also shows significant anti-adhesion activity against *C. jejuni* (Klančnik et al. 2018b). Indeed, this essential oil might inhibit *C. jejuni* adhesion via its inhibition of CmeGH, a *C. jejuni* major facilitator superfamily efflux pump (Jeon et al. 2010). Pinocembrin modulates the metabolic activity of *C. jejuni*. At sub-inhibitory concentrations, pinocembrin did not significantly alter membrane functionality and it increased bacterial fitness. Treatment with pinocembrin evoked decreased expression of ribosomal proteins and down-regulation of several NADH dehydrogenase I chain subunits and proteins involved in iron uptake. This suggests altered protein production and redox cycle and iron metabolism. Interestingly, the chelation of Fe ions during the treatment with pinocembrin increased *C. jejuni* survival, although there was no increase in the formation of reactive oxygen species (Klančnik et al. 2019). Bezek et al. (2016) described an ethanol extract from *E. rutaecarpa*, and its fractions, that reduced QS of *C. jejuni* and also showed anti-adhesion and anti-biofilm effects against *C. jejuni* on a stainless steel surface. The anti-QS effect of this extract was seen at 0.25 × MIC, and was shown through detection of AI-2 activity in the bioluminescence assay using *V. harveyi* (Bassler et al. 1997). Extracts of the fruit peel

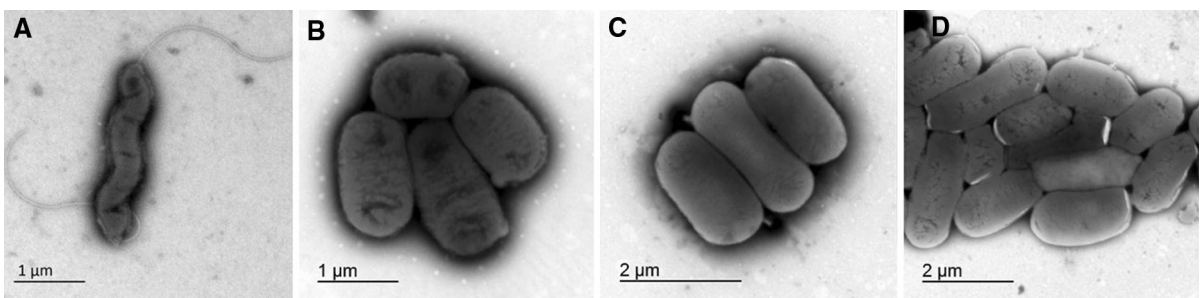

Fig. 6 Representative transmission electron micrographs showing *C. jejuni* K49/4 as the untreated control (**a**) and following treatments with 'Pinot noir' grape skin and seed extract at 200 µg/mL (**b**), and resveratrol at 200 µg/mL (**c**) and 100 µg/mL (**d**). Scale bars: as indicated

from *Citrus limon, Citrus medica* and *Citrus aurantium* were also shown to reduce QS of *C. jejuni*, along with their motility and their expression of the *flaA/B* genes, which are important for the initial adhesion and motility of *C. jejuni* (Castillo et al. 2014). The *C. jejuni* knock-out mutant for the *luxS* gene, which modulates the QS mechanism, showed reduced motility, autoagglutination, adhesion and biofilm formation on a polystyrene surface (Elvers and Park 2002; Jeon et al. 2003; Reeser et al. 2007; Šimunović et al. 2020), which indicated a role for QS in the regulation of these mechanisms (Plummer 2012). Šimunović et al. (2020) have shown a correlation of anti-QS activity of phytochemicals and their anti-adhesion activity against *C. jejuni*.

As can be concluded from the details here, only a few mechanisms are known to be involved in the successful anti-adhesion activities of the phytochemicals against *C. jejuni*. The most important appears to be motility reduction, although efflux pump inhibition, membrane integrity disruption, and QS reduction are also important and might contribute to the mechanisms of action here. Therefore, in future studies, investigations into these anti-adhesion effects of phytochemicals should also include investigation of the potential mechanisms shown here to be involved in these effects.

Conclusions

Infections by *C. jejuni* and its consequences are a major burden on healthcare and food industries. As no known measures to combat this pathogen are effective, the search for alternative strategies is encouraged. We present the reduction or prevention of *Campylobacter* spp. adhesion to abiotic surfaces by phytochemicals as an effective measure for *Campylobacte*r spp. control. Many natural extracts and pure phytochemicals have been well characterized, with known antibacterial and anti-adhesion activities and known mechanisms of action. This type of compounds with known effects on *C. jejuni* motility, efflux pump inhibition, membrane disruption and QS inhibition can be considered and tested as anti-adhesion agents, as these factors influence *C. jejuni* adhesion greatly. An example of effective anti-adhesion agents are thyme extracts as well as the pure compounds carvacrol and thymol. These should be considered for further in vivo testing for effective *C. jejuni* control. Another source of effective anti-adhesion phytochemicals are waste by-products of the agro-food industry. These by-products are a burden for the environment but could be utilized as effective *C. jejuni* adhesion control agents.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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