Preprečevanje adhezije *Campylobacter jejuni* K49/4 na celično kulturo prašičjih črevesnih celic PSI cl1 z uporabo različnih rastlinskih ekstraktov

Prevention of *Campylobacter jejuni* K49/4 adhesion to porcine small intestine cell line PSI cl1 using different plant extracts

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Izvleček

Namen: *Da bi preverili inhibicijo adhezije C. jejuni, smo v poskusih in vitro testirali različne rastlinske ekstrakte na celičnem monosloju celic PSI cl1, in sicer ekstrakt tropin sorte Modri pinot (GSS), ekstrat oljčnih listov (OE) in timijana (TE) ter njegov preostanek po hidrodestilaciji eteričnega olja (TE–R) ter ekstrakt iz semen rastline Alpinia katsumadai (SEE) in njegov preostanek po hidrodestilaciji eteričnega olja (hdSEE–R). Želeli smo preveriti uporabnost odpadnega materiala in stranskih proizvodov agro–živilstva pri preprečevanju adhezije C. jejuni na celično linijo PSI cl1.*

Metode: *Pred poskusom protiadhezijske učinkovitosti ekstraktov smo preverili njihovo protimikrobno delovanje ter citotoksičnost na celični liniji PSI cl1 in s tem določili koncentracijsko območje za testiranje protiadhezijske aktivnosti uporabljenih ekstraktov.*

Abstract

Purpose: *The aim of this study was to investigate the in vitro anti–adhesive properties of chemically characterized ethanolic extracts from waste Pinot noir grape (GSS) skins and seeds, olive tree leaves (OE), thyme (Thymus vulgaris) prior to (TE) and its residue after (TE–R) hydrodistillation of the essential oil, as well as Alpinia katsumadai ethanolic seed extracts (SEE) and its hydrodistillation residue (hdSEE–R) against the pig small intestine epithelial cell line, PSI cl1.*

Methods: *Using PSI cl1, the anti–adhesion activities of these extracts, which normally represent "waste material" and by–products from the agro–food industry, were determined. Initially, the anti–Campylobacter jejuni and cytotoxic activities of GSS, TE, TE–R, SEE and hdSEE–R were determined to avoid any interference in the anti–adhesion assay being used.*

Rezultati: *Najučinkovitejša ekstrakta pri preprečevanju adhezije C. jejuni na celice PSI sta bila SEE in hdSEE–R, sledila sta TE in TE–R, ki sta do 30 % zmanjšala adhezijo bakterije na celično linijo. Najslabšo protiadhezijsko učinkovitost je imel ekstrakt GSS.*

Zaključek: *Odpadni materiali (TE–R in hdSEE–R) ter OE, ekstrakt iz stranskega proizvoda agro–živilske industrije, so se pokazali kot zelo uspešni pri preprečevanju adhezije C. jejuni tudi pri zelo nizkih koncentracijah. Obetavni rezultati študije kažejo možnosti uporabe ekstraktov iz odpadnih rastlinskih materialov na različnih področjih, vključno z industrijo in skrbjo za zdravje ljudi in živali.*

Results: *The A. katsumadai extracts showed the strongest anti–adhesive activities against C. jejuni K49/4. When using TE and TE–R, C. jejuni adhesion to PSI cl1 cells was inhibited by almost 30% over a large concentration range of extracts. GSS extracts had the lowest impact on the adhesion rate of C. jejuni to PSI cl1 cells.*

Conclusion: *Our findings suggest that agro–food waste material and many by–products from the agro–food industry represent sources of bioactive phytochemicals that are effective at low concentrations and could be used as therapeutic agents to prevent bacterial adhesion.*

This represents a step towards the application of new innovative strategies to control Campylobacter contamination and infection in the food chain. We suggest that not only plant extracts, but also waste material and agro–food industry by–products can be used as promising novel therapeutic agents with possible medical and industrial applications.

