UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Dina RAMIĆ

OBVLADOVANJE BIOFILMA BAKTERIJ *Campylobacter jejuni* Z ZAVIRANJEM MEDCELIČNE KOMUNIKACIJE

DOKTORSKA DISERTACIJA

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CONTROL OF *Campylobacter jejuni* BIOFILM BY INHIBITION OF CELL-TO-CELL COMMUNICATION

DOCTORAL DISSERTATION

Ljubljana, 2022

Posvećeno mom Taji…

"The greatest glory in living lies not in never falling, but in rising every time we fall."
- Nelson Mandela -

Na podlagi Statuta Univerze v Ljubljani in po sklepu senata Biotehniške fakultete ter sklepu Komisije za doktorski študij Bioznanosti Univerze v Ljubljani z dne 18. 5. 2021 je bilo potrjeno, da kandidatka izpolnjuje pogoje za opravljanje doktorata znanosti na interdisciplinarnem doktorskem študijskem programu Bioznanosti, znanstveno področje Agroživilska mikrobiologija. Za mentorico je imenovana prof. dr. Sonja Smole Možina. Za somentorja je imenovan doc. dr. Iztok Dogša.

Delo je bilo opravljeno v Laboratoriju za živilsko mikrobiologijo, Katedre za biotehnologijo, mikrobiologijo in varnost živil, Oddelka za živilstvo na Biotehniški fakulteti Univerze v Ljubljani, v Laboratoriju za mikrobno ekologijo in fiziologijo, Oddelka za mikrobiologijo na Biotehniški fakulteti Univerze v Ljubljani, in na Oddelku za farmakognozijo, Fakultete za farmacijo (Univerza v Gradcu, Gradec, Avstrija).

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AI Novi pristopi obvladovanja bakterij *Campylobacter jejuni* v živilski industriji so predmet številnih raziskav. Rastlinski izvlečki predstavljajo obetavne alternativne protimikrobne učinkovine za nadzor biofilma. V tej doktorski nalogi smo pripravili in testirali različne pripravke sivke vrst *L. angustifolia* in *L. x intermedia* proti filmotvornosti bakterij *C. jejuni* na abiotskih površinah. S filmotvornostjo smo se spoprijeli preko tarč, kot so gibljivost, pritrjevanje in medcelična komunikacija bakterij *C. jejuni*. Optimizirali smo metodo gojenja in preučevanja biofilma bakterij *C. jejuni* na abiotski površini in sicer na interfazi trdna površina/tekočina s konfokalno vrstično lasersko mikroskopijo. Poleg vpeljave visoko tlačne tekočinske kromatografije v kombinaciji s fluorescenčnim detektorjem (HPLC-FLD) smo razvili, optimizirali in nadgradili metodo preučevanja medcelične komunikacije bakterij *C. jejuni* z biosenzorjem *V. harveyi* MM30. Nova metoda je v primerjavi s HPLC-FLD natančnejša in občutljivejša. Vsi pripravki sivke so uspešno odstranili zreli biofilm bakterij *C. jejuni* z abiotske površine. Prav tako so vsi pripravki sivke uspešno zavrli gibljivost, pritrjevanje bakterij *C. jejuni* na abiotske površine in filmotvornost. Pripravki sivke niso značilno znižali koncentracije signalnih molekul avtoinduktor-2 (AI-2) v izrabljenem gojišču bakterij *C. jejuni*. Rezultati metode rt-PCR niso pokazali vpliva eteričnega olja sivke na izražanje gena *luxS* pri bakterijah *C. jejuni*. Transkriptomska analiza planktonske kulture bakterij *C. jejuni* po izpostavljenosti eteričnemu olju sivke je pokazala spremembo izražanja številnih genov, pomembnih za gibljivost, filmotvornost, metabolizem železa in stresni odziv bakterij *C. jejuni*. Te lastnosti predstavljajo nove, obetavne tarče za obvladovanje biofilma bakterij *C. jejuni* v živilski industriji.

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AB New approaches are being studied intensively to control *Campylobacter jejuni* in the food industry. Plant extracts represent promising alternative antimicrobial agents for biofilm control. In this doctoral thesis, we prepared and tested different preparations of *Lavandula angustifolia* and *L. x intermedia* against biofilm formation of *C. jejuni* on abiotic surfaces. Biofilm formation was challenged through targets such as motility, attachment and intercellular communication of *C. jejuni*. We optimized the method of culturing and studying the biofilm of *C. jejuni* on an abiotic surface, specifically at the solid/liquid interface, using confocal laser scanning microscopy. We developed, optimized and upgraded a method for studying the intercellular communication of *C. jejuni* with the *V. harveyi* MM30 biosensor strain, which proved to be accurate and more sensitive compared to the high pressure liquid chromatography with fluorescence detector (HPLC-FLD) method. All lavender preparations successfully removed the mature biofilm of *C. jejuni* from the abiotic surface. Likewise, all lavender preparations successfully inhibited the motility, attachment of *C. jejuni* to abiotic surfaces and biofilm formation of *C. jejuni*. Lavander preparations did not lower significantly the concentration of autoinducer-2 (AI-2) signalling molecules in the spent medium of *C. jejuni.* The rt-PCR did not show the significant effect of lavender essential oil on the expression of the *luxS* gene in *C. jejuni*. Transcriptomic analysis of the planktonic culture of *C. jejuni* after exposure to lavender essential oil showed that the expression of many genes important for motility, biofilm formation, iron metabolism and stress response of *C. jejuni* has been changed. These traits represent new, promising targets for *C. jejuni* biofilm control.

KAZALO VSEBINE

KAZALO ZNANSTVENIH DEL

KAZALO PREGLEDNIC

KAZALO SLIK

KAZALO PRILOG

OKRAJŠAVE IN SIMBOLI

AHL	acil-homoserin lakton, signalna molekula
AI-2	avtoinduktor-2, signalna molekula
CFU	enote, ki tvorijo kolonije (angl. *Colony Forming Units*)
CLSM	vrstična konfokalno laserska mikroskopija (angl. *Confocal Laser Scanning Microscopy*)
DMSO	dimetil sulfoksid
DNK	deoksiribonukleinska kislina
eDNK	zunajcelična deoksiribonukleinska kislina
EI	etanolni izvleček
EO	eterično olje
EU	Evropska Unija
GC-MS	plinska kromatografija v kombinaciji z masno spektrometrijo
GRAS	na splošno priznan kot varen (angl. *Generally Recognized As Safe*)
HPLC-FLD	visokotlačna tekočinska kromatografija v kombinaciji s fluorescenčnim detektorjem (angl. *High Pressure Liquid Chromatography with FLuorescence Detector*)
IG	izrabljeno gojišče
LC-MS	tekočinska kromatografija v kombinaciji z masno spektrometrijo (angl. *Liquid Chromatography – Mass Spectrometry*)
luxS	gen, ki sproži sintezo proteina LuxS
MH	Müller-Hinton
MIK	minimalna inhibitorna koncentracija
NCTC	Nacionalna zbirka tipskih kultur (angl. *The National Collection of Type Cultures*)
OD	optična gostota (angl. *Optical Density*)
RNK	ribonukleinska kislina
rt-PCR	verižna reakcija s polimerazo v realnem času
RLE	relativne luminiscenčne enote
VBNC	živo, vendar ne kultivabilno (angl. *Viable But Not Culturable*)

1 UVOD S PREDSTAVITVIJO PROBLEMATIKE, CILJEV IN HIPOTEZ

1.1 UVOD

1.1.1 Rod *Campylobacter*

Kampilobaktre je prvič opisal leta 1886 nemško-avstrijski pediater Theodor Escherich, ki je v vzorcu blata opazil spiralno zvite, ne-kultivabilne bakterije. Na začetku so jih klasificirali v rod *Vibrio* oz. *Helicobacter* in sicer zaradi izredne morfološke podobnosti. Leta 1963 sta Sebald in Véron na podlagi manjšega genoma, ne-fermentativnega metabolizma, ter mikroaerofilnih zahtev gojenja ločila rod *Campylobacter* od rodu *Vibrio*. Posebno pozornost so kampilobaktri pritegnili v sedemdesetih letih, ko so bili izolirani iz blata otrok in odraslih, prvič na selektivnem gojišču za gojenje bakterij rodu *Campylobacter*. To je bil temelj za začetek raziskovanja teh bakterij. V tem obdobju so zaznali kampilobaktre kot pomembne povzročitelje črevesnih okužb pri ljudeh, a niso vedeli, da se ta bakterija prenaša s kontaminirano hrano. Kljub temu je število raziskav te bakterije začelo hitro naraščati (On, 2001; Silva in sod., 2011).

Rod *Campylobacter* se danes uvršča v družino Campylobacteriaceae, red Campylobacterales, razred proteobakterij Epsilonproteobacteria. Rod *Campylobacter* sestavlja 41 vrst (Parte in sod., 2020). Večinoma gre za gibljive, mikroaerofilne (85 % N_2, 5-10 % O_2 in 3-5 % CO_2), po Gramu negativne, tanke, spiralno zvite celice, velikosti od 0,5 do 5 µm, široke od 0,2 do 0,8 µm (Kreling in sod., 2020). Veljajo za občutljive bakterije, ki za svojo rast potrebujejo gojišča z dodatkom aminokislin (serin, aspartat, asparagin in glutamat), piruvata in laktata, saj jih večina ne uporablja sladkorjev kot vir ogljika. So termofilni mikroorganizmi, ki za svojo optimalno rast potrebujejo temperaturo med 37 °C in 42 °C. Ne rastejo pod 30 °C, ker ne vsebujejo beljakovin, ki jih ščitijo pred hladnim šokom (angl. *Cold Shock Proteins*). Vsebujejo encim oksidazo, ki jih ščiti pred kisikovimi radikali (Facciolà in sod., 2017). Znano je, da kampilobaktri v neugodnih razmerah zelo hitro preidejo v ne-kultivabilno stanje (angl. *Viable But Not Culturable*; VBNC), zaradi česar jih je težje izolirati in identificirati (Kassem in sod., 2013).

Bakterije rodu *Campylobacter* veljajo danes za enega od najpogostejših povzročiteljev črevesnih okužb pri ljudeh, ki se prenašajo preko kontaminirane hrane ali vode. Zoonoza, ki jo povzročajo, se imenuje kampilobakterioza, z najpogostejšimi simptomi kot so akutna driska, trebušni krči in visoka vročina. Možni so tudi zapleti, ki vključujejo endokarditis, pljučnico, sepso, ter nevrološke izpade kot je Guillain-Barré sindrom (Igwaran in Okoh, 2019). Njihovi naravni gostitelji so domače rejne živali, predvsem perutnina (piščanci in purani), manj govedo, prašiči, ali ovce. Najdemo jih v prebavilih živali, pri katerih ne povzročajo bolezenskega stanja (Kreling in sod., 2020). Zanimivo je, da piščanci starosti do 7 dni v svojem črevesju ne vsebujejo kampilobaktrov. Še vedno ni znano, kdaj in kako se piščanci okužijo. Predvideva se, da bakterijo lahko prenašajo okužene muhe, ptice ali

glodavci, ter osebje z onesnaženimi, čevlji, orodjem ipd. Ko je piščanec okužen, nadaljnji prenos poteka po fekalno-oralni poti znotraj jate (Agunos in sod., 2014; Cawthraw in Newell, 2010; Gill in sod., 2017; Rukambile in sod., 2019). Kampilobaktri najpogosteje vstopijo v živilsko proizvodno-oskrbovalno (v nad. živilsko) verigo pri zakolu perutnine. Takrat lahko kontaminirajo abiotske površine, ki pridejo v stik z živilskimi izdelki, ter na njih ustvarjajo biofilme ali kolonizirajo že obstoječe biofilme. Odstotek okuženih piščančjih trupov je dokaj visok in se razlikuje v odvisnosti od letnega časa. Slaba higienska praksa, navzkrižna kontaminacija perutninskega mesa in termično neobdelano perutninsko meso so najpogostejše poti, ki pripeljejo do okužbe človeka (Gölz in sod., 2018; Powell in sod., 2012).

Izrazito povečano število kampilobakterioz se beleži že od leta 2005. Leta 2020 je bil *Campylobacter* najpogostejši povzročitelj črevesnih okužb pri ljudeh v Evropski Uniji (EU). Predstavljal je več kot 60 % vseh prijavljenih zoonoz, ki se prenašajo s kontaminirano hrano. Med 2011 in 2020 je število potrjenih primerov kampilobakterioz, prijavljenih v EU, pokazalo jasen sezonski trend, ki je dosegel vrhunec v poletnih mesecih. Večinoma se pri bolnikih izolirajo *C. jejuni* (88,1 %), sledijo mu *C. coli* (10,6 %), *C. fetus* (0,16 %), *C. upsaliensis* (0,11 %) in *C. lari* (0,09 %). V večini primerov je šlo za samolimitirajoče okužbe ljudi, redki so potrebovali hospitalizacijo. Kljub temu je hospitalizacija zaradi kampilobakterioz najpogostejša med zoonozami, ki se prenašajo preko kontaminirane hrane. Najpogostejši vir sporadičnih okužb je bilo perutninsko meso, sledita mu mleko in voda, ki sta pomemben vir izbruhov (EFSA/ECDC, 2021).

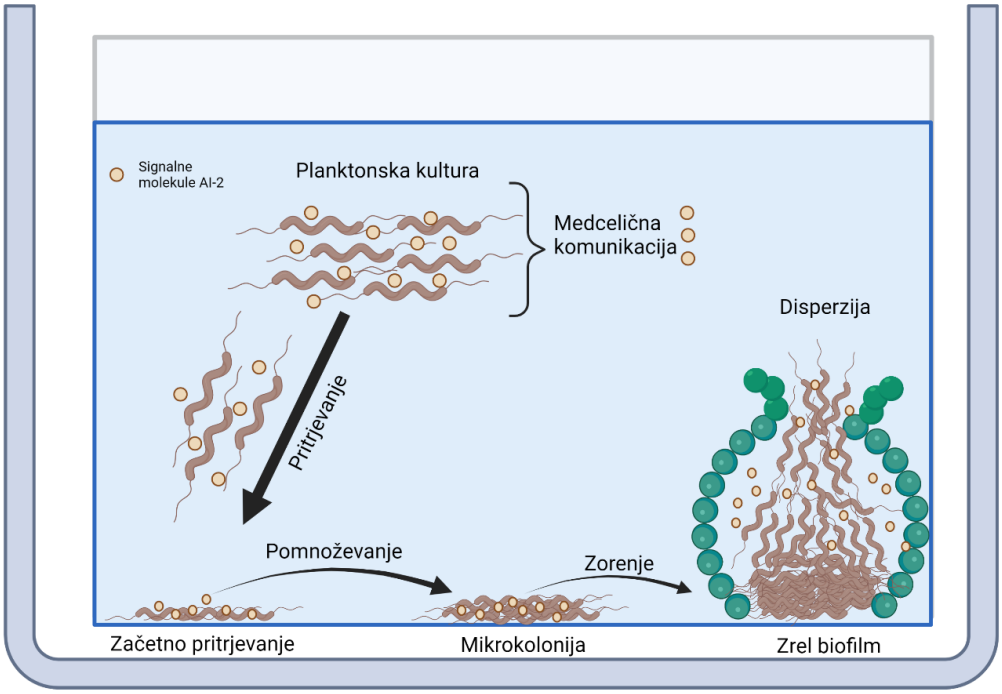
Zaskrbljujoče je tudi hitro naraščajoče število proti antibiotikom odpornih sevov, izoliranih večinoma iz perutnine. Največ izolatov je odpornih proti fluorokinolonom, eritromicinu in tetraciklinom. Trend naraščanja odpornosti je predvsem viden v državah južne in vzhodne Evrope, kar vključuje tudi Slovenijo. Značilni so tudi večkratno odporni sevi proti antibiotikom, kar vključuje kombinirano odpornost proti fluorokinolonom, eritromicinu in tetraciklinom (Rozman in sod., 2018). Mnogo izolatov je odpornih tudi proti razkužilom, ki se največkrat uporabljajo v prehranski industriji, kot so triklosan, trinatrijev fosfat, klorheksidinijev diacetat, benzalkonijev klorid in cetilpiridinijev klorid. Odpornost proti razkužilom je v nekaterih primerih lahko povezana tudi z navzkrižno odpornostjo proti antibiotikom, saj *Campylobacter* pogostokrat uporablja izlivne črpalke CmeABC za črpanje protimikrobnih snovi iz celice (Mavri in sod., 2016). Potrebno je poiskati nove načine obvladovanja kampilobaktra, kar vključuje uporabo alternativnih učinkovin, kot so rastlinski pripravki, ter nove celične tarče, preko katerih bi uspešno zavrli prisotnost te bakterije v živilski verigi.

Kljub celovitemu nadzoru v večini držav članic EU, se predvideva, da je število prijavljenih in potrjenih primerov kampilobakterioz podcenjeno. Čeprav se prisotnost bakterij *Campylobacter* preverja v celotni živilski verigi, kar vključuje primarno proizvodnjo, zakol, predelavo ter prodajo, je število kampilobaktrioz še vedno izrazito visoko (Teunis in sod.,

2013; EFSA/ECDC, 2021). *Campylobacter* »paradoks« je v raziskovalnem svetu zelo dobro znan. Čeprav je izrazito občutljiv in zahteven za gojenje, *Campylobacter* še vedno povzroča številne težave v celotni živilski verigi, posledično tudi v javnem zdravstvu. Pereče vprašanje številnih raziskav je, kako zmanjšati prisotnost kampilobaktra v celotni živilski verigi ter s tem preprečiti nevarnost, ki jo ta bakterija predstavlja za javno zdravstvo.

1.1.2 Biofilmi – neizčrpen vir bakterijskih kontaminacij v živilski verigi

Bakterijske biofilme lahko definiramo kot eno- ali večvrstne strukturirane skupnosti bakterij, pogostokrat obdane s polimernim matriksom, ki ga bakterije same proizvajajo in je sestavljen iz polisaharidov, beljakovin, maščob in zunajcelične DNK (eDNK). Celice so znotraj biofilma pritrjene ena na drugo in/ali na različne površine (Flemming in sod., 2016). Razlikujemo pet faz razvoja biofilma (Slika 1). Prva faza vključuje fazo reverzibilnega pritrjevanja planktonskih celic na površine. Pri tej fazi je izrednega pomena medcelična komunikacija s signalnimi molekulami, ki je bolj podrobno opisana v nadaljevanju te naloge. Druga faza je ireverzibilno pritrjevanje in namnoževanje celic na površinah, kateri sledi faza zorenja biofilma, ki se konča z razvojem zrelega biofilma obdanega z debelim polimernim matriksom. V končni fazi, ki pomeni propad biofilma, pride do razprševanja oz. disperzije posameznih celic iz biofilma (Hassan in sod., 2020). Biofilmi so najbolj razširjena in uspešna oblika življenja na Zemlji, znotraj katere mikroorganizmi kooperativno sodelujejo, izmenjujejo genetsko informacijo, vključno z geni za rezistenco, ter delijo skupne dobrine. Biofilm nudi celicam večjo zaščito pred različnimi okoljskimi stresnimi dejavniki, vključno s protimikrobnimi sredstvi (Flemming in sod., 2016).



Slika 1: Shematski prikaz ravoja bakterijskega biofilma (Prirejeno po Hassan in sod., 2020).

V živilski verigi lahko mikroorganizmi tvorijo biofilme na kontaktnih površinah s hrano (npr. nerjaveče jeklo, steklo, guma, les, različni plastični materiali) in posledično navzkrižno kontaminirajo živilske izdelke, ki so namenjeni končnemu potrošniku. Poleg tega njihov nadzor predstavlja resen izziv, saj povzročajo velike gospodarske in energetske izgube, poškodujejo površine in opremo ter vodijo do stalne kontaminacije živilskih izdelkov (Galié in sod., 2018). Značilni problem živilske verige so patogeni mikroorganizmi, ki predstavljajo veliko tveganje za javno zdravstvo, saj tudi oni lahko ustvarjajo eno- ali večvrstne biofilme na abiotskih površinah ter posledično navzkrižno kontaminirajo živilske izdelke. S tem pa zmanjšujejo kakovost, varnost in obstojnost živilskih izdelkov. Ocenjuje se, da so biofilmi povezani z več kot 65 % okužb ter da njihovo zdravljenje presega milijardo dolarjev letno (Satpathy in sod., 2016).

Filmotvornost je ključna za preživetje bakterij *C. jejuni* izven njihovega naravnega okolja, t.j. črevesa. Lahko tvorijo biofilme na različnih površinah v živilski verigi, kot so nerjaveče jeklo, plastika, steklo in nitrocelulozne membrane. Biofilme kampilobaktrov najdemo tudi v napajalnih sistemih kokošnjakov (Tram in sod., 2020). Stradanje, aerobni stres, prisotnost eDNK, žolčnih soli in mesnega soka povečuje filmotvornost bakterij *C. jejuni*, medtem ko jo osmotski stres močno zmanjšuje. Našteti zunanji dejavniki sprožijo pri tej bakteriji prilagoditvene mehanizme, ki omogočajo izražanje genov, potrebnih za sintezo beljakovin, vključenih v tvorbo površinskih struktur pomembnih za gibljivost, pritrjevanje, virulenco, stresni odziv in protimikrobno odpornost (Püning in sod., 2021). Za filmotvornost bakterij *C. jejuni* se predvideva, da je izrednega pomena tudi medcelično signaliziranje, ki ga omogočajo signalne molekule avtoinduktorji-2 (AI-2), produkti prepisovanja gena *luxS* (več v poglavju 1.1.3) (Plummer, 2012). Ker so bakterije *C. jejuni* mikroaerofilni mikroorganizmi, jih v naravi najpogosteje najdemo znotraj večvrstnih biofilmov, kjer se preživetje tega patogena izboljša. Znotraj biofilmov lahko pride do razvoja lokalnega okolja z gradientom kisika in hranil, kar vpliva na heterogenost celične populacije. Specifično mikrookolje znotraj obstoječega biofilma je lahko odlična niša za mikroorganizme, ki potrebujejo posebne razmere, kot je npr. mikroaerofilna atmosfera (Bridier in sod., 2015). Znano je, da bakterije *C. jejuni* lahko kolonizirajo že obstoječe biofilme ter da v večvrstnih biofilmih z bakterijami *Escherichia coli*, *ginosa*, *Enterococcus faecalis*, *Salmonella enterica* in *Staphylococcus simulans* preživijo dlje časa in proizvedejo več biomase (Feng in sod., 2018; Indikova in sod., 2015; Teh in sod., 2010; Teh in sod., 2019; Zhong in sod., 2020).

1.1.2.1 Metode obvladovanja filmotvornosti v živilski verigi

Živilska industrija običajno uporablja stroškovno učinkovite kemijske (raztopine natrijevega hidroksida ali natrijevega hipoklorita) in fizikalne metode (vroča vodna para in ozon) za odstranjevanje biofilmov (Galié in sod., 2018). Uporabljajo se tudi mehanske tehnike za fizično odstranjevanje ali preprečevanje razvoja biofilma znotraj cevi in na površinah. Kljub temu se filmotvornost s stroškovno učinkovitimi metodami na določenih predelih in pri metodah pakiranja ne da popolnoma kontrolirati. Zato se uporabljajo tudi druge metode, ki

vključujejo uporabo detergentov z vsebnostjo encimov, kot so proteaze, glikozidaze in Dnaze. Površine lahko obdelujemo tudi z različnimi premazi, kot so Ag^{+}, Cu^{2+} ali Zn^{2+} nanodelci ali s protifilmotvornimi polimeri, ki vsebujejo lizocime ali bakteriocine (Galié in sod., 2018).

Nobena od naštetih metod ni popolnoma učinkovita pri preprečevanju razvoja oz. odstranjevanju biofilma. Hkrati je zaskrbljujoče naraščanje odpornosti proti različnim komercialnim antibiotikom in razkuževalnim sredstvom. Zato se danes raziskujejo nove metode preprečevanja razvoja in odstranjevanja biofilma. Vse bolj popularne so t.i. zelene tehnologije, ki so pri potrošnikih bolj priljubljene v primerjavi s sintetičnimi razkužili, ki se pogostokrat uporabljajo v živilski verigi in lahko povečajo količino nepotrebnih kemikalij v okolju (Bridier in sod., 2015). Raziskuje se uporaba bakteriofagov, ki lahko naravno okužijo bakterije in zagotovijo zelo specifično, nestrupeno nadzorovanje biološkega obraščanja različnih mikroorganizmov (Ferriol-González in Domingo-Calap, 2020). Encimi (α-amilaze, β-glukanaze in protezae), ki jih proizvajajo različne probiotične bakterije (*Lactobacillus bulgaricus, Lactococcus lactis, Streptococcus termophilus*) so se pokazali kot učinkovita sredstva za odstranjevanje biofilmskega polimernega matriksa (Borges in sod., 2020). Probiotične bakterije, kot je *Bacillus subtilis*, kažejo tudi na močno delovanje proti razvoju in za odstranjevanje biofilma bakterij *C. jejuni* z abiotskih površin (Erega in sod., 2021; Šimunović in sod., 2022). Številne raziskave testirajo rastlinske pripravke, ki so priznani kot varni (angl. *Generally Recognized As Safe,* GRAS) in imajo širok spekter protimikrobnega delovanja, vključno s preprečevanjem medcelične komunikacije, pritrjevanja in filmotvornosti (Khameneh in sod., 2019; Klančnik in sod., 2021). V naših raziskavah smo uporabili različne pripravke sivke z namenom odstranjevanja in preprečevanja razvoja biofilma bakterij *C. jejuni*.

1.1.2.2 Metode preučevanja filmotvornosti

Pri preučevanju biofilmov najpogosteje določamo število živih bakterijskih celic znotraj biofilma, pokritost površine z biomaso, ter opisujemo strukturo in sestavo biofilma. Za določanje skupne količine biomase najpogosteje uporabljamo posredne metode barvanja, kot je barvanje s kristal vijoličnim barvilom. Za določanje števila živih bakterijskih celic lahko uporabljamo direktne metode, kot so gojitvene metode (določanje števila kolonij) in bolj sodobne metode, kot je pretočna citometrija (Alves in sod., 2020; Azeredo in sod., 2016; Franklin in sod., 2015). Lahko uporabljamo tudi različne tipe mikroskopije. Vrstična elektronska mikroskopija nam omogoča vpogled v strukturo in površino biofilma, prostorsko porazdelitev celic ter lokalizacijo izbranih makromolekul v biofilmu, poda nam lahko tudi informacijo o kemijski sestavi. Konfokalna mikroskopija nam omogoča vpogled v prostorsko strukturo biofilma, kot tudi ločevanje živih od mrtvih celic, ob uporabi specifičnih, fluorescenčnih barvil. Ima sicer slabšo ločljivost od elektronske mikroskopije, a večje vidno polje in omogoča opazovanje nativnih vzorcev. Lahko merimo tudi metabolično

aktivnost bakterij znotraj biofilma s kolorimetričnim določanjem pretvorbe tetrazolijevih soli v formazan (Carrascosa in sod., 2021; Püning in sod., 2021; Relucenti in sod., 2021).

Izbrana tehnika preučevanja biofilma je odvisna od zastavljenih ciljev oz. od tega, kaj želimo pri biofilmu preučevati. Nekatere metode so bolj primerne za kvantifikacijo biomase, medtem ko nam druge lahko podajo tudi podatek o količini živih oz. mrtvih celic. Glede na to, da so bakterije *C. jejuni* izredno zahtevne za gojenje, smo v naših raziskavah morali optimizirati metodo gojenja in preučevanja biofilma bakterij *C. jejuni*. Zato smo uporabili sodobno mikroskopsko tehniko, kot je vrstična konfokalno laserska mikroskopija, ki nam je omogočila vrednotenje živosti celic z metodo barvanja živo/mrtvo (angl. *LIVE/DEAD*) ter vpogled v prostorsko strukturo biofilma. Za analizo slik se pogostokrat uporablja program Fiji ImageJ (NIH, ZDA), s katerim lahko ugotavljamo delež pokritosti abiotske površine na interfazi trda površina/tekočina in razmerje med živimi in mrtvimi celicami (Hympanova in sod., 2020; Terlep in sod., 2022).

1.1.3 Medcelična komunikacija pri bakterijah

Mnogo let se je verjelo, da so bakterije enocelični organizmi, ki se kot posamezniki nepovezano nahajajo v okolju, a v zadnjem desetletju številne raziskave potrjujejo nasprotno. Mikroorganizmi so bitja, ki lahko med seboj komunicirajo, izmenjujejo informacije in ustvarjajo večcelične entitete (Diggle in sod., 2007; Dogša in sod., 2014; Štefanič in sod., 2015; Xavier in sod., 2011). Danes vemo, da so bakterije zmožne sintetizirati, pošiljati, sprejemati in procesirati informacije, ki so podane v obliki različnih signalnih molekul. Naraščanje bakterijske gostote vodi h kopičenju signalnih molekul, ta pa k spremembi obnašanja populacije (Antunes in Ferreira, 2009; Boyer in Wisniewski-Dyé, 2009; Camilli in Bassler, 2006). Ko je ustrezna koncentracija signalnih molekul dosežena, bakterije začnejo koordinirano izločati javne dobrine, kot so zunajcelične beljakovine, polimeri, encimi, eDNK, bakteriocini, ki so dostopne vsem v populaciji. Izločanje javnih dobrin izboljša fitnes oziroma preživetje in razmnoževanje bakterij. Sprememba izražanja genov vpliva tudi na izražanje različnih bakterijskih lastnosti (adaptacij), kot so bioluminiscenca, sporulacija, kompetenca, proizvodnja antibiotikov, filmotvornost in izločanje virulentnih dejavnikov. Bakterijska komunikacija uravnava interferenčno kompeticijo. Naštete lastnosti kažejo na socialno obnašanje bakterij (Michie in sod., 2016; Rutherford in Bassler, 2012; Whiteley in sod., 2017).

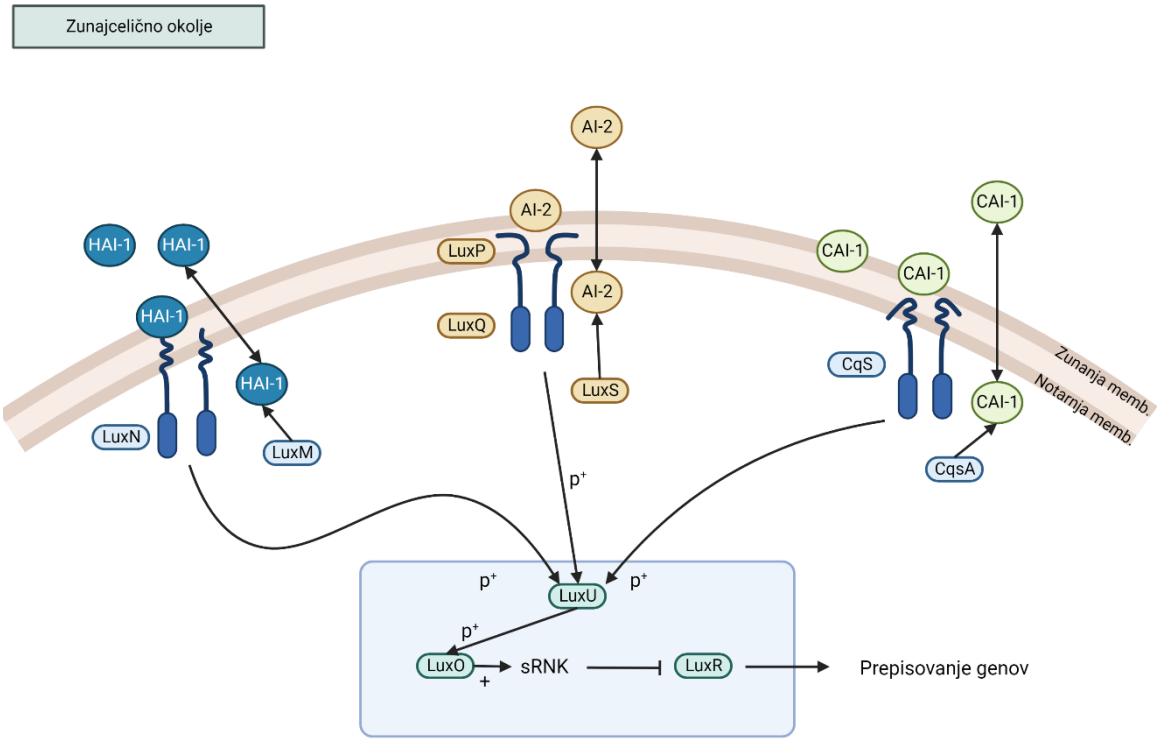
Najbolj raziskana skupina signalnih molekul so acil-homoserin laktoni (AHL), ki jih proizvajajo po Gramu negativne bakterije in so jih prvič zaznali pri bakteriji *Vibrio fischeri* (Boyer in Wisniewski-Dyé, 2009; Eberhard in sod., 1981; Hanzelka in Greenberg, 1996). Razlikujejo se po dolžini ogljikove verige, ki lahko vsebuje od 4 do 18 C atomov, kot tudi po položaju acilne skupine. Vse signalne molekule AHL imajo podobno homoserin lakton obročasto strukturo (Steindler in Venturi, 2007). Po Gramu negativne bakterije običajno vsebujejo encim LuxI, ki omogoča sintezo signalne molekule AHL iz S-adenozilmetionina

(SAM). Receptorski encim LuxR omogoča detekcijo signalnih molekul AHL. Visoka koncentracija signalnih molekul AHL pripelje do konformacijske spremembe receptorskega encima LuxR, ki gre v kompleks s signalno molekulo AHL in vpliva na spremembo izražanja tarčnih genov (Ampomah-Wireko in sod., 2021). Mnoge po Gramu negativne bakterije uporabljajo analogne sisteme LuxI/LuxR, ki vplivajo na izražanje različnih tarčnih genov (Li in Nair, 2012). Za razliko od po Gramu negativnih bakterij, po Gramu pozitivne bakterije proizvajajo signalne molekule, ki so tipično peptidi. Sinteza signalnih peptidov poteka na ribosomih in so naprej podvrženi različnim spremembam, ki so pomembne za dozorevanje teh signalov (Abisado in sod., 2018).

Signalne molekule, ki jih bakterije uporabljajo za med- in znotraj vrstno komunikacijo, se imenujejo avtoinduktorji-2 (AI-2). Lahko jih uporabljajo po Gramu pozitivne in po Gramu negativne bakterije. Encim LuxS, ki je produkt gena *luxS,* je odgovoren za biosintezo signalne molekule AI-2. Prvič so encim LuxS in signalno molekulo AI-2 zaznali pri bakteriji *V. harveyi*. Danes je prisotnost encima LuxS potrjena pri več kot 70 bakterijskih vrstah (Galloway in sod., 2011; Xu in sod., 2017). Encim LuxS iz S-ribozil homocisteina (SRH), ki je stranski produkt molekule SAM, sintetizira S-4,5-dihidroksi-2,3-pentandion [(S)-DPD], ki je prekurzor signalne molekule AI-2 (pro-AI-2). Spontana ciklizacija in hidratacija pro-AI-2 vodi k oblikovanju različnih variacij signalne molekule AI-2, od katerih sta najbolj poznani dve obliki: (2S, 4S)-2-metil-2,3,3,4-tetrahidroksi-tetrahidrofuranborat (S-THMF-borat, BAI-2) in (2R, 4S)-2-metil-2,3,3,4-tetrahidroksitetrahidrofuran (R-THMF, AI-2). Možno je, tudi da se signal AI-2, ki ga proizvaja ena bakterijska vrsta, modificira v signal AI-2, ki ga zaznava druga bakterijska vrsta (Chen in sod., 2002; Federle, 2009; Plummer, 2012; Semmelhack in sod., 2005; Thiel in sod., 2009; Zhao in sod., 2018). Ker je encim LuxS del centralnega metabolizma, vpliva tudi na izražanje različnih genov, ki so vključeni v preživetje, patogenost, gibljivost, filmotvornost, kemotakso, sporulacijo. Pri različnih bakterijah, kot so *Bacillus cereus*, *Borrelia burgdoferi*, *Streptococcus pneumoniae, S. aureus*, *H. pylori, E. coli* in *S. enterica* so dokazali, da medcelična komunikacija s signalno molekulo AI-2 močno vpliva na filmotvornost, proizvodnjo zunajceličnih polimerov, gibljivost in odpornost proti antibiotikom (Pereira in sod., 2013). Pri mutantah *luxS* se spremeni izražanje v povprečju od 1 do 10 % genov celotnega genoma. Prav tako pride do spremembe izražanja prekurzorjev (SAM, SAH, SRH), ki so potrebni za sintezo signalne molekule AI-2. Zmanjša se tudi patogenost, virulenca, gibljivost, pritrjevanje, filmotvornost, ter kemotaksa pri mnogih po Gramu negativnih bakterijah (Papenfort in Bassler, 2016).

Bakterijske vrste *V. harveyi* uporabljajo beljakovinski kompleks LuxP/LuxO za zaznavanje signalnih molekul AI-2. Vezava signalne molekule AI-2 na receptor LuxP pripelje do njegove konformacijske spremembe, kar omogoči nastanek asimetričnega dimera, ki je sestavljen iz dveh monomerov $LuxPQ_p$ ($LuxPQ_p$-$LuxP'Q_p'$) in vpliva na inaktivacijo kinazne aktivnosti beljakovine LuxO. Ko je kinaza LuxO aktivna, dodaja fosfate na beljakovino LuxU, ko je neaktivna, pa jih odceplja (Bassler in sod., 1997; Bassler in Freeman, 1999; Lilley in Bassler, 2000; Waters in Bassler, 2006). Zanimivo je, da bakterije *E. coli* in *S.*

typhimurium uporabljajo receptorsko beljakovino LsrB za zaznavanje signalnih molekul AI-2 (R-THMF), ki se po strukturi razlikuje od signalne molekule AI-2 (S-THMF), ki jo zaznava bakterija *V. harveyi* preko receptorske beljakovine LuxP (Surette in sod., 1999). Pokazali so tudi, da je podobnost sekvenc beljakovin LuxP in LsrB izredno majhna (11% podobnost). Prav tako signalna molekula AI-2 uravnava prepis operona *lsrACDBFGE*, ki ga vsebujeta bakteriji *E. coli* in *S. typhimurium*. Ko enkrat beljakovina LsrB zazna signalno molekulo AI-2, jo transportni sistem Lsr ABC takoj vnese v celico, kjer pride do njene fosforilacije. Fosforilirana signalna molekula AI-2 se veže na beljakovino LsrR, ki naprej uravnava izražanje operona *lsr* (Federle, 2009).



Slika 2: Shematski prikaz medceličnega signaliziranja pri bakterijah *V. harveyi*. Bakterije *V. harveyi* uporabljajo 3 različne signalne molekule za komunikacijo. Signalni molekuli HAI-1 in CAI-1 sta vrstno specifični, medtem ko je signalna molekula AI-2 medvrstna komunikacijska signalna molekula (Prirejeno po Waters in Bassler, 2006).

1.1.3.1 Medcelična komunikacija pri bakterijah *C. jejuni*

Čeprav obstajajo številne raziskave na področju medcelične komunikacije različnih bakterij, so informacije glede medcelične komunikacije bakterij rodu *Campylobacter* še vedno izredno omejene. Znano je, da sta Elvers in Park leta 2002 potrdila prisotnost homolognega gena *luxS* pri bakteriji *C. jejuni*, kot tudi aktivno proizvodnjo signalne molekule AI-2 (Elvers in Park, 2002). Za aktivno izločanje signalnih molekul AI-2 iz celice, bakterije običajno uporabljajo transportne sisteme. Pri bakteriji *C. jejuni* je pokazano, da je transport signalne molekule AI-2 deloma odvisen od ne-kodirajoče RNK (CjNC110). V primeru delecije sekvence CjNC110 je bil opažen zmanjšan odziv poročevalskega seva na zunajcelične

signalne molekule AI-2, a povišen odziv poročevalskega seva na znotrajcelične signalne molekule AI-2, kar nakazuje, da je sekvenca CjNC110 potrebna za transport signalne molekule AI-2 čez celično membrano (Kreuder in sod., 2020). Zaenkrat ni znana receptorska beljakovina za signalno molekulo AI-2 pri bakterijah rodu *Campylobacter.* Predvideva se, da bakterije *C. jejuni* uporabljajo dvokomponentni sistem za zaznavanje signalnih molekul AI-2 (Adler in sod., 2015). Točna koncentracija signalnih molekul AI-2, ki jih tekom rasti proizvajajo bakterije *C. jejuni* ni bila znana in smo jo v tem doktorskem delu uspešno določili z uporabo analitske in biološke metode.

Ker celotnega mehanizma medcelične komunikacije pri bakteriji *C. jejuni* ne poznamo, vpliv signalne molekule AI-2 na fenotip teh bakterij načeloma preučujemo preko delecije gena *luxS*, preko vpliva različnih protimikrobnih snovi na izražanje gena *luxS* ali preko vpliva na sam signal AI-2. Zaenkrat morfoloških sprememb pri mutantah *luxS* niso zaznali (Jeon in sod., 2003; Mou in Plummer, 2016). Hitrost rasti mutante *luxS* načeloma ni različna v primerjavi z divjim tipom (Holmes in sod., 2009; Elvers in Park, 2002). Prisotne pa so fenotipske razlike pri mutantah *luxS*, kar vključuje zmanjšano gibljivost, pritrjevanje, filmotvornost, invazijo in kolonizacijo celičnih kultur, ter oksidativni stres (Kim in sod., 2021; Elvers in Park, 2002; Plummer in sod., 2012; Quiñones in sod., 2009; Reeser in sod., 2007; Šimunović in sod., 2020), medtem ko je kemotaksa proti aminokislinam povečana (Tram et al., 2020). Potrebno je poudariti, da so vse spremembe specifične za posamezne seve ter so možne variacije zaradi samega seva, pogojev gojenja, uporabljene metode preučevanja ali vrste mutageneze (Püning in sod., 2021).

1.1.3.2 Metode preučevanja bakterijske medcelične komunikacije

Za preučevanje medcelične komunikacije bakterij običajno uporabljamo klasične analitske metode ter biološke metode. Klasične analitske metode najpogosteje temeljijo na uporabi kolorimetričnih ter kromatografskih metod, kot so tankoplastna kromatografija, tekočinska in plinska kromatografija brez ali v kombinaciji z masno spektrometrijo, visokotlačna tekočinska kromatografija brez ali v kombinaciji s fluorescenčnim detektorjem. Pri klasičnih analitskih metodah sta pomembna dva ključna koraka in sicer ekstrakcija in detekcija signalnih molekul. Sama priprava vzorca za analitske analize je zahtevna, draga in dolgotrajna, zagotavljajo pa zelo natančne, ponovljive in zanesljive rezultate (Turan in sod., 2017).

Biološke metode temeljijo na uporabi bakterijskih biosenzorskih sevov, ki zelo hitro in z visoko občutljivostjo ugotavljajo prisotnost signalnih molekul. Sama priprava vzorca za biosenzorske metode je hitrejša, cenejša in enostavnejša v primerjavi z analitskimi metodami. Ko biosenzorskem sevu dodamo zunajcelično signalno molekulo, jo biosenzor zazna in spremeni izražanje genov, ki vodijo k spremembi fenotipa, kot je npr. proizvodnja pigmenta violaceina, izražanje luminiscence, fluorescence ali β-galaktozidaze (Delle Side in sod., 2015; Turan in sod., 2017). Bakterija *Vibrio harveyi* predstavlja enega od najpogostejše

uporabljenih bakterijskih biosenzorskih sevov, ki naravno vsebuje sistem medceličnega signaliziranja. Lahko zazna kar tri različne signalne molekule (HAI-1, CAI-1 in AI-2). Ko bakterije *V. harveyi* zaznajo signalne molekule, pride do izražanja luciferaze, encima, ki je odgovoren za emitiranje luminiscence. Za merjenje luminiscence uporabljamo luminometer (Nackerdien in sod., 2008; Zhao in sod., 2018).

Pri preučevanju medcelične komunikacije moramo poznati sistem medceličnega signaliziranja preučevane bakterije oz. signalno molekulo, ki jo tarčna bakterija proizvaja. Potrebno je izbrati ustrezno metodo, s katero bomo zanesljivo zaznali in natančno določili koncentracijo signalne molekule. Ker bakterija *C. jejuni* aktivno proizvaja signalno molekulo AI-2, je najprej potrebno izbrati metodo, ki bo omogočila zaznavanje in merjenje koncentracije teh signalih molekul z visoko občutljivostjo in natančnostjo. V tej doktorski nalogi smo želeli razviti, optimizirati in nadgraditi biosenzorsko metodo detekcije in kvantifikacije signalnih molekul AI-2, ki jih proizvajajo bakterije *C. jejuni*. Vpeljali smo tudi direktno, kemijsko-analitsko metodo in sicer visoko tlačno tekočinsko kromatografijo (HPLC) v kombinaciji s fluorescenčnim detektorjem (FLD), s katero smo ovrednotili točnost kvantifikacije signalnih molekul AI-2 z biosenzorsko metodo.

1.1.4 Rastlinski pripravki kot obetavne bioaktivne učinkovine za obvladovanje bakterijske filmotvornosti

Rastlinski pripravki imajo pomembno vlogo v tradicionalni medicini že od antičnih časov. Danes postajajo vse bolj priljubljeni zaradi prepoznavnosti kot varni izdelki za človeka in okolje. Stopnjuje se negativni odnos potrošnikov do uporabe kemično sintetiziranih spojin v živilski verigi. Zato znanstveniki v rastlinskih pripravkih danes vidijo obetaven vir številnih bioaktivnih učinkovin za boj proti različnim škodljivim mikroorganizmom (Afonso in sod., 2022; Mishra in sod., 2020; Negi, 2012). Moderne tehnologije omogočajo varno in učinkovito ekstrakcijo fitofarmacevtskih učinkovin iz rastlin. Uporabljajo se ekstrakti cvetov, listov, celih zeli, korenin in odpadnega rastlinskega materiala po npr. destilaciji eteričnega olja, kot tudi čiste spojine, ki se izolirajo iz rastlinskih pripravkov (Das in sod., 2010; Khan in sod., 2013). Večina raziskav temelji na določanju protimikrobnega učinka rastlinskih pripravkov v planktonskih kulturah. Nujno je raziskave usmeriti na raziskovanje učinka proti biofilmom, ki so najbolj razširjena in uspešna bakterijska oblika življenja na Zemlji (Flemming in Wuertz, 2019). Razvoj biofilma vključuje pet glavnih faz, ki so bile opisane v poglavju 1.1.2. Rastlinske pripravke lahko uporabimo za obvladovanje vseh pet faz razvoja biofilma, med katerimi sta najbolj kritična faza pritrjevanja bakterijskih celic na abiotske oz. biotske površine, in faza zorenja biofilma, kjer se razvija njegova struktura. Obetavna tarča, s katero bi lahko zavrli filmotvornost, je sistem medcelične komunikacije, ki v splošnem spodbuja in koordinira razvoj biofilma (Klančnik in sod., 2021; Machado in sod., 2020).

Protimikrobnost rastlinskim pripravkom dajejo sekundarni metaboliti, ki jih lahko razdelimo na fenole, terpene, spojine, ki vsebujejo dušik (alkaloidi, cianogeni glukozidi, kanavanin) ter spojine, ki vsebujejo žveplo (glutation, glukozinolati, fitoaleksini, tionini, defenzini, aliin) (Jamwal in sod., 2018). V tej doktorski nalogi smo se osredotočili na etanolne izvlečke in eterična olja, ki so večinoma sestavljena iz fenolov. Fenoli so velika skupina spojin, ki vključujejo fenolne kisline, kinone, flavonoide, flavone, flavonone, tanine in kumarine (Alara in sod., 2021). Njihov glavni protibakterijski učinek temelji na omejevanju dostopnosti hranil, poškodovanju membrane in DNK, ter vezavi na adhezine, celično steno in beljakovine. Delujejo tudi kot inhibitorji efluksnih črpalk, zmanjšujejo proizvodnjo zunajceličnega polisaharidnega matriksa, ter zmanjšujejo izražanje genov, ki so pomembni za sintezo bičkov, pilov in fimbrijev (Mishra in sod., 2020; Vaou in sod., 2021). Za eterična olja je pokazano, da modificirajo celično steno in membrano, kar povzroča izgubo znotraj celičnih molekul kot so nukelinske kisline in beljakovine. To posledično lahko vpliva na funkcionalnost respiratorne verige in sinteze adenozintrifosfata (ATP) (Angane in sod., 2022). Eterična olja motijo in zavirajo sistem medcelične komunikacije z inhibicijo sinteze signalnih molekul in vezave na receptorje signalnih molekul, z razgradnjo signalnih molekul in s preprečevanjem izločanja signalnih molekul (El-Tarabily in sod., 2021).

Posebno bogate s fenolnimi spojinami in eteričnimi olji so rastline družine *Lamiaceae.* V družino *Lamiaceae* spadajo mnogi rodovi rastlin, znani po številnih bioaktivnih snoveh, kot so npr. *Lavandula*, *Thymus*, *Origanum*, *Rosmarinus*, *Mentha, Ocimum, Salvia* in *Clinopodium* (Saleem in sod., 2009). Vse so znane tudi po protibakterijskem učinku proti različnim po Gramu pozitivnim (*Streptococcus pyogenes, S. aureus, Listeria monocytogenes*) in po Gramu negativnim bakterijam (*E. coli*, *Salmonella typhimurium*, *Legionella pneumophila*) (Chassagne in sod., 2021). Močan protifilmotvorni učinek proti bakterijam *S. aureus, Streptoccocus epidermidis, P. aeruginosa, Pseudomonas florescens, B. cereus* in *B. subtilis* so pokazali rastlinski pripravki vste *Rosmarinus officinalis* L. Prav tako so rastlinski pripravki vrste *Thymus vulgaris* L. pokazali močan učinek na gibljivost, medcelično komunikacijo, filmotvornost in izražanje genov, pomembnih za sintezo bičkov pri bakterijah *P. flourescens* in *Chromobacterium violaceum.* Protifilmotvoren učinek proti številnim bakterijam so pokazale tudi čiste spojine karvakrol, eugenol, timol, linalol, cinamilaldehid, ki so glavne komponente eteričnih olj omenjenih rastlin (Ćirić in sod., 2019).

Protibakterijski kot tudi protifilmotvorni učinek proti bakteriji *C. jejuni* so pokazali rastlinski pripravki različnih vrst (*Achillea millefolium, Coriandrum sativum, Euodia ruticarpa, Juniperus communis, Lavandula hybrida, Olea europaea, Origanum vulgare, R. officinalis, T. vulgaris*), kot tudi čiste spojine, ki jih najdemo v teh pripravkih (karvakrol, trans-cinamaldehid, eugenol, linalol, resveratrol, ɣ-terpinen) (Bezek in sod., 2016; Duarte in sod., 2016; Šimunović in sod., 2020; Wagle in sod., 2019). Med njimi so tudi pripravki, ki so vplivali na sistem medcelične komunikacije bakterij *C. jejuni*. Nobena raziskava ni podala odgovora, ali rastlinski pripravki delujejo na znižanje koncentracije signalnih molekul, na

izražanje gena *luxS* ali na receptor za zaznavanje signalnih molekul. Študija, ki so jo izvedli Šimunović in sod. (2020), nam je služila kot izhodišče, ki nas je usmerila v nadaljne raziskave na področju preučevanja vpliva rastlinskih pripravkov na filmotvornost in medcelično komunikacijo bakterij *C. jejuni*. Med številnimi rastlinskimi pripravki, ki so jih Šimunović in sod. (2020) testirali, se je sivka izkazala kot obetavno protibakterijsko sredstvo z učinkom proti medcelični komunikaciji bakterij *C. jejuni*. Zato smo v tej doktorski nalogi uporabili pripravke sivke vrst *Lavandula angustifolia* in *Lavandula x intermedi*a in jih testirali proti biofilmu bakterij *C. jejuni*.

1.2 NAMEN DOKTORSKE NALOGE

Namen doktorske naloge je bil določiti učinek rastlinskih pripravkov sivke vrst *L. angustifolia* in *L. x intermedia* v subinhibitornih koncentracijah na medcelično komunikacijo, pritrjevanje, filmotvornost in odstranjevanje biofilma bakterij *C. jejuni* z abiotskih površin, kot tudi njihovo medsebojno povezanost.

S tem namenom smo iz cvetov sivke vrst *L. angustifolia* in *L. x intermedia* pripravili eterična olja in etanolne izvlečke ter jim določili kemijski profil z uporabo tekočinske (LC) in plinske kromatografije (GC) v kombinaciji z masno spektrometrijo (MS). Posebno pozornost smo posvetili odpadnemu materialu, ki preostane po destilaciji eteričnega olja sivke in še vedno vsebuje bioaktivne spojine. Zato smo tudi odpadni material uporabili za pripravo etanolnih izvlečkov. Recikliranje odpadnega materiala združuje ekonomičnost in okoljevarstvo, dva vidika, ki sta izrednega pomena za prihodnost. Glede na ugotovljen kemijski profil pripravljenega materiala smo izbrali glavne čiste spojine, ki smo jih uporabili v bioloških poskusih.

Ker je končni cilj zavreti filmotvornost bakterij *C. jejuni*, je potrebno raziskati mehanizme, ki so v filmotvornost bakterij vpleteni. To so mehanizmi medcelične komunikacije, gibljivosti in začetnega pritrjevanja, brez katerih je razvoj biofilma okrnjen. Namen dela je torej spremljati filmotvornost bakterij *C. jejuni* ob dodatku rastlinskih pripravkov sivke do 72 h. Za obvladovanje filmotvornih lastnosti smo uporabili subinhibitorne koncentracije rastlinskih pripravkov, saj ne želimo vplivati na rast bakterij, ampak samo na njihove filmotvorne lastnosti. Za odstranjevanje zrelih biofilmov smo uporabili različne koncentracije rastlinskih pripravkov sivke, saj je znano, da so biofilmske celice kar 1000-krat bolj odporne proti protimikrobnem sredstvom v primerjavi s planktonskimi celicami.

Campylobacter jejuni je zahtevna bakterija za laboratorijsko gojenje. Prav tako je zelo zahtevno gojenje biofilma teh bakterij. Zato smo najprej optimizirali metodo gojenja in preučevanja biofilma bakterij *C. jejuni* na interfazi tekočina/trda površina z vrstično konfokalno lasersko mikroskopijo. Želeli smo zagotoviti tudi ustrezno preučevanje sistema medcelične komunikacije bakterij *C. jejuni*. Iz tega razloga smo razvili, optimizirali in nadgradili metodo detekcije in kvantifikacije signalnih molekul AI-2, ki jih proizvaja

bakterija *C. jejuni* s posredno, biosenzorsko metodo in z direktno, kemijsko-analitsko metodo, HPLC-FLD.

1.3 RAZISKOVALNE HIPOTEZE

Raziskovalne hipoteze, ki smo jih postavili pred začetkom eksperimentalnega dela, so bile naslednje:

- Rastlinski pripravki sivke in njihove izbrane čiste spojine v subinhibitornih koncentracijah zmanjšujejo koncentracijo signalnih molekul AI-2 v izrabljenem gojišču bakterij *C. jejuni*.
- Zmanjšanje koncentracije signalnih molekul AI-2 v izrabljenem gojišču bakterij *C. jejuni* je posledica učinka rastlinskih pripravkov sivke na izražanje gena *luxS*.
- Zmanjšanje koncentracije signalnih molekul AI-2 korelira z zaviranjem pritrjevanja *C. jejuni* na abiotske površine z rastlinskimi pripravki sivke.
- Zmanjšanje koncentracije signalnih molekul AI-2 korelira z učinkovitostjo odstranjevanja zrelega biofilma *C. jejuni* z abiotskih površin z rastlinskimi pripravki sivke.

2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Protifilmotvorni učinek pripravkov sivke vrste *Lavandula angustifolia* proti bakteriji *Campylobacter jejuni*

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Raziskovalci intenzivno proučujejo nove pristope obvladovanja filmotvornosti bakterij *Campylobacter jejuni* v živilski industriji. Naravni pripravki, posebej rastlinski izvlečki, predstavljajo obetavne alternativne protimikrobne učinkovine za nadzor biofilma. Posušene cvetove sivke vrste *Lavandula angustifolia* smo uporabili za proizvodnjo eteričnega olja (LEO) in etanolnega izvlečka (LEF), kot tudi za proizvodnjo etanolnega izvlečka iz odpadnega materiala, pridobljenega po destilaciji eteričnega olja (LEW). Za nadaljnje testiranje smo uporabili tudi sedem glavnih čistih spojin, ki smo jih s kemijsko karakterizacijo določili v teh pripravkih. Pripravke sivke smo uporabili za odstranjevanje biofilma bakterij *C. jejuni* z abiotske površine. Za preučevanje molekularnih mehanizmov, na katerih temelji delovanje pripravka LEO proti pritrjevanju in gibljivosti bakterij *C. jejuni*, smo uporabili sekvenciranje naslednje generacije. Kemijska analiza pripravka LEO je pokazala, da so 1,8-cineol, linalol in linalil acetat njegove glavne spojine. Glavne sestavine pripravkov LEF in LEW so bili glikozidi fenolnih kislin, flavonoidi so bili redko prisotni. Minimalne inhibitorne koncentracije pripravkov sivke in čistih spojin proti bakteriji *C. jejuni* so znašale med 0,2 mg/mL in 1 mg/mL. Pripravek LEO je imel najmočnejši učinek na odstranjevanje biofilma bakterij *C. jejuni*. Učinek pripravkov sivke na zmanjšanje pritrjevanja bakterij *C. jejuni* je bil okoli 1 $\log_{10}$ CFU/ml, kar je v skladu s priporočili Evropske agencije za varnost hrane. Pripravki sivke so zmanjšali gibljivost bakterij *C. jejuni* za skoraj 50 %, kar posledično lahko vpliva na filmotvornost. Pridobljeni podatki so bili v skladu z analizo transkriptoma bakterij *C. jejuni*, ki je pokazala, da je pripravek LEO znižal izražanje genov, pomembnih za filmotvornost. Tudi pripravek LEW je pokazal dober protibakterijski učinek in učinek proti biofilmu, zlasti proti mehanizmom pritrjevanja in gibljivosti. Ta raziskava predstavlja inovativen pristop za boj proti biofilmom bakterij *C. jejuni* z uporabo alternativnih strategij in s tem potencial za razvoj novih učinkovitih sredstev, ki omogočajo preprečevanje in odstranjevanje biofilma.

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Antibiofilm Potential of *Lavandula* Preparations against *Campylobacter jejuni*

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ABSTRACT New approaches for the control of *Campylobacter jejuni* biofilms in the food industry are being studied intensively. Natural products are promising alternative antimicrobial substances to control biofilm production, with particular emphasis on plant extracts. Dried flowers of *Lavandula angustifolia* were used to produce essential oil (LEO), an ethanol extract (LEF), and an ethanol extract of *Lavandula* postdistillation waste material (LEW). The chemical compositions determined for these *Lavandula* preparations included seven major compounds that were selected for further testing. These were tested against *C. jejuni* for biofilm degradation and removal. Next-generation sequencing was used to study the molecular mechanisms underlying LEO actions against *C. jejuni* adhesion and motility. Analysis of LEO revealed 1,8-cineol, linalool, and linalyl acetate as the main components. For LEF and LEW, the main components were phenolic acid glycosides, with flavonoids rarely present. The MICs of the *Lavandula* preparations and pure compounds against *C. jejuni* ranged from 0.2 mg/ml to 1 mg/ml. LEO showed the strongest biofilm degradation. The reduction of *C. jejuni* adhesion was ≥1 $\log_{10}$ CFU/ml, which satisfies European Food Safety Authority recommendations. *Lavandula* preparations reduced *C. jejuni* motility by almost 50%, which consequently can impact biofilm formation. These data are in line with the transcriptome analysis of *C. jejuni*, which indicated that LEO downregulated genes important for biofilm formation. LEW also showed good antibacterial and antibiofilm effects, particularly against adhesion and motility mechanisms. This defines an innovative approach using alternative strategies and novel targets to combat bacterial biofilm formation and, hence, the potential to develop new effective agents with biofilm-degrading activities.

IMPORTANCE The *Lavandula* preparations used in this study are found to be effective against *C. jejuni*, a common foodborne pathogen. They show antibiofilm properties at subinhibitory concentrations in terms of promoting biofilm degradation and inhibiting cell adhesion and motility, which are involved in the initial steps of biofilm formation. These results are confirmed by transcriptome analysis, which highlights the effect of *Lavandula* essential oil on *C. jejuni* biofilm properties. We show that the waste material from the hydrodistillation of *Lavandula* has particular antibiofilm effects, suggesting that it has potential for reuse for industrial purposes. This study highlights the need for efforts directed toward such innovative approaches and alternative strategies against biofilm formation and maintenance by developing new naturally derived agents with antibiofilm activities.

KEYWORDS *Lavandula* preparations, antibiofilm activity, *Campylobacter jejuni*

The food industry is in a constant battle against economic losses caused by the formation and prevalence of bacterial biofilms on food manufacturing surfaces. Moreover, bacterial biofilms, as a persistent form of bacterial lifestyle in food processing environments, are a significant public health problem (1). One of the most prevalent foodborne

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pathogens and the major cause of human intestinal infections in developed countries is *Campylobacter jejuni* (2, 3). Human infections occur mainly by consumption of undercooked meat, especially poultry, or food that has been in contact with a contaminated surface (4).

Campylobacter jejuni can form biofilms on different abiotic surfaces used in the food industry (e.g., polystyrene, glass, and stainless steel), which is crucial for its environmental survival under various conditions, and thus remains a significant food-safety challenge in the food industry (5). A major problem is also the widespread antibiotic resistance of *C. jejuni* to a wide range of antibiotics as well as resistance to various biocides used for disinfection in the food industry (6–8).

Cost-effective chemical and physical methods are used for biofilm eradication in the food industry; however, these show only limited success (9). As biofilms contribute to bacterial pathogenicity and resistance against antibiotics and biocides, innovative strategies are needed to inhibit the bacterial properties involved (e.g., motility and adhesion) or to degrade or remove biofilms as a control against foodborne pathogens. Natural products have been shown to represent promising alternatives that can be effective antimicrobial agents against numerous bacterial targets that are important for biofilm formation and do not create selection pressure for the emergence of further antibiotic-resistant bacteria (10).

One such commonly used crop worldwide belongs to the *Lamiaceae* family and the genus *Lavandula*, some strains of which are *Lavandula angustifolia* Mill (true lavender, *Lamiaceae*), *Lavandula latifolia* Medik (spike lavender), and *Lavandula* × *intermedia* Emeric ex Loisel (lavandin) (11). These have been used since ancient times for medicinal purposes and more recently in industrial processes (12, 13).

The genus *Lavandula* grows around the Mediterranean and in southern Europe, northeastern Africa, the Middle East, southwest Asia, and southeast India (14). These aromatic plants have a rich essential oil content, for which sedative, carminative, antidepressive, antispasmodic, antimicrobial, and anti-inflammatory effects have been reported (15, 16). Along with one of their main components, linalool (17, 18), lavender and lavandin essential oils can have strong antibiofilm effects against the bacteria *Staphylococcus aureus*, *Escherichia coli*, and *Campylobacter* spp. (19–21). However, there have not been any studies that have investigated the potential molecular mechanisms of the antibiofilm actions of *Lavandula* essential oil, and so the present study investigates this.

In addition to alternative strategies using low doses of bioactive phytochemicals to control bacterial biofilms, this study has focused also on the use of by-products of essential oil distillation. More recently, the demand for *Lavandula* essential oils has grown hugely, which has resulted in greater cultivation and production of these plants. Approximately 200 tons of lavender oil and 1,200 tons of lavandin oil are produced annually, and there has been a concomitant increase in the by-products that are accumulated as waste materials and hydrolates or aromatic waters after essential oil production (22–24). These by-products and especially waste from the agro-food industry also represent a potentially important source of effective phytochemicals (10). For example, waste materials from spike lavender have antioxidant properties (13), and lavender hydrolates have antifungal and antibacterial properties that enhance the flavor and taste of foods (25). Reuse of these waste materials will partially solve one of the major problems—their disposal (26). In addition, the reuse of such a material is environmentally friendly, as the use of disinfectants, which are commonly used in the food industry, can lead to additional unnecessary chemicals in the environment (8). However, current studies have been focused only on planktonic cell suspensions, and there have not been any reports on antibiofilm effects of such *Lavandula* by-products. This is important, as biofilms are the most prevailing growth lifestyle in bacteria, and it is well known that in biofilms, the effectiveness of antimicrobials is significantly reduced (up to 1,000-fold) compared to that in the planktonic state (27).

In this study, *Lavandula* preparations and selected pure compounds were used to determine whether they have antibiofilm effects against *C. jejuni* NCTC 11168. Dried

TABLE 1 Identification of the main common flavonoids in the *Lavandula* ethanolic extract and in the ethanolic extract of waste material from hydrodistillation

No.	Compound identified	Full scan MS (*m/z*)	Fragment ions (MS^2; *m/z*)[a]	UV maximum (nm)[b]
1	Coumaric acid hexoside I	325 $[M-H]^-$	163 (100), 119 (25)	263, 290 sh
2	Caffeic acid hexoside	387 $[M+HCOOH-H]^-$	341 (100), 207 (25)	302
3	Ferulic acid hexoside I	355 $[M-H]^-$	193 (100), 149 (20)	302
4	Coumaric acid hexoside II	371 $[M+HCOOH-H]^-$, 325 $[M-H]^-$	325 (100)	277, 290 sh
5	Ferulic acid hexoside II	401 $[M+HCOOH-H]^-$, 355 $[M-H]^-$	355 (100)	295, 319
6	Apigenin-7-*O*-glucoside	431 $[M-H]^-$	269 (100)	268, 334
7	Rosmarinic acid	359 $[M-H]^-$	161 (100), 179 (30), 223 (10)	292 sh, 328

[a]Numbers in parentheses are percent relative intensities.
[b]sh, shoulder.

flowers of *Lavandula angustifolia* were used to produce an essential oil (LEO), an ethanol extract (LEF), and the ethanol extract of *Lavandula* postdistillation waste material (LEW). First, degradation of *C. jejuni* biofilms formed on an abiotic surface using these *Lavandula* preparations was investigated. Furthermore, strategies to target motility and adhesion of *C. jejuni* were introduced, with the aim to prevent or reduce biofilm formation in its early stages. Finally, an RNA sequencing (RNA-seq) approach was used to better understand the molecular mechanisms underlying the actions of LEO against *C. jejuni* biofilms and thus indicate innovative *Campylobacter* control strategies.

RESULTS

Dried flowers of *Lavandula* were used to produce LEO, the essential oil, LEF, an ethanol extract of flowers prior to distillation, and LEW, an ethanol extract of *Lavandula* postdistillation waste material. Each of these was initially analyzed for their chemical composition. Then, the main pure compounds were selected and included in the anti-*Campylobacter* analysis and MIC determinations, with the aim to compare the activities of the *Lavandula* preparations with those of the pure compounds. This analysis started with the innovative strategy of degradation and removal of these preformed biofilms of *C. jejuni* NCTC 11168. Second, the first phases of biofilm formation in which *C. jejuni* adhesion and motility are the predominant factors were targeted. Finally, a molecular level evaluation of the mode of action of LEO was performed.

Phytochemical analysis and identification of flavonoids. The ethanolic extract of the *Lavandula* flowers, LEF, and the ethanolic extract of *Lavandula* postdistillation waste material, LEW, were analyzed for their phenolic compound contents using liquid chromatography-photo diode array-electrospray ionization mass spectrometry. LEF and LEW had phenolic acid glycosides as their major constituents. In addition, rosmarinic acid and the flavone apigenin-7-*O*-glucoside were detected. Flavonoids only had a minor role in the composition of LEF and LEW. Table 1 summarizes the peaks that were identified for both LEF and LEW. Figure S1 in the supplemental material illustrates a representative UV chromatogram. Compound 4 in Table 1 is most probably identical to the *o*-coumaric acid-2-*O*-glucoside previously described for *L. angustifolia* (28), as indicated by the UV spectral data; however, *m*-coumaric acid as an aglycon cannot be excluded at this point.

According to the gas chromatography-mass spectrometry analysis, the *Lavandula* essential oil, LEO, was particularly rich in 1,8-cineol, a cyclic monoterpene (36.1%), and the terpene alcohol linalool (15.8%). LEO was also rich in the furanoids *cis*-linalool oxide (7.9%) and *trans*-linalool oxide (5.9%). Two other main components were linalyl acetate (6.4%) and the monoterpenoid camphor (5.9%). Other components identified at >1% were α-terpineol, octen-3-yl-1-acetate, lavandulyl acetate and thymol, with the content of borneol close to 1% (Table 2).

According to these analyses, 1,8-cineol, linalool, linalyl acetate, camphor, (−)-borneol, *p*-coumaric acid, and *trans*-ferulic acid were selected as the individual pure compounds to be included in further analysis.

TABLE 2 Identification of the main components of the *Lavandula* essential oil

Retention time (min.)	Retention index[a]	Compound identified[b]	Quantification (% of total)[c]
5.50	932	α-Pinene	0.27
5.90	946	Camphene	0.17
6.68	975	β-Pinene	0.39
7.79	1012	Hexyl acetate	0.20
8.18	1023	*p*-Cymene	0.32
8.41	1030	1,8-Cineol	36.11
9.88	1071	*cis*-Linalool oxide (furanoid)	7.90
10.47	1087	*trans*-Linalool oxide (furanoid)	5.87
10.95	1100	Linalool	15.79
11.43	1112	Octen-3-yl-1-acetate	1.14
12.65	1142	Camphor	5.93
13.12	1153	Nerol oxide	0.61
13.53	1163	Borneol	0.97
13.90	1172	*cis*-Linalool oxide (pyranoid)	0.66
14.03	1175	Terpinen-4-ol	0.56
14.60	1189	α-Terpineol	2.19
14.72	1192	Hexyl butanoate	0.44
16.63	1237	Cuminaldehyde plus hexyl-2-methyl butanoate	0.43
17.41	1256	Linalyl acetate	6.37
18.91	1291	Lavandulyl acetate	1.62
18.97	1293	Thymol	1.41
22.00	1365	Neryl acetate	0.32
22.80	1384	Geranyl acetate	0.61
30.57	1579	Caryophyllene oxide	0.97
34.39	1682	α-Bisabolol	0.21

[a]Linear retention index relative to *n*-alkanes on HP-5-MS column.
[b]Compounds identified by mass spectral libraries (63–65; laboratory database) and retention index (RI) comparison (63).
[c]Quantification by normalization (area percent method) without considering calibration factors.

Anti-*Campylobacter* activity of *Lavandula* preparations and pure compounds. To evaluate the anti-*Campylobacter* activities of the *Lavandula* preparations and the seven chosen pure compounds as the main phytochemical compounds of LEO, LEF, and LEW, their MICs against *C. jejuni* NCTC 11168 were determined (Table 3). LEO and the pure compounds linalool, (−)-borneol, camphor, and 1,8-cineol had medium anti-*Campylobacter* activities, with MICs against *C. jejuni* NCTC 11168 of 0.25 mg/ml, with the last two compounds with MICs of 0.5 mg/ml. A stronger anti-*Campylobacter* effect was seen for linalyl acetate, with a MIC against *C. jejuni* NCTC 11168 of 0.2 mg/ml. Interestingly, LEW had the same anti-*Campylobacter* activities as LEF and also with *p*-coumaric acid and *trans*-ferulic acid, with MICs of 1 mg/ml.

Degradation of preformed biofilms by the *Lavandula* preparations. To determine the effects of these *Lavandula* preparations on *C. jejuni* biofilms, a novel approach was developed based on culturing *C. jejuni* at the air-liquid interface for 48 h. After treatment with the

TABLE 3 MICs against *C. jejuni* NCTC 11168 for the *Lavandula* preparations (LEO, LEF, and LEW) and pure compounds

Treatment	MIC (mg/liter)
Lavandula essential oil	0.25 ± 0.06
Lavandula ethanol extract	1.00 ± 0.25
Lavandula waste material	1.00 ± 0.25
Linalool	0.25 ± 0.06
Linalyl acetate	0.20 ± 0.05
Camphor	0.50 ± 0.12
(−)-Borneol	0.25 ± 0.06
1,8-Cineol	0.50 ± 0.12
p-Coumaric acid	1.00 ± 0.25
trans-Ferulic acid	1.00 ± 0.25

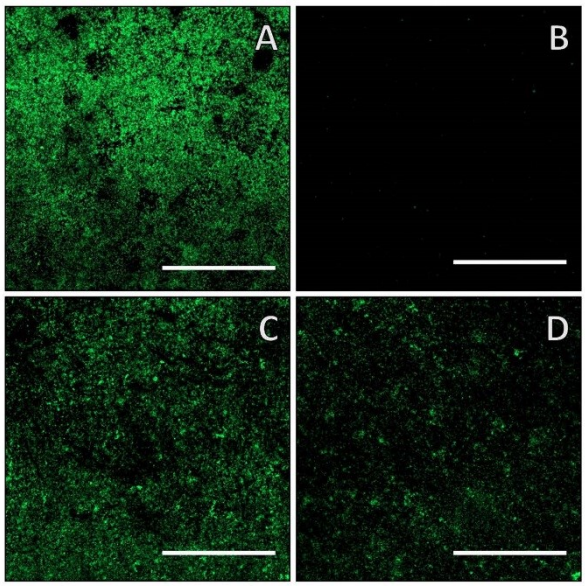

FIG 1 Representative fluorescence microscopy images of *C. jejuni* NCTC 11168 biofilms grown on glass surfaces treated with different *Lavandula* preparations. (A) Positive control incubated in MH broth with addition of *Campylobacter* growth supplement for 72 h in a microaerobic atmosphere at 42°C. (B to D) After 48 h of growth, the mature biofilms were exposed to the *Lavandula* preparations for 24 h: essential oil (LEO) (B), ethanol extract (LEF) (C), and ethanol extract of *Lavandula* postdistillation waste material (LEW) (D), all here at 2× MIC (see Table 3), under the same conditions as for panel A. Scale bars, 200 μm.

Lavandula preparations, this allowed measurement of the relative area of the glass surface covered by the biofilm by using confocal laser scanning microscopy. The images of the untreated cultures showed the biofilm structure of the viable bacteria, which were mainly dispersed into small microcolonies (Fig. 1). The influence of different concentrations of LEO, LEF, and LEW on the biofilm structure and the cell viabilities were determined after 24 h of treatments (Fig. 2). Interestingly, when the biofilms were exposed to LEO, LEF, and LEW, there was significant degradation of the biofilms across all of these treatments. LEO was the most effective, as it effectively removed the bacterial biofilms. LEF and LEW had similar effects for *C. jejuni* biofilm removal (Fig. 2). After the treatments of the *C. jejuni* biofilms with LEF and LEW at 8× MIC, the relative surface cover with live cells was 0.016 ± 0.005 to 0.031 ± 0.002, respectively. Surprisingly, for LEO, the same was seen for concentrations as low as 1× MIC and 2× MIC, with a relative surface cover of 0.043 ± 0.005 and almost zero, respectively. Interestingly, even for LEO at 0.5× MIC, the relative cover of the glass surface was affected, with a relative surface cover of 0.38 ± 0.15. Additionally, the lowest concentration of LEW was more effective than LEF, with relative surface cover for LEW at 2× MIC of 0.51 ± 0.09 compared with LEF at 2× MIC of 0.88 ± 0.01.

Modulation of *Campylobacter* adhesion by *Lavandula* preparations and pure compounds. *Campylobacter jejuni* adhesion to an abiotic surface was used as a strategy to target one of the first steps of biofilm formation. The effects of LEO, LEF, and LEW and the pure compounds on adhesion of *C. jejuni* NCTC 11168 to a polystyrene surface was measured as the numbers of adhered cells (CFU/ml) after 4 h, 8 h, and 24 h of treatment with subinhibitory concentrations (Fig. 3). Different times were selected to show these effects on adhesion to the abiotic surface and for the mechanisms involved, which are important for reversible (4 h and 8 h) and irreversible (24 h) attachment.

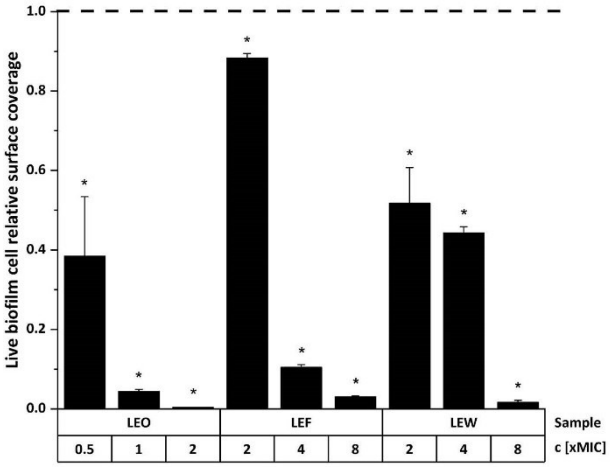

FIG 2 Relative *C. jejuni* NCTC 11168 biofilm coverages of the glass surface following exposure to the *Lavandula* preparations, in comparison to the untreated *C. jejuni* control (dashed line). After 48 h of growth, the mature biofilms were exposed to the *Lavandula* preparations for 24 h: LEO (essential oil) at 0.5×, 1×, and 2× MIC; LEF (ethanol extract) and LEW (ethanol extract of *Lavandula* postdistillation waste material) at 2×, 4×, and 8× MIC. Data are relative values of biofilm cover of the glass surface presented as means ±standard deviations. *, $P < 0.05$ versus control.

The relative adhesion of the *C. jejuni* cells was significantly lower after treatments with all of these *Lavandula* preparations, with the strongest effects seen after 4 h (Fig. 3). The most effective treatments here were with LEW at 4 h, when the relative adhesion was 0.002 ± 0.001 (Fig. 3A) and 0.04 ± 0.01 for LEF at 8 h (Fig. 3B). These data also indicated that LEO, LEF, and LEW all had comparable effects on *C. jejuni* adhesion to a polystyrene surface after 24 h of incubation, where the relative adhesion was 0.08 ± 0.07 for all *Lavandula* preparations (Fig. 3C).

Interestingly, the relative adhesion after the treatments with LEO, LEF, and LEW was comparable and in the same ranges as those for camphor, (−)-borneol and *p*-coumaric acid at 4 h and 24 h of treatment and for *p*-coumaric acid and *trans*-ferulic acid at 8 h of treatment. Also, the other pure compounds, linalool, linalyl acetate, and 1,8-cineol, were more effective after 4 h, where the relative adhesion was 0.21 ± 0.08 for linalool and 0.17 ± 0.05 for both linalyl acetate and 1,8-cineol (Fig. 3A).

Modulation of *Campylobacter* motility by *Lavandula* preparations and pure compounds. As one of the first steps that are important for biofilm formation, cell motility was also targeted. The influence of LEO, LEF, and LEW and the pure compounds was measured on soft agar and compared with untreated cells. Their subinhibitory concentrations all significantly decreased *C. jejuni* NCTC 11168 motility (Fig. 4). LEO, LEF, and LEW had comparable effects on *C. jejuni* relative motility, at 0.47 ± 0.03, 0.45 ± 0.10, and 0.45 ± 0.09, respectively, in comparison with the motility of untreated *C. jejuni* colonies. The greatest antimotility effect of the pure compounds was seen for (−)-borneol, at 0.41 ± 0.03 relative to the motility of the untreated *C. jejuni* colonies.

Effects of LEO on the transcriptome of *C. jejuni* NCTC 11168. It is important to evaluate the mechanisms and the potential targets behind these reductions in biofilm-related contamination and for eradication of biofilm-related infections. According to the effects on *C. jejuni* biofilm degradation and on motility and adhesion in the early phase of biofilm formation, we selected LEO to extend this study to the molecular mechanisms behind these actions against *C. jejuni* NCTC 11168. For the biofilms, LEO at its MIC was already sufficient, while to effectively reduce *C. jejuni* motility and adhesion to polystyrene,

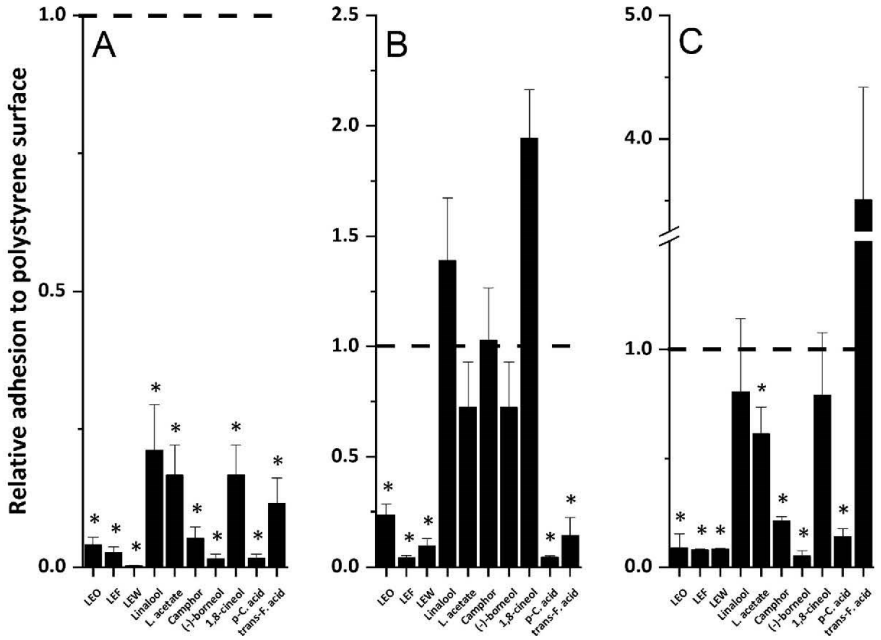

FIG 3 Relative adhesion of *C. jejuni* NCTC 11168 to a polystyrene surface after 4 h (A), 8 h (B), and 24 h (C) of exposure to the *Lavandula* preparations and pure compounds (as indicated) at 0.25× MIC in comparison to that of untreated *C. jejuni* controls (dashed lines). Attached cells were suspended by sonication, and their concentrations were determined by plate counting. Data are relative values of adhered cells as means ±standard deviations. * $P < 0.05$ versus control. LEO, essential oil; LEF, ethanol extract; LEW, ethanol extract of *Lavandula* postdistillation waste material.