INTRODUCTION

Campylobacter jejuni is one of the most common causes of bacterial–associated diarrhea in the industrialized world, and is associated with Guillain–Barré syndrome (1). Risk assessment studies concluded that the highest risk of *Campylobacter* spp. infection resulted from the handling of raw or undercooked poultry meats and resultant cross–contamination (2). An alarming increase in the prevalence of antibiotic–resistant bacterial strains has been seen, primarily due to excessive and often unnecessary use of antibiotics in humans and animals. This has been observed in *Campylobacter* spp., which are highly sensitive to environmental stress but have evolved mechanisms for survival both inside and outside of a host (3,4). In the food industry, the problem arises in the same way. Modern commercial food production facilitates the emergence and spread of bacterial resistance through the intensive use of antimicrobial agents for cleaning and through international trade of raw materials and food products. Attachment of *C. jejuni* in the gut is an essential step in the infection of the human host, and represents an important virulence mechanism for *C. jejuni* pathogenesis and transmission (5). Thus, the targeting of bacterial attachment through a mechanism that is not related to bacterial growth inhibition might be of paramount importance in the control of *Campylobacter* contamination.

Herbs are a source of a large variety of active compounds that have the potential to inhibit *Campylobacter* adhesion to intestinal mucosa, prevent colonization in poultry, and reduce transmission to humans (5). Anti–adhesive agents like those produced by herbs have a major advantage in combating infections without the selection pressure that results in the emergence of resistant bacteria, while also not causing deleterious effects to the host microbiota (6). Furthermore, agro–food and pharmaceutical industry by–products and waste materials present a high economic and environmental burden. Grape skins and seeds, and olive leaves are such by–products of the wine and olive oil industries and could present a reasonable source of bioactive phytochemicals. *Alpinia katsumadai* is used in Chinese medicine as an anti–emetic to increase appetite, and in animal feed to facilitate rapid growth of domestic animals, which contributes to the requirements for more nat-

ural and less chemically treated products for animal breeding (7, 8).

The aim of our study was to investigate the *in vitro* anti–adhesive properties of chemically characterized ethanolic extracts from grape skins and seeds (*Vitis vinifera*, GSS), thyme (*Thymus vulgaris*) prior to (TE) and after hydrodistillation of the essential oil (TER)—since this material is usually unused waste after production of thyme essential oil — as well as from leaves of the olive tree (*Olea europea*, OE) and *A. katsumadai* seed ethanol extract (SEE) and its leftovers after hydrodistillation of the essential oil (hdSEE–R). These extracts were studied as potential anti–*Campylobacter* actives in terms of growth inhibition and anti–adhesion activity against *C. jejuni* in pig epithelial cells (PSI cl1).

MATERIALS AND METHODS

Preparation of extracts

Pinot noir waste skins and seeds were collected from a commercial winery and freeze dried at –45 °C and 20 Pa (Lio 5P; Kambič, Slovenia). Prior to the extraction, the samples were powdered with liquid nitrogen in a mortar, and mixed with the extraction solvents at a concentration of 50 mg mL^{-1}. The extractions were performed for 12 h at 4 °C after 40 min of ultrasonication in a water bath (Sonorex; Bandelin, Germany). Sonication was then repeated for an additional 40 min the following day. The ethanolic extracts of thyme and olive leaves were prepared from 21 g whole aerial parts of thyme (*T. vulgaris L.*) (Slovenia) and 10 g olive tree leaves (*O. europaea* L.) (Slovenia). In the case of thyme (TE), the plant material was extracted with 45% ethanol for 24 h according to the Pharmacopoeia protocol (9). Post–distillation waste extract (TER) was prepared after hydrodistillation of the essential oil in a Clevenger type apparatus. Dry plant material was hydrodistilled, then dried and extracted with 45% ethanol for 24 h. Air–dried olive leaves were milled and mixed with 70% ethanol, and the mixture was extracted overnight on an orbital shaker, at 25 °C. The olive leaf extract was subsequently extracted with hexane to remove the leaf waxes. The macerated samples were then filtered and the solvent was evaporated at 40 °C under reduced pressure. Any remaining solvent was removed with N_2. The *A. katsumadai* seed ethanol extract, SEE, was prepared from 400 g dried seeds (Plantasia, Oberndorf, Austria), with 1500 mL 96% ethanol. The extraction was carried out at room temperature for 24 h, after which, the solvent was evaporated off under reduced pressure at 40 °C. SEE was then hydrodistilled for 2 h using a Clevenger–type apparatus. The residue from SEE hydrodistillation, hdSEE–R, was freeze–dried.

Bacterial strain and growth conditions

The poultry meat isolate *C. jejuni* K49/4 was grown overnight on Columbia agar (Oxoid, Hampshire, UK) at 42 °C microaerobically (5% O_2, 10% CO_2, 85% N_2). The cultivation media used were Mueller Hinton broth and agar (MHB, MHA; Oxoid) supplemented with 5% defibrinated horse blood (Oxoid). To produce exponential–phase *C. jejuni* cells, cultures were grown microaerobically at 42 °C for 9 h in MHB. The bacterial number was determined with spectrophotometric analysis using measurements in the absorbance mode at a wavelength of 600 nm. For the antimicrobial activity assays, *C. jejuni* cultures were diluted in MHB to 2×10^5 CFU mL^{-1}, and to 10^8 CFU mL^{-1} for infection of the cell line in cell culture media.

PSI CELLS

The normal PSI epithelium–derived cell line (PSI cl1) was obtained from an adult pig, as previously described (10). Cells were classified as cryptic, incompletely differentiated enterocytes and represent a model close to humans in terms of genome, organ development, anatomy, physiology, metabolism, and intestine–microbe interactions (11–13). The cell lines are not of tumor origin and are therefore a better *in vitro* model for studying the pathogen–host interactions than human tumorigenic cell lines such as Caco–2. PSI cl1 was grown to confluence in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, St. Louis, MO, USA), supplemented with 5% fetal calf serum (BioWhittaker Europe, Essen, Germany), 2 mM L–glutamine, 100 U mL^{-1} penicillin, and 1 mg mL^{-1} streptomycin, in tissue–culture flasks at 37 °C in a 5 % humidified CO_2 atmosphere. For all experimental

assays, 96–well microplates were seeded with approximately 5.0 × 10^5 PSI cells mL^{-1}, and incubated for 24 h, to form a confluent monolayer.

Antimicrobial susceptibility testing

Stock solutions of each extract (GSS, OE, TE, TE–R, SEE, hdSEE–R) were prepared in dimethyl sulfoxide (DMSO; Sigma–Aldrich). A suspension of *C. jejuni* (2×10^5 CFU mL) in MHB was prepared in 96–well microplates to which different concentrations (from 0.02 mg mL^{-1} to 10 mg mL^{-1}) of extracts were added to assess their antimicrobial activity. The microdilution method was used to measure the minimal inhibitory concentrations (MICs), with the addition of the BacTiter–Glo reagent (Promega, Madison, WI, USA) and macrodilution methods were used for kinetics of inhibition as previously described (14). In addition, control wells were prepared with MHB culture medium as well as MHB medium and DMSO solvent without the addition of extract. A suspension of only *C. jejuni* in MHB served as the growth control. The antimicrobial effectiveness was determined as a percentage of growth inhibition after 24 h, and was calculated as follows: I = [(C – T)/C] ×100, where C is the cell concentration under the control treatment, and T is the cell concentrations under the treatment with the extracts. The plating medium was Campylosel blood (Biomerieux, Marcy l'Etoile, France). All measurements were carried out in triplicate.

Evaluation of cytotoxicity

The cytotoxic effects of GSS, OE, TE, TE–R, SEE and hdSEE–R were determined using the 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide (MTT) colorimetric assay, as described by Mossmann (15). The wells with cells and cell culture medium served as negative controls, and wells with DMSO in cell culture medium (0.1 mg mL^{-1} final concentration) was used as a positive control. A control with DMSO at the concentration corresponding to the highest quantity present (0.025 mg mL^{-1}) was used to demonstrate that DMSO is not toxic to these cells at the concentrations used in the assays. The extent of MTT reduction was determined spectrophotometrically at 570 nm using a Multiscan reader (Thermo Fisher Scientific, Waltham, MA, USA). The experiments were carried out in triplicate. The cytotoxicity was expressed as the concentration of extract that inhibited cell growth by 50% (IC50). The average absorbance of the control wells was assumed to be 100%, and the IC50 values were calculated for each test extract.