LEO was effective at subinhibitory concentrations (i.e., 0.25× MIC). Thus, the mechanism of these LEO actions against *C. jejuni* were evaluated with the major focus on the genes modulated and the cell processes they are involved in.

Campylobacter jejuni NCTC 11168 in exponential growth phase was treated with LEO for 30 min and with a dimethyl sulfoxide (DMSO) control to eliminate any influence of the solvent used for LEO. The transcriptomic analysis was for the differentially expressed genes in comparison to expression in control cultures ($P \leq 0.05$), with a view to predicting their generic functions and, of greater interest, their known functions. Strain-specific RNA-seq was analyzed using Ion Torrent technology. The reads were mapped to the annotated *Campylobacter jejuni* subsp. *jejuni* reference genome, with the data available at NCBI under BioProject accession number PRJNA57587.

(i) Differential gene expression for *C. jejuni* NCTC 11168 treated with LEO. Detailed analysis showed that 326 genes were differentially expressed in *C. jejuni* NCTC 11168 when treated with LEO, with normalisation to the DMSO control ($\log_2$ fold change [$\log_2$FC] ≥ 1; $P \leq 0.05$). Among these, 138 genes were upregulated and 188 genes were downregulated (Table S1).

The LEO treatment upregulated 42 genes that are involved in the synthesis of ribosomal and export proteins in *C. jejuni* (Table S1; Fig. S2). This led to global changes in gene expression of various pathways, including the tricarboxylic acid (TCA) cycle and those of pyruvate metabolism, nicotinate, and nicotinamide metabolism, folate biosynthesis, aromatic amino acid biosynthesis, and terpenoid and porphyrin biosynthesis (see Fig. S2). The genes in the main efflux system of *C. jejuni* involved in β-lactam resistance were also upregulated (i.e., *cmeA*, *cmeB*, and *cmeC*) (Fig. S2).

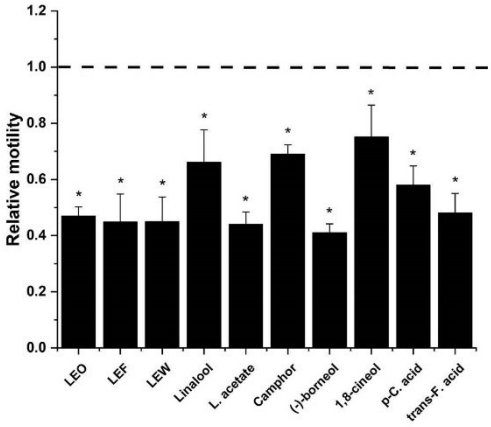

FIG 4 Relative motility of *C. jejuni* NCTC 11168 after exposure to the *Lavandula* preparations and pure compounds (as indicated) at 0.25× MIC (see Table 3) in comparison to that of the untreated *C. jejuni* control (dashed line). The diameters of swarming colonies were measured after 48 h of incubation on soft agar. Data are relative values as means ± standard deviations. *, $P < 0.05$ versus control. LEO, essential oil; LEF, ethanol extract; LEW, ethanol extract of *Lavandula* postdistillation waste material.

Of the 188 downregulated genes, 73 are involved in synthesis of transmembrane proteins and are important for the transport of different proteins and ions. Many genes involved in the iron uptake system were also downregulated, including the ABC transporters and TonB box (i.e., *chuA*, *chuB*, *tonB3*, *tonB2*, *exbB1*, *exbB2*, *Cj0178*, and *ceuC*) (Table S1; Fig. S2). Moreover, four genes involved in stress defense were downregulated (i.e., *cstA*, *hrcA*, *dcuA*, and *ppk*) (Table S1).

It is worth noting that 24 of the differentially expressed genes are involved in *C. jejuni* motility and biofilm formation (Table 4). Most of the genes that are involved in

TABLE 4 Differentially expressed genes that are important for biofilm formation of *C. jejuni* NCTC 11168[a]

Gene	Log_2 fold change	Description
folK	−1.9	2-Amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase, with genes important for flagellar synthesis
motA	−1.1	Flagellar motor proton channel
flaG	−1.0	Flagellar protein
Cj0719c	−1.2	Involved in PLP[b] homeostasis, with genes important for synthesis of flagella
pseF	−1.3	Pseudaminic acid cytidylyltransferase, catalyzes the final step in the biosynthesis of pseudaminic acid, a sialic-acid-like sugar that is used to modify flagellin
maf4	−1.4	Uncharacterized protein, with *flaA* gene, which enables synthesis of flagellin
flgG	−1.0	Flagellar basal-body rod protein
flgI	−1.1	Flagellar P-ring protein
Cj1467	−1.4	Uncharacterized protein, with genes important for synthesis of flagella
fliQ	−1.1	Flagellar biosynthetic protein
kpsM	−1.9	Capsule polysaccharide export system inner membrane protein
kpsS	−1.2	Polysaccharide modification protein
murB	−1.0	UDP-*N*-acetylenolpyruvoylglucosamine reductase, involved in cell wall formation
mrdB	−1.2	RodA protein homolog, involved in cell wall formation
bamD	−2.1	Outer membrane protein
waaC	−1.2	Lipopolysaccharide heptosyltransferase
lgt	−1.5	Prolipoprotein diacylglyceryl transferase
Cj0262	1.0	Methyl-accepting chemotaxis signal transduction protein
lpxA	1.3	Involved in biosynthesis of lipid A
peB3	1.9	Major antigenic peptide, with *flaA* and *flaC* genes, which enable synthesis of flagellin
peB2	1.0	Major antigenic peptide

[a]Differential expression was defined as a false discovery rate *P* value of ≤0.05.
[b]PLP, pyridoxal 5′-phosphate.

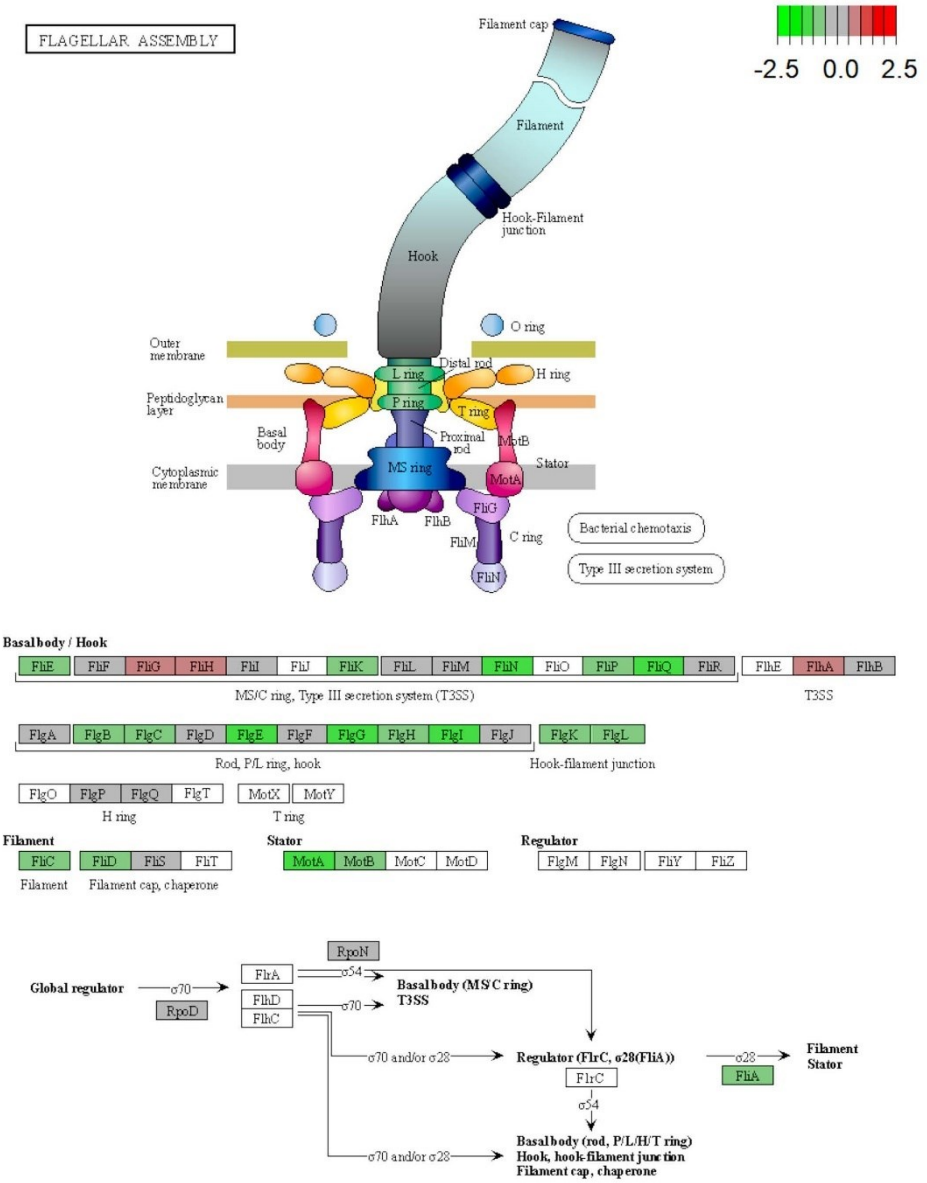

FIG 5 Pathway map of the *C. jejuni* NCTC 11168 flagellar assembly (Cje02040). This assembly showed upregulated (red) and downregulated (green) genes in *C. jejuni* after exposure to the essential oil LEO at 0.25× MIC for 30 min (see Table 3). Those genes are essential for locomotion and flagellum-dependent motility, which represent two features necessary for biofilm formation of *C. jejuni*. KEGG map of differentially expressed genes involved in flagellar assembly was constructed using Pathview (70) and the KEGG genome database (https://www.genome.jp/kegg/pathway.html).

TABLE 5 Gene ontology categories that are significantly enriched in terms of biological processes and molecular functions differentially regulated between LEO-treated samples and control samples

GO code	Term	No. of genes differentially expressed[a]	FDR *P* value	Regulation
Biological process				
0040011	Locomotion	5/32	0.054026	Down
0071973, 0097588, 0001539, 0048870, 0006928	Cilium or flagellum-dependent cell motility	3/23	0.012148	Down
0065007	Biological regulation	19/83	0.062751	Down
0044271	Cellular nitrogen compound biosynthetic process	65/183	1.62E−10	Up
0006430	Transfer RNA aminoacylation	2/22	0.006264	Up
0006807	Nitrogen compound metabolic process	87/433	2.16E−08	Up
Molecular function				
0046873	Metal ion transmembrane transporter activity	2/16	0.061105	Down
0015103	Organic anion transmembrane transporter activity	4/10	0.085316	Down
0019843	rRNA binding	27/39	1.51E−15	Up
0003735	Structural constituent of ribosome	36/52	1.00E−15	Up
0005198	Structural molecule activity	37/59	1.14E−14	Up
0097159, 1901363	Organic cyclic compound binding	93/436	0.006437	Up
0003676	Nucleic acid binding	54/175	4.16E−05	Up
0000049	tRNA binding	6/18	0.009744	Up
0005488	Binding	113/588	0.048065	Up
0140101	Catalytic activity, acting on a tRNA	2/43	0.048065	Up
0004812, 0016875	Aminoacyl-tRNA ligase activity	2/22	0.005088	Up
0140098	Catalytic activity, acting on RNA	9/64	0.069198	Up

[a]Absolute $\log_2$ fold change of ≥1; false discovery rate [FDR] *P* value of ≤0.05 relative to total number of genes in enriched category.

flagellar assembly of *C. jejuni* were downregulated, and 18 of them had a fold change lower than −1 ($\log_2$FC) (Fig. 5).

(ii) Gene ontology enrichment analysis. Gene ontology (GO) enrichment analysis of the significant differentially expressed genes (FC ≥ 1, $P \leq 0.05$) in these LEO-treated samples compared with expression in controls showed the following two categories (Table 5): (i) biological processes with upregulation that included cellular nitrogen compound biosynthetic processes, tRNA aminoacylation, and nitrogen compound metabolic processes, and with downregulation for locomotion, cilium or flagellum-dependent cell motility, and biological regulation, and (ii) molecular functions with upregulation that included catalytic activity, nucleic acid and organic cyclic compound binding, and structural molecules, and with downregulation for transmembrane transport.

DISCUSSION

In the present study, *Lavandula* preparations (i.e., LEO, LEF, and LEW) were used, with the aim to modulate the *Campylobacter* properties that are responsible for the initial phase of the multifactorial event of biofilm formation on abiotic polystyrene and glass surfaces. In doing so, we used a new approach of culturing and studying biofilms formed at the air-liquid interface. Also, we investigated LEO, LEF, and LEW as effective control strategies for biofilm degradation and removal under conditions that do not provide selection pressure for further development of antibiotic resistance of *Campylobacter* spp.

The occurrence of persistent and resistant *C. jejuni* cells in their biofilm form is associated worldwide with severe infections and gastrointestinal diseases in humans (4). The attachment of biofilms to different abiotic surfaces is responsible for major problems in the food industry, as the reversible binding turns into irreversible bonding and biofilm formation, with possible further dispersion and contamination of the food matrix (9, 29). Therefore, alternative strategies to either remove preformed biofilms or inhibit biofilm formation based on alternative antimicrobial agents of natural origin represent an innovative control approach, as we have described here.

For this purpose, dried flowers of *Lavandula angustifolia* were used to produce LEO, the essential oil, LEF, the ethanol extract of flowers prior to distillation, and LEW, the

ethanol extract of *Lavandula* postdistillation waste material. Interestingly, LEF and LEW had similar chemical compositions. As expected, their main constituents were phenols, with the detection of rosmarinic acid, the flavone apigenin-7-*O*-glucoside, and a number of phenolic acid glycosides. Torras-Claveria et al. (24) reported a comparable composition for *Lavandula* × *intermedia* waste material, which in their case contained glucosides of coumaric, ferulic, and caffeic acids in addition to rosmarinic acid, chlorogenic acid, and a number of flavone glycosides. As the identification in the present study was derived from mass spectrometry and UV data, the sugar moieties were designated only as hexosides, although glucosidation is most likely to occur, according to other reports for *Lavandula* spp. (24, 28). The complete chemical profile was determined here for the essential oil, LEO, with 1,8-cineol, linalool, and linalyl acetate as the major compounds. This confirms the origin of LEO as the genus *Lavandula* (17), although the high levels of 1,8-cineol and linalool oxides indicate a high degree of oxidation for LEO, most likely due to extended storage after purchase of the plant material. On this basis, the chemical composition of LEO was only partially comparable with those in extensive previous studies in which linalool and linalyl acetate have been seen as the main components (12, 30–32). According to the chemical compositions of LEO, LEF, and LEW, the following seven main compounds were selected for further testing: 1,8-cineol, linalool, linalyl acetate, camphor, (−)-borneol, *p*-coumaric acid, and *trans*-ferulic acid. The antibacterial effects of these pure compounds were then compared with the antibacterial effects of the *Lavandula* preparations (LEO, LEF, and LEW) against *C. jejuni*.

Linalyl acetate had the strongest effect, while (−)-borneol, camphor, and 1,8-cineol had comparable antibacterial effects to that of LEO. Similarly, Blažeković et al. (12) showed that essential oils of *L.* × *intermedia* Budrovka and *L. angustifolia*, as well as linalool and linalyl acetate, have antibacterial effects against numerous Gram-positive and Gram-negative bacteria. For comparison, the *Lavandula* preparations and pure compounds used in the present study had stronger antibacterial effects against the Gram-negative *C. jejuni*. However, according to the higher levels of oxidized compounds in LEO than in the essential oils from *L.* × *intermedia* Budrovka and *L. angustifolia* tested by Blažeković et al. (12), a stronger antimicrobial effect might have been expected. At the same time, antibacterial effects of plant preparations can depend strongly on the type and storage of the plant material, the method of preparation of the plant material, the type of microorganisms used, the inoculum volumes, and the culture medium used, along with the pH, temperature, and incubation time (33, 34).

The moderate but comparable antibacterial effects of the ethanol extract LEF and the waste material LEW, as well as for *p*-coumaric acid and *trans*-ferulic acid, showed that LEW contains a diverse pool of bioactive compounds with antimicrobial properties. This is consistent with previous studies on postdistillation thyme waste, pinot noir grape skins and seeds, and postdistillation juniper fruit waste, where the advantages of agricultural waste material as promising antimicrobial agents with potential industrial applications were demonstrated (35, 36).

The *Lavandula* preparations in this study were effective for degradation and removal of the *C. jejuni* biofilms. LEO had the strongest effects on these mature *C. jejuni* biofilms. Similarly, Dănilă et al. (37) reported that lavender essential oil is an effective antibiofilm agent against *Staphylococcus epidermidis* clinical strains. It has also already been shown that various essential oils are good antibiofilm agents on various surfaces (38, 39). As showed here, LEO is rich in secondary metabolites, which are probably responsible for the degradation of the extracellular polymer matrix of the biofilms (40). Part of the *C. jejuni* polymers in the extracellular matrix can also form a capsule (41). Indeed, this study has shown that the two major proteins that are involved in the formation of the capsule in *C. jejuni* (i.e., KpsM and KpsS) were downregulated. High levels of numerous secondary metabolites might also modulate the synthesis and activity of enzymes to cause capsule degradation while disturbing the uptake of nutrients. These can also alter the pH gradient of the cell cytoplasm and thus cause cell death (42).

This was also confirmed by GO analysis of *C. jejuni* NCTC 11168, where LEO was seen to modify different biological and metabolic processes. In addition, it was shown that LEF and LEW have comparable effects to each other on biofilm removal, which might be due to their similar chemical compositions. Particularly good antibiofilm effects of this *Lavandula* agriculture waste material, LEW, emphasize its potential as an antimicrobial agent with possible industrial applications for precoating abiotic surfaces and for their disinfection.

The *Lavandula* preparations were successful for the degradation of these mature *C. jejuni* biofilms and were further used to determine whether they can be used to prevent biofilm formation. The first phase of biofilm formation is the attachment of the cells to the surface (29). Here, these *Lavandula* preparations reduced the adhesion of *C. jejuni* to polystyrene after 4, 8, and 24 h of incubation. These reductions in the adhesion were by $\geq$1 $\log_{10}$ CFU/ml, which satisfies the recommendations proposed by the European Food Safety Authority (3). These results are also comparable to the effects of lavandin essential oil and ethanol extracts from *Urtica dioica* on the adhesion of *C. jejuni* (21).

To potentially focus on the components of these *Lavandula* preparations that are particularly important for this reduced *C. jejuni* adhesion, the seven major components as pure compounds were also tested, with various effects seen. For example, (−)-borneol reduced *C. jejuni* adhesion the most after 24 h of treatment, although this effect was weaker after 4 h and 8 h. On the other hand, *trans*-ferulic acid reduced *C. jejuni* adhesion after 4 h and 8 h of treatment, but this was lost after 24 h. These differing phenomena might be due to effects of the pure compounds on different targets at different times. It is interesting to note, however, that these main components from the essential oil and ethanol extracts did not have as good of effects against *C. jejuni* adhesion as the *Lavandula* preparations. Likewise, Duarte et al. (20) showed that coriander essential oil had greater effects against *C. jejuni* and *Campylobacter coli* adhesion than the pure compound linalool. This indicates that it is the combination of the individual components in these *Lavandula* preparations that is important for these antiadhesion effects.

Furthermore, the effects of the *Lavandula* preparations and pure compounds were monitored for *C. jejuni* motility, which is important for both colonization of surfaces and survival in the environment through biofilm formation (10, 43). All three of the *Lavandula* preparations and all pure compounds indeed reduced the motility of *C. jejuni*. The *Lavandula* preparations all had similar effects on *C. jejuni* motility. Linalyl acetate and (−)-borneol had similar effects as LEO, while LEO had stronger antimotility effects than linalool and camphor. This also suggests that the combination of the pure compounds in LEO is important for these antimotility effects. For LEF and LEW, these both had stronger antimotility effects than *p*-coumaric and *trans*-ferulic acids, which suggests that these two compounds only contribute to the higher antimotility effects of the combined components of LEF and LEW.

The RNA-seq data provided new insights into the influence of LEO on gene expression in *C. jejuni*. It can be noted that many ribosomal genes were upregulated, which could be the result of a global change in the transcriptome that caused transcriptional reprogramming. Transcriptional reprogramming might indicate that the bacteria are under intense stress (44). When bacteria are under stress, the stringent response is activated, which affects the expression of ribosomal genes that are necessary for stress resistance (45). Upregulation of the CmeABC efflux system confirms that *C. jejuni* was under severe stress while exposed to LEO. Nevertheless, it can be seen that this transcriptional reprogramming affected the gene expression of many pathways, including the TCA cycle, pyruvate metabolism, nicotinate and nicotinamide metabolism, folate biosynthesis, aromatic amino acid biosynthesis, and terpenoid and porphyrin biosynthesis (see Fig. S2 in the supplemental material). Many transmembrane proteins were downregulated, including proteins involved in the iron-uptake system. Askoura et al. (46) have shown that iron acquisition genes are downregulated during acid stress, and

so it can be hypothesized that exposure of *C. jejuni* to LEO also led to stress responses in these bacteria. It is important to note that the iron-uptake system is involved in the colonization, biofilm formation, and pathogenicity of this pathogen (47, 48), and so downregulation of this pathway might lead to reduction of these processes. Gene *cstA*, which encodes a carbon starvation protein, was also downregulated, and this gene is known to be involved in *C. jejuni* motility and autoagglutination (49). Three other downregulated genes are also important for the stress response of *C. jejuni*: *ppk*, *dcuA*, and *hrcA* (50, 51). Downregulation of these genes might also contribute to biofilm reduction.

The analysis here of the transcriptome of *C. jejuni* NCTC 11168 following treatment with LEO showed that most of the genes involved in flagellar assembly and modification of flagella were downregulated (Fig. 5), which helps to explain these observations at the physiological level. Some of the downregulated genes (e.g., *flhK*, *Cj0719c*, *maf4*, and *Cj1467*) are also part of a network of genes that are important for flagellar synthesis. It is known that *C. jejuni* requires functional flagella for initial attachment and further biofilm formation (52), and so it is necessary to modulate genes involved in flagellar assembly to reduce biofilm formation. Motility in *Campylobacter* is regulated by a chemotactic signaling system that allows the organisms to follow favorable chemical gradients in environments (53). It is interesting to note that *Cj0262c* was upregulated, which is the gene that encodes the transducer-like protein and is involved in chemotaxis of *C. jejuni* (54). Furthermore, its closely related Tlp1-3 transducer-like proteins that are also involved in the *C. jejuni* chemotaxis sensory system, motility, and colonization (55) were not differentially expressed. From the results obtained at the molecular and physiological level, it can be concluded that LEO did not influence chemotactic behavior and, consequently, chemotactic motility of *C. jejuni*.

Furthermore, LEO also reduced the expression of genes that are important for formation of proteins and lipopolysaccharides of the outer bacterial membrane (i.e., *kpsM*, *kpsS*, *bamD*, *waaC*, and *lgt*) and are involved in the initial attachment of these cells to abiotic and biotic surfaces (56). In contrast, upregulation was seen for four of the main genes that are involved in the synthesis of outer membrane proteins and are important for cell adhesion and biofilm formation (i.e., *peb2*, *peb3*, *Omp50*, and *porA*) (57). Overall, these data indicate that downregulation of the flagellar genes, together with lower expression of genes involved in the iron-uptake system, in stress defense, and thus, in reduced cell adhesion and motility are the most interesting targets to combat biofilm formation. These data confirm that bacteria are under severe stress while exposed to such plant materials. These insights into complete transcriptome analysis open up new aspects and possibilities for research of new targets that should help in the control of this pathogen.

The results of the present study show the potential use of this *Lavandula* ethanol extract of postdistillation waste material, LEW, against preformed mature biofilms as well as against the adhesion and motility mechanisms involved in biofilm formation. These results offer new solutions for the use of such plant waste materials that still contain large amounts of bioactive molecules. The reuse of LEW might thus provide economic benefits in different industrial fields (e.g., pharmaceuticals and food industry) and might also help solve one of the global problems here: waste disposal (58, 59).

Conclusion. *Campylobacter jejuni* is shown to be sensitive to *Lavandula* preparations and selected pure compounds. *Lavandula* preparations are shown to be particularly effective in the fight against one of the world's most common foodborne pathogens, *C. jejuni*. The *Lavandula* preparations have relatively potent antibiofilm properties. Moreover, both physiological and molecular approaches confirmed modulation of the first steps in biofilm formation (i.e., adhesion and motility). Also, it has been shown that the postdistillation waste material of *Lavandula* flowers has particular antibiofilm effects against *C. jejuni*, which suggests that such waste material can be reused for industrial purposes. Therefore, further efforts can now be directed toward such innovative

approaches for alternative strategies and novel targets against bacterial biofilms to find and develop new and effective agents with antibiofilm activities.

MATERIALS AND METHODS

Chemicals. Muller-Hinton (MH) agar was from bioMérieux (Marcy-l'Etoile, France), MH broth was from Oxoid (Hampshire, UK), and Karmali agar was from Biolife (Milan, Italy). Glycerol solution was from Kemika (Zagreb, Croatia), *Campylobacter* growth supplement and phosphate-buffered saline (PBS) were from Oxoid, kanamycin, dimethyl sulfoxide (DMSO), resazurin, menadione, propidium iodide (PI), and TRI reagent were from Sigma-Aldrich (Steinheim, Germany), SYTO9 was from Thermo Fisher (Waltham, USA), and ethanol was from Merck (Darmstadt, Germany). PureLink DNase and PureLink RNA minikits, Qubit RNA HS, Ion Total RNA-seq kits v2, RNA-seq barcode BC primer, and Ion PI Hi-Q sequencing 200 kits were from Thermo Fisher Scientific (Carlsbad, CA, USA), NEXTflex poly(A) magnetic beads were from Perkin Elmer (Waltham, MA, USA), and high-sensitivity DNA kits were from Agilent Technologies (Santa Clara, CA, USA). The pure compounds linalool and 1,8-cineol were from Symrise (Holzminden, Germany), linalyl acetate and *p*-coumaric acid were from Honeywell Fluka (Charlotte, NC, USA), camphor was from (ICN Biomedicals Inc., Aurora, OH, USA), and (−)-borneol and *trans*-ferulic acid were from Carl Roth (Karlsruhe, Germany). The total ion chromatograms from the gas chromatography-mass spectrometry analysis showed the following purities: linalool, 94.88%; 1,8-cineol, 99.85%; linalyl acetate, 95.64%; camphor, 94.77%; and (−)-borneol, 96.65%. The photodiode array (PDA) total scan chromatograms from the high-pressure liquid chromatography (HPLC)-UV showed that *p*-coumaric acid consisted of the *trans* (98.33%) and *cis* (1.53%) isomers, while ferulic acid also consisted of the *trans* (97.87%) and *cis* (1.85%) isomers.

Lavandula. Dried flowers of *Lavandula angustifolia* were from Kottas Heilkräuter (Vienna, Austria; control number KLA91232), from which the LEO, LEF, and LEW were produced. LEO was prepared by hydrodistillation (60), with 2 h distillation of 30 g flowers in 1 liter water in a Neo-Clevenger-type distiller, and then stored at 4°C. LEF and LEW were prepared by 4-h to 6-h ethanol extraction (Soxhlet extraction) of 20 g flowers or *Lavandula* postdistillation waste material in 500 ml 96% ethanol. These were then concentrated in a rotary evaporator (Laborota 4000; Heidolph Instruments, Germany) at 40°C and 17.5 kPa pressure and stored at 4°C.

Phytochemical analysis of LEF and LEW. Identification of the flavonoids in LEF and LEW, and determination of the purities of the *p*-coumaric and ferulic acid, were carried out using liquid chromatography-photo diode array-electrospray ionization mass spectrometry. The dry ethanol extracts from the flowers and the waste material (5 mg) were dissolved in 1 ml ethanol and centrifuged prior to analysis. This analysis was performed on an HPLC system (Ultimate RS 3000 Dionex) that included a pump, autosampler, column compartment, and photodiode array detector, which was coupled to a mass spectrometer (LTQ XL; Thermo Scientific, Waltham, USA). The column (Luna phenyl-hexyl, 5 μm, 250 by 2 mm; Phenomenex, Torrance, CA, USA) was used with a gradient elution of 0.1% formic acid in water (eluent A) and acetonitrile (eluent B) as follows: 0→20 min, 10%→70% B; 20→40 min, 70%→100% B; 40→45 min, 100% B; 45→45.5 min, 100%→10% B; and 45.5→52 min, 10% B. The flow rate was 0.250 ml/min, and the column was maintained at 35°C.

Mass spectra were recorded in negative ion mode for the *m/z* range from 50 to 2,000 atomic mass units (amu), with data-dependent fragmentation (normalized collision energy, 35%). Mass spectrometry conditions were as follows: capillary temperature, 350°C; source temperature, 300°C; sheath and auxiliary gas flow, 40 and 10 arbitrary units (machine settings), respectively; source voltage, 3.5 kV; and capillary voltage, −17 V. The compounds eluted were determined by their ultraviolent and mass spectra and in comparison with the literature (24, 61, 62).

Gas chromatography-mass spectrometry analysis of LEO. Identification of the main compounds in LEO and determination of the purities of the linalool, 1,8-cineol, linalyl acetate, camphor, and (−)-borneol were carried out by gas chromatography-mass spectrometry. LEO (10 μl) was dissolved in 990 μl hexane and further analyzed by gas chromatography (7890 A; Agilent Technologies, Santa Clara, CA, USA) and mass spectrometry (5975 C VL MSD; Agilent Technologies, USA), operating at 70 eV, with an ion source temperature of 230°C and interface temperature of 280°C. A split injection (injection volume, 0.2 μl; split ratio, 50:1) at a 240°C injector temperature was used for LEO. A fused silica capillary column was used (5% phenyl, 95% methyl polysiloxane; HP-5MS, 30 m by 250 μm by 0.25 μm; Agilent J & W, USA). The temperature program was 1 min at 60°C and then raised to 220°C at 3°C/min. The carrier gas was helium 5.6 at a flow rate of 0.9 ml/min. Data acquisition was performed using Agilent GC/MSD ChemStation version E.02.02 for the mass scan range of 40 U to 400 U. The compounds were identified by their retention indices according to Adams (63) and by comparing their mass spectra with spectral data libraries (63–65) and the laboratory own database.

Bacterial strains and growth conditions. *Campylobacter jejuni* NCTC 11168 (National Collection of Type Culture) was used in this study. The strain was stored at −80°C in 20% glycerol and 80% MH broth. Prior to the experiments, the strain was subcultivated on Karmali agar for 24 h at 42°C under microaerobic conditions (85% N_2, 5% O_2, 10% CO_2). The strain was further subcultured microaerobically in MH broth for 24 h at 42°C, and bacterial counts were determined by spectrophotometric measurements of absorbance at 600 nm. The inoculum was prepared in MH broth at 10^5 CFU/ml for determination of the MICs as well as for the assays that targeted *C. jejuni* motility and adhesion. For counting, the *C. jejuni* NCTC 11168 strain was plated on MH agar under the appropriate conditions (as described above), and the colonies were counted and expressed as CFU/ml.

Antimicrobial susceptibility. The MICs were determined by the broth microdilution method, as previously described (66). Stock solutions of LEO, LEF, and LEW *Lavandula* preparations and the pure compounds linalool, linalyl acetate, camphor, (−)-borneol, 1,8-cineol, *p*-coumaric acid, and *trans*-ferulic acid were prepared in DMSO at 40 mg/ml. The DMSO in the MH broth did not exceed 1%.

Targeting *Campylobacter* biofilm degradation. To determine the effects of the *Lavandula* preparations for degradation and removal of *C. jejuni* biofilms, a novel approach based on culturing *C. jejuni* on an air-liquid interface was developed, with measurement of the relative glass surface coverage after treatment with the *Lavandula* preparations. Optimal *C. jejuni* growth conditions were established on the air-liquid interface (e.g., optimal O_2 concentration). Twelve-well microtiter plates (Sarstedt, Nümbrecht, Germany) were used, with the following added to each well: 3.0 ml MH broth, supplemented with 0.4% (vol/vol) *Campylobacter* growth supplement (which contained 0.125 g sodium pyruvate, 0.125 g sodium metabisulfite, and 0.125 g ferrous sulfate). The medium was inoculated with a 1% (vol/vol) *C. jejuni* NCTC 11168 overnight culture that contained approximately 1×10^8 CFU/ml. Autoclaved microscopy coverslips (20 by 20 mm; Brand, Wertheim, Germany) were used as the model for the glass surface, which were inserted and tilted to the side of each microtiter well after inoculation of the medium. To prevent desiccation and to minimize the proportion of dead cells, the cultures were incubated without shaking in a microaerobic atmosphere for 72 h at 42°C, in a damp environment. The spent medium in each well was replaced with fresh medium every 24 h.

Visible biofilms were formed at the air-liquid interface on the glass coverslips after 48 h of incubation and were then treated for 24 h with LEO at 0.5× MIC, MIC, or 2× MIC, as well as with LEF and LEW ethanol extracts at 2× MIC, 4× MIC, and 8× MIC, according to the MICs determine as bacterial suspensions (see "Bacterial strains and growth conditions" and "Antimicrobial susceptibility"). Nontreated biofilms from *C. jejuni* NCTC 11168 were used as the negative controls. The biofilms were analyzed under confocal microscopy after 72 h of incubation.

After incubation, the microscopy coverslips were first washed in PBS to remove weakly adhered cells. This was performed by carefully and slowly submerging the microscopy coverslips in PBS twice in a row by holding them with forceps. The excess liquid was then removed by tapping the edge of the glass on a paper towel. The microscopy coverslips were then stained with 50 μl of a mixture of 20 mM PI and 5 mM SYTO 9 for 15 min (Live/Dead test) (57) in an aerobic atmosphere at room temperature in the dark. The mixture of the PI solution and SYTO 9 was prepared by adding 3 μl PI and 3 μl SYTO 9 to 1 ml physiological solution. PI cannot enter intact cells, while dead cells with a compromised cell membrane allow the passage of PI. When PI binds to DNA, it emits red fluorescence upon excitation. On the other hand, SYTO 9 stains DNA in cells green, regardless of their membrane integrity. Thus, dead cells appear under fluorescence microscopy as red and only those that are only green are alive (57).

Confocal microscopy (LSM 800 Axio Observer Z1 inverted microscope; Zeiss, Germany) was performed using the 20× (numerical aperture [NA], 0.4) lens objective. SYTO 9 fluorescence was excited with a 488-nm diode laser, and PI fluorescence was excited with a 561-nm diode laser, operated at 0.5% power. We always considered the upper 600 μm of the biofilm-covered glass surface, starting at the air-liquid interface in the direction toward the liquid. Experiments were performed in three or more biological replicates, and 10 images were acquired for each sample (5 by 2 mosaic images; total analyzed surface, 1470 μm by 607 μm). In controls, this contained >400,000 viable cells. The images for each slide were analyzed using Fiji software (version 1.52c) to obtain the biofilm surface cover. Individual channel images were converted to binary format by manually setting the threshold value that fully separated the biofilm from the background. Random noise was reduced for all of the images using the despeckle function. The biofilm surface coverage was obtained by using our ImageJ custom macro, where surface coverage of biofilm was calculated by first summing the areas covered by green cells (SYTO 9) and red cells (PI) and then subtracting the area of cells that were simultaneously green and red. The area covered by red cells was under 2% in all cases.

Targeting *Campylobacter* adhesion. The adhesion of *C. jejuni* NCTC 11168 was analyzed under treatments with the *Lavandula* preparations and pure compounds at subinhibitory concentrations. Inocula were prepared as described above (see "Bacterial strains and growth conditions") and treated with the LEO, LEF, and LEW *Lavandula* preparations and pure compounds at 0.25× MIC. They were then transferred (200 μl) to 96-well polystyrene microtiter plates (Nunc 266 120 polystyrene plates; Nunc, Denmark) and incubated microaerobically at 42°C for 4, 8, and 24 h. The supernatants with nonadherent cells were removed from each well and rinsed three times with PBS. Then, 200 μl PBS was added, with sonication for 10 min (28 kHz, 300 W; Iskra PIO, Šentjernej, Slovenia). The adhesion of cells was examined as CFU per milliliter, as previously described (35). The untreated culture acted as the negative control.

Targeting *Campylobacter* motility. Antimotility assays were performed as previously described (21). Briefly, *C. jejuni* NCTC 11168 cultures were treated with LEO, LEF, and LEW *Lavandula* preparations and pure compounds at 0.25× MIC for 24 h in a microaerobic atmosphere at 42°C. After treatment, 1 μl of the treated cultures was placed in the middle of plated soft agar. The plates were incubated for 48 h in a microaerobic atmosphere at 42°C. After this incubation, the diameters of the swarming colonies were measured. The untreated culture acted as the negative control.

RNA sequencing. (i) RNA isolation and quantification. The molecular mechanisms of LEO action in *C. jejuni* NCTC 11168 were analyzed for treatment with subinhibitory concentrations of the LEO *Lavandula* preparation. *C. jejuni* NCTC 11168 was grown microaerobically in 42 ml of MH broth for 16 h at 42°C to the middle exponential phase and then treated with LEO at 0.25× MIC for 30 min. The negative control was the culture treated with 1% DMSO under the same conditions, and the positive control was the nontreated culture. The cells were harvested by centrifugation (5,000 × *g*, 5 min, 4°C), and resuspended to a 1-ml cell suspension, which contained approximately 10^9 CFU/ml. Cell lysis for isolation of

total RNA was performed using RNA isolation (TRI) reagent (Sigma-Aldrich), DNase treatment using PureLink DNase kits (Thermo Fisher Scientific), and purification of isolated RNA using PureLink RNA mini-kits (Thermo Fisher Scientific), according to the manufacturers' instructions. Quantification and qualification of the total RNA quality were determined using Qubit RNA HS assay kits (Thermo Fisher Scientific) and fluorimeter measurements (Qubit v4; Thermo Fisher Scientific). mRNA was enriched from the total RNA using magnetic beads (NEXTflex poly(A); Perkin Elmer).

(ii) Ion Torrent library preparation and sequencing. For next-generation RNA-sequencing (RNA-seq), transcriptome libraries were constructed using Ion Total RNA-seq kit v2 (Thermo Fisher Scientific). Briefly, mRNA samples were enzymatically fragmented and purified using magnetic beads. Afterwards, ion adaptors were hybridized onto the fragmented mRNAs and ligated, and reverse transcription was performed. The prepared cDNA samples were purified using magnetic beads, and each cDNA sample was barcoded with an Ion Xpress RNA-Seq barcode BC primer (Thermo Fisher Scientific). The cDNA libraries were purified, and the concentration and size distribution of cDNA libraries were determined using a 2100 Bioanalyzer and high-sensitivity DNA kits (Agilent Technologies). The barcoded cDNA libraries prepared were diluted to the same molar concentrations, pooled in equal volumes, and amplified using an Ion OneTouch 2 system with the accompanying Ion PI Hi-Q OT2 200 kits. Sequencing was performed on an Ion Proton system, using Ion PI Hi-Q sequencing 200 kits (Thermo Fisher Scientific). The datasets generated and analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) repository (https://www.ncbi.nlm.nih.gov/sra/) under BioProject accession PRJNA747749 and SRA accession numbers SRR15183216, SRR15183214, SRR15183213, and SRR15183212.

(iii) Data analysis. The bioinformatics analysis was performed using a CLC Genomics Workbench (version 12.0.3) and a CLC Genomics server (version 11.0.2). Prior to the differential expression analysis, quality control of the sequencing reads and trimming of the adapter sequences were performed using the "trim reads" tool. Sequencing reads from each library were subjected to differential expression analysis using the RNA-Seq Analysis 2.21 tool (CLC Genomics server, 20.0.2). *Campylobacter jejuni* subsp. *jejuni* NCTC 11168 (ATCC 700819) complete genome sequence and genome annotations from the NCBI Nucleotide database (accession number NC_002163.1) were used as the reference genome sequences (67). To compare gene expression between samples treated with LEO and the controls, the "differential expression in two groups 1.1" tool was used. Genes with absolute $\log_2$ fold change of ≥ 1 and a false-discovery rate (FDR) P value of ≤ 0.05 were considered differentially expressed. Differentially expressed genes were further analyzed via the STRING Consortium 2020, which provides functional enrichment analysis of protein-protein interaction networks in the STRING mapper tool (https://string-db.org/cgi/input.pl?sessionId=oO8HWWKYl5Fd&input_page_show_search=on). To construct a network of differentially expressed genes using Cytoscape, the NCBI protein identifiers of differentially expressed genes were searched in String database for the organism *Campylobacter jejuni* subsp. *jejuni* NCTC 11168 (ATCC 700819), with a cutoff of 0.8 confidence (score) and a maximum of 10 additional interactors. Afterwards, the network was clustered using the MCL Cluster algorithm implemented in the Cytoscape plugin clusterMaker (68) to determine clusters and functional interactions. For each cluster, the built-in functional enrichment available from the stringApp was used to obtain enriched terms.