Anti-adhesion, invasion and intracellular survival of *C. jejuni*

For the infection of PSI cells, *C. jejuni* were diluted in cell culture media to 10^8 CFU mL^{-1}. Stock solutions of extracts were prepared in DMSO, further diluted in cell culture medium, and added directly to the cell monolayers together with *C. jejuni* from the exponential growth phase. The cells were incubated at 37 °C in 5% CO_2 for 2 h to allow adhesion and invasion. The control wells were prepared by adding only bacterial inoculum to cell cultures, without extracts. A control with bacterial inoculum and DMSO in amounts corresponding to the highest quantity present (0.025 mg mL^{-1}) was also used to demonstrate that there was no inhibition by DMSO at the concentrations used in the assays. After washing twice with 200 µL DMEM without antibiotic, DMEM containing 100 µg mL^{-1} gentamicin was added to determine the number of invaded *C. jejuni*. After a 1 h incubation, the monolayers were lysed with 300 µL 1 mL L–1 (v/v) Triton–X100. The numbers of intracellular bacteria were determined through culturability assays at 3, 5, 10 and 24 h post–infection. The total number of adherent and internalized bacteria were determined simultaneously by performing the invasion assay but without gentamicin treatment (15). The difference between the numbers of total and intracellular bacteria was calculated to obtain the number of adherent *C. jejuni* cells. All experiments were repeated three times and the data presented as mean ± standard deviation of the adherent and internalized *C. jejuni*.

Statistical analysis

The data were analyzed with the Predictive Analytics Software statistics 202 software, version 18.0 (IBM Corp., Armonk, NY, USA). One–way ANOVA followed by Dunnett's multiple comparison tests was per-

formed to compare the C. *jejuni* counts between the control and all the test extracts in the anti–adhesion assays. The results were considered significant at P≤0.001.

RESULTS

Antimicrobial susceptibility testing

The MICs of plant extracts against C. *jejuni* were determined with the microdilution method. These defined the antimicrobial activities for GSS, TE and OE at 1.25 mg mL^{-1}. In contrast, TE–R had greater antimicrobial activity, with a MIC of 0.625 mg mL^{-1} (Table 1), which increased as a consequence of hydrodistillation of TE. When comparing the antimicrobial activities of GSS, TE, TE–R and OE against C. *jejuni*, expressed as percentages of C. *jejuni* growth inhibition after 24 h (Fig. 1), the extracts caused less than 20% growth inhibition at these previously determined MICs (as determined from the kinetics of the inactivation curves; data not shown). Thus, for the evaluation of minimal inhibitory effects, C. *jejuni* survival must remain < 80%. However, this did indicate more efficient inhibition of C. *jejuni* growth with TE–R, compared to TE and OE. SEE had the highest antimicrobial activity, which did not change significantly during the process of hydrodistillaton, as seen by MICs of 0.25 mg mL^{-1} for SEE and 0.5 mg mL^{-1} for hdSEE–R. For both SEE and hdSEE–R, at the concentration of 0.625 mg mL^{-1} or higher, there was strong inhibition of C. *jejuni* growth by over 50%, compared to control.

Cytotoxic activity of extracts on PSI cells

The cytotoxic activities of extracts were expressed as percentages of inhibition of cell viability for the PSI cells. When the concentration of GSS extract was <2.5 mg mL^{-1}, there were no cytotoxic effects on the PSI cells, and lower GSS extract concentration of 1.25 mg mL^{-1} showed a cytotoxicity of only about 5%. At 5 mg mL^{-1}, GSS extract showed 30% cytotoxicity for the PSI cells. Concentrations of TE, TE–R and OE lower than 1.25 mg mL^{-1} did not have any detectable cytotoxic effect on the PSI cells. When the concentration of TE–R, TE and OE was doubled to 2.5 mg mL^{-1}, 20% cytotoxicity was observed for the PSI cells with TE–R, and 10% with TE and OE. Concentrations of 5 mg mL^{-1} showed that only TE–R exceeded its IC50 (i.e., >50 % cell mortality), thus confirming that the TE–R was more toxic for the PSI cells at high concentrations (Fig. 2). With SEE and hdSEE–R at <0.625 mg mL^{-1}, no cytotoxic effects were observed, and both showed IC50 values of ~5 mg mL^{-1} and 2.5 mg mL^{-1}, respectively. However, there was no cytotoxic influence in the following anti–adhesion tests, as these were performed using much lower concentrations of plant extracts.