Gene enrichment analysis was performed using GO_MWU (https://github.com/z0on/GO_MWU) (69), which uses Mann-Whitney U tests and the Benjamini-Hochberg (B-H) FDR corrections of P values to define which enriched GO categories are significantly represented by either upregulated or downregulated genes. GO categories with B-H FDR P values of <0.1 were considered significantly enriched by either upregulated or downregulated genes.

Statistical analysis. All of the experiments were carried out in triplicates as three or more independent experiments. The data are expressed as means ±standard deviations, with analysis using Origin 2018 (OriginLab, Northampton, MA, USA). Statistical analysis was performed in IBM SPSS Statistics 23 (Statsoft Inc., Tulsa, OK, USA). To determine distribution of data, a Kolmogorov-Smirnov test of normality was performed and statistical significances were determined using Mann-Whitney tests for two independent means. Data were accepted as significant at a P value of <0.05.

Data availability. The datasets generated and analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) repository under the BioProject accession PRJNA747749 and SRA accession numbers SRR15183216, SRR15183214, SRR15183213, and SRR15183212.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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D.R. conceived and conducted experiments. F.B. and U.K. analyzed data. A.K., I.D., and S.S.M. designed and coordinated research. D.R. wrote the manuscript. All authors have read and approved the manuscript.

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REFERENCES

1. Srey S, Jahid IK, Ha SD. 2013. Biofilm formation in food industries: a food safety concern. Food Control 31:572–585. https://doi.org/10.1016/j.foodcont.2012.12.001.
2. Bridier A, Sanchez-Vizuete P, Guilbaud M, Piard JC, Naïtali M, Briandet R. 2015. Biofilm-associated persistence of food-borne pathogens. Food Microbiol 45:167–178. https://doi.org/10.1016/j.fm.2014.04.015.
3. European Food Safety Authority, European Centre for Disease Prevention and Control. 2019. The European Union One Health 2018 zoonoses report. EFSA J 17:e05926. https://doi.org/10.2903/j.efsa.2019.5926.
4. Gölz G, Kittler S, Malakauskas M, Alter T. 2018. Survival of *Campylobacter* in the food chain and the environment. Curr Clin Micro Rep 5:126–134. https://doi.org/10.1007/s40588-018-0092-z.
5. Tram G, Day CJ, Korolik V. 2020. Bridging the gap: a role for *Campylobacter jejuni* biofilms. Microorganisms 8:452. https://doi.org/10.3390/microorganisms8030452.
6. Mavri A, Smole Možina S. 2013. Development of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* adapted to biocides. Int J Food Microbiol 160:304–312. https://doi.org/10.1016/j.ijfoodmicro.2012.11.006.
7. García-Sánchez L, Melero B, Jaime I, Rossi M, Ortega I, Rovira J. 2019. Biofilm formation, virulence and antimicrobial resistance of different *Campylobacter jejuni* isolates from a poultry slaughterhouse. Food Microbiol 83:193–199. https://doi.org/10.1016/j.fm.2019.05.016.
8. Beier RC, Byrd JA, Andrews K, Caldwell D, Crippen TL, Anderson RC, Nisbet DJ. 2021. Disinfectant and antimicrobial susceptibility studies of the food-borne pathogen *Campylobacter jejuni* isolated from the litter of broiler chicken houses. Poult Sci 100:1024–1033. https://doi.org/10.1016/j.psj.2020.10.045.
9. Galié S, García-Gutiérrez C, Miguélez EM, Villar CJ, Lombó F. 2018. Biofilms in the food industry: health aspects and control methods. Front Microbiol 9:898. https://doi.org/10.3389/fmicb.2018.00898.
10. Klančnik A, Šimunović K, Sterniša M, Ramić D, Smole Možina S, Bucar F. 2021. Anti-adhesion activity of phytochemicals to prevent *Campylobacter jejuni* biofilm formation on abiotic surfaces. Phytochem Rev 20:55–84. https://doi.org/10.1007/s11101-020-09669-6.
11. Slavov AM, Karneva KB, Vasileva IN, Denev PN, Denkova RS, Shikov VT, Manolova MN, Lazarova YL, Ivanova VN. 2018. Valorization of lavender waste – obtaining and characteristics of polyphenol rich extracts. Food Sci Appl Biotechnol 1:11–18. https://doi.org/10.30721/fsab2018.v1.i1.5.
12. Blažeković B, Yang W, Wang Y, Li C, Kindl M, Pepeljnjak S, Vladimir-Knežević S. 2018. Chemical composition, antimicrobial and antioxidant activities of essential oils of *Lavandula* × *intermedia* 'Budrovka' and *L. angustifolia* cultivated in Croatia. Ind Crops Prod 123:173–182. https://doi.org/10.1016/j.indcrop.2018.06.041.
13. Méndez-Tovar I, Herrero B, Pérez-Magariño S, Pereira JA, Asensio S-, Manzanera MC. 2015. By-product of *Lavandula latifolia* essential oil distillation as source of antioxidants. J Food Drug Anal 23:225–233. https://doi.org/10.1016/j.jfda.2014.07.003.
14. Camen D, Hadaruga N, Luca R, Dobrei A, Nistor E, Posta D, Dobrei A, Velicevici G, Petcov A, Sala F. 2016. Research concerning the influence of fertilization on some physiological processes and biochemical composition of lavender (*Lavandula angustifolia* L.). Agric Agric Sci Procedia 10:198–205. https://doi.org/10.1016/j.aaspro.2016.09.053.
15. Raut JS, Karuppayil SM. 2014. A status review on the medicinal properties of essential oils. Ind Crops Prod 62:250–264. https://doi.org/10.1016/j.indcrop.2014.05.055.
16. Vodnar DC, Călinoiu LF, Dulf FV, Ştefănescu BE, Crişan G, Socaciu C. 2017. Identification of the bioactive compounds and antioxidant, antimutagenic and antimicrobial activities of thermally processed agro-industrial waste. Food Chem 231:131–140. https://doi.org/10.1016/j.foodchem.2017.03.131.
17. Carrasco A, Martinez-Gutierrez R, Tomas V, Tudela J. 2016. *Lavandula angustifolia* and *Lavandula latifolia* essential oils from Spain: aromatic profile and bioactivities. Planta Med 82:163–170. https://doi.org/10.1055/s-0035-1558095.
18. Martucci JF, Gende LB, Neira LM, Ruseckaite RA. 2015. Oregano and lavender essential oils as antioxidant and antimicrobial additives of biogenic gelatin films. Ind Crops Prod 71:205–213. https://doi.org/10.1016/j.indcrop.2015.03.079.
19. Budzyńska A, Wieckowska-Szakiel M, Sadowska B, Kalemba D, Rózalska B. 2011. Antibiofilm activity of selected plant essential oils and their major components. Pol J Microbiol 60:35–41. https://doi.org/10.33073/pjm-2011-005.
20. Duarte A, Luís Â, Oleastro M, Domingues FC. 2016. Antioxidant properties of coriander essential oil and linalool and their potential to control *Campylobacter* spp. Food Control 61:115–122. https://doi.org/10.1016/j.foodcont.2015.09.033.
21. Šimunović K, Ramić D, Xu C, Smole Možina S. 1975. Modulation of *Campylobacter jejuni* motility, adhesion to polystyrene surfaces, and invasion of INT407 cells by quorum-sensing inhibition. Microorganisms 8:104. https://doi.org/10.3390/microorganisms8010104.
22. Kivrak Ş. 2018. Essential oil composition and antioxidant activities of eight cultivars of lavender and lavandin from western Anatolia. Ind Crops Prod 117:88–96. https://doi.org/10.1016/j.indcrop.2018.02.089.
23. Prusinowska R, Śmigielski K, Stobiecka A, Kunicka-Styczyńska A. 2016. Hydrolates from lavender (*Lavandula angustifolia*) - their chemical composition as well as aromatic, antimicrobial and antioxidant properties. Nat Prod Res 30:386–393. https://doi.org/10.1080/14786419.2015.1016939.
24. Torras-Claveria L, Jauregui O, Bastida J, Codina C, Viladomat F. 2007. Antioxidant activity and phenolic composition of lavandin (*Lavandula x intermedia* Emeric ex Loiseleur) waste. J Agric Food Chem 55:8436–8443. https://doi.org/10.1021/jf070236n.
25. Śmigielski KB, Prusinowska R, Krosowiak K, Sikora M. 2013. Comparison of qualitative and quantitative chemical composition of hydrolate and essential oils of lavender (*Lavandula angustifolia*). J Essent Oil Res 25:291–299. https://doi.org/10.1080/10412905.2013.775080.
26. Guil-Guerrero JL, Ramos L, Moreno C, Zúñiga-Paredes JC, Carlosama-Yepez M, Ruales P. 2016. Antimicrobial activity of plant-food by-products: a review focusing on the tropics. Livest Sci 189:32–49. https://doi.org/10.1016/j.livsci.2016.04.021.
27. Stewart PS. 2015. Antimicrobial tolerance in biofilms. Microbiol Spectr 3:MB-0010-2014. https://doi.org/10.1128/microbiolspec.MB-0010-2014.
28. Areias FM, Valentão P, Andrade PB, Moreira MM, Amaral J, Seabra RM. 2000. HPLC/DAD analysis of phenolic compounds from lavender and its application to quality control. J Liq Chromatogr Relat Technol 23:2563–2572. https://doi.org/10.1081/JLC-100100510.
29. Teh AHT, Lee SM, Dykes GA. 2014. Does *Campylobacter jejuni* form biofilms in food-related environments? Appl Environ Microbiol 80:5154–5160. https://doi.org/10.1128/AEM.01493-14.
30. De Rapper S, Viljoen A, Van Vuuren S. 2016. The *in vitro* antimicrobial effects of *Lavandula angustifolia* essential oil in combination with conventional antimicrobial agents. Evid Based Complement Alternat Med 2016:2752739. https://doi.org/10.1155/2016/2752739.
31. Lis-Balchin M, Hart S. 1999. Studies on the mode of action of the essential oil of lavender (*Lavandula angustifolia* P Miller). Phytother Res 13:540–542. https://doi.org/10.1002/(SICI)1099-1573(199909)13:6<540::AID-PTR523>3.0.CO;2-I.
32. Nikolić M, Jovanović KK, Marković T, Marković D, Gligorijević N, Radulović S, Soković M. 2014. Chemical composition, antimicrobial, and cytotoxic properties of five *Lamiaceae* essential oils. Ind Crops Prod 61:225–232. https://doi.org/10.1016/j.indcrop.2014.07.011.
33. Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods - a review. Int J Food Microbiol 94:223–253. https://doi.org/10.1016/j.ijfoodmicro.2004.03.022.

34. Calo JR, Crandall PG, O'Bryan CA, Ricke SC. 2015. Essential oils as antimicrobials in food systems - a review. Food Control 54:111–119. https://doi.org/10.1016/j.foodcont.2014.12.040.
35. Šikić Pogačar M, Klančnik A, Bucar F, Langerholc T, Smole Možina S. 2016. Anti-adhesion activity of thyme (*Thymus vulgaris* L.) extract, thyme post-distillation waste, and olive (*Olea europea* L.) leaf extract against *Campylobacter jejuni* on polystyrene and intestine epithelial cells. J Sci Food Agric 96:2723–2730. https://doi.org/10.1002/jsfa.7391.
36. Klančnik A, Šikić Pogačar M, Trošt K, Tušek Žnidarič M, Mozetič Vodopivec B, Smole Možina S. 2017. Anti-*Campylobacter* activity of resveratrol and an extract from waste Pinot noir grape skins and seeds, and resistance of *Campylobacter jejuni* planktonic and biofilm cells, mediated via the CmeABC efflux pump. J Appl Microbiol 122:65–77. https://doi.org/10.1111/jam.13315.
37. Dănilă E, Moldovan Z, Popa M, Chifiriuc MC, Kaya AD, Kaya MA. 2018. Chemical composition, antimicrobial and antibiofilm efficacy of *C. limon* and *L. angustifolia* EOs and of their mixtures against *Staphylococcus epidermidis* clinical strains. Ind Crops Prod 122:483–492. https://doi.org/10.1016/j.indcrop.2018.06.019.
38. Bazargani MM, Rohloff J. 2016. Antibiofilm activity of essential oils and plant extracts against *Staphylococcus aureus* and *Escherichia coli* biofilms. Food Control 61:156–164. https://doi.org/10.1016/j.foodcont.2015.09.036.
39. Kerekes EB, Deák É, Takó M, Tserennadmid R, Petkovits T, Vágvölgyi C, Krisch J. 2013. Antibiofilm forming and anti-quorum sensing activity of selected essential oils and their main components on food-related micro-organisms. J Appl Microbiol 115:933–942. https://doi.org/10.1111/jam.12289.
40. Wagle BR, Upadhyay A, Upadhyaya I, Shrestha S, Arsi K, Liyanage R, Venkitanarayanan K, Donoghue DJ, Donoghue AM. 2019. *trans*-Cinnamaldehyde, eugenol and carvacrol reduce *Campylobacter jejuni* biofilms and modulate expression of select genes and proteins. Front Microbiol 10:1837. https://doi.org/10.3389/fmicb.2019.01837.
41. Rubinchik S, Seddon AM, Karlyshev AV. 2014. A negative effect of *Campylobacter* capsule on bacterial interaction with an analogue of a host cell receptor. BMC Microbiol 14:141. https://doi.org/10.1186/1471-2180-14-141.
42. Pisoschi AM, Pop A, Georgescu C, Turcuş V, Olah NK, Mathe E. 2018. An overview of natural antimicrobials role in food. Eur J Med Chem 143:922–935. https://doi.org/10.1016/j.ejmech.2017.11.095.
43. Reuter M, Ultee E, Toseafa Y, Tan A, Van Vliet AHM. 2020. Inactivation of the core *cheVAWY* chemotaxis genes disrupts chemotactic motility and organised biofilm formation in *Campylobacter jejuni*. FEMS Microbiol Lett 367:fnaa198. https://doi.org/10.1093/femsle/fnaa198.
44. Aseev LV, Koledinskaya LS, Boni IV. 2016. Regulation of ribosomal protein operons *rplM-rpsI*, *rpmB-rpmG*, and *rplU-rpmA* at the transcriptional and translational levels. J Bacteriol 198:2494–2502. https://doi.org/10.1128/JB.00187-16.
45. Boutte CC, Crosson S. 2013. Bacterial lifestyle shapes stringent response activation. Trends Microbiol 21:174–180. https://doi.org/10.1016/j.tim.2013.01.002.
46. Askoura M, Youns M, Halim Hegazy WA. 2020. Investigating the influence of iron on *Campylobacter jejuni* transcriptome in response to acid stress. Microb Pathog 138:103777. https://doi.org/10.1016/j.micpath.2019.103777.
47. Miller CE, Williams PH, Ketley JM. 2009. Pumping iron: mechanisms for iron uptake by *Campylobacter*. Microbiology (Reading) 155:3157–3165. https://doi.org/10.1099/mic.0.032425-0.
48. Püning C, Su Y, Lu X, Gölz G. 2021. Molecular mechanisms of *Campylobacter* biofilm formation and quorum sensing. *In* Backert S (ed), Fighting *Campylobacter* infections. Curr Top Microbiol and Immunol, vol 431. Springer, Cham, Switzerland. https://doi.org/10.1007/978-3-030-65481-8_11.
49. Rasmussen JJ, Vegge CS, Frøkiær H, Howlett RM, Krogfelt KA, Kelly DJ, Ingmer H. 2013. *Campylobacter jejuni* carbon starvation protein A (CstA) is involved in peptide utilization, motility and agglutination, and has a role in stimulation of dendritic cells. J Med Microbiol 62:1135–1143. https://doi.org/10.1099/jmm.0.059345-0.
50. Drozd M, Chandrashekhar K, Rajashekara G. 2014. Polyphosphate-mediated modulation of *Campylobacter jejuni* biofilm growth and stability. Virulence 5:680–690. https://doi.org/10.4161/viru.34348.
51. Kim SH, Chelliah R, Ramakrishnan SR, Perumal AS, Bang WS, Rubab M, Daliri EBM, Barathikannan K, Elahi F, Park E, Jo HY, Hwang SB, Oh DH. 2020. Review on stress tolerance in *Campylobacter jejuni*. Front Cell Infect Microbiol 10:596570. https://doi.org/10.3389/fcimb.2020.596570.
52. Guerry P. 2007. *Campylobacter* flagella: not just for motility. Trends Microbiol 15:456–461. https://doi.org/10.1016/j.tim.2007.09.006.
53. Chandrashekhar K, Kassem II, Rajashekara G. 2017. *Campylobacter jejuni* transducer like proteins: chemotaxis and beyond. Gut Microbes 8:323–334. https://doi.org/10.1080/19490976.2017.1279380.
54. Chandrashekhar K, Srivastava V, Hwang S, Jeon B, Ryu S, Rajashekara G. 2018. Transducer-like protein in *Campylobacter jejuni* with a role in mediating chemotaxis to iron and phosphate. Front Microbiol 9:2674. https://doi.org/10.3389/fmicb.2018.02674.
55. Rahman H, King RM, Shewell LK, Semchenko EA, Hartley-Tassell LE, Wilson JC, Day CJ, Korolik V. 2014. Characterisation of a multi-ligand binding chemoreceptor CcmL (Tlp3) of *Campylobacter jejuni*. PLoS Pathog 10:e1003822. https://doi.org/10.1371/journal.ppat.1003822.
56. Naito M, Frirdich E, Fields JA, Pryjma M, Li J, Cameron A, Gilbert M, Thompson SA, Gaynor EC. 2010. Effects of sequential *Campylobacter jejuni* 81-176 lipooligosaccharide core truncations on biofilm formation, stress survival, and pathogenesis. J Bacteriol 192:2182–2192. https://doi.org/10.1128/JB.01222-09.
57. Asakura H, Yamasaki M, Yamamoto S, Igimi S. 2007. Deletion of *peb4* gene impairs cell adhesion and biofilm formation in *Campylobacter jejuni*. FEMS Microbiol Lett 275:278–285. https://doi.org/10.1111/j.1574-6968.2007.00893.x.
58. Cvetanović A, Švarc-Gajić J, Zeković Z, Mašković P, Đurović S, Zengin G, Delerue-Matos C, Lozano-Sánchez J, Jakšić A. 2017. Chemical and biological insights on *Aronia* stems extracts obtained by different extraction techniques: from wastes to functional products. J Supercrit Fluids 128:173–181. https://doi.org/10.1016/j.supflu.2017.05.023.
59. Kabir F, Tow WW, Hamauzu Y, Katayama S, Tanaka S, Nakamura S. 2015. Antioxidant and cytoprotective activities of extracts prepared from fruit and vegetable wastes and by-products. Food Chem 167:358–362. https://doi.org/10.1016/j.foodchem.2014.06.099.
60. Meyer-Warnod B. 1984. Natural essential oils: extraction processes and application to some major oils. Perfum Flavorist 9:93–104.
61. Çelik SE, Tufan AN, Bekdeser B, Özyürek M, Güçlü K, Apak R. 2017. Identification and determination of phenolics in Lamiaceae species by UPLC-DAD-ESI-MS/MS. J Chromatogr Sci 55:291–300. https://doi.org/10.1093/chromsci/bmw184.
62. Lopes CL, Pereira E, Soković M, Carvalho AM, Barata AM, Lopes V, Rocha F, Calhelha RC, Barros L, Ferreira ICFR. 2018. Phenolic composition and bioactivity of *Lavandula pedunculata* (Mill.) Cav. Samples from different geographical origin. Molecules 23:1037. https://doi.org/10.3390/molecules23051037.
63. Adams RP. 2007. Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy. 4th ed. Allured Pub Corp., Carol Stream, IL.
64. NIST. 2006. NIST mass spectral library. Rev 2005. D.05.01, US Department of Commerce. https://chemdata.nist.gov/.
65. Joulain D, König W. 1998. The atlas of spectral data of sesquiterpene hydrocarbons. E. B. Verlag, Hamburg, Germany.
66. Klančnik A, Guzej B, Kolar MH, Abramovic H, Možina S. 2009. *In vitro* antimicrobial and antioxidant activity of commercial rosemary extract formulations. J Food Prot 72:1744–1752. https://doi.org/10.4315/0362-028x-72.8.1744.
67. Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Chillingworth T, Davies RM, Feltwell T, Holroyd S, Jagels K, Karlyshev AV, Moule S, Pallen MJ, Penn CW, Quail MA, Rajandream MA, Rutherford KM, van Vliet AH, Whitehead S, Barrell BG. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403:665–668. https://doi.org/10.1038/35001088.
68. Morris JH, Apeltsin L, Newman AM, Baumbach J, Wittkop T, Su G, Bader GD, Ferrin TE. 2011. clusterMaker: a multi-algorithm clustering plugin for Cytoscape. BMC Bioinformatics 12:436. https://doi.org/10.1186/1471-2105-12-436.
69. Wright RM, Aglyamova GV, Meyer E, Matz MV. 2015. Gene expression associated with white syndromes in a reef building coral, *Acropora hyacinthus*. BMC Genomics 16:371. https://doi.org/10.1186/s12864-015-1540-2.
70. Luo W, Brouwer C. 2013. Pathview: an R/Bioconductor package for pathway-based data integration and visualization. Bioinformatics 29:1830–1831. https://doi.org/10.1093/bioinformatics/btt285.

2.1.2 Pojasnitev komunikacijskega sistema AI-2 bakterij *Campylobacter jejuni* s kvantitativno biosenzorsko metodo

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S hrano prenosljiva, patogena bakterija *Campylobacter jejuni* proizvaja medvrstno signalno molekulo avtoinduktor-2 (AI-2). Signalna molekula AI-2 lahko sproži povečano kolonizacijo in filmotvornost, kar predstavlja resno tveganje za javno zdravstvo. Do danes je komunikacijski sistem bakterij *C. jejuni* le delno raziskan, saj je detekcijo in kvantifikacijo avtoinduktorskih signalnih molekul AI-2 v kompleksnih gojiščih zelo težko izvesti. V tej raziskavi smo razvili biosenzorsko metodo, ki vključuje biosenzorski sev *Vibrio harveyi* za natančno kvantifikacijo in spremljanje proizvodnje signalih molekul AI-2 bakterije *C. jejuni* 81-176 v definiranem rastnem gojišču in v modelnem živilskem matriksu (5 % piščančji sok). Testiranih je bilo več biosenzorskih sevov *V. harveyi*, vendar je najobčutljivejši odziv na signalne molekule AI-2 imel biosenzorski sev *V. harveyi* MM30, zaradi sposobnosti samoojačanja odziva. Koncentracije signalnih molekul AI-2, izmerjene z razvito biosenzorsko metodo, smo potrdili z neodvisno analitsko metodo HPLC-FLD, ki smo jo prvič vpeljali za analizo signalnih molekul bakterij *Campylobacter*. Koncentracija AI-2, ki jo proizvaja *C. jejuni* 81-176 v modelnem živilskem matriksu, je bila približno 5-krat višja kot v definiranem rastnem gojišču, pri enaki gostoti celic. Tudi naraščanje koncentracije signalnih molekul AI-2 pri bakteriji *C. jejuni* je linearno z gostoto celic, kar nakazuje na to, da signalne molekule AI-2 predstavljajo metabolne stranske produkte pri bakterijah *C. jejuni* in ne prave molekule za zaznavanje kvoruma. Razvita biosenzorska metoda je zelo občutljiva, kar dokazuje zmanjšana meje detekcije (za faktor 100) v primerjavi z metodo HPLC-FLD in omogoča kvantifikacijo signalnih molekul AI-2 v kompleksnih gojiščih, kot je hrana. Nova spoznanja bodo pripomogla izboljšati kakovost in varnost proizvodnje hrane.

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Elucidation of the AI-2 communication system in the food-borne pathogen *Campylobacter jejuni* by whole-cell-based biosensor quantification

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ABSTRACT

The food-borne pathogen *Campylobacter jejuni* produces autoinducer-2 (AI-2) as an interspecies signalling molecule. AI-2 can trigger enhanced colonisation and biofilm formation, and this poses a serious risk to public health. To date, this communication system of *C. jejuni* is only partially understood, as detection and quantification of such autoinducer signalling molecules in complex media is hard to achieve. We have developed a whole-cell *Vibrio harveyi*-based biosensor assay to accurately quantify and follow production of AI-2 by *C. jejuni* 81–176 in a defined growth medium and in a model food system. Several *V. harveyi* strains were tested, but the most sensitive bioluminescent response to *C. jejuni* AI-2 was achieved with *V. harveyi* MM30, likely due to its ability to self-amplify the response to AI-2. The AI-2 concentrations measured by this biosensor were confirmed using an independent analytical method, HPLC-FLD, which we introduced for *Campylobacter* analytics for the first time. The AI-2 concentration produced by *C. jejuni* 81–176 in the model food system was ~5-fold that in the defined growth medium, at the same cell density. Together with the linear increments in AI-2 concentrations with cell density, this suggests that in *C. jejuni*, AI-2 represents a metabolic by-product rather than a true quorum-sensing molecule. This biosensor method is highly sensitive, as shown by the reduction in the limit of detection (by a factor of 100) compared to HPLC-FLD, and it enables quantification of AI-2 in complex matrices, such as food, which will help to improve the quality and safety of food production.

1. Introduction

Bacterial communication is 'multilingual', which indicates that bacteria can produce different signalling molecules to communicate among themselves to carry out a wide range of complex social behaviours that can be guided by quorum sensing (Abisado et al., 2018). Sending, receiving and processing information in the form of these 'autoinducer' signalling molecules enables a single-cell organism to behave as a multicellular entity, and promotes better survival in a complex environment (Hooshangi and Bentley, 2008). Quorum sensing affects bacterial processes such as bioluminescence, secretion of virulence factors, production of public goods and biofilm formation (Papenfort and Bassler, 2016).

The autoinducer-2 (AI-2) signalling molecule can be considered as a 'universal language' for interspecies communication (Galloway et al., 2010). The enzyme LuxS is responsible for AI-2 biosynthesis, and to date, AI-2 has been found in over 70 bacterial species (Lowery et al., 2008; Vendeville et al., 2005). LuxS synthesises 4,5-dihydroxy-2, 3-pentandion (DPD), which undergoes spontaneous rearrangements to form a variety of DPD derivatives that interconvert and exist in equilibrium, known as the AI-2 pool (Galloway et al., 2010; Miller et al., 2004; Semmelhack et al., 2005). If the *luxS* gene is inactivated, AI-2 production is eliminated (Xavier and Bassler, 2003).

The classical analytical methods for detection and quantification of autoinducer signalling molecules are based on colorimetric, liquid and gas chromatography techniques (Campagna et al., 2009; Thiel et al., 2009; Wattanavanitchakorn et al., 2014; Xu et al., 2017). Biosensor-based methods include use of engineered fluorescent and luminescent bacterial sensors, and they are commonly used for simple, sensitive and rapid signalling molecule detection (Chen et al., 2002; Dogsa et al., 2021; Miller et al., 2004; Rajamani and Sayre, 2018; Schauder et al., 2001; Servinsky et al., 2015; Surette et al., 1999; Taga and Xavier, 2011; Vilchez et al., 2007; Zhu and Pei, 2008). One of the most often used biosensors is *Vibrio harveyi*, where quorum sensing provides the biological background, as it has three different auto-inducer signalling molecules: HAI-1, CAI-1 and AI-2. Such

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whole-cell-based biosensors sense the signalling molecules and respond by emitting luminescence, which can then be measured using a luminometer (Zhao et al., 2018).

Campylobacter jejuni is the most prevalent bacterial food-borne pathogen in the industrial world. It can reduce the hygiene conditions of food, and represents a severe public health risk (Bridier et al., 2015; EFSA/ECDC, 2021). *C. jejuni* has been reported to have a *luxS* gene homologue and to actively produce AI-2 (Elvers and Park, 2002). No AI-2 receptor homologue has been identified in *Campylobacter* spp. to date (Püning et al., 2021), although an AI-2 uptake assay has indicated that *C. jejuni* might respond to AI-2 through a two-component regulatory system (Adler et al., 2015). A recent study showed that AI-2 export in *C. jejuni* is modulated by the small non-coding RNA CjNC110 (Kreuder et al., 2020). It is believed that *C. jejuni* biofilm formation can be enhanced (Teh et al., 2014) or reduced (Erega et al., 2021) by the presence of other bacterial species. Absence of the *luxS* gene in *C. jejuni* results in reduced colonisation and biofilm formation on different surfaces (Klančnik et al., 2021). On the other hand, LuxS also has a central role as a metabolic enzyme in the activated methyl cycle that is responsible for generation of S-adenosyl-L-methionine, where AI-2 represents a by-product. It is not clear if the observed phenotypes of *luxS* mutants are a consequence of changes in the methyl cycle or the lack of AI-2 (Holmes et al., 2009).

However, no model food system for studying *C. jejuni* intercellular signalling has been established yet. *C. jejuni* is one of the microbes that pose a significant food safety risk on chicken meat, along with *Salmonella enterica, Listeria monocytogenes* and *Escherichia coli*. In general, for *C. jejuni, S. enterica, L. monocytogenes* and *E. coli* studies of survivability and biofilm formation, raw poultry products, including chicken juice, are frequently used in different forms and different concentrations, respectively (Birk et al., 2003, Klančnik et al., 2014; Lee et al., 2021; Piskernik et al., 2011). It was shown that organic material residues like food particles and chicken juice can act as a conditioning layer on surfaces, which can increase bacterial adhesion (Arnold and Bailey, 2000; Brown et al., 2014; Li et al., 2017; Melo et al., 2017).

As there remains a lack of information on the concentration and dynamics of AI-2 production by *C. jejuni* during growth, our first objective was to develop a whole-cell biosensor method for *C. jejuni* AI-2 quantification in growth medium. In addition, a model food system for investigation of these aspects was defined too. The development of whole-cell biosensor method was based on *V. harveyi* biosensor strain selection, using various modelling and experimental approaches. Secondly, HPLC with fluorescence detection (HPLC-FLD) was used here for the first time for *C. jejuni* AI-2 detection and quantification. Finally, the dynamics of AI-2 synthesis as a function of cell density were determined using both approaches.

2. Materials and methods

2.1. Bacterial strains

Campylobacter jejuni 81–176 and *C. jejuni* 81-176Δ*luxS* were used in this study. To generate the *luxS* mutant, the *C. jejuni* 81–176 recipient strain was transformed (Wang and Taylor, 1990) by genomic DNA prepared from the *luxS* mutant described previously (Plummer et al., 2012). For the biosensor assay, four different *V. harveyi* strains were used: BB170, MM30, BB152 and MM32 (Bassler et al., 1997). For bacterial growth conditions, see Supplementary Methods, Bacterial growth conditions. For details about *V. harveyi* quorum sensing mechanism and *V. harveyi* strains, see Supplementary Methods, *Vibrio harveyi* quorum sensing mechanism and *Vibrio harveyi* biosensor strains.

2.2. Preparation of C. jejuni 81–176 and C. jejuni 81-176ΔluxS spent growth medium

Overnight cultures of *C. jejuni* 81–176 and *C. jejuni* 81-176Δ*luxS* were inoculated into Mueller–Hinton (MH) broth to OD_{600} = 0.1 a.u. Value OD_{600} = 0.1 a.u. corresponds to (1.1 ± 0.1) x 10^7 CFU/mL (Supplementary Table S1). For further experiments, the cultures were diluted 100-fold in fresh growth medium. This fresh growth medium was either the defined growth medium (fresh MH broth) or the model food system (5% sterile chicken juice in fresh MH broth). The cultures were incubated microaerobically at 42 °C for up to 48 h, with regular sampling. After these incubations, the culture samples were filtered through 0.2-μm syringe filters, to provide the spent growth medium (SGM) without bacteria. The SGM was stored at −80 °C until use.

2.3. Selection of the V. harveyi biosensor strain for quantitative AI-2 bioassay

The biosensor assay, based on the procedure by Sivakumar et al. (2011), was used to test the response levels to AI-2 in SGM from *C. jejuni* 81–176 in four different *V. harveyi* biosensor strains (BB170, MM30, BB152 and MM32). Fresh MH broth and SGM from *C. jejuni* 81-176Δ*luxS*, which did not contain any AI-2, were used as the negative controls (for details, see Supplementary Methods, Biosensor assay). Based on the results of this biosensor assay, the biosensor strain with best response characteristics was selected for quantitative AI-2 bioassay.

2.4. Biosensor-based quantification of AI-2 concentrations in defined growth medium and a model food system

Current quantitative AI-2 bioassays have relatively low LOD and/or provide only relative AI-2 amounts (Sivakumar et al., 2011; Taga and Xavier, 2011). To develop sensitive, quantitative AI-2 bioassay we followed the general procedures for quantification of the concentrations of signalling molecules in absolute units using whole-cell-based biosensors as described by Dogsa et al. (2021). First, we obtained the medium that contained the AI-2 (i.e., experimental SGM; from *C. jejuni* 81–176 cultures) and the medium that did not contain the AI-2 (i.e., control SGM; from *C. jejuni* 81-176Δ*luxS* cultures). To obtain AI-2 concentration gradient the experimental SGM was diluted with control SGM. Next, the AI-2 dilution series was mixed with fresh autoinducer bioassay growth medium that was inoculated with the biosensor, *V. harveyi* MM30. After the incubation, the biosensor bioluminescent response was measured (see Supplementary Methods, Quantitative AI-2 bioassay).

This quantitative AI-2 bioassay provides the bioluminescence responses of the *V. harveyi* MM30 biosensor as two datasets: as a function of the AI-2 concentration in the experimental SGM, and in medium containing a known AI-2 concentration (see Supplementary Methods, Quantitative AI-2 bioassay). Data were fitted to Equation (1), which is the Hill modelling equation adopted for AI-2 (Chu et al., 2009; Dogsa et al., 2021):

$$W([AI\text{-}2]) = \frac{W_{max}[AI\text{-}2]^n}{Km^n + SM[AI\text{-}2]^n} \quad (1)$$

where W is the maximum normalised resposne to AI-2 at the concetration *[AI-2]*, *Km* is the AI-2 concentration at which half of the maximum response is achieved, and n (the Hill coefficient) describes the cooperativity between the transcriptional activators.

After data were fitted, the sample and calibration curves were obtained. The standard for calibration was DPD (Carbosynth, UK) across a range of known concentrations. DPD is a precursor of AI-2 and it is used as a standard for AI-2 (Song et al., 2014). The concentrations of AI-2 in the SGM were calculated in absolute units based on the comparison of the experimental samples with the calibration curves (based on the middle section of the sigmoid curve). Thus, possible saturation and consequent underestimation of AI-2 concentrations was avoided.

2.5. Detection and quantification of AI-2 with HPLC-FLD

The AI-2 produced by *C. jejuni* 81–176 in the MH broth were detected by HPLC-FLD here, for the first time, following the protocol of Song et al. (2014) used for detection and quantification of AI-2 in *E. coli* and *V. harveyi*. Briefly, the metabolic precursor of AI-2, DPD that exists in an equilibrium with AI-2 in water (Galloway et al., 2010; Miller et al., 2004; Semmelhack et al., 2005), was used as the HPLC standard. To obtain positive control, the MH broth was spiked with DPD. *C. jejuni* 81-176Δ*luxS* SGM was used as the negative control (i.e., control SGM; with no AI-2). The working solutions of DPD (range, 0.3–2.5 μM) were obtained through dilution in MH broth of the stock solution of 800 μM DPD. To quantify the concentrations of AI-2 produced by *C. jejuni* 81–176 in MH broth, the calibration curve was constructed using DPD at the above concentrations. To detect the AI-2 by FLD, the samples were derivatized at 90 °C for 40 min using 0.2 mg/mL diaminonaphthalene (DAN) (Sigma Aldrich, Steinheim am Albuch, Germany) in aqueous solution. The obtained fluorescent derivative product, 1-(3-methyl-bezo [g]quinoxalin-2-yl)-ethane-1,2-diol, has the excitation and emission wavelengths at 271 and 503 nm, respectively (Lee et al., 2021; Song et al., 2014). The DAN stock solution was prepared by dissolving 10 mg DAN in 50 mL 0.1 M HCl. After the samples were cooled to room temperature, they were analysed using HPLC-FLD: the samples (20 μL) were injected into the HPLC system (Knauer, Berlin, Germany) equipped with a spectrofluorometric detector (Shimadzu RF-551; Kyoto, Japan). Separation was achieved on C-18 reverse-phase column (Nucleodur; 150 × 4.6 mm; 5 μm; Macherey-Nagel) maintained at 30 °C.

2.6. Statistical analysis

All of the experiments were carried out as three or more (biological) independent experiments, with each independent experiment consisting of three or more technical replicates. The data are expressed as means ± standard deviation. Statistical analysis was performed using OriginPro (OriginLab, Northampton, USA), with unpaired Student's t-tests (two sided). Data were accepted as significant at $p < 0.05$. The model fits are shown with 95% confidence levels. Fitting of biosensor response data was also performed in OriginPro, using reduced χ^2 and R^2 as measures for quality of fits. To minimise the reduced χ^2, the Levenberg-Marquardt method was applied. The AI-2 concentrations and their lower limit of detection (LOD) for the biosensor assays were calculated from the calibration curves, with LOD defined as the minimal AI-2 concentrations that induced a response in the biosensor *V. harveyi* MM30 that significantly differed from the response of the blank. Therefore, the LOD was determined as the AI-2 concentrations at the response equal to the response of the blank +3 standard deviations of the response of the blank.

3. Results

3.1. Selection of the most suitable biosensor V. harveyi strain for studying bacterial communication in C. jejuni

To quantify AI-2 produced by *C. jejuni* 81–176, the biosensor with the best characteristics in response to AI-2 was needed. For this purpose, four different *V. harveyi* biosensor strains were tested: BB170, MM30, BB152 and MM32 (for details see Supplementary Methods, Biosensor assay). The negative controls were fresh MH broth (medium control) and *C. jejuni* 81-176Δ*luxS* SGM (control SGM), both of which contained no AI-2.

All four *V. harveyi* biosensors showed increased bioluminescence after addition of fresh MH broth, *C. jejuni* 81-176Δ*luxS* SGM, and experimental SGM from *C. jejuni* 81–176 (Fig. 1). Of note, there were no statistically significant differences between the responses of the biosensors to the medium control and the control SGM ($p > 0.05$), which confirmed that this *C. jejuni* 81-176Δ*luxS* mutant did not produce AI-2.

The largest differences between the responses to the control and experimental SGM were observed with the biosensor *V. harveyi* MM30 (Fig. 1B). These differences exceeded 2 $\log_{10}$ in the time frame of 4 h–8 h, when *V. harveyi* MM30 was in exponential growth phase (Supplemental Fig. S1). Indeed, they were significantly different even after 8 h of incubation ($p < 0.05$), when *V. harveyi* MM30 was in the stationary growth phase (Supplementary Fig. S1). For the other biosensors, these differences were less pronounced, which suggested that *V. harveyi* MM30 was the most sensitive to the AI-2 in the *C. jejuni* 81–176 SGM, and it was therefore selected for the further analysis.

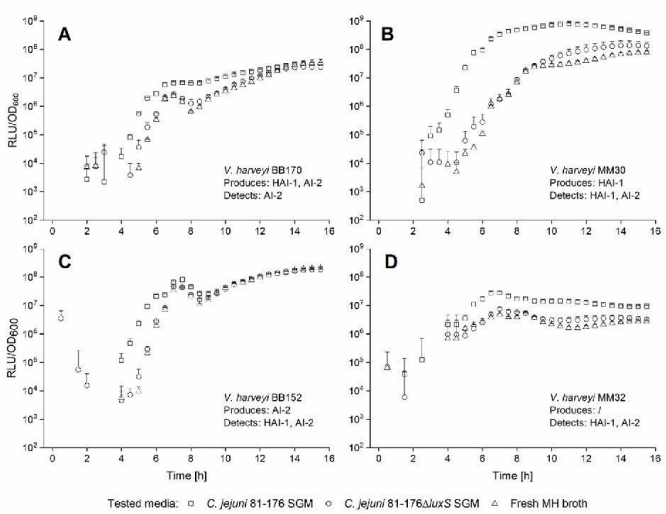

Fig. 1. Bioluminescence responses of the *Vibrio* biosensor strains. *V. harveyi* BB170 (**A**), *V. harveyi* MM30 (**B**), *V. harveyi* BB152 (**C**) and *V. harveyi* MM32 (**D**) responses to addition of fresh MH broth (△, medium control) and to spent growth medium from *C. jejuni* 81-176Δ*luxS* (AI-2 production deficient strain) (○, negative control) and spent growth medium from *C. jejuni* 81–176 (AI-2 producing strain) (□, experimental SGM). Bioluminescence was measured for 15 h at 30 °C under an aerobic atmosphere. Relative luminescence units were normalised to OD_{600}, and data are means ± standard deviation of 3 biological replicates, each consisting of 8 technical replicates. For clarity, only positive standard deviation is shown, and only every other measured point is shown. Production or detection of two signalling molecules HAI-1 or AI-2 by V. *harveyi* is indicated; for details see Supplementary Methods, *Vibrio harveyi* quorum sensing mechanism and *Vibrio harveyi* biosensor strains.