Anti-adhesion activity on cell cultures

GSS extracts reduced C. *jejuni* adhesion to PSI cells by 10% at 50 µg mL^{-1} and 100 µg mL^{-1}, although this did not reach statistical significance. Interestingly, the highest concentration of GSS extract used (200 µg mL^{-1}) showed only a 5% reduction in C. *jejuni* adhe-

Table 1. *Characteristics of extracts (Minimal inhibitory concentration (MIC), cytotoxic influence and anti-adhesion activity)*

	GSS	OE	TE	TE-R	SEE	hdSEE-R
MIC (mg mL-1)	1.25	1.25	1.25	0.625	0.25	0.5
Cytotoxicity (mg mL-1)	> 1.25	> 2.5	> 2.5	> 2.5	> 2.5	> 2.5
Anti-adhesion activity* (µg mL-1)	≥ 50	≥ 0.78	≥ 0.78	≥ 0.78	≥ 0.78	≥ 0.78

The concentration of particular extract that significantly inhibited adhesion of C. *jejuni* to PSI cl1 cells.

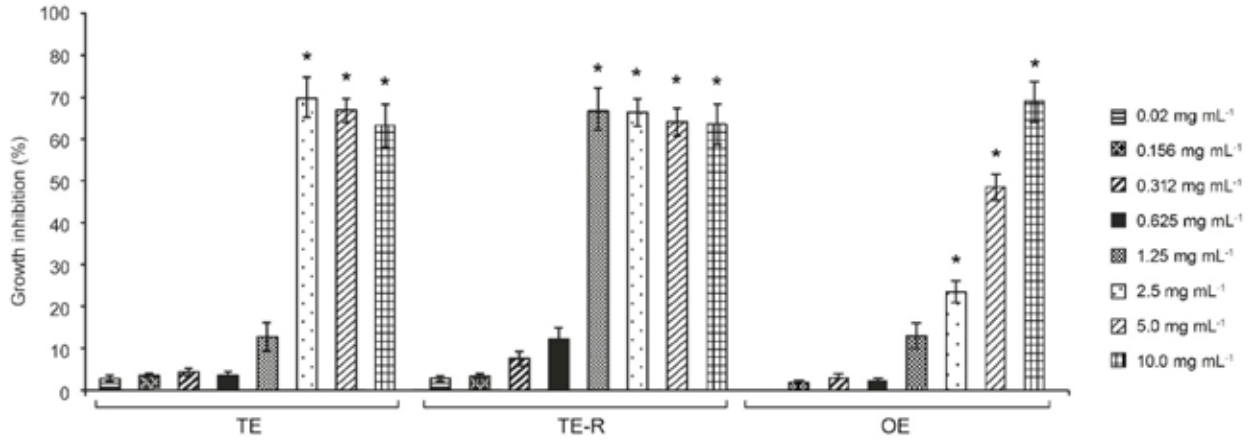

Figure1. *Inhibitory effect of extracts from thyme prior to (TE), its residue (TE-R) after hydrodistillation of the essential oil and olive tree leaves (OE) at different concentrations on C. jejuni K49/4 following 24 h of incubation.*

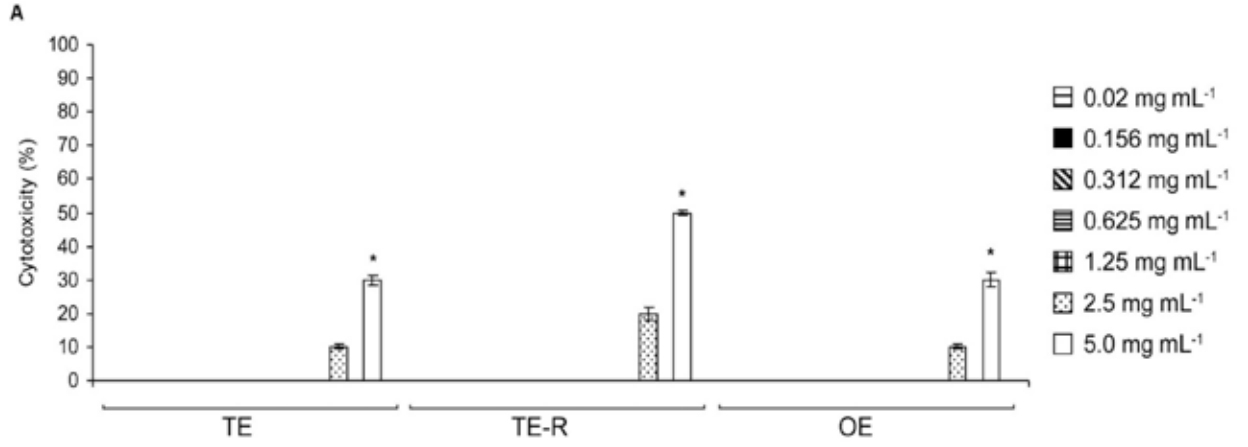

Figure2. *Cytotoxic activity of different concentrations of extracts from thyme prior to (TE), its residue (TE-R) after hydrodistillation of the essential oil and olive tree leaves (OE) on pig small-intestine (PSI cl1) epithelial cell lines. Data are means ± standard deviation. *, $P \leq 0.001$, versus control.*

sion to the PSI cells (Fig. 3). The adhesion activity of C. *jejuni* towards the PSI cells was significantly reduced by the TE, TE–R and OE over a large concentration range of these extracts (0.78 µg mL^{-1} to 200 µg mL^{-1}). They resulted in up to 30% reduction in C. *jejuni* adhesion, compared to the untreated control. Anti–adhesion activity of SEE and hdSEE–R on C. *jejuni* was observed over a large concentration range of these extracts, from 0.2 to 50 µg mL^{-1} (Fig. 3). When compared to control, a statistically significant 20% reduction in the C. *jejuni* adhesiveness was confirmed; however, no dose–dependency could be observed, except for the lowest dose (0.2 µg mL^{-1}), which showed no significant reduction of C. *jejuni* adhesion. For both SEE and hdSEE–R, 12.5 µg mL^{-1} showed the strongest anti–adhesion activity against C. *jejuni*, with inhibition of over 20 %.