3.2. Whole-cell-based biosensor quantification of C. jejuni AI-2

The aim of developing the quantitative whole-cell-based biosensor assay was to obtain a reliable, accurate, and sensitive method for measuring the concentrations of AI-2 produced by *C. jejuni* 81–176. The optimal time for incubation of the biosensor in the presence of AI-2 was determined for quantification of the biosensor response to AI-2 by monitoring the growth and bioluminescence responses of the biosensor up to 15 h after addition of DPD (positive control) or experimental SGM. After 8 h of incubation, the bioluminescence response of the *V. harveyi* MM30 biosensor was saturated (i.e., there was no further increase in bioluminescence) and showed the largest differences from the controls (Fig. 1B). Therefore, this time point was chosen for AI-2 quantification in the further analysis.

After incubation of the biosensor in the presence of several dilutions of experimental SGM, the response levels as a function of relative dilutions were obtained using a luminometer. The response levels are expressed as the relative luminescence units per cell, normalised to the maximum response, W_{max}. This thus provided a relative measure, W, of how strongly the biosensor cells responded to the signalling molecule; i.e., to added DPD or to AI-2 produced by *C. jejuni*. This is shown as a function of exogenously added AI-2 (Fig. 2). The sigmoidal functions, which represent the Hill equation (see Equation (1)), can describe the relationships between transcription factors and promoter activities and can be applied to such whole-cell-based biosensors (Chu et al., 2009; Dogsa et al., 2021). In this case, LuxR is a transcription factor that binds directly to the promoter region of the *lux* operon that codes for luciferase. The resulting luciferase production thus defines the bioluminescence (Bassler et al., 1997) as a measure of promoter activity; i.e., the response level W. As the LuxR concentration is dependent on the AI-2 concentration, it can be reasonably expected that the response level W will follow Equation (1).

Campylobacter jejuni 81–176 was grown either in defined growth medium (fresh MH broth) or in a model food system (5% chicken juice in MH broth). During growth, the corresponding SGM that contained unknown concentrations of AI-2 was periodically collected and diluted in the control SGM from *C. jejuni* 81-176Δ*luxS* cultivated in defined growth medium, or in the model food system, with the AI-2 concentrations determined using the biosensor *V. harveyi* MM30. The calibration curve was obtained for determination of these AI-2 concentrations in absolute units. The standard molecule DPD was diluted in control SGM, cultivated in the defined growth medium or in the model food system, and also tested using the biosensor *V. harveyi* MM30 (Fig. 2A). The example in Fig. 2B of the biosensor *V. harveyi* MM30 maximum normalised responses to the experimental SGM alone (i.e., without added standard DPD) was measured as relative luminescence units per OD_{600}. The fitting parameters are given in Supplementary Table S2. For AI-2 quantification, a comparison of non-normalised responses was carried out (Supplementary Fig. S2). Only the linear parts of the curves were considered for AI-2 concentration determination. The concentration of AI-2 in the experimental (i.e., *C. jejuni* 81–176) SGM is shown as an example in Supplementary Fig. S2, and it was (1.21 ± 0.05) μM.

The same procedure was performed for *C. jejuni* 81–176 cultivated in the model food system. As shown in Fig. 3 and Supplementary Table S3, the modelling equation (Equation (1)) was also successfully applied here. As illustrated in Supplementary Fig. S3, the concentration of AI-2 in the model food system was (6.1 ± 2.3) μM. This concentration was about 5-fold higher than those in the defined experimental SGM at the same time, even though the growth curves of *C. jejuni* 81–176 in the defined growth medium and in the model food system were comparable (Supplementary Fig. S4).

3.3. Detection of AI-2 produced by C. jejuni 81–176 using HPLC-FLD

To verify the whole-cell-based biosensor quantification approach, the comparison to an independent analytical method was performed. We have used HPLC-FLD, introduced for the first time to detect AI-2 produced by *C. jejuni* 81–176. The metabolic precursor of AI-2, DPD, was used as the positive control, with DPD (1.875 μM) added to the MH broth to detect it and verify the identity of the peak. The control SGM from *C. jejuni* 81-176Δ*luxS* was used as the negative control (no signal present). Typical chromatograms of MH broth spiked with DPD (1.875 μM), *C. jejuni* 81-176Δ*luxS* SGM and *C. jejuni* 81–176 SGM are shown in Fig. 4. Good separation of the derivate product from the background of the complex matrix (i.e., MH broth) was achieved, as no extraneous peaks were observed at the corresponding retention time (approximately 7.9 min). The absence of this chromatographic peak in the SGM of *C. jejuni* 81-176Δ*luxS* confirms that this mutant does not produce AI-2. The presence of the same chromatographic peak at 7.9 min in the SGM of *C. jejuni* 81–176 confirms the DPD/AI-2 is released from *C. jejuni* 81–176.

The HPLC-FLD method for *C. jejuni* 81–176 AI-2 detection and

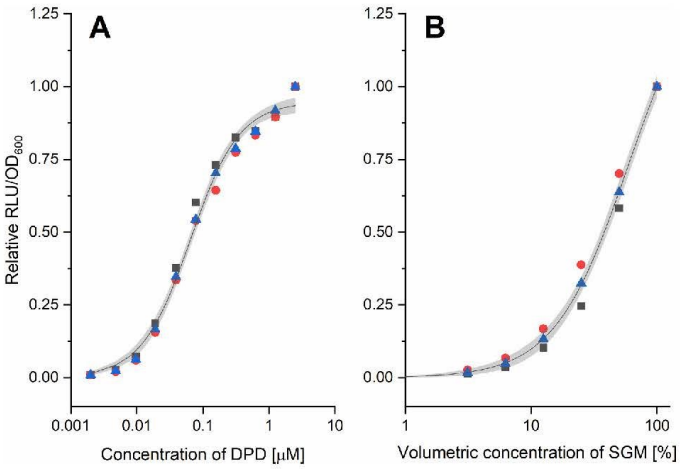

Fig. 2. Signalling molecule concentrations determined in medium using the biosensor-based assay. Maximum normalised responses of *V. harveyi* MM30 to exogenously added DPD at different concentrations (**A**; i.e., positive control) and to *C. jejuni* 81–176 SGM after 20 h of growth (**B**; i.e., experimental SGM). Data are means ± standard deviation of 3 biological replicates, each consisting of 8 technical replicates. Grey lines, best concatenated fit according to Equation (1), with 95% confidence interval are shown. For calculation of AI-2 concentrations, the relevant non-normalised data for the *V. harveyi* MM30 responses were used (Supplementary Fig. S2).

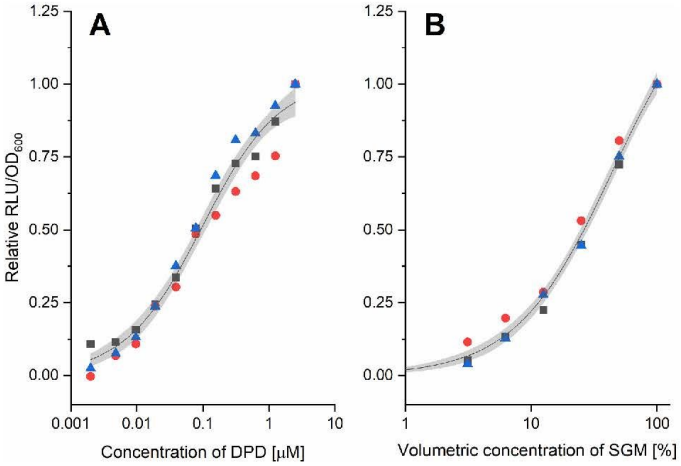

Fig. 3. Signalling molecule concentrations determined in the model food system using the biosensor-based assay. Maximum normalised responses of *V. harveyi* MM30 to exogenously added DPD at different concentrations (**A**; i.e., positive control; prepared in *C. jejuni* 81-176Δ*luxS*, cultivated in the model food system for 24 h), and to *C. jejuni* 81–176 SGM cultivated in the model food system (**B**; i.e., experimental SGM). Data are means ± standard deviation of 3 biological replicates, each consisting of 8 technical replicates. Grey lines, best concatenated fit according to Equation (1) with 95% confidence interval are shown. For calculation of AI-2 concentrations, the relevant non-normalised data for the *V. harveyi* MM30 responses were used (Supplementary Fig. S3).

quantification enabled the parallel validation of the results obtained by the biosensor method.

3.4. Dynamics of *C. jejuni* 81–176 AI-2 synthesis

The concentrations of AI-2 in the SGM of *C. jejuni* 81–176 and the growth of *C. jejuni* 81–176 over time were investigated. AI-2 concentrations were determined and compared using the two different analytical methods, as the biosensor-based (*V. harveyi* MM30) and the classical (HPLC-FLD) methods. To calculate the concentrations of AI-2 using the biosensor-based method, a mathematical model was used as described previously. To calculate the concentrations of AI-2 determined by HPLC-FLD, a calibration curve was determined using the standard signalling molecule DPD at known concentrations (Supplementary Fig. S5).

The concentrations of AI-2 in the *C. jejuni* 81–176 SGM followed the *C. jejuni* 81–176 cell concentrations and reached a maximum after 20 h (Fig. 5). At this time, the HPLC-FLD indicated a concentration of (1.60 ± 0.16) µM, while the biosensor method indicated (1.21 ± 0.05) µM (Table 1). After that, both the signalling molecule concentrations and the *C. jejuni* 81–176 cell concentrations decreased. It is important to note that in the *C. jejuni* 81–176 SGM the AI-2 concentrations have increased linearly with the cell concentrations (Supplementary Fig. S7).

The differences in the concentrations of the signalling molecules obtained by each of these analytical methods were mostly within the experimental error (Table 1). Thus, there were no statistically significant differences between the AI-2 concentrations obtained by the biosensor assay and the independent HPLC-FLD method at any time ($p > 0.05$). This confirmed that the biosensor assay is an accurate method that allows quantification of such signalling molecules. Moreover, the biosensor method made it possible to measure the concentrations of AI-2 in the of *C. jejuni* 81–176 SGM after 4 h of incubation, at only (0.008 ± 0.001) µM, while HPLC-FLD did not detect such low signalling molecule concentrations (Table 1). Here, the LOD of the biosensor method was lower than the LOD of the classical HPLC-FLD method by a factor of about 100 (Fig. 5).

4. Discussion

In the present study, a reliable and sensitive biological method was developed to detect and quantify *C. jejuni* AI-2 produced in a defined growth medium and in a model food system. The data obtained by the biosensor method were verified using the independent method of HPLC-FLD. The proposed approach is a methodological novelty in the field of bacterial communication in *C. jejuni*, and enabled us to measure the precise concentrations of AI-2 produced in the SGM of *C. jejuni* for the first time.

Vibrio harveyi whole-cell biosensors are most often used for qualitative detection of signalling molecules produced by different bacteria (Delle Side et al., 2015), as these biosensors are non-pathogenic and are easy to cultivate (Montánchez and Kaberdin, 2020). To upgrade the method for this quantitative detection of signalling molecules in *C. jejuni*, the SGM of non-producing *C. jejuni* 81-176Δ*luxS* (control SGM) and AI-2 producing *C. jejuni* 81–176 (experimental SGM) were initially examined using four different *V. harveyi* biosensors. The response of these biosensors was measured as the bioluminescence intensities per biosensor unit. In principle, it is expected that the most suitable AI-2 biosensor will respond only to AI-2, as this will result in a high ratio of response induced by AI-2 proficient medium against AI-2 deficient medium. However, in *V. harveyi*, expression of bioluminescence can be triggered by three different signalling molecules: HAI-1, CAI-1 and AI-2 (Papenfort and Bassler, 2016), although the triple HAI-1$^-$, CAI-1$^-$ and AI-2$^-$ mutant has a modest growth defect (Waters and Bassler, 2006) and thus, is not suitable for studying the communication systems of other bacteria. Moreover, it is important to note that CAI-1 is a strain-specific signalling molecule, playing a minor role in *V. harveyi* quorum-sensing gene expression and triggers bioluminescence at lower cell densities (Waters and Bassler, 2006). HAI-1 and AI-2 are the major signalling molecules that trigger strong bioluminescence responses of *V. harveyi* at higher cell densities (Anetzberger et al., 2009), which is important to measure their concentrations reliably. The detection of HAI-1 by all of the biosensor strains tested here should not be problematic, as HAI-1 was not detected in the *C. jejuni* strain used (see Sections 3.1, 3.3). This leads us to the expectation that the best candidate for an AI-2 biosensor for *C. jejuni* is *V. harveyi* MM32, as this mutant does not produce signalling molecules AI-2 and HAI-1 due to the lack of functional enzymes (LuxS and LuxM) responsible for their synthesis. Indeed, AI-2 producing *V. harveyi* strains showed low or non-significant differences between the AI-2 deficient and AI-2 proficient SGM, which was not the case for *V. harveyi* MM32 (Fig. 1). However, of the strains tested here, *V. harveyi* MM30 was even better. Not only did it produce the strongest response to AI-2 from *C. jejuni* 81–176 SGM, but it also had the highest

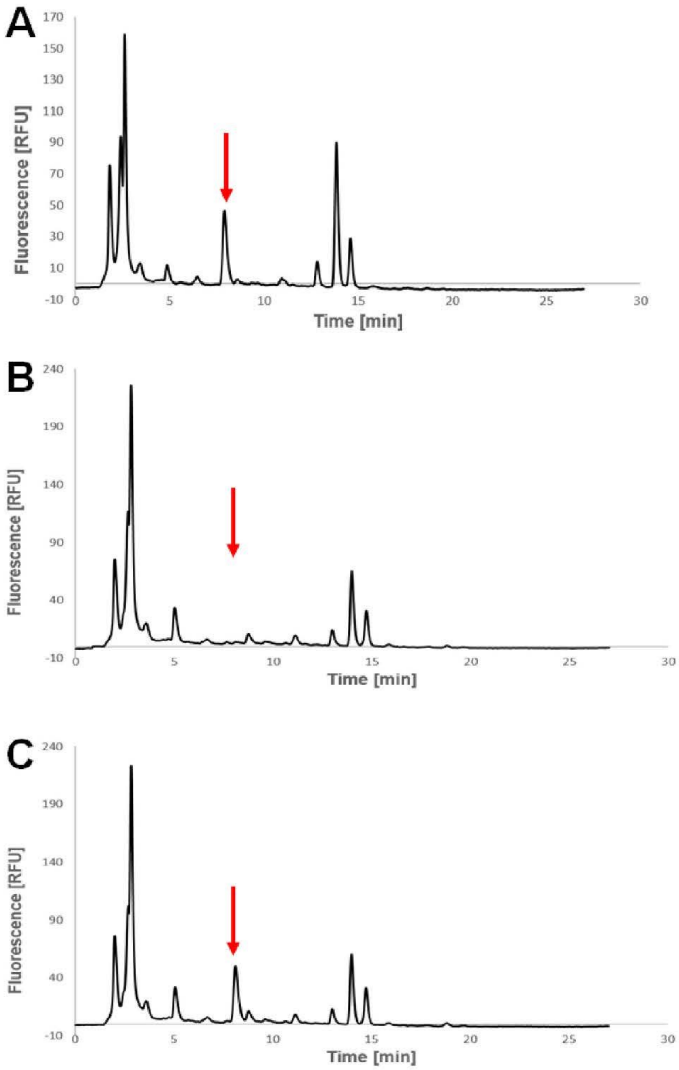

Fig. 4. HPLC with fluorescence detection for identification of AI-2. Representative chromatograms are shown for: MH broth spiked with DPD (1.875 μM) that exists in equilibrium with AI-2 in aqueous environment (A, positive control), *C. jejuni* 81-176Δ*luxS* SGM (B, negative control) and *C. jejuni* 81–176 SGM (C, experimental SGM). All the samples were derivatized by diaminonaphthalene (DAN), to obtain fluorescently labelled DPD product, 1-(3-methyl-bezo[g] quinoxalin-2-yl)-ethane-1,2-diol. The arrow indicates the retention time of approximately 7.9 min corresponding to the fluorescently labelled DPD derivative product. The chromatogram of *C. jejuni* 81-176Δ*luxS* SGM (B) also confirms that this mutant does not produce AI-2. Additional control chromatograms are shown in Supplementary Fig. S6.

ratio to the control, AI-2 deficient, *C. jejuni* 81-176Δ*luxS* SGM (100:1). The explanation for this can be found in the production of HAI-1 by V. *harveyi* MM30, as this can amplify the effects of AI-2 on the bioluminescent response. This is in agreement with Anetzberger et al. (2012) and Aqawi et al. (2020), who reported that induction of bioluminescence in *V. harveyi* by HAI-1 is dependent on the presence of other autoinducers.

This self-amplification of the response to AI-2 by V. *harveyi* MM30 enabled the measurement of lower AI-2 concentrations (by a factor of 100) compared to the reference HPLC-FLD method. This helped to define the linear relationship between AI-2 concentration and *C. jejuni* 81–176 cell density over extended ranges of cell densities. This linear relationship indicates that the information about cell density is "ideally" encoded; i.e., the signalling molecule per cell is constant regardless of the cell density (Drees et al., 2014). On the other hand, it is hard to achieve a switch-like response characteristic for true quorum sensing (Hense and Schuster, 2015) in a system where the signalling molecules follow a linear cell density response, even if the response system is ultra-sensitive (Dogsa et al., 2021). Also, we show here that the AI-2 concentration in *C. jejuni* 81–176 is growth medium dependent, even though the growth curves (and thus the cell densities) were the same. In addition, the linearity in AI-2 production implies that there is no specific regulation of AI-2 production in *C. jejuni* (i.e., AI-2 is constitutively produced) (Dogsa et al., 2021).

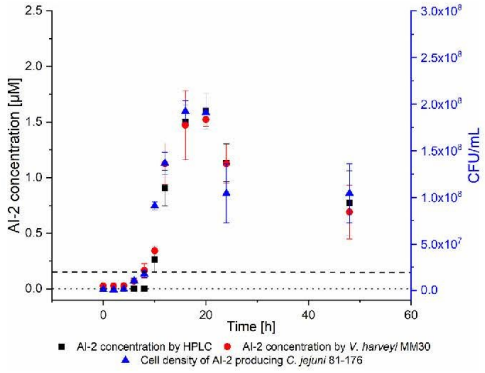

Fig. 5. Dynamics of AI-2 synthesis in spent growth medium from *C. jejuni* 81–176 according to the biosensor (*V. harveyi* MM30 circles) and HPLC with fluorescence detection (squares). Spent growth medium from *C. jejuni* 81–176 was collected at different times. CFU/mL was also recorded for each sample, as a measure of viable *C. jejuni* 81–176 cells during their growth. The limits of detection for the biosensor method (dotted line) and the HPLC method (dashed line) are also shown and correspond to 0.001 μM and 0.15 μM, respectively. Data are means ± standard deviation.

Table 1
AI-2 production by *C. jejuni* 81–176 according to the detection and quantification methods.

Time	AI-2 concentration (μM)	
(h)	HPLC with fluorescence detection	*Vibrio harveyi* MM30 biosensor
0	<LOD	<LOD
2	<LOD	<LOD
4	<LOD	0.008 ± 0.001
6	<LOD	0.034 ± 0.002
8	<LOD	0.11 ± 0.05
10	0.26 ± 0.12	0.25 ± 0.07
12	0.91 ± 0.16	0.89 ± 0.15
16	1.50 ± 0.16	1.17 ± 0.25
20	1.60 ± 0.16	1.21 ± 0.05
24	1.13 ± 0.17	0.89 ± 0.10
48	0.77 ± 0.29	0.54 ± 0.20

Data are means ± standard deviation.
LOD, limit of detection.

Altogether, these data are in favour of the hypothesis of Holmes et al. (2009), who suggested that AI-2 in *C. jejuni* merely represents a metabolic by-product that is produced in a crucial central metabolic pathway, i.e., the methyl cycle, that is driven by LuxS (Pereira et al., 2013). Nevertheless, on the basis that AI-2 is an interspecies signalling molecule, the AI-2 produced by *C. jejuni* has the potential to induce the phenotypic response in other bacterial species. Consequently, this will impact upon inter-species interactions, such as those in mixed species biofilms that are believed to enhance *C. jejuni* survival and virulence (Teh et al., 2014).

In the future, we can envision the integration of such whole-cell biosensor assay into microfluidic systems, which have great potential for multiplex monitoring of various contaminants (Cao et al., 2021). In the case of food, one such contaminant could be AI-2 signalling molecules, which can indicate microbial food contamination. Combined with the simultaneous monitoring of some other food quality control parameters with microfluidic devices, the ability to monitor food spoilage online can be improved.

5. Conclusion

In this study we developed a whole-cell based biosensor assay for simple, safe, rapid, sensitive and accurate AI-2 quantification in complex matrices. This biological approach enabled the quantification of AI-2 in complex media from *C. jejuni*, a known food borne pathogen, the knowledge of which can help to improve the quality and safety of food production processes. This AI-2 production in *C. jejuni* was growth medium dependent. Together with the linear dependence of *C. jejuni* AI-2 with culture cell density, this supports the hypothesis that AI-2 is a metabolic by-product of *C. jejuni*, rather than a true quorum-sensing signalling molecule in *C. jejuni*.

CRediT authorship contribution statement

DR conceived and conducted experiments and wrote original draft. DR and SSM made conceptualization. DR and ID set methodology and analysed data. ID supervised research. ID, AK and SSM coordinated research, reviewed and edited draft. SSM provided funding acquisition. All authors read and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2022.114439.

References

Abisado, R.G., Benomar, S., Klaus, J.R., Dandekar, A.A., Chandler, J.R., 2018. Bacterial quorum sensing and microbial community interactions. mBio 9 (3). https://doi.org/10.1128/MBIO.02331-17 e02331-17.

Adler, L., Alter, T., Sharbati, S., Gölz, G., 2015. The signalling molecule autoinducer-2 is not internalised in *Campylobacter jejuni*. Berl. Munch. Tierarztl. Wochenschr. 128 (3–4), 111–116. PMID: 25876270.

Anetzberger, C., Pirch, T., Jung, K., 2009. Heterogeneity in quorum sensing-regulated bioluminescence of *Vibrio harveyi*. Mol. Microbiol. 73, 267–277. https://doi.org/10.1111/J.1365-2958.2009.06768.X.

Anetzberger, C., Reiger, M., Fekete, A., Schell, U., Stambrau, N., Plener, L., Kopka, J., Schmitt-Kopplin, P., Hilbi, H., Jung, K., 2012. Autoinducers act as biological timers in *Vibrio harveyi*. PLoS One 7, e48310. https://doi.org/10.1371/JOURNAL.PONE.0048310.

Aqawi, M., Gallily, R., Sionov, R.V., Zaks, B., Friedman, M., Steinberg, D., 2020. Cannabigerol prevents quorum sensing and biofilm formation of *Vibrio harveyi*. Front. Microbiol. 858 https://doi.org/10.3389/FMICB.2020.00858.

Arnold, J.W., Bailey, G.W., 2000. Surface finishes on stainless steel reduce bacterial attachment and early biofilm formation: scanning electron and atomic force microscopy study. Poultry Sci. 79, 1839–1845. https://doi.org/10.1093/ps/79.12.1839.

Bassler, B.L., Greenberg, E.P., Stevens, A.M., 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. J. Bacteriol. 179, 4043–4045. https://doi.org/10.1128/JB.179.12.4043-4045.1997.

Birk, T., Ingmer, H., Andersen, M.T., Jørgensen, K., Brøndsted, L., 2003. Chicken juice, a food-based model system suitable to study survival of *Campylobacter jejuni*. Lett. Appl. Microbiol. 38, 66–71. https://doi.org/10.1046/j.1472-765X.2003.01446.x.

Bridier, A., Sanchez-Vizuete, P., Guilbaud, M., Piard, J.C., Naïtali, M., Briandet, R., 2015. Biofilm-associated persistence of food-borne pathogens. Food Microbiol. 45, 167–178. https://doi.org/10.1016/j.fm.2014.04.015.

Brown, H.L., Reuter, M., Salt, L.J., Cross, K.L., Betts, R.P., van Vliet, A.H.M., 2014. Chicken juice enhances surface attachment and biofilm formation of *Campylobacter jejuni*. Appl. Environ. Microbiol. 80, 7053–7060. https://doi.org/10.1128/AEM.02614-14.

Campagna, S.R., Gooding, J.R., May, A.L., 2009. Direct quantitation of the quorum sensing signal, autoinducer-2, in clinically relevant samples by liquid chromatography–tandem mass spectrometry. Anal. Chem. 81, 6374–6381. https://doi.org/10.1021/AC900824J.

Cao, Y., Zhang, B., Zhu, Z., Xin, X., Wu, H., Chen, B., 2021. Microfluidic based whole-cell biosensors for simultaneously on-site monitoring of multiple environmental contaminants. Front. Bioeng. Biotechnol. 9, 6221108 https://doi.org/10.3389/fbioe.2021.622108.

Chen, X., Schauder, S., Potier, N., Van Dorsselaer, A., Pelczer, I., Bassler, B.L., Hughson, F.M., 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415 (6871), 545–549. https://doi.org/10.1038/415545a.

Chu, D., Zabet, N.R., Mitavskiy, B., 2009. Models of transcription factor binding: sensitivity of activation functions to model assumptions. J. Theor. Biol. 257, 419–429. https://doi.org/10.1016/J.JTBI.2008.11.026.

Delle Side, D., Giuffreda, E., Tredici, S.M., Talà, A., Pennetta, C., Alifano, P., 2015. Quorum sensing: complexity in the bacterial world. Chaos, Solit. Fractals 81, 551–555. https://doi.org/10.1016/J.CHAOS.2015.05.011.

Dogsa, I., Spacapan, M., Dragoš, A., Danevčič, T., Pandur, Ž., Mandic-Mulec, I., 2021. Peptide signaling without feedback in signal production operates as a true quorum sensing communication system in *Bacillus subtilis*. Commun. Biol. 41 (4), 1–12. https://doi.org/10.1038/s42003-020-01553-5, 2021.

Drees, B., Reiger, M., Jung, K., Bischofs, I.B., 2014. A modular view of the diversity of cell-density-encoding schemes in bacterial quorum-sensing systems. Biophys. J. 107, 266–277. https://doi.org/10.1016/J.BPJ.2014.05.031.

EFSA/ECDC, The European Union One Health 2020 Zoonoses Report, 2021. EFSA J. 17 https://doi.org/10.2903/J.EFSA.2021.6971.

Elvers, K.T., Park, S.F., 2002. Quorum sensing in *Campylobacter jejuni*: detection of a luxS encoded signalling molecule. Microbiology 148, 1475–1481. https://doi.org/10.1099/00221287-148-5-1475.

Erega, A., Stefanic, P., Dogsa, I., Danevčič, T., Simunovic, K., Klančnik, A., Smole Možina, S., Mandic Mulec, I., 2021. Bacillaene mediates the inhibitory effect of *Bacillus subtilis* on *Campylobacter jejuni* biofilms. Appl. Environ. Microbiol. 87 https://doi.org/10.1128/AEM.02955-20.

Galloway, W.R.J.D., Hodgkinson, J.T., Bowden, S.D., Welch, M., Spring, D.R., 2010. Quorum sensing in Gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways. Chem. Rev. 111, 28–67. https://doi.org/10.1021/CR100109T.

Hense, B.A., Schuster, M., 2015. Core principles of bacterial autoinducer systems. Microbiol. Mol. Biol. Rev. 79, 153–169. https://doi.org/10.1128/MMBR.00024-14.

Holmes, K., Tavender, T.J., Winzer, K., Wells, J.M., Hardie, K.R., 2009. AI-2 does not function as a quorum sensing molecule in *Campylobacter jejuni* during exponential growth in vitro. BMC Microbiol. 91 (9), 1–11. https://doi.org/10.1186/1471-2180-9-214.

Hooshangi, S., Bentley, W.E., 2008. From unicellular properties to multicellular behavior: bacteria quorum sensing circuitry and applications. Curr. Opin. Biotechnol. 19, 550–555. https://doi.org/10.1016/J.COPBIO.2008.10.007.

Klančnik, A., Piskernik, S., Bucar, F., Vučković, D., Smole Možina, S., Jeršek, B., 2014. Reduction of microbiological risk in minced meat by a combination of natural antimicrobials. J. Sci. Food Agric. 94, 2758–2765. https://doi.org/10.1002/jsfa.6621.

Klančnik, A., Šimunović, K., Sterniša, M., Ramić, D., Smole Možina, S., Bucar, F., 2021. Anti-adhesion activity of phytochemicals to prevent *Campylobacter jejuni* biofilm formation on abiotic surfaces. Phytochem Rev. 55–84. https://doi.org/10.1007/s11101-020-09669-6.

Kreuder, A.J., Ruddell, B., Mou, K., Hassall, A., Zhang, Q., Plummer, P.J., 2020. Small noncoding RNA cjnc110 influences motility, autoagglutination, ai-2 localization, hydrogen peroxide sensitivity, and chicken colonization in *Campylobacter jejuni*. Infect. Immun. https://doi.org/10.1128/IAI.00245-20.

Lee, D.U., Park, Y.J., Yu, H.H., Jung, S.C., Park, J.H., Lee, D.H., Lee, N.K., Paik, H.D., 2021. Antimicrobial and antibiofilm effect of ε-Polylysine against *Salmonella Enteritidis*, *Listeria monocytogenes*, and *Escherichia coli* in tryptic soy broth and chicken juice. Foods 10, 2211. https://doi.org/10.3390/foods10092211.

Li, J., Feng, J., Ma, L., de la Fuente Núñez, C., Gölz, G., Lu, X., 2017. Effects of meat juice on biofilm formation of *Campylobacter* and *Salmonella*. Int. J. Food Microbiol. 253, 20–28. https://doi.org/10.1016/j.ijfoodmicro.2017.04.013.

Lowery, C.A., Dickerson, T.J., Janda, K.D., 2008. Interspecies and interkingdom communication mediated by bacterial quorum sensing. Chem. Soc. Rev. 37, 1337–1346. https://doi.org/10.1039/B702781H.

Melo, R.T., Mendonça, E.P., Monteiro, G.P., Siqueira, M.C., Pereira, C.B., Peres, P.A.B.M., Fernandez, H., Rossi, D.A., 2017. Intrinsic and extrinsic aspects on *Campylobacter jejuni* biofilms. Front. Microbiol. 8, 1332. https://doi.org/10.3389/fmicb.2017.01332.

Miller, S.T., Xavier, K.B., Campagna, S.R., Taga, M.E., Semmelhack, M.F., Bassler, B.L., Hughson, F.M., 2004. *Salmonella typhimurium* recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. Mol. Cell. 15, 677–687. https://doi.org/10.1016/j.molcel.2004.07.020.

Montánchez, I., Kaberdin, V.R., 2020. *Vibrio harveyi*: a brief survey of general characteristics and recent epidemiological traits associated with climate change. Mar. Environ. Res. 154, 104850 https://doi.org/10.1016/J.MARENVRES.2019.104850.

Papenfort, K., Bassler, B.L., 2016. Quorum sensing signal-response systems in Gram-negative bacteria. Nat. Rev. Microbiol. 14, 576–588. https://doi.org/10.1038/NRMICRO.2016.89.

Pereira, C.S., Thompson, J.A., Xavier, K.B., 2013. AI-2-mediated signalling in bacteria. FEMS Microbiol. Rev. 37, 156–181. https://doi.org/10.1111/J.1574-6976.2012.00345.X.

Piskernik, S., Klančnik, A., Tandrup Riedel, C., Brøndsted, L., Smole Možina, S., 2011. Reduction of *Campylobacter jejuni* by natural antimicrobials in chicken meat-related conditions. Food Control 22, 718–724. https://doi.org/10.1016/j.foodcont.2010.11.002.

Plummer, P., Sahin, O., Burrough, E., Sippy, R., Mou, K., Rabenold, J., Yaeger, M., Zhang, Q., 2012. Critical role of LuxS in the virulence of *Campylobacter jejuni* in a Guinea pig model of abortion. Infect. Immun. 80, 585. https://doi.org/10.1128/IAI.05766-11.

Püning, C., Su, Y., Lu, X., Gölz, G., 2021. Molecular mechanisms of *Campylobacter* biofilm formation and quorum sensing. Curr. Top. Microbiol. Immunol. 431 https://doi.org/10.1007/978-3-030-65481-8_11.

Rajamani, S., Sayre, R., 2018. Biosensors for the detection and quantification of AI-2 class quorum-sensing compounds. Methods Mol. Biol. 1673, 73–88. https://doi.org/10.1007/978-1-4939-7309-5_6.

Schauder, S., Shokat, K., Surette, M.G., Bassler, B.L., 2001. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol. Microbiol. 41 (2), 463–476. https://doi.org/10.1046/j.1365-2958.2001.02532.x.

Semmelhack, M.F., Campagna, S.R., Federle, M.J., Bassler, B.L., 2005. An expeditious synthesis of DPD and boron binding studies. Org. Lett. 7, 569–572. https://doi.org/10.1021/OL047695J.

Servinsky, M.D., Terrell, J.L., Tsao, C.-Y., Wu, H.-C., Quan, D.N., Zargar, A., Allen, P.C., Byrd, C.M., Sund, C.J., Bentley, W.E., 2015. Directed assembly of a bacterial quorum. ISME J. 101 (10), 158–169. https://doi.org/10.1038/ismej.2015.89, 2016.

Sivakumar, K.K., Jesudhasan, P.R., Pillai, S.D., 2011. Detection of autoinducer (AI-2)-like activity in food samples. Methods Mol. Biol. 692, 71–82. https://doi.org/10.1007/978-1-60761-971-0_6.

Song, X.N., Qiu, H. Bin, Xiao, X., Cheng, Y.Y., Li, W.W., Sheng, G.P., Li, X.Y., Yu, H.Q., 2014. Determination of autoinducer-2 in biological samples by high-performance liquid chromatography with fluorescence detection using pre-column derivatization. J. Chromatogr. A 1361, 162–168. https://doi.org/10.1016/J.CHROMA.2014.07.103.

Surette, M.G., Miller, M.B., Bassler, B.L., 1999. Quorum sensing in Escherichia coli, Salmonella typhimurium, and Vibrio harveyi: a new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci. Unit. States Am. 96 (4), 1639–1644. https://doi.org/10.1073/pnas.96.4.1639.

Taga, M.E., Xavier, K.B., 2011. Methods for analysis of bacterial autoinducer-2 production. Curr. Protoc. Microbiol., 22045583 https://doi.org/10.1002/9780471729259.mc01e01s23.

Teh, A.H.T., Lee, S.M., Dykes, G.A., 2014. Does *Campylobacter jejuni* form biofilms in food-related environments? Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.01493-14.

Thiel, V., Vilchez, R., Sztajer, H., Wagner-Döbler, I., Schulz, S., 2009. Identification, quantification, and determination of the absolute configuration of the bacterial quorum-sensing signal autoinducer-2 by gas chromatography–mass spectrometry. Chembiochem 10, 479–485. https://doi.org/10.1002/CBIC.200800606.

Vendeville, A., Winzer, K., Heurlier, K., Tang, C.M., Hardie, K.R., 2005. Making "sense" of metabolism: autoinducer-2, LuxS and pathogenic bacteria. Nat. Rev. Microbiol. 3, 383–396. https://doi.org/10.1038/NRMICRO1146.

Vilchez, R., Lemme, A., Thiel, V., Schulz, S., Sztajer, H., Wagner-Döbler, I., 2007. Analysing traces of autoinducer-2 requires standardization of the *Vibrio harveyi* bioassay. Anal. Bioanal. Chem. 387, 489–496. https://doi.org/10.1007/s00216-006-0824-4.

Wang, Y., Taylor, D.E., 1990. Natural transformation in *Campylobacter* species. J. Bacteriol. 172, 949–955. https://doi.org/10.1128/JB.172.2.949-955.1990.

Waters, C.M., Bassler, B.L., 2006. The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. https://doi.org/10.1101/gad.1466506.

Wattanavanitchakorn, S., Prakitchaiwattana, C., Thamyongkit, P., 2014. Rapid and simple colorimetric method for the quantification of AI-2 produced from *Salmonella* Typhimurium. J. Microbiol. Methods 99, 15–21. https://doi.org/10.1016/J.MIMET.2014.01.014.

Xavier, K.B., Bassler, B.L., 2003. LuxS quorum sensing: more than just a numbers game. Curr. Opin. Microbiol. 6, 191–197. https://doi.org/10.1016/S1369-5274(03)00028-6.

Xu, F., Song, X., Cai, P., Sheng, G., Yu, H., 2017. Quantitative determination of AI-2 quorum-sensing signal of bacteria using high performance liquid chromatography–tandem mass spectrometry. J. Environ. Sci. 52, 204–209. https://doi.org/10.1016/J.JES.2016.04.018.

Zhao, J., Quan, C., Jin, L., Chen, M., 2018. Production, detection and application perspectives of quorum sensing autoinducer-2 in bacteria. J. Biotechnol. 268, 53–60. https://doi.org/10.1016/J.JBIOTEC.2018.01.009.

Zhu, J., Pei, D., 2008. A LuxP-based fluorescent sensor for bacterial autoinducer II. ACS Chem. Biol. 3, 110–119. https://doi.org/10.1021/CB7002048.

2.1.3 Kontrola filmotvornosti bakterij *Campylobacter jejuni* z eteričnimi olji in etanolnimi izvlečki iz odpadnega materiala sivke vrste *Lavandula x intermedia* (lavandin)

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Živilska industrija se nenehno spopada z izrazito razširjeno, s hrano prenosljivo, filmotvorno bakterijo *Campylobacter jejuni*. Za nadzor biofilmov v celotni živilski industriji se uporabljajo različni pristopi, vendar nobeden ni popolnoma učinkovit. V tej študiji smo pripravili in določili kemijski profil eteričnih olj (EO), etanolnih izvlečkov cvetov pred destilacijo (EF) in etanolnih izvlečkov odpadnega materiala pridobljenega po destilaciji eteričnega olja (EWM) iz sivke vrste *Lavandula* × *intermedia*, podvrste 'Bila', 'Budrovka' St Nicholas in 'Budrovka', ki so bili nadalje uporabljeni za zmanjšanje medcelične signalizacije, pritrjevanja in filmotvornosti bakterij *C. jejuni* ter za testiranje njihovega antioksidativnega učinka. Glikozidi hidroksicimetne kisline so bili glavne spojine obeh vrst etanolnih izvlečkov lavandina, medtem ko so bili linalol, linalil acetat, 1,8-cineol in kamfor glavne spojine EO lavandina. Testirana EO so pokazala najmočnejše protibakterijsko delovanje z minimalno inhibitorno koncentracijo 0,25 mg/mL. Pripravki EF so se izkazali kot bolj učinkoviti pri zmanjševanju medcelične signalizacije in pritrjevanja bakterij *C. jejuni* v primerjavi s pripravki EO in EWM, medtem ko so EO pokazala nekoliko boljši učinek proti filmotvornosti. Zanimivo je, da smo največjo antioksidativno aktivnost ugotovili pri pripravkih EWM. Potrdili smo pozitivno in zmerno korelacijo med zmanjšanjem medcelične signalizacije in pritrjevanjem bakterij *C. jejuni,* ter med pritrjevanjem in filmotvornostjo. Rezultati nakazujejo, da so nove bakterijske tarče zanimive za nadzor filmotvornosti z alternativnimi naravnimi sredstvi v celotni živilski industriji.

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Campylobacter jejuni Biofilm Control with Lavandin Essential Oils and By-Products

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Abstract: The food industry is constantly struggling with one of the most prevalent biofilm-forming and food-borne pathogenic bacteria, *Campylobacter jejuni*. Different approaches are used to control biofilms in the food production chain, but none is fully effective. In this study, we aim to produce and determine the chemical profile of essential oils (EOs), ethanolic extracts of flowers prior to distillation (EFs), and ethanolic extracts of post-distillation waste material (EWMs) from *Lavandula* × *intermedia* 'Bila', 'Budrovka' St Nicholas and 'Budrovka', which were further used to reduce C. *jejuni* intercellular signaling, adhesion, and biofilm formation, as well as to test their antioxidant activity. Glycosides of hydroxycinnamic acids were the major constituents of both types of lavandin ethanolic extract, while linalool, linalyl acetate, 1,8-cineol, and camphor were the major compounds found in lavandin EOs. Tested EOs showed the best antibacterial activity with a minimal inhibitory concentration of 0.25 mg/mL. Lavandin EFs proved more effective in reducing C. *jejuni* intercellular signaling and adhesion compared to lavandin EOs and EWMs, while lavandin EOs showed a slightly better effect against biofilm formation. Interestingly, the best antioxidant activity was determined for lavandin EWMs. A positive and moderate correlation was found between the reduction of C. *jejuni* intercellular signaling and adhesion, as well as between adhesion and biofilm formation. These findings mean novel bacterial targets are of interest for biofilm control with alternative natural agents throughout the whole food production chain.

Keywords: *Campylobacter jejuni*; biofilm; adhesion; intercellular signaling; lavandin formulations

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1. Introduction

Microbial biofilms are the predominant form of bacterial lifestyle in industrial environments and protect bacteria from physical trauma, desiccation, and antimicrobial agents [1]. Numerous reports have found that food-borne pathogens persist on food contact surfaces (e.g., plastic, steel, glass, rubber, and wood) in the form of biofilms and affect the quality, quantity, and safety of food products. Moreover, their control is a serious challenge in the food production chain because they cause huge economic and energy losses, damage surfaces and equipment, and lead to continuous contamination of food, posing a major ongoing public health risk [2].

Pathogenic bacteria *Campylobacter jejuni* are one of the most common bacterial agents of self-limiting gastrointestinal diseases in humans, but they can also cause more serious neurological disorders, such as Guillain–Barré syndrome [3]. Contaminated surfaces and undercooked poultry meat are the most common vectors of pathogen transmission to humans. Campylobacters represent a severe public health burden in the European Union, where they caused approximately 121.000 intestinal infections in 2020, leading to huge

economic losses [4]. A global concern is also the increasing prevalence of antibiotic-resistant and biocide-resistant strains of *C. jejuni*, isolated mainly from poultry [5,6].

Although Campylobacters are considered susceptible bacteria, they survive in environments outside their natural habitat, i.e., intestines. Properties such as intercellular signaling with AI-2 signaling molecule, motility, and chemotaxis allow them to form biofilms or colonize existing biofilms on abiotic surfaces such as polystyrene, glass, and stainless steel. Within biofilms, Campylobacters are protected from antimicrobial agents that penetrate the biofilm matrix slowly and poorly [7]. Various approaches are used to control Campylobacters in the food production chain, but none is fully effective [8]. Therefore, the right approach is needed to control Campylobacters and their biofilm formation in the food production chain at all stages, from primary production to slaughter, processing, and sale of meat. With increasing concerns about antibiotic resistance and environmental impacts, conventional antibiotics, biocides, and preservation methods are being replaced with naturally occurring alternatives that are recognized as safe (GRAS) and have a broad spectrum of antimicrobial activity, including prevention of intercellular signaling, adhesion, and biofilm formation (interrelated bacterial characteristics that are necessary for biofilm establishment) [9–12].

The plants from the *Lamiaceae* family, genus *Lavandula*, are an inexhaustible source of biologically active phytochemicals with great antimicrobial, anti-biofilm, and antioxidant properties. The genus *Lavandula* includes 39 species, numerous hybrids, and about 400 registered cultivars grown generally in the Mediterranean region [13]. Three *Lavandula* species are used for commercial essential oil (EO) production: *Lavandula angustifolia* Mill. (true lavender), *Lavandula* × *intermedia* Emeric ex Loisel syn. *L. hybrida* L. (lavandin) and *Lavandula latifolia* Medicus (spike lavender) [14]. Nowadays, the cultivation of lavandin, a natural sterile hybrid derived from a cross between *L. angustifolia* and *L. latifolia*, has become increasingly popular because of higher EO yields in comparison to true lavender (120 kg/ha compared to 40 kg/ha). It is preferred for personal care and hygiene products, industrial and household cleaners, as well as for antiseptics, antifungals, and insecticides [15,16]. In this study, flowers of indigenous Croatian cultivars *Lavandula* × *intermedia* 'Bila', 'Budrovka' St Nicholas (SN), and 'Budrovka' were used for the first time to prepare EOs and ethanolic extracts in order to test their anti-biofilm and antioxidant activity, features important for ensuring food safety and quality.

Higher production of EO consequently resulted in increased accumulation of by-products, i.e., waste materials and hydrolates gained after EO distillation [17,18]. These by-products remain a potentially important source of potent phytochemicals, as shown by the results for spike lavender (which have great antioxidant properties) and lavender hydrolates (which have antifungal and antibacterial properties) [19,20]. The reuse of such natural waste material is environmentally friendly, which makes it more popular than synthetic disinfectants that are often used in the food industry and can lead to additional unnecessary chemicals in the environment [21]. Our previous study has shown that *L. angustifolia* waste material had a promising anti-biofilm effect against pre-established *C. jejuni* biofilms [22], but in this study, lavandin waste materials were used to target *C. jejuni* properties, i.e., intercellular signaling, adhesion, and biofilm formation to prevent *C. jejuni* biofilm establishment.

The aim of this study was to find potential antimicrobials that will be able to prevent or reduce *C. jejuni* National Collection of Type Culture (NCTC) 11168 biofilm formation on an abiotic surface. For that purpose, dried flowers of *Lavandula* × *intermedia* 'Bila', 'Budrovka' SN, and 'Budrovka' were used to produce EOs, ethanolic extracts of flowers prior to distillation (EFs), and ethanolic extracts of post-distillation waste material (EWMs). Afterward, their chemical characterization was performed. *C. jejuni* intercellular signaling and adhesion were used as targets to prevent or reduce biofilm formation in its early stages. Further, *C. jejuni* biofilm formation, with the addition of lavandin formulations at subinhibitory concentration, was monitored in order to determine how preventive measures affect *C. jejuni* biofilm establishment. A correlation between intercellular signaling, adhesion

and biofilm formation was determined. Finally, the antioxidant activity of *L.* × *intermedia* formulations was also explored.

2. Results

2.1. Chemical Composition of Lavandin Ethanolic Extracts and EOs

The lavandin ethanolic extracts (EFs and EWMs) were analyzed for their phenolic compounds using LC-PDA-ESI-MS analysis. Glycosides of hydroxycinnamic acids were the major constituents of both types of lavandin ethanolic extract. Rosmarinic acid was found in both types of ethanolic extracts; salvianolic A and 3-(3,4-dihydroxyphenyl) lactic acid were only detected in the EWMs. The latter can be easily derived from rosmarinic acid by ester hydrolysis. In addition, flavones apigenin-7-O-glucoside and ladanein were detected. Flavonoids only had a minor role in the composition of the lavandin ethanolic extracts (EFs and EWMs). Table 1 summarizes the peaks that were identified for both types of lavandin ethanolic extract. It is interesting to note that the yield of ethanol extraction was higher for waste materials (EWMs) than for dried flowers (EFs) (Figure S1). Supplementary Figures S2 and S3 illustrate representative UV chromatograms for EFs and EWMs, respectively. To see the difference in composition between both types of extracts, EF and EWM, a comparison of peak areas of individual compounds, i.e., quantification of their relative amounts, is presented in Supplementary Figure S4.

Table 1. Identification of the main common phenolic compounds in the lavandin ethanolic extract (EF) and lavandin (EWM).

No.	Rt	Compound Identified	Full Scan MS (*m*/*z*)	Fragment Ions (MS^2; *m*/*z*)	UV Maximum (nm)
1	4.97	3-(3,4-Dihydroxyphenyl)lactic acid *	395 $[M+HCOOH-H]^-$, 197 $[M-H]^-$	197 (100)	225 sh, 281
2	8.34	Coumaric acid hexoside I	325 $[M-H]^-$	163 (100), 119 (25)	263, 290 sh
3	10.23	Ferulic acid hexoside I	355 $[M-H]^-$	193 (100), 149 (20)	302
4	10.23	Caffeic acid hexoside	387 $[M+HCOOH-H]^-$	341 (100), 207 (25)	302
5	12.13	Coumaric acid hexoside II	371 $[M+HCOOH-H]^-$, 325 $[M-H]^-$	325 (100)	277, 290 sh
6	14.21	Ferulic acid hexoside II	401 $[M+HCOOH-H]^-$, 355 $[M-H]^-$	355 (100)	295, 319
7	18.87	Apigenin-7-O-glucoside	431 $[M-H]^-$	269 (100)	268, 334
8	19.60	Rosmarinic acid	359 $[M-H]^-$	161 (100), 179 (30), 223 (10)	292 sh, 328
9	23.96	Salvianolic acid A *	493 $[M-H]^-$	295 (100), 313 (10)	287, 340 sh
10	30.24	Ladanein (5,6-di-OH-7,4′-dimethoxy flavone)	315 $[M-H]^-$	300 (100)	284, 333

* Not detected in lavandin ethanolic extract (EF).

According to the gas chromatography–mass spectrometry (GC–MS) analysis, used lavandin EOs belong to *Lavandula* × *intermedia* 'Bila', L. × intermedia 'Budrovka' SN, and L. × intermedia 'Budrovka' (Table 2). Linalool was the most represented terpene alcohol in the chemical composition of tested EOs, where lavandin EO 'Bila' contained 40.4%, lavandin EO 'Budrovka' SN 43.1%, and lavandin EO 'Budrovka' 47.2% linalool. Major differences were observed in the content of linalyl acetate, where lavandin EO 'Bila' contained 6.6%, lavandin EO 'Budrovka' SN 5.3%, and lavandin EO 'Budrovka' 26.7% linalyl acetate. Interestingly, lavandin EO 'Bila' and lavandin EO 'Budrovka' SN were comparable in composition, whereas lavandin EO 'Budrovka' showed remarkable differences, aside from its higher linalyl acetate content and its low content of lavandulol, endo-borneol, (Z)-β-ocimene, camphor, terpinene-4-ol and (E)-β-farnesene. In contrast, the content of 1,8-cineol was similar in all EOs (Table 2).

Table 2. Identification of the main components of the lavandin EOs.

Retention Time	Retention Index [a]	Compound [b]	Quantification of Total [c]		
			Lavandula × *Intermedia* 'Bila'	*Lavandula* × *Intermedia* 'Budrovka' SN	*Lavandula* × *Intermedia* 'Budrovka'
5.126	926	α-Thujene	0.086	0.139	0.012
5.301	932	α-Pinene	0.635	0.813	0.317
5.680	946	Camphene	0.248	0.33	0.031
6.349	973	Sabinene	0.173	0.196	0.178
6.442	975	β-Pinene	0.882	0.98	0.685
6.710	979	3-Octanon	tr	tr	0.601
6.851	991	Myrcen	0.27	0.311	0.343
7.449	1010	Δ3-Carene	0.235	0.222	tr
7.925	1023	p-Cymene	0.275	0.263	0.132
8.126	1030	1,8-Cineol	14.091	12.585	14.211
8.378	1037	(Z)-β-Ocimene	2.565	4.047	0.44
8.754	1047	(E)-β-Ocimene	0.128	0.27	0.046
9.115	1058	γ-Terpinene	0.104	0.165	tr
9.392	1065	cis-Sabinenhydrate	0.059	0.088	0.025
9.595	1071	cis-Linalool oxide (furanoid)	tr	0.027	0.116
10.187	1087	trans-Linalool oxide (furanoid)	tr	tr	0.097
10.198	1088	Terpinolene *	0.14	0.22	n.d.
10.662	1100	Linalool	40.412	43.058	47.206
11.141	1112	Octen-3-yl-1-acetate	0.05	0.024	0.235
12.323	1142	Camphor	2.998	1.371	0.383
12.616	1150	Hexyl-2-methylpropanoate	0.099	0.087	0.135
13.209	1163	endo-Borneol + Lavandulol	13.946	14.053	0.121
13.706	1175	Terpinen-4-ol	8.37	8.813	0.962
14.271	1190	α-Terpineol	1.159	0.827	1.106
14.418	1192	Hexylbutanoate	0.753	0.739	0.628
15.793	1227	endo-Bornylformiate *	0.23	0.171	n.d.
16.290	1237	Hexyl-2-methylbutanoate	0.206	0.194	0.276
16.497	1242	Hexylisovalerate	0.058	0.047	0.128
17.069	1256	Linalylacetate	6.645	5.264	26.709
18.577	1291	Lavandulylacetate	0.879	0.693	0.105
22.607/22.676	1387/1390	Hexylhexanoate + 7-epi-Sesquiphellandrene	0.148	0.118	0.069
23.773	1419	trans-Caryophyllene	0.663	0.625	1.704
25.423	1457	(E)-β-Farnesene	2.611	2.151	0.123
25.782	1466	Lavandulyl-butanoate *	0.102	0.051	n.d.
26.265	1480	Germacrene D	0.357	0.431	0.162
27.549	1510	Lavandulyl-isovalerate *	0.309	0.219	n.d.
30.174	1579	trans-Caryophyllenoxide	tr	0.018	0.341
34.016	1682	α-Bisabolol	tr	0.033	0.89

[a] Linear Retention Index relative to n-alkanes on HP-5-MS column; [b] Compounds identified by mass spectral libraries [23,24]; [c] Quantification by normalization (area percent method) without considering calibration factors. tr—in traces; n.d.—not determined; * not determined in *Lavandula* × *Intermedia* 'Budrovka'.

2.2. *Anti-Campylobacter Activity of Lavandin Formulations*

In order to evaluate the anti-Campylobacter activities of the lavandin EOs and ethanolic extracts (EFs and EWMs), their minimal inhibitory concentration (MIC) against *C. jejuni* NCTC 11,168 and *C. jejuni* 11168ΔluxS were determined (Table 3). The lavandin formulations showed antimicrobial efficacy against *C. jejuni* NCTC 11168 at a concentration ranging between 0.25–1 mg/mL. The most favorable effect was shown by samples from the group of EOs: the MICs for all EOs were 0.25 mg/mL. Slightly weaker antimicrobial activity was shown by a group of samples of ethanolic extracts, where lavandin EFs showed better

performance compared to lavandin EWMs (Table 3). The same MIC values were also determined against C. *jejuni* 11168ΔluxS, where concentration ranged between 0.25–1 mg/mL. Here, samples of EOs showed the best antimicrobial activity, while lavandin EWM proved to be less effective (Table 3).

Table 3. Minimal inhibitory and subinhibitory concentration determined against *C. jejuni* NCTC 11168 and *C. jejuni* 11168ΔluxS for the lavandin formulations (EOs, EFs and EWMs).

	***C. jejuni* NCTC 11168**		***C. jejuni* 11168ΔluxS**	
Sample	**MIC (mg/mL)**	**0.25 × MIC (mg/mL)**	**MIC (mg/mL)**	**0.25 × MIC (mg/mL)**
EO 'Bila'	0.25	0.062	0.25	0.062
EO 'Budrovka' SN	0.25	0.062	0.25	0.062
EO 'Budrovka'	0.25	0.062	0.25	0.062
EF 'Bila'	0.5	0.125	0.25	0.062
EF 'Budrovka' SN	1	0.25	0.5	0.125
EF 'Budrovka'	0.5	0.125	0.5	0.125
EWM 'Bila'	1	0.25	1	0.25
EWM 'Budrovka' SN	1	0.25	1	0.25
EWM 'Budrovka'	1	0.25	1	0.25

Determining the antibacterial activity of the lavandin formulations was crucial for further experiments. Based on the determined MIC values, a subinhibitory concentration (0.25 × MIC) was calculated to avoid the effect of the lavandin formulations on bacterial growth during the experiments (Table 3) [25]. Thus, the focus of this study was to monitor potential changes in *C. jejuni* properties that are important for biofilm establishment, i.e., intercellular signaling, adhesion, and biofilm formation, while exposed to lavandin formulations at subinhibitory concentration.

2.3. Modulation of Campylobacter Intercellular Signaling by Lavandin Formulations

The effect of lavandin formulations on the intercellular signaling of *C. jejuni* was verified indirectly by measuring the emitted bioluminescence of the biosensor strain *Vibrio harveyi* MM30. This biosensor was chosen because of its mutation in the luxS gene, which consequently does not synthesize AI-2 signaling molecules but can detect external AI-2 released into the growth medium by *C. jejuni* [26,27]. The intensity of the bioluminescence signal is proportional to the signal concentration in the tested spent medium (SM) [27].

C. jejuni NCTC 11168 and *C. jejuni* 11168ΔluxS were cultivated without or with the addition of the lavandin EOs and ethanolic extracts (EFs and EWMs) at a subinhibitory concentration (0.25 × MIC). Before determining the effect of lavandin formulations on the intercellular signaling of *C. jejuni*, the colony-forming units (CFUs) were determined to verify the effect on bacterial growth. Indeed, the used formulations did not significantly affect the growth of *C. jejuni* NCTC 11168 and *C. jejuni* 11168ΔluxS at a concentration of 0.25 × MIC ($p > 0.05$) (Supplementary Table S1). SMs of *C. jejuni* NCTC 11168 and *C. jejuni* 11168ΔluxS were further analyzed with the biosensor *V. harveyi* MM30, and emitted bioluminescence was measured for 15 h. The reduction of bioluminescence was calculated using Equation (1). This was the indirect measure for the reduction of *C. jejuni* intercellular signaling.

All lavandin EFs significantly reduced *C. jejuni* intercellular signaling ($p < 0.05$) (Figure 1). Lavandin EWM 'Bila' and EWM 'Budrovka' SN were also successful in reducing *C. jejuni* intercellular signaling ($p < 0.05$), while lavandin EOs did not have a significant effect on *C. jejuni* intercellular signaling ($p > 0.05$) (Figure 1). Lavandin EFs 'Bila' and 'Budrovka' and lavandin EWM 'Bila' reduced *C. jejuni* intercellular signaling by approximately 95% (Supplementary Table S2).

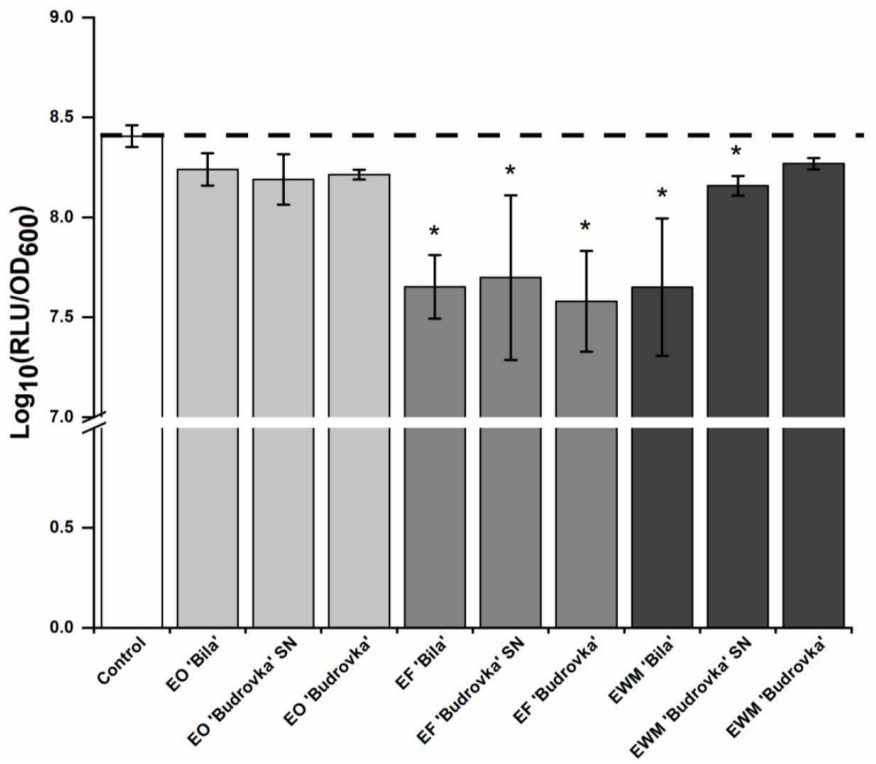

Figure 1. Effect of different lavandin formulations (EOs, EFs and EWMs) at subinhibitory concentration (0.25 × MIC) on the intercellular signaling of *C. jejuni* NCTC 11168. *C. jejuni* NCTC 11168 was cultivated for 24 h without or with the addition of lavandin formulations at subinhibitory concentration. Afterwards, SMs were prepared and added to *V. harveyi* MM30 biosensor strain. *V. harveyi* MM30 bioluminescence response was the indirect measure for *C. jejuni* intercellular signaling. Log_{10} average values ± SD are shown. * $p < 0.05$, vs. control. (EO, essential oil; EF, ethanolic extract prior to distillation; EWM, ethanolic extract of post-distillation waste material).

2.4. Modulation of Campylobacter Adhesion by Lavandin Formulations

Lavandin EOs, EFs, and EWMs were used at a subinhibitory concentration (0.25 × MIC) in order to prevent the adhesion of *C. jejuni* to a polystyrene surface. Results are shown in Figure 2. The adhesion of *C. jejuni* to a polystyrene surface was significantly reduced by all lavandin EOs and ethanolic extracts (EFs and EWMs), with the exception of lavandin EWM 'Budrovka' SN ($p < 0.05$). Within the exact group of lavandin formulations, EOs, EFs and EWMs had a similar effect on *C. jejuni* adhesion (Figure 2).

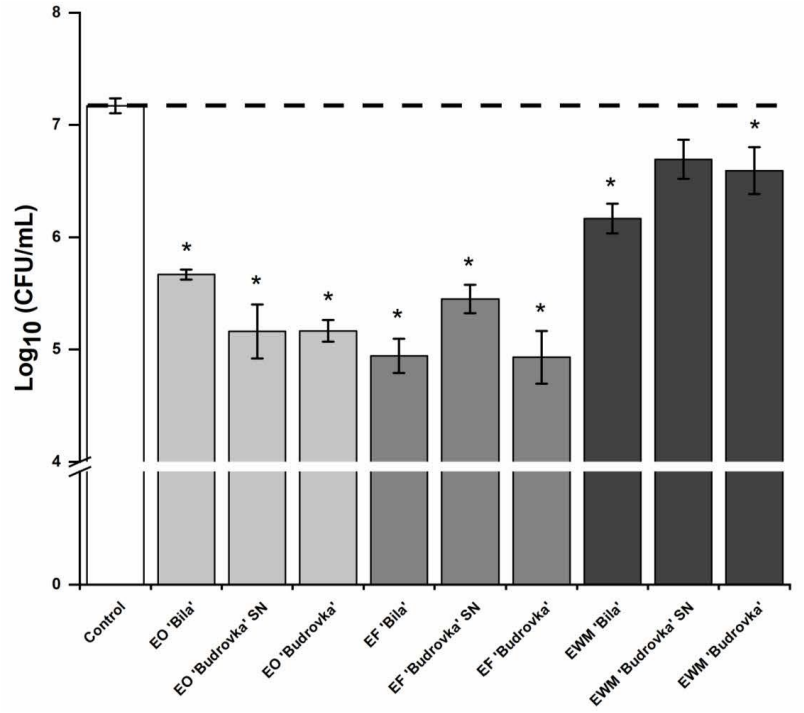

Figure 2. Effect of different lavandin formulations (EOs, EFs and EWMs) at subinhibitory concentration (0.25 × MIC) on the adhesion of *C. jejuni* NCTC 11168 to a polystyrene surface. *C. jejuni* NCTC 11168 was cultivated in a polystyrene microtiter plate without or with the addition of lavandin formulations at subinhibitory concentration in a micro-aerobic atmosphere at 42 °C for 24 h. Attached cells were suspended by sonication and their concentration was determined by plate counting. Log_{10} average values ± SD are shown. * $p < 0.05$, vs. control. (EO, essential oil; EF, ethanolic extract prior to distillation; EWM, ethanolic extract of post-distillation waste material).

Lavandin EF 'Bila' and 'Budrovka' proved slightly more effective, with an almost 99% reduction of adhesion (i.e., a reduction of 2 log_{10}). Lavandin EF 'Bila' was more effective on *C. jejuni* adhesion compared to EO and EWM 'Bila' ($p < 0.05$). There were no significant differences in the effects of EO and EWM 'Bila' ($p > 0.05$). Lavandin EO 'Budrovka' SN and 'Budrovka' were more effective than the lavandin EWMs ($p < 0.05$), but there were significant differences compared to EFs 'Budrovka' SN and 'Budrovka' ($p > 0.05$). All lavandin EFs were more effective in reducing *C. jejuni* adhesion to the polystyrene surface compared to the lavandin EWMs ($p < 0.05$).

2.5. *Modulation of Campylobacter Biofilm Formation by Lavandin Formulations*

The biofilm formation of *C. jejuni* was observed on a glass surface, which presented the model of an abiotic surface. Lavandin formulations at subinhibitory concentration were used in order to prevent biofilm formation. Coverage of the glass surface with *C. jejuni* NCTC 11,168 biofilm at air/liquid interface was determined after 72 h of exposure to lavandin formulations. Every 24 h, the SM was replaced with a fresh one, where lavandin formulations at subinhibitory concentration were added. This step was important

because, in this way, C. *jejuni* was constantly exposed to the same concentration and form of lavandin formulations.

All lavandin EOs and ethanolic extracts (EFs) significantly reduced the biofilm formation of C. *jejuni* on the glass surface ($p < 0.05$) (Figure 3). Among the lavandin EWMs, only lavandin EWM 'Bila' significantly reduced biofilm formation ($p < 0.05$). Once again, it is obvious that, within the exact group of lavandin formulations, EOs, EFs and EWMs had a similar effect on C. *jejuni* biofilm formation.

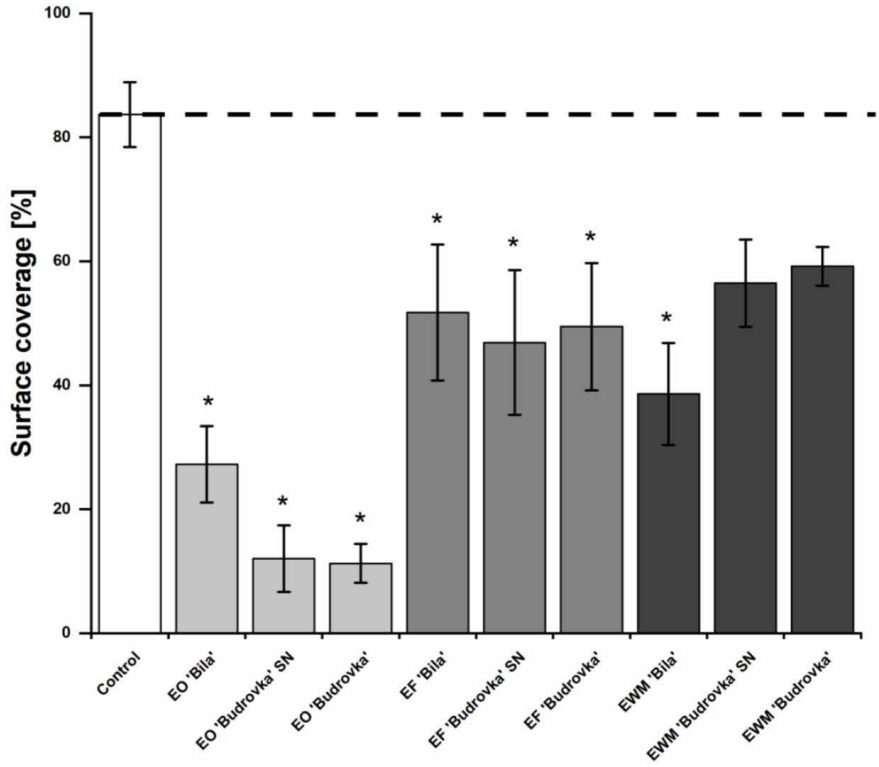

Figure 3. Effect of different lavandin formulations (EOs, EFs and EWMs) at subinhibitory concentration (0.25 × MIC) on biofilm formation of C. *jejuni* NCTC 11168. C. *jejuni* NCTC 11,168 was cultivated on a glass surface without or with the addition of lavandin formulations at subinhibitory concentration in a micro-aerobic atmosphere at 42 °C for 72 h. Every 24 h, the SM was replaced with a fresh Muller–Hinton (MH) broth, where lavandin formulations at subinhibitory concentration were added. After 72 h of incubation, microscopic slides were rinsed and stained with 1% [*v*/*v*] crystal violet. Biofilms were examined at air/liquid interface and surface coverage was measured. Average values ± SD are shown. * $p < 0.05$, vs. control. (EO, essential oil; EF, ethanolic extract prior to distillation; EWM, ethanolic extract of post-distillation waste material).

Lavandin EOs proved most effective against C. *jejuni* biofilm formation, where lavandin EO 'Budrovka' SN and 'Budrovka' reduced the biofilm formation by approximately 85%. Interestingly, lavandin EO 'Bila' reduced biofilm formation by approximately 67%, and its effect was not significantly different from the effect of lavandin EF 'Bila' and EWM 'Bila' ($p > 0.05$).

Lavandin ethanolic extracts (EFs and EWMs) had a weaker effect on *C. jejuni* biofilm formation. Among the ethanolic extracts, the most effective was lavandin EWM 'Bila', with a biofilm reduction of approximately 44%. The effect of lavandin EWM 'Bila' was not significantly different from the effect of lavandin EF 'Bila'.

2.6. Correlation between MIC and C. jejuni Intercellular Signaling, Adhesion and Biofilm Formation

In order to test whether the measured parameter MIC and *C. jejuni* properties—i.e., intercellular signaling, adhesion, and biofilm formation—were in correlation, Pearson's correlation test was used (Figure 4). There was a weak and negative correlation ($p < 0.01$) between MIC and *C. jejuni* intercellular signaling (Figure 4), meaning that the increment of MIC leads to the reduction of intercellular signaling. A medium and positive correlation ($p < 0.01$) was found between *C. jejuni* intercellular signaling and adhesion (Figure 4), meaning that the reduction of intercellular signaling leads to the reduction of adhesion. A medium and positive correlation ($p < 0.01$) was also observed for *C. jejuni* adhesion and biofilm formation (Figure 4), meaning that the reduction of adhesion leads to the reduction of biofilm formation.

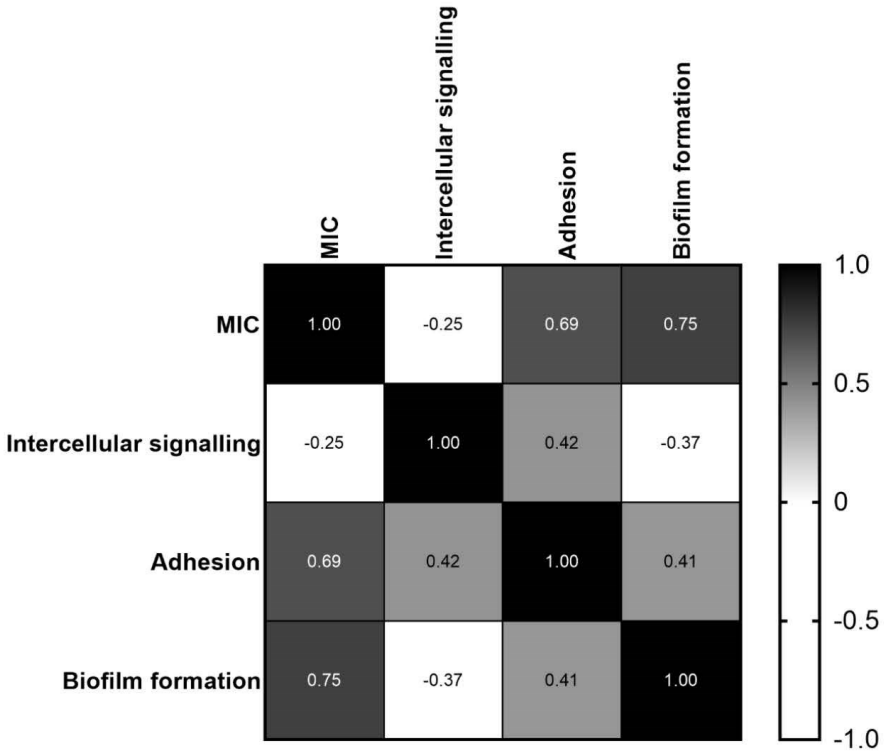

Figure 4. Correlation between MIC of lavandin formulations, *C. jejuni* intercellular signaling, adhesion and biofilm formation. Pearson's correlation test was used in order to determine the correlation among variables. For the Pearson correlation, an absolute value of 1 indicates a perfect linear relationship. A correlation close to 0 indicates no linear relationship between the variables. The sign of the coefficient indicates the direction of the relationship. Correlation was significant at the level < 0.01.

2.7. Antioxidant Activity of Lavandin Formulations

The antioxidant activity of the lavandin formulations was determined using the DPPH radical scavenging assay. Lavandin EOs were tested at a concentration of 40 mg/mL. Lavandin EO 'Bila' and 'Budrovka' showed similar results, with lavandin EO 'Bila' having a scavenging activity of 75.03 ± 11.33% and lavandin EO 'Budrovka' having a scavenging activity of 70.85 ± 2.80%. Lavandin EO 'Budrovka' SN had better antioxidant activity, with 90.17% ± 0.45% scavenging activity.

The lavandin ethanolic extracts (EFs and EWMs) were tested for antioxidant activity at a much lower concentration compared to the lavandin EOs. EFs were tested at a concentration of 2.5 mg/mL, while EWMs were tested at a concentration of 1 mg/mL. EF 'Bila' and 'Budrovka' SN had similar results, with EF 'Bila' having a scavenging activity of 86.73 ± 2.81% and EF 'Budrovka' SN of 83.54 ± 4.58%. EF 'Budrovka' had the best antioxidant activity among the lavandin EFs, with a scavenging activity of 93.87 ± 0.67%.

All three EWMs had a similar scavenging activity, with lavandin EWM 'Bila' having a scavenging activity of 92.16 ± 1.73, EWM 'Budrovka' SN of 94.38 ± 1.19, and EWM 'Budrovka' of 92.94 ± 0.38%.

3. Discussion

Current measures that are used to combat the persistence of *C. jejuni* in the food production chain are not fully effective, so there is a need for new approaches to control *C. jejuni* across the whole food production chain from farm to fork. In order to find potential antimicrobials that will be able to prevent or reduce *C. jejuni* biofilm establishment on abiotic surfaces, dried flowers of *Lavandula* × *intermedia* 'Bila', 'Budrovka SN, and 'Budrovka' were used to prepare EOs, ethanolic extracts of lavandin flowers prior to distillation (EFs) and ethanolic extracts of lavandin post-distillation waste material (EWMs). Prepared lavandin formulations were used to target *C. jejuni* intercellular signaling, adhesion, and biofilm formation in order to combat biofilm establishment. Subinhibitory concentration was used to avoid effects on *C. jejuni* growth.

Prior to the experiments at the biological level, the chemical composition of lavandin EOs, EFs and EWMs was investigated to gain insight into the chemical profile of prepared lavandin formulations. EFs and EWMs had a similar chemical composition, where phenols were the major compounds detected. However, it is important to note that the extraction yield was higher for waste material compared to flowers prior to the distillation (Supplementary Figure S1). This can be due to better accessibility of waste material to the solvent, as by hydrodistillation, the plant material was cooked for the time of hydrodistillation. Therefore, the plant cellular matrix was better solubilized, and extractive compounds were more easily available. Moreover, 3-(3,4-dihydroxyphenyl)lactic acid, the hydrolysis product of rosmarinic acid, as well as salvianolic acid A, could only be detected in the extract from waste materials after hydrodistillation, indicating that artifact formation during hydrodistillation has to be taken into account. In addition, the flavones apigenin-7-O-glucoside and ladanein were detected. A comparable composition was reported for *L.* × *intermedia* waste material, where chlorogenic acid and a number of flavone glycosides were also found [18]. A similar composition for *Lavandula* ethanolic extracts was also reported in our previous study [22]. Mass spectrometry and UV-VIS data were used for the identification of phenols, so the sugar moieties were only designated as hexosides; however, according to other reports for *Lavandula* spp., glucosidation is most likely to occur [18,28].

The complete chemical profile was determined for all three lavandin EOs. Linalool, linalyl acetate, 1,8-cineol, and camphor were the major compounds found in tested EOs. This confirms that tested EOs belonged to the genus *Lavandula*, more specifically to the hybrid *L.* × *intermedia* [29,30]. The EO of *L.* × *intermedia* 'Budrovka' contained the highest percentage of linalool (47.2%) and linlyl acetate (26.7%) compared to the percentage of linalool and linalyl acetate found in *L.* × *intermedia* 'Bila' and 'Budrovka' SN (40.4–43.1% and 5.3–6.6%, respectively). This is especially interesting because *L.* × *intermedia* 'Budrovka' and 'Budrovka' SN were cultivated at the same geographic location but in different fields,

which indicates that ontogenetic and morphogenetic factors can also influence the chemical variability, either to a quantitative or qualitative extent [31].

Among tested lavandin formulations, all three lavandin EOs were shown to possess the best antibacterial activity, with MIC values of 0.25 mg/mL. The antibacterial activity of *L.* × *intermedia* 'Budrovka' and *L. angustifolia* EOs were observed against different Gram-positive and Gram-negative bacteria [29]. In our previous study [22], the same MIC was determined for *L. angustifolia* EO against *C. jejuni*. Regarding the information provided, *C. jejuni* seems most sensitive to *Lavandula* formulations among all tested bacteria. This could be due to the type of microorganism, the inoculum volumes, and the culture medium used, together with the pH, temperature, and incubation time. Type and storage, as well as the method for plant formulation preparation, can also influence antimicrobial activity [32].

EFs and EWMs had a moderate but comparable effect against *C. jejuni*, although weaker than EOs. It is most likely that this can be explained by the glycosidic nature of the major constituents of the ethanolic extracts resulting in decreased cell wall permeability. Nevertheless, EFs and EWMs still contain a diverse pool of bioactive compounds and are effective antibacterial agents. This is in agreement with previous studies on post-distillation thyme waste, pinot noir grape skins and seeds, juniper fruit waste, and *Lavandula* waste material, which also showed great antimicrobial activity [22,33,34].

In further experiments, lavandin formulations were used at a subinhibitory concentration as a novel approach to control biofilm development, focusing on *C. jejuni* properties: intercellular signaling and adhesion. All lavandin EFs and lavandin EWMs 'Bila' and 'Budrovka' SN significantly reduced *C. jejuni* intercellular signaling, proving to be more effective than the lavandin EOs. It is hypothesized that the signaling molecules were bound to the solid aggregates of the precipitate in the samples of the ethanolic extracts, which became apparent during the preparation of the SMs and were removed along with the bacteria during filtration. Moreover, the process of intercellular signaling can be disrupted by different mechanisms: reducing the activity of receptor protein or synthase; inhibiting the production of signaling molecules; degrading the signaling molecules; mimicking the signaling molecules primarily by using the analogs of signal molecules (e.g., secondary metabolites of natural formulations) [35]. A comparable result was reported for *L. hybrida* EO at subinhibitory concentration, which inhibited the intercellular signaling of *C. jejuni* by approximately 66% [25]. Similarly, a strong effect on the inhibition of *C. jejuni* intercellular signaling was also found for the ethanolic fruit extract *Euodia ruticarpa*, which showed a reduction of more than 90% [36].

Adhesion is a bacterial feature affected by intercellular signaling. It is crucial for the development of *C. jejuni* biofilm, so it is necessary to inhibit it in order to prevent biofilm establishment [9]. A statistically significant effect on the reduction of *C. jejuni* adhesion to a polystyrene surface was confirmed for all tested lavandin preparations, with the exception of the lavandin EWM 'Budrovka' SN. There were no statistically significant differences between the anti-adhesion effect of lavandin EOs and EFs. Moreover, a slightly better effect could be observed for lavandin EF 'Bila' and 'Budrovka' compared to their EOs, where lavandin EF 'Budrovka' reduced adhesion by >99%. This is comparable with the results gained for thyme ethanolic extracts [33]. An excellent anti-adhesion effect was also observed for the *L. hybrida* EO, which reduced the adhesion of *C. jejuni* to the polystyrene surface by 96% [25]. This is comparable to the observed results for the lavandin EOs. Similar results for lavender formulations were shown in our previous study [22], where it was confirmed that lavender EOs were able to affect the expression of genes carrying the transcript for the outer membrane proteins involved in the initial adhesion of *C. jejuni* to contact surfaces. Moreover, a moderate and positive correlation was found between intercellular signaling and adhesion, indicating that a decrease in intercellular signaling leads to a decrease in the adhesion of bacterial cells to abiotic surfaces. These results are supported by the research by [25], who also found a correlation between the reduction of intercellular signaling and the reduction of adhesion. Altogether, these findings confirm that formulations from the genus *Lavandula* have great anti-adhesion potential against

C. jejuni and that intercellular signaling is an important target of *Lavandula* preparations to combat the adhesion of *C. jejuni*.