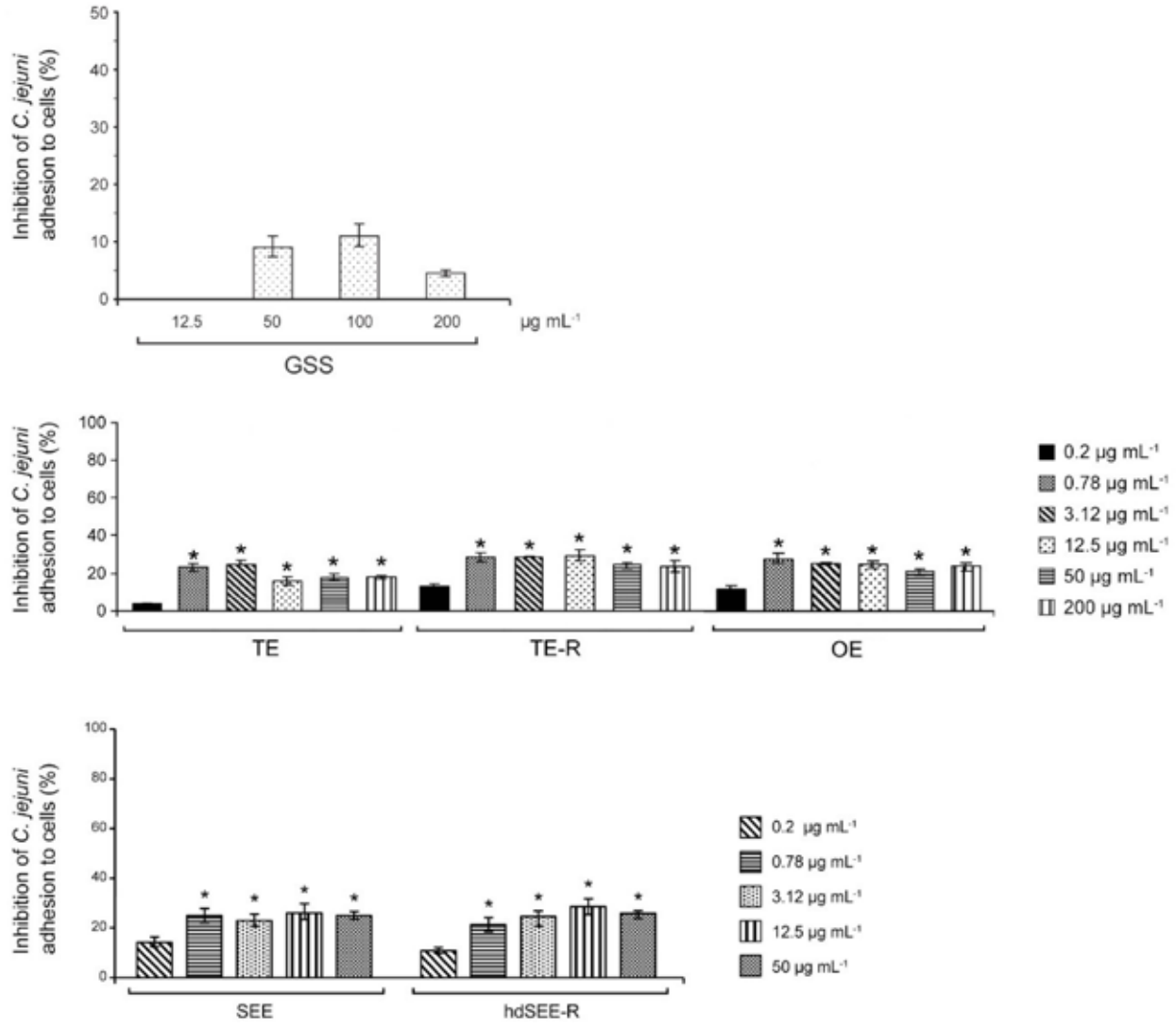

Figure 3. *Anti-adhesion of different concentrations of extracts from waste Pinot noir grape (GSS) skins and seeds, thyme (Thymus vulgaris) prior to (TE) and its residue (TE-R) after hydrodistillation of the essential oil, as well as Alpinia katsumadai ethanolic seed extract (SEE) and its hydrodistillation residue (hdSEE-R) on C. jejuni food isolate K49/4 in pig small-intestine (PSI cl1) epithelial cells. Data are means ± standard deviation. *, P ≤0.001, versus control.*

DISCUSSION

Given the public health significance of *Campylobacter* infections, it is important to understand how this bacterium survives in the environment and enters the food chain (17). We focused our study on the potential uses of agro–food by–products and waste as anti–adhesive compounds. We tested Pinot noir grapes, (GSS); olive leaves (OE); thyme (TE) and its waste following hydrodistillation (TE–R), *A. katsumadai* seed extracts (SEE) and the waste material left after hydrodistillation (hdSEE–R). Collecting the waste that was left after the preparation of essential oils from TE and SEE, which contain potential bioactive phytochemicals, resulted in maximum anti–adhesive benefit, as essential oil production remains high cost, uses large amounts of water, and leaves solid residue (18).

In the present study, we showed that the GSS extract

had moderate anti–*Campylobacter* activity, which was comparable to those of more well–known sources of plant phenolic compounds, like wine (19) and grape skin, as tested against different foodborne pathogens (20). TE showed moderate anti–*Campylobacter* activity, which was comparable to several herb extracts (21). TE–R had a 2–fold lower MIC than the TE, and a higher impact on *C. jejuni* growth, which was not reflected in different quantities of the major compounds. Also, in the case of the OE, we confirmed that antimicrobial activities were not reduced in agricultural residual material providing a new use for what is normally regarded as a waste product. MICs of SEE and hdSEE–R confirmed the pronounced to moderate activities that these extracts have against Gram–negative *Campylobacter* spp. (22). Furthermore, no cytotoxic activities were observed at the concentrations defined for the MIC of GSS extract. The cytotoxic assays testing TE, TE–R and OE did not reveal any appreciable cytotoxic activities against PSI cells at concentrations that inhibit the *Campylobacter* growth by > 10%. SEE and hdSEE–R also did not show any appreciable cytotoxic activities against PSI cells at the MICs for *C. jejuni*.

Gastrointestinal tract infections are generally initiated by attachment of pathogenic bacterial cells to the human intestinal mucus. The attachment step is an important therapeutic target and its disruption can eliminate infection at the early stages. Adherence involves interactions between complementary molecules on the surfaces of the bacteria and the host epithelium. The overall specificity of a bacterium for a particular host is therefore contingent on the presence of definitive oligosaccharide receptors (23). Anti–adhesion or anti–biofilm formation activities have been shown 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 (24–26). In our study, the reduction of adhesion to cell cultures by more than 20% is significant since *in vivo* adhesion could be even lower due to host cell defenses, such as the mucus layer that forms a barrier and can limit access of microbes to the epithelial cell surface, and peristalsis, which mechanically removes unattached bacteria (27). We showed for the first time that the OE, TE, TE–R, SEE and hdSEE–R are effective for inhibition of *C. jejuni* adhesion, and thus, for biofilm formation, although they did not inhibit *C. jejuni* growth or kill *C. jejuni* cells at concentrations tested for their anti–adhesion activity. Also, the concentrations of the extracts that had anti–adhesion activity did not measurably alter *C. jejuni* growth. The tested extracts can thus be considered as potential new anti–virulence compounds that inhibit bacterial adhesion rather than bacterial growth. Our results demonstrated reduced *C. jejuni* attachment with TE and SEE and interestingly, also with their waste material left after the hydrodistillation of essential oil (22, 27). This indicates that the volatiles present in the extract itself, but not in the waste material, do not significantly contribute to the observed anti–adhesion effects. The SEE and hdSEE–R extracts showed strong anti–adhesion activities against *C. jejuni* on PSI cells even at very low concentrations where antimicrobial and cytotoxic effects can be excluded as having any influence on the anti–adhesion activities of extracts. These anti–adhesion activities were stable across a large concentration range, which is an important characteristic that makes their use possible in different applications. By inhibiting adhesion of *Campylobacter* to eukaryotic cells, we can potentially also modulate *Campylobacter* pathogenicity.

CONCLUSION

Campylobacter spp. are extremely susceptible to a wide variety of food–processing methods and environmental stresses. However, at the same time, they are increasingly resistant to constantly changing environments and to antimicrobials, compromising the effectiveness of current methods for controlling *Campylobacter* in the food industry. In this study, we have presented an alternative strategy for the safe control of *Campylobacter* contamination by targeting their adhesion properties. We suggest that bioactive plant extracts and their waste material, and agricultural by–products can be used as promising novel therapeutic

agents, with possible medical and industrial applications. GSS, TE and TE–R, along with the OE, SEE and hdSEE–R can prevent non–specific and specific cell adhesion of *Campylobacter* to biotic surfaces, and by extension, also inhibit bacterial colonization on epithelial cells. Presumably through inhibition of essential bacterial enzymes, blockage of receptors and bacterial adhesins, these extracts have a potential for prevention and treatment of *Campylobacter* infections. To our knowledge, this is the first study demonstrating the anti–adhesion potential of materials produced as a by–product of essential oil production. It indicates the potential use of such bioactive waste as a new antimicrobial and anti–adhesion agent.

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