It is clear that the lavandin formulations successfully reduced *C. jejuni* adhesion, but the most important question was whether the lavandin formulations could reduce biofilm development even after 72 h of incubation. Indeed, all lavandin EOs and EFs, as well as lavandin EWM 'Bila', significantly reduced the biofilm development of *C. jejuni* on a glass surface even after 72 h of incubation ($p < 0.05$). A moderate and positive correlation was found between reducing adhesion and biofilm formation, confirming that reducing adhesion is a crucial step in combating bacterial biofilm, but a correlation between reducing intercellular signaling and biofilm was not found, which was expected, as biofilm formation is a multifactorial event and does not only depend on intercellular signaling. If we consider all the facts together, it is evident that intercellular signaling is the primary mechanism that needs to be reduced in order to reduce adhesion and, consequently, biofilm establishment. The lavandin EOs had the most favorable inhibitory effect against biofilm establishment. The latter can be attributed to the higher content of bioactive secondary metabolites in EO. It is interesting that there were no significant differences between the effects of lavandin EO, EF, and EWM 'Bila', indicating that waste material can match EO in its effects. Studies have also shown the anti-biofilm activity of linalool against different bacteria [9,29], but not as good as for EO. Naturally, linalool is one of the major compounds found in the tested EOs, but it is important to emphasize that the action of EO comes from the action of all the bioactive compounds found in EO [22].

Finally, the antioxidant activity of prepared lavandin formulations was tested. The antioxidant activity of natural compounds is important because it can reduce the oxidation of food products that come to consumers, thus improving food quality [37]. Among all the lavandin formulations, lavandin EWM had the best antioxidant activity and scavenging activity of more than 90% at a concentration of 1 mg/mL. In order to gain a similar effect with lavandin EFs and EOs, a concentration 2.5 times or 40 times higher had to be used. Similar results were found for the ethanolic extract *Ocimum basilicum*, which had better antioxidant activity than the EO of *O. basilicum* [38]. The better antioxidant activity of lavandin ethanolic extract formulations can be attributed to their strongly different chemical composition compared to EOs. For example, ladanein, which was found in the tested ethanolic extracts, is known to be a good antioxidant agent [39]. Moreover, lavandin EWMs had a relatively higher concentration of some identified compounds than EFs (concluded from the mass spectrometry and UV-VIS data), which can explain their better antioxidant activity. Similar results were found for lavandin 'Budrovka' EO, which had an IC_{50} value of 21.6 mg/mL [29]. By comparing our results with the research carried out on lavandin 'Sumiens', 'Super A' and 'Grosso', it was recognized that tested cultivars 'Bila', 'Budrovka' SN, and 'Budrovka' had an antioxidant activity that was twice as good [40]. Such an effect is probably the result of synergistic interactions between EOs constituents, as linalool and linlyl acetate had much higher IC_{50} values (218.6 mg/mL or 157.1 mg/mL, respectively) than lavandin EO [29].

4. Material and Methods

4.1. Chemicals

The MH agar was from BioMéroeux (Marcy-l'Étoile, France), the MH broth was from Oxoid (Hampshire, UK) and the Karmali agar was from Biolife (Milan, Italy). The glycerol solution was from Kemika (Zagreb, Croatia), the phosphate-buffered saline (PBS) was from Oxoid, and the kanamycin, dimethylsulphoxide (DMSO), resazurin, menadione, and Folin–Ciocalteu reagent were from Sigma Aldrich (Steinheim, Germany). The sodium chloride, magnesium sulfate heptahydrate, L-arginine, and 96% ethanol were from Merck (Darmstadt, Germany). The casamino acid was from Thermo Fisher Scientific (Carlsbad, CA, USA).

4.2. Lavandin Formulations

Three *Lavandula* × *intermedia* cultivars ('Bila', 'Budrovka' SN and 'Budrovka') were used in this study. *Lavandula* × *intermedia* 'Bila' was cultivated in Spodnje Pitve, Hvar, Croatia (43°09′06″ N, 16°40′35″ E), while *L.* × *intermedia* 'Budrovka' SN and *L.* × *intermedia* 'Budrovka' were cultivated in Jelsa, Hvar, Croatia (43°09′23″ N, 16°41′04″ E). The samples were collected in the afternoon hours during July 2019. Dried flowers were used to prepare the lavandin EOs and ethanolic extracts (EFs).

The EOs were prepared by hydrodistillation [41], with about 200 g of flowers distilled in two liters of water in a Clevenger-type apparatus for three to four hours and then stored at 4 °C. The waste material obtained after the hydrodistillation of the lavandin flowers was also used for the preparation of the ethanolic extracts (EWMs).

The ethanolic extracts from the lavandin dried flowers (EFs) and waste material (EWMs) gained after the hydrodistillation process were prepared by a four-to-six-hour ethanol extraction (Soxhlet extraction) of 20 g dried flowers in 150 mL 96% ethanol. These were then concentrated in a rotary evaporator (Laborota 4000; Heidolph Instruments, Germany) at 40 °C and 175 mbar pressure and stored at 4 °C.

4.3. Phytochemical Analysis of Lavandin Ethanolic Extracts

The identification of the phenolic compounds in lavandin ethanolic extracts (EFs and EWMs) was carried out using liquid chromatography—photo diode array—electrospray ionization mass spectrometry (LC-MS) following the protocol described in [22] (for details, see Supplementary Methods, LC-MS Conditions). The compounds eluted were determined by their UV-VIS and mass spectra, in comparison with the literature [18,42–46].

4.4. GC–MS Analysis of Lavandin EOs

The identification of the main compounds in lavandin EOs was carried out by GC–MS following the protocol described in [19] (for details, see Supplementary Methods, GC–MS Conditions). The compounds were identified by their retention indices according to [44] and by comparing their mass spectra with spectral data libraries [23,24,47] and with the laboratory's own database.

4.5. Bacterial Strains and Growth Conditions

C. jejuni NCTC 11168 and *C. jejuni* 11168ΔluxS [48] were used in this study. The strains were stored at −80 °C in a 20% glycerol and 80% MH broth. Prior to the experiments, *C. jejuni* NCTC 11168 was subcultivated on Karmali agar, and *C. jejuni* 11168ΔluxS on an MH agar supplemented with 30 mg/L kanamycin for 24 h at 42 °C under micro-aerobic conditions (85% N_2, 5% O_2, 10% CO_2). The strains were further subcultured in an MH broth under the same conditions, and bacterial OD was determined by spectrophotometric measurements of absorbance at 600 nm after the incubation. The inoculum was prepared in an MH broth at 10^5 CFU/mL for determination of the MICs and assays that targeted *C. jejuni* intercellular signaling and adhesion. For counting, strains were plated on an MH agar under conditions described above in this section, and the colonies were counted and expressed as CFU/mL.

For the autoinducer-2 bioassay, the biosensor strain *V. harveyi* MM30 [22] was used. The strain was stored at −80 °C in a 20% glycerol and 80% autoinducer bioassay (AB) medium composed of NaCl [0.02 g/L], MgSO4 + 7 H2O [0.01 g/L], casamino acid [0.002 g/L], PBS [1 M], L-arginine [0.1 M] and glycerol [50% (*v*/*v*)] [49]. Prior to the experiments, strains were subcultured for 16 h aerobically at 30 °C in an AB liquid medium.

4.6. Antimicrobial Potential of Lavandin Formulations

The MICs against *C. jejuni* NCTC 11168 and *C. jejuni* 11168Δ*luxS* were determined by the broth microdilution method, as previously described [50]. Stock solutions of lavandin EOs and ethanolic extracts (EFs and EWMs) were prepared in DMSO at 40 mg/mL. Serial dilutions of stock solutions were performed in an MH broth in a 96-well microtiter plate

(NUNC 266 120 polystyrene plates; Nunc, Denmark), after which bacterial inoculum was added, prepared as described in Section 2.5. During experiments, the DMSO in the MH broth did not exceed a concentration of 1% [*v*/*v*], which did not influence the growth of bacteria [25]. In further experiments, a concentration of 0.25 × MIC was used, as this concentration was the first concentration that did not influence the growth of *C. jejuni* [25].

4.7. Targeting Intercellular Signaling of C. jejuni

Overnight cultures of *C. jejuni* NCTC 11168 and *C. jejuni* 11168ΔluxS were inoculated in an MH broth until OD600 0.1 (10^7 CFU/mL). For further experiments, the cultures were diluted 100-fold in an MH broth, and an MH broth supplemented with lavandin formulations (EOs and ethanolic extracts [EFs and EWMs]) at subinhibitory concentration (0.25 × MIC). The cultures were further incubated for 24 h at 42 °C under micro-aerobic conditions. After the incubation, bacterial growth was determined using the plate counting method as described previously (Section 2.5), and cultures were further filtrated through 0.2 µm syringe filters to gain an SM where no bacteria were present. SMs were stored at −80 °C until further experiments.

V. harveyi MM30 was used for the biosensor assay in order to test the effect of lavandin formulations on *C. jejuni* intercellular signaling. An overnight culture of *V. harveyi* MM30 was diluted 5000-fold in an AB liquid medium to contain approximately 10^3 CFU/mL and was used in further experiment. *C. jejuni* NCTC 11168 SM (CJ–SM) and *C. jejuni* 11168ΔluxS SM (LUXS–SM), untreated or treated with lavandin formulations at subinhibitory concentration, were added to the suspension of the biosensor to a final concentration of 5% (*v*/*v*) of each (i.e., 10 µL of CJ–SM, 10 µL of LUXS–SM and 180 µL of the biosensor strain) in 96-well white microtiter plates with a transparent bottom (Greiner Bio-One, Kremsmünster, Austria). LUXS–SM, untreated or treated with lavandin formulations at subinhibitory concentration, was used as the blank [5% (*v*/*v*) LUXS–SM, 95% (*v*/*v*) AB medium], while the negative control was a 5% (*v*/*v*) LUXS–SM and 95% (*v*/*v*) *V. harveyi* suspension. The relative luminescence signals, expressed as relative luminescence units (RLU), and a growth of *V. harveyi* MM30, expressed as OD600, after the addition of CJ–SM (treated or untreated), LUXS–SM (treated or untreated), and MH broth, were measured with a microplate reader (Varioskan Lux, Thermo Scientific, Waltham, MA, USA) at 30 min intervals over 15 h at 30 °C.

The measured values (RLU and OD600) of LUXS–SM (treated or untreated), used as blank, were deducted from the values (RLU and OD600) gained for the bioluminescence response and the growth of the *V. harveyi* MM30 biosensor after the addition of CJ–SM and LUXS–SM (both treated or untreated). Results from the bioluminescence response were normalized with OD600. The reduction of bioluminescence was calculated by Equation (1):

$$\%\ bioluminescence\ reduction = 100 - \left(\frac{11168T - luxST}{11168C - luxSC}\right) \times 100\%, \quad (1)$$

where 11168T is the normalized bioluminescence response of *V. harveyi* MM30 after the addition of CJ–SM treated with lavandin formulations at subinhibitory concentration, luxST is the normalized bioluminescence response of *V. harveyi* MM30 after the addition of LUXS–SM treated with lavandin formulations at subinhibitory concentration, 11168C is the normalized bioluminescence response of *V. harveyi* MM30 after the addition of untreated CJ–SM and luxSC is the normalized bioluminescence response of *V. harveyi* MM30 after the addition of untreated LUXS–SM.

4.8. Anti-Adhesion Potential of Lavandin Formulations

The adhesion of *C. jejuni* NCTC 11168 was investigated under treatments with lavandin formulations at subinhibitory concentration. Inoculum was prepared as described in Section 2.5 and treated with lavandin EOs, and ethanolic extracts (EFs and EWMs) at 0.25 × MIC Polystyrene microtiter plates with 96 wells (Nunc 266 120 polystyrene plates; Nunc, Denmark) were prepared as described in [22] and were incubated for 24 h. The ad-

hesion of cells was determined as CFU/mL, as previously described [22,33]. The untreated culture was used as a negative control.

4.9. Anti-Biofilm Potential of Lavandin Formulations

The anti-biofilm potential of lavandin EOs and ethanolic extracts (EFs and EWMs) was evaluated according to the previously reported method [22]. Briefly, in a 50 mL centrifuge tube (Sarstedt, Nümbrecht, Germany), 20 mL of MH broth supplemented with lavandin formulations at subinhibitory concentration (0.25 × MIC) were added and were inoculated with 5% [*v*/*v*] inoculum of *C. jejuni* overnight culture (10^8 CFU/mL). Autoclaved microscope slides (26 × 76 mm; Deltalab, Barcelona, Spain) were used as a model for a glass surface and were vertically inserted into the centrifuge tube after inoculation of the medium. The cultures were incubated without shaking in a micro-aerobic atmosphere for 72 h at 42 °C, in a damp environment. After 24 h, microscopic slides were transferred to new centrifuge tube where 20 mL of fresh MH broth, supplemented with lavandin formulations at subinhibitory concentration (0.25 × MIC), were added. The same was repeated after an additional 24 h of incubation. Untreated cultures were used as negative controls. After 72 h of incubation, the microscopic slides were rinsed three times with PBS to remove weakly adhered cells and stained with a 1% [*v*/*v*] crystal violet stain. The biofilms on the air/liquid interface were investigated with a light microscope (DM750, Leica, Germany) equipped with a camera (ICC50 W, Leica, Germany) under a 400× *g* magnification. For each sample, five technical replicates of vertically connected images of biofilm at the air/liquid interface were captured (5 × 1 mosaic images; total analyzed surface per image, 1600 µm × 1200 µm). The images were processed using the Fiji program [51] as described in [22], with minor modifications. Based on the image processing, the surface coverage in percent was determined.

4.10. Free Radical Scavenging Activity Assay (DPPH Assay)

The free radical scavenging activities of the lavandin EOs and ethanolic extracts (EFs and EWMs) were evaluated using the stable DPPH radicals as previously described [29]. Briefly, the DPPH was prepared at a concentration of 0.2 mg/mL in 96% ethanol. The lavandin EOs were assayed at a concentration of 40 mg/mL in 96% ethanol, lavandin ethanolic extracts (EFs) at a concentration of 2.5 mg/mL in 96% ethanol and lavandin ethanol extracts (EWMs) at a concentration of 1 mg/mL in 96% ethanol. We added 20 µL of DPPH to 60 µL of the lavandin samples (EOs, EFs, and EWMs) in a non-sterile 96-well polystyrene microtiter plate (Brand, Wertheim, Germany). For blank, 20 µL of 96% ethanol was added to 60 µL of the lavandin samples (EOs, EFs, EWMs), and for a negative control, 20 µL of DPPH solution was added to 60 µL of the 96% ethanol. The microtiter plate was shaken for 1 min at 600 rpm in a microplate shaker (Eppendorf, Hamburg, Germany) and incubated for 30 min in the dark at room temperature. After incubation, the absorbance was measured at 517 nm on the microplate reader. The scavenging activity was calculated using the following Equation (2):

$$\%\ SA = \left(1 - \left(\frac{A\ (sample) - (A(blank)}{A(neg.control)}\right)\right) \times 100 \quad (2)$$

4.11. Statistical Analysis

All the experiments were carried out in triplicate as three or more independent experiments. The data are expressed as means ± standard deviation, with analysis using Origin 2018 (OriginLab, Northampton, MA, USA). Statistical analysis was performed using IBM SPSS Statistics 23 (Statsoft Inc., Tulsa, OK, USA). In order to determine the distribution and homogeneity of the data, the Kolmogorov–Smirnov test of normality and the homogeneity of variances test were performed. The data were normally distributed and variances were equal across groups. Statistical significances were determined using the One-Way ANOVA test. Data were accepted as significant at $p < 0.05$. Pearson's correlation

test was used to determine the correlation between MIC and *C. jejuni* intercellular signaling, adhesion, and biofilm formation. The correlation was significant at the level $p < 0.01$.

5. Conclusions

This comparative study aimed to find antimicrobials that were potentially able to reduce *C. jejuni* biofilm establishment on abiotic surfaces. Prepared EOs, EFs, and EWMs of *Lavandula* × *intermedia* 'Bila', 'Budrovka' SN, and 'Budrovka' showed great antibacterial activity against one of the major food-borne pathogens, *C. jejuni*. Their effect against *C. jejuni* intercellular signaling, adhesion, and biofilm formation at subinhibitory concentration was confirmed, making lavandin formulations antimicrobial agents that can be used as an innovative approach to control *C. jejuni* biofilm development. Moreover, a correlation was confirmed between the reduction of *C. jejuni* intercellular signaling and the reduction of *C. jejuni* adhesion, two interrelated properties that can be easily controlled simultaneously. It is important to emphasize that lavandin ethanolic extracts showed better activity against *C. jejuni* intercellular signaling and adhesion, as well as better antioxidant activity, which makes them competitive with EOs. These findings mean that novel bacterial targets are of interest for biofilm control with alternative natural agents throughout the whole food production chain.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antibiotics11070854/s1: Method S1. LC-MS Conditions; Method S2. GC–MS Conditions; Table S1. Growth of *C. jejuni* NCTC 11168 and *C. jejuni* 11168ΔluxS in MH broth without or with the addition of lavandin formulations (EOs, EFs and EWMs) at subinhibitory concentration (0.25 × MIC) after 24 h of incubation in a micro-aerobic atmosphere. Average values of CFU/mL are shown ± SD; Table S2. Reduction of *C. jejuni* NCTC 11168 intercellular signaling after the addition of lavandin formulations (EOs, EFs and EWMs) at subinhibitory concentration (0.25 × MIC). Average values in % are shown ± SD; Figure S1. Yield of ethanol extraction for lavandin ethanolic extracts, i.e., lavandin EFs and lavandin EWMs. Figure S2. HPLC chromatogram (UV 310 nm) of the ethanolic extract of lavandin flowers prior to distillation [EFs]. * not identified (no significant ionization in ESI positive and negative mode); compound 1 was not detected; for identity of compounds 2–10, please refer to Table 1. Figure S3. HPLC chromatogram (UV 320 nm) of the ethanolic extract of lavandin postdistillation waste materials (EWMs). * not identified (no significant ionization in ESI positive and negative mode); compound 1 (3-(3,4-OH-phenyl)lactic acid was not detectable at 310 nm, but was detected in the ESI-MS base peak chromatogram; for identity of compounds 2–10, please refer to Table 1. Figure S4. Comparison of peak areas of compounds 2–10 in EF and EWM samples. Peak areas were calculated from UV chromatograms at 310 nm.

Author Contributions: D.R. and J.O. conceived and conducted the experiments. F.B. and D.R. analyzed the data. S.S.M. designed and coordinated the research. D.R. wrote the manuscript. S.S.M., B.J. and M.J. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Elgamoudi, B.A.; Korolik, V. *Campylobacter* biofilms: Potential of natural compounds to disrupt *Campylobacter jejuni* transmission. *Int. J. Mol. Sci.* **2021**, *22*, 12159. [CrossRef] [PubMed]
2. Galié, S.; García-Gutiérrez, C.; Miguélez, E.M.; Villar, C.J.; Lombó, F. Biofilms in the food industry: Health aspects and control methods. *Front. Microbiol.* **2018**, *9*, 898. [CrossRef] [PubMed]
3. Igwaran, A.; Okoh, A.I. Human campylobacteriosis: A public health concern of global importance. *Heliyon* **2019**, *5*, e02814. [CrossRef] [PubMed]
4. EFSA; ECDC. The European Union One Health 2020 Zoonoses Report. *EFSA J.* **2021**, *19*, 6971. [CrossRef]

5. Mavri, A.; Smole Možina, S. Development of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* adapted to biocides. *Int. J. Food Microbiol.* **2013**, *160*, 304–312. [CrossRef]
6. Rozman, V.; Matijašić, B.B.; Smole Možina, S. Antimicrobial Resistance of Common Zoonotic Bacteria in the Food Chain: An Emerging Threat. In *Antimicrobial Resistance—A Global Threat*; Kumar, Y., Ed.; Intech Open: London, UK, 2018. [CrossRef]
7. Püning, C.; Su, Y.; Lu, X.; Gölz, G. Molecular mechanisms of *Campylobacter* biofilm formation and quorum sensing. *Curr. Top. Microbiol. Immunol.* **2021**, *431*, 293–319. [CrossRef]
8. Larsen, M.H.; Dalmasso, M.; Ingmer, H.; Langsrud, S.; Malakauskas, M.; Mader, A.; Møretrø, T.; Možina, S.S.; Rychli, K.; Wagner, M.; et al. Persistence of foodborne pathogens and their control in primary and secondary food production chains. *Food Control* **2014**, *44*, 92–109. [CrossRef]
9. Klančnik, A.; Šimunović, K.; Sterniša, M.; Ramić, D.; Smole Možina, S.; Bucar, F. Anti-adhesion activity of phytochemicals to prevent *Campylobacter jejuni* biofilm formation on abiotic surfaces. *Phytochem. Rev.* **2021**, *20*, 55–84. [CrossRef]
10. Kampf, G. Biocidal agents used for disinfection can enhance antibiotic resistance in Gram-negative species. *Antibiotics* **2018**, *7*, 110. [CrossRef]
11. Breijyeh, Z.; Jubeh, B.; Karaman, R. Resistance of Gram-negative bacteria to current antibacterial agents and approaches to resolve it. *Molecules* **2020**, *25*, 1340. [CrossRef]
12. Giacometti, F.; Shirzad-Aski, H.; Ferreira, S. Antimicrobials and food-related stresses as selective factors for antibiotic resistance along the farm to fork continuum. *Antibiotics* **2021**, *10*, 671. [CrossRef] [PubMed]
13. Salehi, B.; Mnayer, D.; Özçelik, B.; Altin, G.; Kasapoğlu, K.N.; Daskaya-Dikmen, C.; Sharifi-Rad, M.; Selamoglu, Z.; Acharya, K.; Sen, S.; et al. Plants of the genus *Lavandula*: From farm to pharmacy. *Nat. Prod. Commun.* **2018**, *13*, 1385–1402. [CrossRef]
14. Lesage-Meessen, L.; Bou, M.; Sigoillot, J.C.; Faulds, C.B.; Lomascolo, A. Essential oils and distilled straws of lavender and lavandin: A review of current use and potential application in white biotechnology. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 3375–3385. [CrossRef]
15. Bajalan, I.; Pirbalouti, A.G. Variation in chemical composition of essential oil of populations of *Lavandula* × *intermedia* collected from Western Iran. *Ind. Crops Prod.* **2015**, *69*, 344–347. [CrossRef]
16. Kara, N.; Baydar, H. Determination of lavender and lavandin cultivars (*Lavandula* sp.) containing high quality essential oil in Isparta, Turkey. *Turk. J. F. Crops* **2013**, *18*, 58–65.
17. Prusinowska, R.; Śmigielski, K.; Stobiecka, A.; Kunicka-Styczyńska, A. Hydrolates from Lavender (*Lavandula angustifolia*)–Their chemical composition as well as aromatic, antimicrobial and antioxidant properties. *Nat. Prod. Res.* **2016**, *30*, 386–393. [CrossRef] [PubMed]
18. Torras-Claveria, L.; Jauregui, O.; Bastida, J.; Codina, C.; Viladomat, F. Antioxidant activity and phenolic composition of lavandin (*Lavandula* × *intermedia* Emeric Ex Loiseleur) waste. *J. Agric. Food Chem.* **2007**, *55*, 8436–8443. [CrossRef]
19. Méndez-Tovar, I.; Herrero, B.; Pérez-Magariño, S.; Pereira, J.A.; Manzanera, M.C.A.S. By-product of *Lavandula latifolia* essential oil distillation as source of antioxidants. *J. Food Drug Anal.* **2015**, *23*, 225–233. [CrossRef]
20. Śmigielski, K.B.; Prusinowska, R.; Krosowiak, K.; Sikora, M. Comparison of qualitative and quantitative chemical composition of hydrolate and essential oils of lavender (*Lavandula Angustifolia*). *J. Essent. Oil Res.* **2013**, *25*, 291–299. [CrossRef]
21. Bridier, A.; Sanchez-Vizuete, P.; Guilbaud, M.; Piard, J.C.; Naïtali, M.; Briandet, R. Biofilm-associated persistence of food-borne pathogens. *Food Microbiol.* **2015**, *45*, 167–178. [CrossRef]
22. Ramić, D.; Bucar, F.; Kunej, U.; Dogša, I.; Klančnik, A.; Smole Možina, S. Antibiofilm potential of *Lavandula* preparations against *Campylobacter jejuni*. *Appl. Environ. Microbiol.* **2021**, *87*, AEM0109921. [CrossRef] [PubMed]
23. Adams, R.P. *Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectroscopy*, 4th ed.; Allured Publishing Corporation: Carol Stream, IL, USA, 2007.
24. NIST. *Mass Spectrometry Data Center*; NIST: Gaithersburg, MD, USA. Available online: https://chemdata.nist.gov/ (accessed on 4 June 2021).
25. Šimunović, K.; Ramić, D.; Xu, C.; Smole Možina, S. Modulation of *Campylobacter jejuni* motility, adhesion to polystyrene surfaces, and invasion of Int407 cells by quorum-sensing inhibition. *Microorganisms* **2020**, *8*, 104. [CrossRef] [PubMed]
26. Bassler, B.L.; Greenberg, E.P.; Stevens, A.M. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J. Bacteriol.* **1997**, *179*, 4043–4045. [CrossRef] [PubMed]
27. Ramić, D.; Klančnik, A.; Smole Možina, S.; Dogša, I. Elucidation of the AI-2 communication system in the food-borne pathogen *Campylobacter jejuni* by whole-cell-based biosensor quantification. *Biosens. Bioelectron.* **2022**, *212*, 114439. [CrossRef]
28. Areias, F.M.; Valentão, P.; Andrade, P.B.; Moreira, M.M.; Amaral, J.; Seabra, R.M. HPLC/DAD analysis of phenolic compounds from Lavender and its application to quality control. *J. Liq. Chromatogr. Relat. Technol.* **2000**, *23*, 2563–2572. [CrossRef]
29. Blažeković, B.; Yang, W.; Wang, Y.; Li, C.; Kindl, M.; Pepeljnjak, S.; Vladimir-Knežević, S. Chemical composition, antimicrobial and antioxidant activities of essential oils of *Lavandula* × *intermedia* 'Budrovka' and *L. angustifolia* cultivated in Croatia. *Ind. Crops Prod.* **2018**, *123*, 173–182. [CrossRef]
30. Jug-Dujaković, M.; Ninčević, T.; Grdiša, M.; Liber, Z.; Šatović, Z. Genetic analysis of Lavandin (*Lavandula* × *intermedia* Emeric Ex Loisel.) landraces from the island of Hvar, Croatia. In *Book of Abstracts–8th CMAPSEEC*; Ibraliu: Alba, TX, USA, 2014.
31. Aprotosoaie, A.C.; Gille, E.; Trifan, A.; Luca, V.S.; Miron, A. Essential oils of *Lavandula* genus: A systematic review of their chemistry. *Phytochem. Rev.* **2017**, *16*, 761–799. [CrossRef]

32. Burt, S. Essential oils: Their antibacterial properties and potential applications in foods–A review. *Int. J. Food Microbiol.* **2004**, *94*, 223–253. [CrossRef]
33. Pogačar, M.Š.; Klančnik, A.; Bucar, F.; Langerholc, T.; Smole Možina, S. Anti-adhesion activity of thyme (*Thymus vulgaris* L.) extract, thyme post-distillation waste, and olive (*Olea europea* L.) leaf extract against *Campylobacter jejuni* on polystyrene and intestine epithelial cells. *J. Sci. Food Agric.* **2016**, *96*, 2723–2730. [CrossRef]
34. Klančnik, A.; Pogačar, M.Š.; Trošt, K.; Žnidarič, M.T.; Vodopivec, B.M.; Smole Možina, S. Anti-*Campylobacter* activity of resveratrol and an extract from waste pinot noir grape skins and seeds, and resistance of *Campylobacter jejuni* planktonic and biofilm cells, mediated via the CmeABC efflux pump. *J. Appl. Microbiol.* **2017**, *122*, 65–77. [CrossRef]
35. Kalia, V.C. Quorum sensing inhibitors: An overview. *Biotechnol. Adv.* **2013**, *31*, 224–245. [CrossRef] [PubMed]
36. Bezek, K.; Kurinčič, M.; Knauder, E.; Klančnik, A.; Raspor, P.; Bucar, F.; Smole Možina, S. Attenuation of adhesion, biofilm formation and quorum sensing of *Campylobacter jejuni* by *Euodia ruticarpa*. *Phyther. Res.* **2016**, *30*, 1527–1532. [CrossRef] [PubMed]
37. Karabagias, I.; Badeka, A.; Kontominas, M.G. Shelf life extension of lamb meat using thyme or oregano essential oils and modified atmosphere packaging. *Meat Sci.* **2011**, *88*, 109–116. [CrossRef] [PubMed]
38. Rezzoug, M.; Bakchiche, B.; Gherib, A.; Roberta, A.; Kilinçarslan, Ö.; Mammadov, R.; Bardaweel, S.K. Chemical composition and bioactivity of essential oils and ethanolic extracts of *Ocimum basilicum* L. and *Thymus algeriensis* Boiss. & Reut. from the Algerian Saharan Atlas. *BMC Complement. Altern. Med.* **2019**, *19*, 146. [CrossRef]
39. Etsassala, N.G.E.R.; Adeloye, A.O.; El-Halawany, A.; Hussein, A.A.; Iwuoha, E.I. Investigation of in-vitro antioxidant and electrochemical activities of isolated compounds from *Salvia chamelaeagnea* P.J. Bergius extract. *Antioxidants* **2019**, *8*, 98. [CrossRef]
40. Pistelli, L.; Najar, B.; Giovanelli, S.; Lorenzini, L.; Tavarini, S.; Angelini, L.G. Agronomic and phytochemical evaluation of Lavandin and Lavender cultivars cultivated in the Tyrrhenian area of Tuscany (Italy). *Ind. Crops Prod.* **2017**, *109*, 37–44. [CrossRef]
41. Meyer-Warnod, B. Natural essential oils: Extraction processes application to some major oil. *Perfum. Flavorist* **1984**, *9*, 93–104.
42. Çelik, S.E.; Tufan, A.N.; Bekdeşler, B.; Özyürek, M.; Güçllü, K.; Apak, R. Identification and determination of phenolics in *Lamiaceae* species by UPLC-DAD-ESI-MS/MS. *J. Chromatogr. Sci.* **2017**, *55*, 291–300. [CrossRef]
43. Héral, B.; Stierlin, É.; Fernandez, X.; Michel, T. Phytochemicals from the genus *Lavandula*: A review. *Phytochem. Rev.* **2021**, *20*, 751–771. [CrossRef]
44. Lesage-Meessen, L.; Bou, M.; Ginies, C.; Chevret, D.; Navarro, D.; Drula, E.; Bonnin, E.; Del Río, J.C.; Odinot, E.; Bisotto, A.; et al. Lavender- and lavandin-distilled straws: An untapped feedstock with great potential for the production of high-added value compounds and fungal enzymes. *Biotechnol. Biofuels* **2018**, *11*, 217. [CrossRef]
45. Lopes, C.L.; Pereira, E.; Soković, M.; Carvalho, A.M.; Barata, A.M.; Lopes, V.; Rocha, F.; Calhelha, R.C.; Barros, L.; Ferreira, I.C.F.R. Phenolic composition and bioactivity of *Lavandula pedunculata* (Mill.) Cav. Samples from different geographical origin. *Molecules* **2018**, *23*, 1037. [CrossRef] [PubMed]
46. Zhang, Y.; Xiong, H.; Xu, X.; Xue, X.; Liu, M.; Xu, S.; Liu, H.; Gao, Y.; Zhang, H.; Li, X. Compounds identification in *Semen cuscutae* by ultra-high-performance liquid chromatography (UPLCs) coupled to electrospray ionization mass spectrometry. *Molecules* **2018**, *23*, 1199. [CrossRef] [PubMed]
47. Joulain, D.; König, W. *The Atlas of Spectral Data of Sesquiterpene Hydrocarbons*; E.B.-Verlag: Hamburg, Germany, 1998.
48. Plummer, P.J. LuxS and quorum-sensing in *Campylobacter*. *Front. Cell. Infect. Microbiol.* **2012**, *2*, 22. [CrossRef] [PubMed]
49. Sivakumar, K.K.; Jesudhasan, P.R.; Pillai, S.D. Detection of autoinducer (AI-2)-like activity in food samples. *Methods Mol. Biol.* **2011**, *692*, 71–82. [CrossRef] [PubMed]
50. Klančnik, A.; Guzej, B.; Kolar, M.H.; Abramovic, H.; Smole Možina, S. In vitro antimicrobial and antioxidant activity of commercial rosemary extract formulations. *J. Food Prot.* **2009**, *72*, 1744–1752. [CrossRef]
51. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; KAynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; et al. Fiji: An open-source platform for biological-image analysis. *Nat. Methods* **2012**, *9*, 676–682. [CrossRef]

2.2 OSTALO POVEZOVALNO DELO

2.2.1 Določanje koncentracije signalnih molekul AI-2 in izražanja gena *luxS* pri bakterijah *Campylobacter jejuni* po tretiranju s pripravki sivke

Bakterije *C. jejuni* so zmožne medcelične komunikacije preko signalnih molekul AI-2, ki jih pri tej bakteriji sintetizira gen *luxS* (Plummer, 2012). Signalne molekule AI-2 so pomembne za medvrstno komunikacijo med bakterijami (Federle, 2009). Mnoge raziskave so pokazale, da delecija gena *luxS* pri bakteriji *C. jejuni* vpliva na različne fenotipske lastnosti, med katerimi so gibljivost, pritrjevanje na abiotske in biotske površine, filmotvornost, kemotaksa, virulenca in patogenost (Kim in sod., 2021; Elvers in Park, 2002; Plummer in sod., 2012; Quiñones in sod., 2009; Reeser in sod., 2007; Šimunović in sod., 2020). Zaradi teh lastnosti bakterije *C. jejuni* predstavljajo resno težavo v živilski industriji in javnem zdravstvu. Pomembno je poiskati načine modulacije gena *luxS*, saj predvidevamo, da bi z moduliranjem gena *luxS* lahko zmanjšali filmotvornost bakterij *C. jejuni* in s tem posledično nevarnost, ki jo ta bakterija predstavlja za živilsko industrijo in javno zdravstvo.

Rastlinski pripravki veljajo za obetavne alternativne, protimikrobne učinkovine, ki se lahko uporabljajo za moduliranje medcelične komunikacije pri različnih bakterijah. Številne raziskave so pokazale, da rastlinski pripravki zmanjšujejo medcelično signaliziranje bakterij *P. aeuruginosa*, *E. coli*, *S. typhimurium*, *L. monocytogenes*, *S. aureus* (Guglielmi in sod., 2020). Pripravki različnih rastlinskih vrst, kot so *A. millefolium, C. sativum, E. ruticarpa, J. communis, L. hybrida, O. europaea, O. vulgare, R. officinalis* in *T. vulgaris* vplivajo na sistem medcelične komunikacije bakterij *C. jejuni* (Bezek in sod., 2016; Duarte in sod., 2016; Šimunović in sod., 2020; Wagle in sod., 2019). Obstaja tudi korelacija med zmanjšanjem odziva poročevalskega seva *V. harveyi* na izrabljeno gojišče bakterij *C. jejuni* gojenih z rastlinskimi pripravki in zmanjšanjem gibljivosti in pritrjevanjem bakterij *C. jejuni* na abiotske površine po tretiranju z rastlinskimi pripravki (Šimunović in sod., 2020). Pri tem ni jasno ali delujejo pripravki na znižanje koncentracije signalnih molekul AI-2, na izražanje gena *luxS* ali na receptor za zaznavanje signalnih molekul AI-2.

Zanimalo nas je, ali rastlinski pripravki sivke vplivajo na zmanjšanje koncentracije signalnih molekul AI-2, ki jih proizvajajo bakterije *C. jejuni*, kot tudi ali vplivajo na izražanje gena *luxS* pri bakteriji *C. jejuni*. Določili smo koncentracijo signalnih molekul v izrabljenem gojišču bakterij *C. jejuni* brez in s tretiranjem s pripravki sivke (eterično olje, etanolni izvlečki iz cvetov in odpadka: čisti spojini linalol in linalil acetat), kot tudi učinek eteričnega olja sivke, linalola in linalil acetata na izražanje gena *luxS* To nam je pomagalo razumeti mehanizem delovanja sivke na medcelično komunikacijo bakterij *C. jejuni*.

Material in metode

Pripravki sivke

V poskusih smo uporabili pripravke sivke vrste *L. angustifolia* in sicer eterično olje (EO) sivke, etanolni izvleček (EI) iz cvetov sivke, etanolni izvleček (EI) iz odpadnega materiala (odpadek), ki preostane po destilaciji eteričnega olja, kot tudi čisti spojini, linalol in linalil acetat, ki sta glavni sestavini eteričnega olja sivke. Postopek priprave eteričnega olja in etanolnih izvlečkov, kot tudi kemijska karakterizacija pripravkov sivke sta opisana v članku »Protifilmotvorni učinek pripravkov sivke vrste *Lavandula angustifolia* proti bakteriji *Campylobacter jejuni*«, ki je del te doktorske disertacije.

Bakterijski sevi

V poskusih smo uporabljali bakterijske seve *C. jejuni* NCTC 11168 (referenčni sev), *C. jejuni* 11168Δ*luxS* (mutanta, ki ne proizvaja signalne molekule AI-2; negativna kontrola) in poročevalski sev *V. harveyi* MM30. Pogoji gojenja naštetih bakterijskih sevov so opisani v člankih »Razjasnitev komunikacijskega sistema patogenih, s hrano prenosljivih bakterij *Campylobacter jejuni* preko kvantifikacije signalnih molekul AI-2 z biosenzorsko metodo« in »Kontrola filmotvornosti bakterij *Campylobacter jejuni* z eteričnimi olji in etanolnimi izvlečki iz odpadnega materiala sivke vrste *Lavandula x intermedia* (lavandin)«, ki sta del te doktorske disertacije.

Merjenje odziva poročevalskega seva *V. harveyi* MM30 po dodatku izrabljenega gojišča bakterij *C. jejuni,* gojenega s pripravki sivke in čistimi spojinami v subinhibitornih koncentracijah

Merjenje bioluminiscenčnega odziva poročevalskega seva *V. harveyi* se običajno uporablja kot posredna metoda za preučevanje medcelične komunikacije drugih bakterij (Zhao in sod., 2018). Zato smo vpliv pripravkov sivke na medcelično komunikacijo bakterij *C. jejuni* najprej ovrednotili preko merjenja bioluminiscenčnega odziva poročevalskega seva *V. harveyi* MM30 na čitalcu mikrotitrskih plošč (Varioskan Lux, Thermo Scientific, Waltham, MA, USA). Poročevalskemu sevu *V. harveyi* MM30 smo dodali izrabljeno gojišče (IG) bakterij *C. jejuni* NCTC 11168 in bakterij *C. jejuni* 11168Δ*luxS*, ki so bili gojeni z dodatkom pripravkov sivke vrste *L. angustifolia* (EO in EI) in čistimi spojinami (linalol in linalil acetat). Postopka gojenja in priprave IG bakterij *C. jejuni* NCTC 11168 in bakterij *C. jejuni* 11168Δ*luxS*, kot tudi postopek merjenja odziva poročevalskega seva *V. harveyi* MM30 po dodatku IG bakterij *C. jejuni* NCTC 11168 in *C. jejuni* 11168Δ*luxS* sta opisana v člankih »Razjasnitev komunikacijskega sistema patogenih, s hrano prenosljivih bakterij *Campylobacter jejuni* preko kvantifikacije signalnih molekul AI-2 z biosenzorsko metodo« in »Kontrola filmotvornosti bakterij *Campylobacter jejuni* z eteričnimi olji in etanolnimi izvlečki iz odpadnega materiala sivke vrste *Lavandula x intermedia* (lavandin)«, ki sta del te doktorske disertacije. Ker nismo želeli vplivati na rast bakterij *C. jejuni*, ampak samo na

njihove fenotipske lastnosti, smo uporabljali subinhibitorne koncentracije pripravkov sivke in čistih spojin v koncentraciji 1/4 minimalne inhibitorne koncentracije (MIK). Postopek določanja minimalne inhibitorne koncentracije, kot tudi vrednosti koncentracij MIK za testirane pripravke sta navedeni v članku »Protifilmotvorni učinek pripravkov sivke vrste *Lavandula angustifolia* proti bakteriji *Campylobacter jejuni*«, ki je del te doktorske disertacije. Poskusi so bili narejeni v treh bioloških ponovitvah.

Določanje vpliva pripravkov sivke in čistih spojin na koncentracijo signalnih molekul AI-2 v izrabljenem gojišču bakterij *C. jejuni* z metodo HPLC-FLD

Vpliv pripravkov sivke vrste *L. angustifolia* (EO in EI) in čistih spojin (linaol in linalil acetat) na koncentracijo signalnih molekul AI-2 smo določili pri bakterijah *C. jejuni* NCTC 11168. Za negativno kontrolo smo uporabili mutanto *C. jejuni* 11168Δ*luxS*, saj ta ne proizvaja signalne molekule AI-2. Najprej smo pripravili IG bakterij *C. jejuni* NCTC 11168 in *C. jejuni* 11168Δ*luxS*, ki smo jih 24 h gojili z dodatkom pripravkov sivke in čistih spojin v subinhibitornih koncentracijah. Postopka gojenja in pripave IG bakterij *C. jejuni* sta opisana v člankih »Razjasnitev komunikacijskega sistema patogenih, s hrano prenosljivih bakterij *Campylobacter jejuni* preko kvantifikacije signalnih molekul AI-2 z biosenzorsko metodo« in »Kontrola filmotvornosti bakterij *Campylobacter jejuni* z eteričnimi olji in etanolnimi izvlečki iz odpadnega materiala sivke vrste *Lavandula x intermedia* (lavandin)«, ki sta del te doktorske disertacije. Z metodo visoko tlačne tekočinske kromatografije v kombinaciji s fluorescenčnim detektrojem (HPLC-FLD) smo določili koncentracijo signalnih molekul AI-2 v IG bakterij *C. jejuni,* netretiranih in tretiranih s pripravki sivke in čistimi spojinami. Za pripravo umeritvne krivulje smo uporabili standardno molekulo DPD [(S)-4,5-dihidrksi-2,3-pentandion] (Carbosynth, Velika Britanija), ki je prekurzor signalne molekule AI-2. Protokol, ki smo ga uporabljali pri izvedbi metode HPLC-FLD, je opisan v članku »Razjasnitev komunikacijskega sistema patogenih, s hrano prenosljivih bakterij *Campylobacter jejuni* preko kvantifikacije signalnih molekul AI-2 z biosenzorsko metodo«, ki je del te doktorske disertacije. Poskusi so bili narejeni v treh bioloških ponovitvah.

Določanje vpliva eteričnega olja sivke, linalola in linalil acetata na izražanje gena *luxS* z metodo PCR v realnem času (rtPCR)

Izražanje gena *luxS* smo določali v planktonski kulturi bakterij *C. jejuni* NCTC 11168 s pomočjo metode PCR (verižna rekacija s polimerazo) v realnem času (rtPCR) na sistemu rt-PCR (ViiA 7 Real-Time PCR System, Thermo Fisher Scinetific, Applied Biosystems, CA, ZDA). Bakterije *C. jejuni* smo gojili 16 h v tekočem gojišču Mueller Hinton (MH; Oxoid, Velika Britanija), pri temperaturi 42 °C, v mikroaerofilni atmosferi (5 % O_2, 10 % CO_2 in 85 % N_2). Po 16-ih urah smo bakterijskim kulturam dodali EO sivke vrste *L. angustifolia*, linalol ali linalil acetat v subinhibitornih koncentracijah (1/4 MIK) in nadaljevali z gojenjem 30 minut v zgoraj navedenih pogojih. Kot negativno kontrolo smo uporabili bakterijsko kulturo *C. jejuni*, ki je bila gojena 30 min z dodatkom 1 % raztopine dimetil sulfoksid

(DMSO; Merck, Nemčija), saj so bile založne raztopine izbranih pripravkov pripravljene v raztopini DMSO. Koncentracija raztopine DMSO v bakterijskih kulturah, kjer smo dodali izbrane pripravke, ni presegala 1 %. Po 30 minutni inkubaciji smo celice koncentrirali s centrifugiranjem pri pogojih 5000 x g, 5 min, 4 °C, ter smo jih resuspendirali v 1 mL tekočega gojišča MH do koncentracije celic 10^9 CFU/mL. Izolacijo in čiščenje celokupne RNK smo izvedli z uporabo reagenta TRIzol (Sigma Aldrich, ZDA) in kompleta PureLink RNA mini kit (Thermo Fisher Scientific, ZDA) po navodilih proizvajalcev. Encim DNazo smo uporabili za čiščenje izolirane RNK od genomske DNK in sicer ob uporabi kita PureLink DNase (Thermo Fisher Scientific, ZDA) po navodilih proizvajalca. Kvantifikacijo RNK smo izvedli s fluorometrijo na fluorometru Qubit 4 (Thermo Fisher Scientific, Applied Biosystems, CA, ZDA) ob uporabi kita Qubit RNA HS (Thermo Fisher Scientific, ZDA) po navodilih proizvajalca. Z uporabo magnetnih kroglic NEXTflex PolyA (Perkin Elmer, ZDA) smo iz celokupne RNK izolirali mRNK po navodilih proizvajalca.

Za izvedbo metode rtPCR smo najprej konstruirali unikatne začetne oligonukleotide ter sondo FAM-MGB (TaqMAN, Thermo Fisher Scientific, ZDA) (Preglednica 1) s programom BLAST® (National Center for Biotechnology Information, ZDA). Referenčne gene, s katerimi smo izvedli normalizacijo podatkov, smo izbrali na podlagi rezultatov sekveniranja RNK, opisanih v članku »Protifilmotvorni učinek pripravkov sivke vrste *Lavandula angustifolia* proti bakteriji *Campylobacter jejuni*«, ki je del te doktorske disertacije. Izbrali smo gena, ki nista kazala sprememb na nivoju diferencialnega izraženja genov, to sta bila gena *ilvC* in *rpoA*.

Preglednica 1: Seznam uporabljenih začetnih oligonukleotidov.

Ime gena	**F začetni oligonukleotid**	**R začetni oligonukleotid**	**Sonda**
ilvC	TGCAGAATACGGCGATTACATCA	CATCGCTTTTTTAGTCTCT TCAGTG	CAGGGCCAAAG ATTAT
rpoA	GTCTTGAAAAAGCAGGAGTGGTTT	AAGCCCTGCAAGTTCACTT ACA	CTCATCAAAGC AAGCTC
luxS	AGGATTTATGAGAGATCATCTTAAT TCAAATTCAGTT	CAATACTTTTCTCATCAGG TGTTCCAAT	ACCCGTGCGAC AACC

Za pripravo reakcijske mešanice, ki smo jo uporabili pri izvedbi metode rtPCR, smo izbrali mešanico komponent TaqMan universal master mix II with UNG (Thermo Fisher Scientific, ZDA). Reakcijsko mešanico smo pripravili po navodilih proizvajalca. Pogoji pomnoževanja, ki smo jih izbrali za izvedbo rtPCR, so podani v preglednici 2.

Preglednica 2: Pogoji pomnoževanja na rtPCR.

Korak	**Temperatura [°C]**	**Čas [min:s]**	**Št. ciklov**
Inkubacija UNG	50	2:0	1
Encimska aktivacija	95	10:0	1
Denaturacija	95	0:15	40
Prileganje	60	1:0	

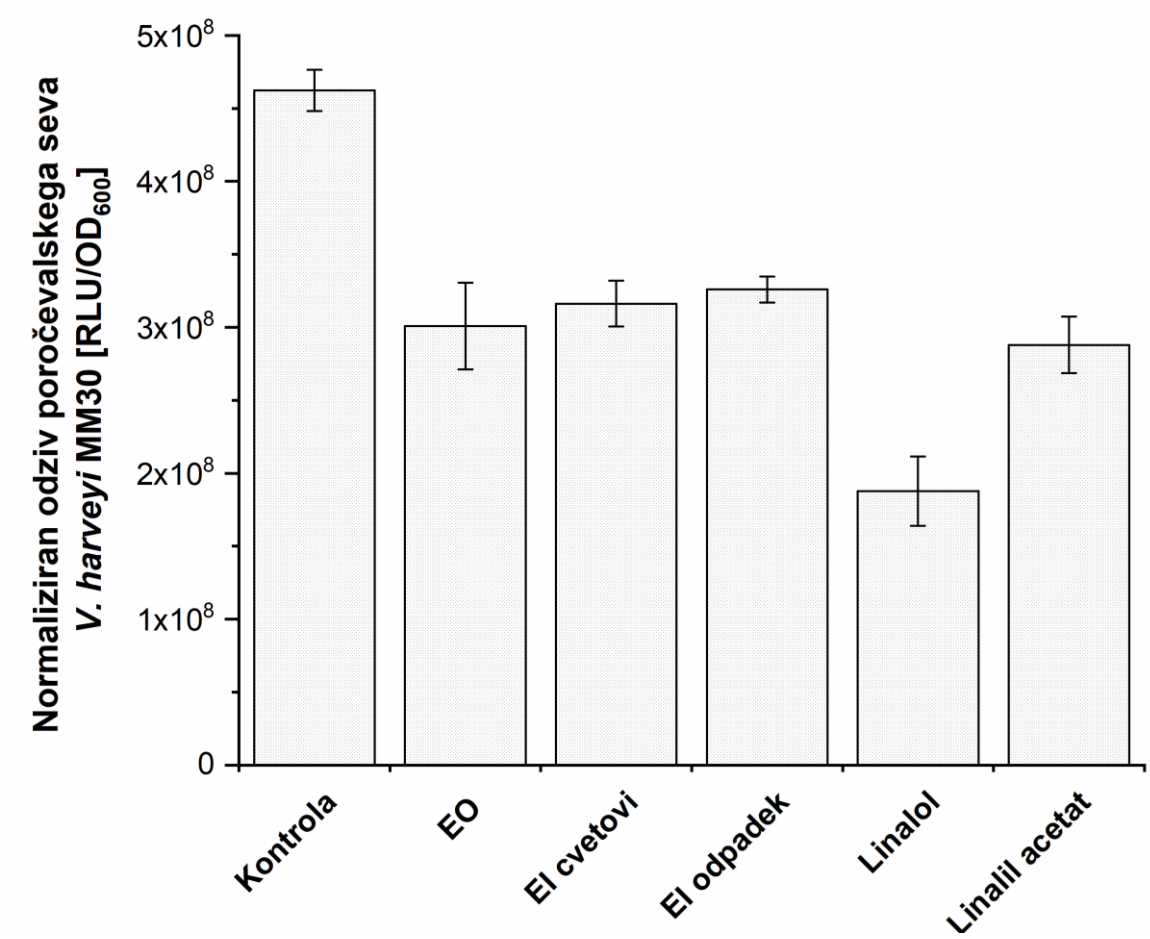
Učinkovitost pomnoževanja smo preračunali na podlagi umeritvene krivulje, za katero smo naredili redčitveno vrsto in sicer za vse vzorce. Po analizi podatkov, pridobljenih z rtPCR, smo ustreznost referenčnih genov preverili z uporabo programa Reffinder (Xie in sod., 2012). Za določanje sprememb v izražanju genov smo uporabili metodo Pfaffl (Pfaffl, 2001). Normalizacijo podatkov smo izvedli, kot jo opisujejo Vandesompele in sod. (2002). Poskusi so bili narejeni v treh bioloških ponovitvah.

Rezultati

Odziv poročevalskega seva *V. harveyi* MM30 na izrabljeno gojišče bakterij *C. jejuni*, gojenih s pripravki sivke in čistimi spojinami v subinhibitornih koncentracijah

Vpliv pripravkov sivke vrste *L. angustifolia* (EO in EI) ter čistih spojin (linalol in linalil acetat) na medcelično komunikacijo bakterij *C. jejuni* smo najprej ovrednotili preko uporabe posredne metode merjenja bioluminiscenčnega odziva poročevalskega seva *V. harveyi* MM30 na IG bakterij *C. jejuni* NCTC 11168, ki so bile gojene z dodatkom izbranih pripravkov v subinhibitornih koncentracijah (1/4 MIK). V teh koncentracijah pripravki sivke in čiste spojine ne vplivajo na rast bakterij *C. jejuni*, kar smo preverili z določanjem CFU/mL. Vplivajo na fenotipske lastnosti, ki jih te bakterije izražajo.

Na sliki 2 lahko opazimo, da je bil bioluminiscenčni odziv poročevalskega seva *V. harveyi* MM30 manjši po dodatku vseh IG bakterij *C. jejuni* NCTC 11168, gojenih z dodatkom pripravkov sivke ali čistih spojin v primerjavi z bioluminiscenčnim odzivom poročevalskega seva *V. harveyi* MM30 po dodatku IG bakterij *C. jejuni* NCTC 11168, gojenih brez dodatka pripravkov sivke ali čistih spojin (kontrola) ($p < 0,05$). Najmočnejši učinek je imela čista spojina linalol. Predvidevali smo, da so pripravki sivke in čiste spojine vplivali na zmanjšanje koncentracije signalnih molekul AI-2, ki jih bakterije *C. jejuni* proizvajajo v času svoje rasti. Predvidevali smo tudi, da pripravki sivke in čiste spojine vplivajo na izražanje gena *luxS* pri bakteriji *C. jejuni*, kar lahko posledično vpliva na zmanjšanje koncentracije signalnih molekul AI-2. Zato smo v nadaljnjih poskusih določili koncentracijo signalnih molekul AI-2 z metodo HPLC-FLD v IG bakterij *C. jejuni* NCTC 11168, ki so bile gojene brez ali z dodatkom subinhibitornih koncentracij pripravkov sivke in čistih spojin. Preverili smo tudi vpliv eteričnega olja sivke, linalola in linalil acetata v subinhibitornih koncentracijah na izražanje gena *luxS* pri bakterijah *C. jejuni* NCTC 11168 z metodo rt-PCR.

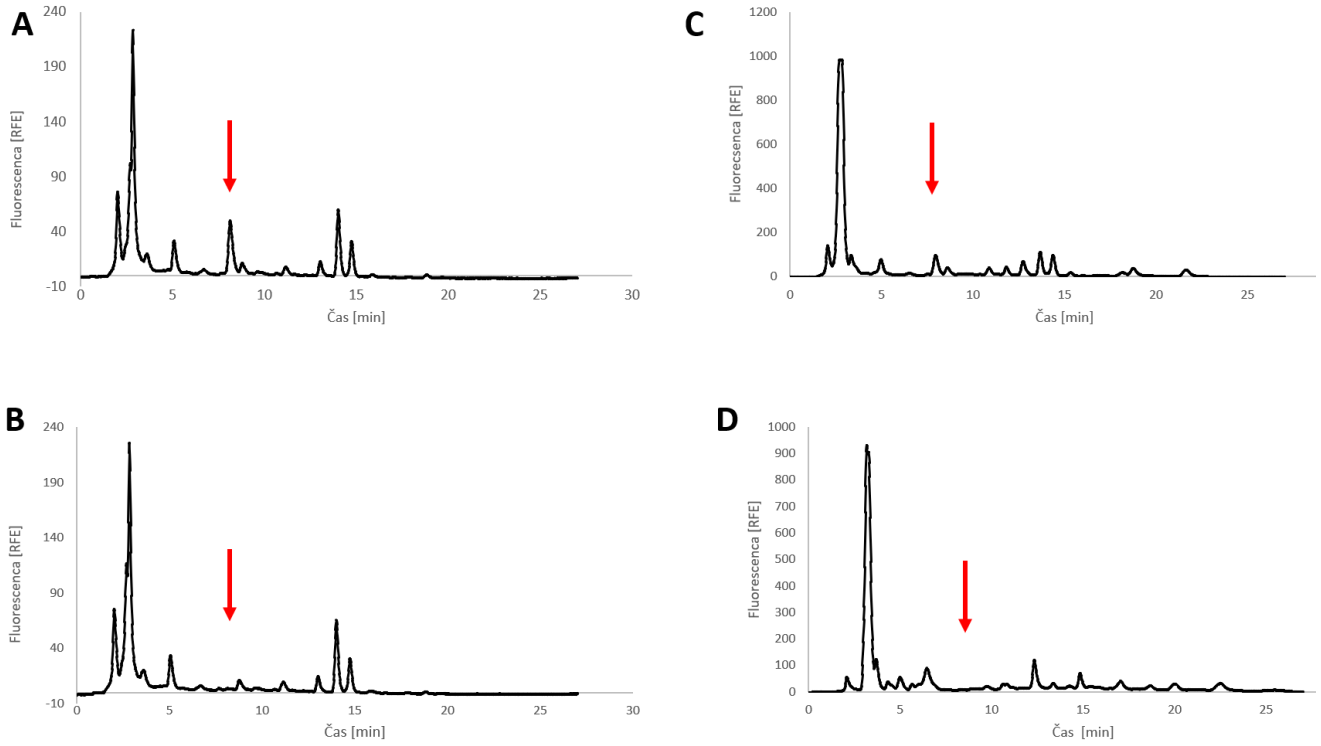


Slika 3: Bioluminiscenčni odziv poročevalskega seva *V. havreyi* MM30, ki jo spodbudi dodatek IG bakterij *C. jejuni* NCTC 11168, ki so bile gojene brez (kontrola) ali z dodatkom pripravkov sivke vrste *L. angustifolia* [EO (eterično olje), EI (etanolni izvleček)] ali čistih spojin (linalol in linalil acetat) v subinhibitornih koncentracijah (1/4 MIK). Prikazane so normalizirane povprečne vrednosti bioluminiscence v relativnih luminiscenčnih enotah [RLE] deljene z optično gostoto [OD_{600}], ki je bila izmerjena za vsak vzorec ± standardni odklon. Od vrednosti bioluminiscence *V. harveyi* MM30, ki jo spodbudi IG bakterij *C. jejuni* NCTC 11168, ki so bile gojene brez ali z dodatkom pripravkov sivke ali čistih spojin v subinhibitornih koncentracijah smo odšteli vrednosti bioluminiscence *V. harveyi* MM30, ki jo spodbudi IG bakterij *C. jejuni* 11168Δ*luxS*, ki so bile gojene brez ali z dodatkom pripravkov sivke ali čistih spojin v subinhibitornih koncentracijah.

Vpliv pripravkov sivke in čistih spojin na koncentracijo signalnih molekul AI-2, ki jih proizvajajo bakterije *C. jejuni*

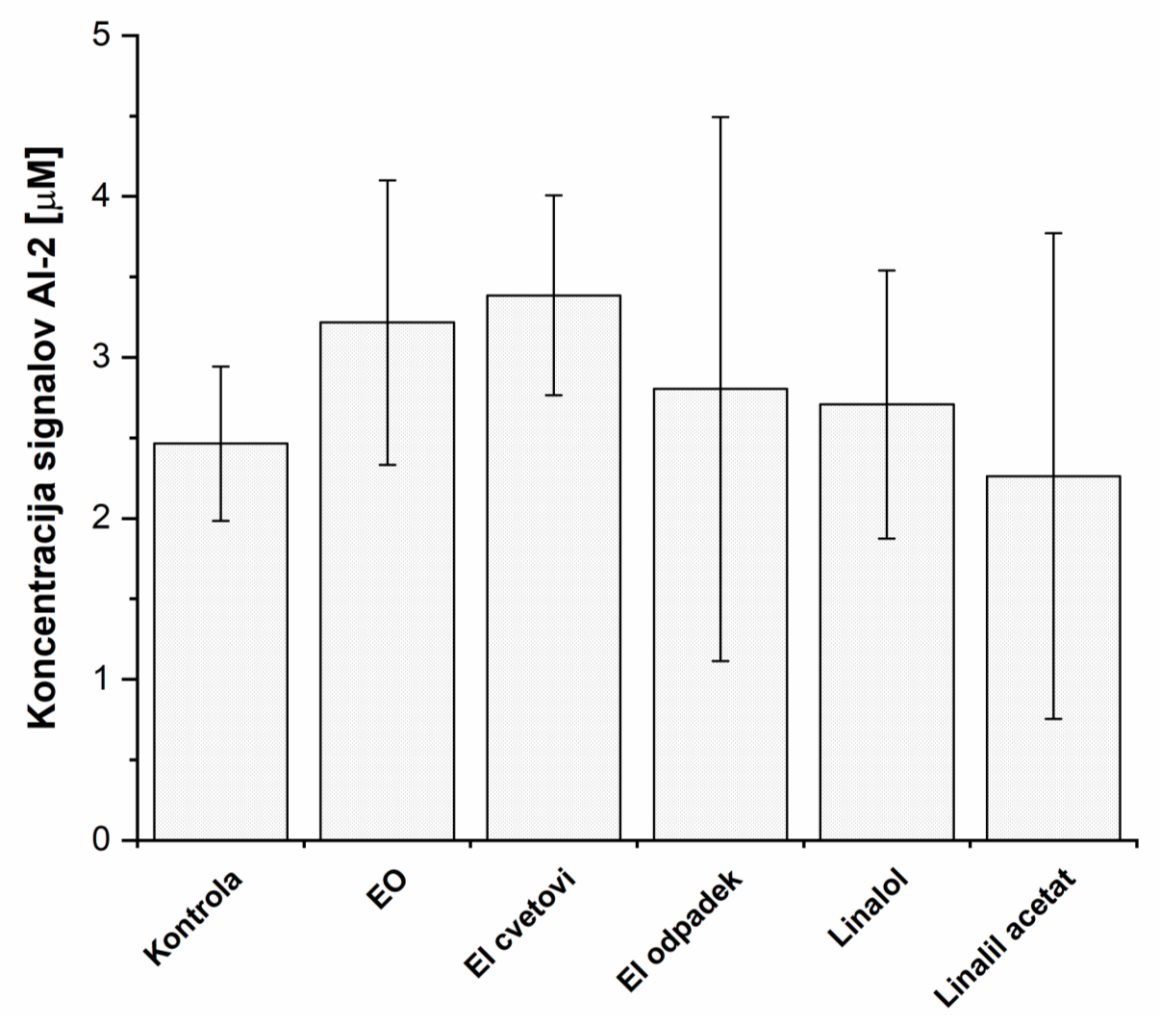
Koncentracijo signalnih molekul AI-2, ki jih proizvajajo bakterije *C. jejuni* NCTC 11168 smo določili z metodo HPLC-FLD. Koncentracijo signalnih molekul AI-2 smo merili v IG bakterij *C. jejuni* NCTC 11168, ki so bile gojene brez ali z dodatkom pripravkov sivke vrste *L. angustifolia* (EO in EI) in čistih spojin (linalol in linalil acetat) v subinhibitornih koncentracijah (1/4 MIK). Kot negativno kontrolo smo uporabili IG bakterij *C. jejuni* 11168Δ*luxS*, ki ne proizvajajo signalne molekule AI-2. Tudi bakterije *C. jejuni* 11168Δ*luxS* smo gojili brez ali z dodatkom pripravkov sivke vrste *L. angustifolia* ali čistih spojin v subinihibitornih koncentracijah (1/4 MIK). Na sliki 3 so prikazani tipični kromatogrami IG

bakterij *C. jejuni* NCTC 11168 in *C. jejuni* 11168Δ*luxS,* gojenih v tekočem gojišču MH, kot tudi kromatogrami IG bakterij *C. jejuni* NCTC 11168 in *C. jejuni* 11168Δ*luxS,* gojenih v tekočem gojišču MH z dodatkom eteričnega olja sivke v subinhibitorni koncentraciji.



Slika 4: Kromatogrami izrabljenih gojišč: bakterijski sev *C. jejuni* NCTC 11168, gojen v tekočem gojišču MH (A), bakterijski sev *C. jejuni* 11168Δ*luxS* ,gojen v tekočem gojišču MH (B), bakterijski sev *C. jejuni* NCTC 11168, gojen v tekočem gojišču MH z dodatkom eteričnega olja sivke vrste *L. angustifolia* v subinhibitorni koncentraciji (C), bakterijski sev *C. jejuni* 11168Δ*luxS,* gojen v tekočem gojišču MH z dodatkom eteričnega olja sivke vrste *L. angustifolia* v subinhibitorni koncentraciji (D). Rdeča puščica prikazuje retencijski čas signalne molekule AI-2 (7,9 min kot smo opisali v članku »Razjasnitev komunikacijskega sistema patogenih, s hrano prenosljivih bakterij *Campylobacter jejuni* preko kvantifikacije signalnih molekul AI-2 z biosenzorsko metodo«, ki je tel te doktorske disertacije), ki jo proizvajajo bakterije *C. jejuni* NCTC 11168 (A in C) oz. odsotnost signalne molekule AI-2 pri bakteriji *C. jejuni* 11168Δ*luxS* (B in D).

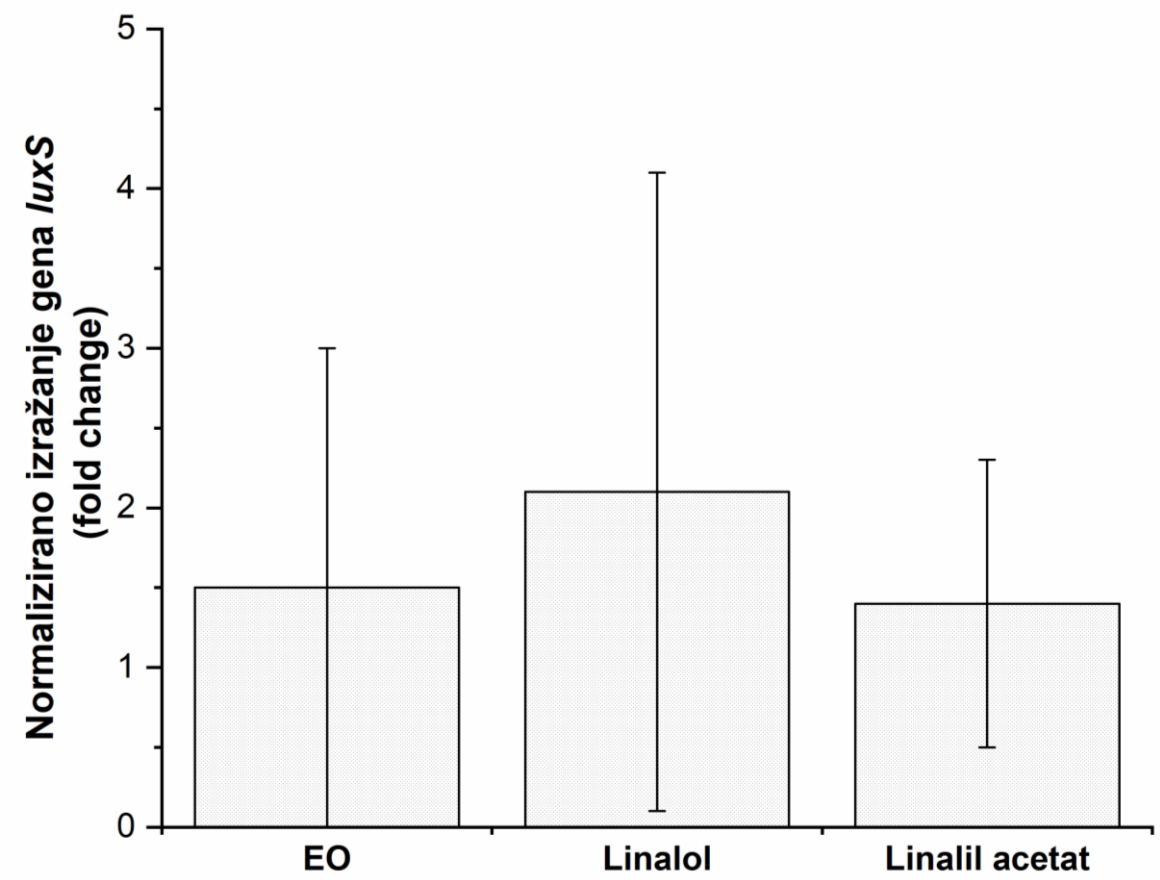
Pri določanju koncentracije signalnih molekul AI-2, ki jih proizvajajo bakterije *C. jejuni* NCTC 11168 z metodo HPLC-FLD smo ugotovili, da se koncentracija signalnih molekul AI-2 v IG bakterij *C. jejuni* NCTC 11168, ki so bile gojene brez pripravkov sivke (kontrola) in čistih spojin statistično ne razlikuje od koncentracije signalnih molekul AI-2 v IG bakterij *C. jejuni* NCTC 11168, ki so bile gojene z dodatkom pripravkov sivke ali čistih spojin v subinhibitornih koncentracijah ($p > 0,05$) (Slika 3).



Slika 5: Koncentracija signalnih molekul AI-2 v IG bakterij *C. jejuni* NCTC 11168 gojenih brez (kontrola) ali z dodatkom pripravkov sivke vrste *L. angustifolia* [EO (eterično olje), EI (etanolni izvleček)] ali čistih spojin (linalol in linalil acetat) v subinhibitornih koncentracijah (1/4 MIK). Koncentracija signalnih molekul AI-2 je določena z metodo HPLC-FLD. Prikazane so povprečne vrednosti v μM ±standardni odklon.

Izražanje gena *luxS* pri bakteriji *C. jejuni* po dodatku eteričnega olja sivke, linalola in linalil acetata v subinhibitornih koncentracijah

Izražanje gena *luxS* pri bakteriji *C. jejuni* NCTC 11168 po dodatku eteričnega olja sivke vrste *L. angustifolia*, linalola in linalil acetata v subinhibitornih koncentracijah (1/4 MIK) smo določili z metodo rt-PCR. Slika 5 prikazuje diferencialno izražanje gena *luxS* po dodatku eteričnega olja sivke, linalola in linalil acetata v subinhibitornih koncentracijah. Lahko sklepamo, da nobeno tretiranje ni statistično značilno vplivalo na diferencialno izražanje gena *luxS* pri bakterijah *C. jejuni* NCTC 11168 ($p > 0,05$). Primerjava je narejena na izražanje gena *luxS* pri negativni kontroli, ki je v tem primeru bila planktonska kultura bakterij *C. jejuni* NCTC 11168 tretirana z 1 % raztopino DMSO.



Slika 6: Normalizirano izražanje gena *luxS* pri bakterijah *C. jejuni* NCTC 11168 po tretiranju z eteričnim oljem (EO) sivke vrste *L. angustifolia*, linalolom in linalil acetatom v subinhibitornih koncentracijah (1/4 MIK). Za določanje sprememb v izražanju genov smo uporabili metodo Pfaffl (Pfaffl, 2001). Normalizacijo podatkov smo izvedli, kot jo opisujejo Vandesompele in sod. (2002). Prikazne so povprečne vrednosti izražanja gena *luxS* ± standardni odklon.

3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

Bakterije *C. jejuni* veljajo za izredno občutljive bakterije, a so kljub temu ena izmed najbolj razširjenih patogenih bakterijskih vrst v celotni živilski verigi. Izrazito razširjenost jim med drugim omogočajo tudi biofilmi, ki predstavljajo edinstveno strategijo preživetja mikroorganizmov zaradi zaščite pred neugodnimi okoljskimi stresnimi dejavniki (EFSA/ECDC, 2020; Klančnik in sod., 2021). *Campylobacter jejuni* je zaradi lastnosti kot so medcelična komunikacija, gibljivost, kemotaksa ter pritrjevanje na različne površine zmožen tvoriti enovrstne, kot tudi večvrstne biofilme na različnih abiotskih površinah, ki jih najdemo v industrijskem okolju. Zmožen je tudi kolonizirati že obstoječe biofilme (Teh in sod., 2010; Tram in sod., 2020). Filmotvornost bakterij je tesno povezana tudi z razvojem odpornosti proti antibiotikom in biocidom, saj znotraj biofilma prihaja do pogostejše izmenjave genetskega materiala, kar vključuje tudi gene za odpornost proti antibiotikom. Poleg tega, antibiotiki in biocidi počasneje prodirajo v biofilm, zaradi česar subletalne koncentracije antibiotikov in biocidov upočasnjujejo rast biofilmskih celic ter spodbujajo mutacije, prenos in izražanje genov za odpornost (Klančnik in sod., 2021). Prevalenca proti antibiotikom in biocidom odpornih sevov bakterij *C. jejuni*, izoliranih predvsem iz perutnine, je visoka in še narašča, kar predstavlja globalni problem (EFSA/ECDC, 2021; Mavri in Smole Možina, 2013; Rozman in sod., 2018). Nujno je poiskati učinkovit pristop za obvladovanje kampilobaktrov v živilski verigi, kot tudi izbrati alternativne učinkovine s potencialnim protifilmotvornim učinkom. Ta je lahko kurativni, kar pomeni, da odstranjujemo že obstoječi biofilm ali preventivni, kjer poskušamo omejiti začetno pritrjevanje bakterij kot predstopnjo filmotvornosti, in nadaljnjo rast biofilma. S tem preprečimo prilagajanje bakterij in nadaljnje razmnoževanje in širjenje v živilski verigi.

Prvi cilj te doktorske naloge je bila optimizacija metode gojenja biofilmov bakterij *C. jejuni* na abiotskih površinah. Kljub temu, da je *C. jejuni* filmotvorna bakterija, je zaradi njenih posebnih gojitvenih zahtev težko nagojiti zrel biofilm. Zato smo biofilme bakterij *C. jejuni* gojili na interfazi trdna površina/tekočina, kjer je zrastlo največ biomase (Priloga D). Na interfazi trdna površina/tekočina je koncentracija O_2 za rast bakterij *C. jejuni* optimalna, saj koncentracija O_2 z globino upada, kar ustreza bakterijam *C. jejuni*, ki so mikroaerofilne bakterije (Stewart, 2012). Za preučevanje biofilmov smo uporabili konfokalno vrstično lasersko mikroskopijo, katera nam je omogočila vpogled v prostorsko strukturo biofilma, kot tudi ovrednotenje živosti celic ob uporabi metode barvanja živo/mrtvo (angl. *LIVE/DEAD*). Prednost te metode je zaznavanje tudi celic VBNC (živo, vendar ne kultivabilno), ki jih z gojitvenimi metodami ne moremo zaznati. *Campylobacter jejuni* pogostokrat v neugodnih, stresnih razmerah preide v stanje VBNC, a je še vedno prisoten in lahko naprej navzkrižno kontaminira površine in prehranske izdelke (Kim in sod., 2021). Zelo je pomembno pridobiti podatek o živosti celic, saj so žive celice problematične in se lahko naprej razmnožujejo ter širijo.

Ko smo metodo gojenja in preučevanja biofilma optimizirali, smo lahko opazovali tudi odstranjevalni učinek pripravkov sivke. Naši rezultati so pokazali, da je med testiranimi pripravki (eterično olje, etanolni izvlečki iz cvetov in odpadnega materiala) sivke vrste *L. angustifolia,* eterično olje najbolj učinkovalo na odstranjevaje zrelega biofilma bakterij *C. jejuni* z abiotske površine. Eterična olja so izrazito bogata s sekundarimi metaboliti, ki sinergistično prispevajo k protimikrobnem delovanju (Dorman in Deans, 2000). Wagle in sod. (2019) so pokazali, da *trans*-cinamaldehid, eugenol in karvakrol odstranjujejo zrele biofilme bakterij *C. jejuni* s površine polistirena in nerjavečega jekla na način, da razgrajujejo komponente zunajceličnega matriksa. Rastlinski sekundarni metaboliti lahko vplivajo tudi na proizvodnjo in aktivnost encimov, ki so pomembni za sintezo zunajceličnega matriksa. Hkrati pa lahko motijo vnos hranil, spreminjajo pH gradient celične citoplazme ter povzročajo celično smrt (Pisoschi in sod., 2018). Zanimivo je tudi, da smo pri pripravkom izpostavljenih vzorcih opazili, da je delež pritrjenih celic manjši ali enak kot 3 %, kar pomeni, da pripravki niso le uničili celic, ampak so jih tudi odstranili s površine. To je lahko posledica spremembe površinske napetosti samih celic in/ali površine, kot tudi vpliva na elektrostatske in van der Waalsove sile med celicam in površino (Roy in sod., 2018). Potrebno je poudariti, da je tudi odpadni material sivke pokazal odstranjevalni učinek, kar pomeni, da bi z optimizacijo postopka priprave izvlečkov iz odpadnega materiala lahko ponovno uporabili odpadek v industrijske namene ter s tem izboljšali ekonomičnost proizvodnje eteričnega olja. S tem bi tudi izboljšali ravnanje z odpadki, saj bi jih ponovno uporabili in s tem zagotovili okoljevarstvene izboljšave. Iz pridobljenih rezultatov je jasno, da rastlinski pripravki sivke odstranjujejo zrele biofilme bakterij *C. jejuni*, kar pomeni, da bi se lahko uporabljali kot alternativni, kurativni načini odstranjevanja biofilmov v živilski verigi.

V nadaljnjih poskusih smo želeli preveriti, ali bi se lahko rastlinski pripravki sivke uporabljali za preventivne namene in sicer za preprečevanje ali zmanjševanje filmotvornosti bakterij *C. jejuni*. Da bi zmanjšali filmotvornost, je potrebno vplivati na ključne korake, ki do filmotvornosti vodijo, to so gibljivost, začetno pritrjevanje in sistem medcelične komunikacije. Gibljivost, ki jo pri bakterijah *C. jejuni* poganjata dva polarno nameščena bička, je nujna za začetno pritrjevanje in nadaljnjo filmotvornost (Guerry, 2007). Gibanje z bički omogoča bakterijam, da premagajo elektrostatične odbojne sile med celicami in površino (Gutman in sod., 2013). Že zmanjšana gibljivost lahko vodi k zmanjševanju pritrjevanja in filmotvornosti bakterij *C. jejuni*. Po izpostavljenosti bakterij *C. jejuni* subinhibitornim koncentracijam pripravkov sivke vrste *L. angustifolia* in čistim spojinam (linalol, linalil acetat, kamfor, borneol, 1,8-cineol, *trans*-ferulna kislina, *p*-kumarna kislina) smo ugotovili, da se gibljivost teh bakterij na poltrdnem gojišču značilno zmanjša ($p < 0,05$). Med testiranimi učinkovinami so pripravki sivke (eterično olje in etanolni izvlečki) imeli najmočnejši učinek na zmanjšanje gibljivosti, kar je lahko posledica sinergističnega delovanja sekundarnih metabolitov. Podobne rezultate so dobili Castillo in sod. (2014), ki so potrdili vpliv različnih citrusov na gibljivost bakterij *C. jejuni*.

Skladno s pričakovanji so pripravki sivke vrst *L. angustifolia* in *L. x intermedia* zmanjšali pritrjevanje bakterij *C. jejuni* na abiotske površine. Med delovanjem pripravkov eteričnih olj in etanolnih izvlečkov ni bilo bistvene razlike, kar potrjuje koristnost odpadnega materiala in potencial za ponovno uporabo v industrijske namene. Uporabnost odpadkov timijana in oljčnih listov proti pritrjevanju bakterij *C. jejuni* na abiotske površine so potrdili tudi Šikić Pogačar in sod. (2016). Šimunović in sod. (2020) so pokazali, da kar devet različnih rastlinskih pripravkov zmanjšuje pritrjevanje bakterij *C. jejuni* na abiotske površine. V naši raziskavi smo pokazali, da pripravki sivke vrste *L. angustifolia* zmanjšujejo pritrjevanje bakterij *C. jejuni* po 4, 8 in 24 h izpostavljenosti pripravkom ter da je njihov učinek močnejši v primerjavi s čistimi spojinami, kar ponovno nakazuje na pomemben sinergistični učinek čistih spojin v pripravkih. Enako so opazili Duarte in sod. (2016), ki so pokazali, da eterično olje koriandra močnejše zmanjšuje pritrjevanje bakterij *C. jejuni* in *C. coli* na abiotske površine v primerjavi z linalolom, ki je ena izmed glavnih spojin eteričnega olja sivke in koriandra. Prav tako so eterična olja in etanolni izvlečki sivke vrste *L. x intermedia* zavrli filmotvornost bakterij *C. jejuni* po 72 h, s čimer smo potrdili, da lahko pripravki sivke dlje časa omejujejo filmotvornost bakterij *C. jejuni*.

S transkriptomsko analizo smo pokazali, da eterično olje sivke vrste *L. angustifolia* v subinhibitorni koncentraciji vpliva na spremembo izražanja kar 326 genov, od tega zmanjšuje izražanje 188 genov v primerjavi s kontrolo. Med temi geni je kar 24 genov vključenih v mehanizem gibljivosti in filmotvornosti bakterij *C. jejuni*. Kot že omenjeno, za pritrjevanje in nadaljnjo filmotvornost bakterije *C. jejuni* potrebujejo funkcionalne bičke, zato je zelo pomembno modulirati izražanje genov, ki so potrebni za sintezo in modifikacijo bičkov, če želimo zmanjšati filmotvornost teh bakterij. Opazili smo tudi zmanjšano izražanje genov, ki vplivajo na sintezo beljakovin in lipopolisaharidov zunanje bakterijske membrane, kot so *kpsM, kpsS, bamD, waaC*, in *lgt*. Vsi našteti geni so vključeni v proces pritrjevanja bakterij *C. jejuni* na abiotske površine (Naito in sod., 2010). Podatki, ki smo jih pridobili s sekvenciranjem RNK, kažejo tudi na to, da so bakterije *C. jejuni* ob dodatku eteričnega olja sivke *L. angustifolia* doživele kar obsežno transkriptomsko reprogramiranje, kar je posledica odziva bakterij na stres (Aseev in sod., 2016). Opazili smo povečano izražanje sistema črpalk CmeABC, ki jih bakterije *C. jejuni* uporabljajo za aktivno izločanje protimikrobnih snovi, kar potrjuje stresno stanje bakterij *C. jejuni* (Lin in sod., 2002). Mnogi geni za kodiranje transmembranskih beljakovin so se slabše izražali, vključno z geni, ki so pomembni za kodiranje beljakovin za vnos železa. Pokazano je, da je sistem za vnos železa pri kampilobaktru vpleten v kolonizacijo, filmotvornost in patogenost teh bakterij ter da se v stresnih razmerah izražanje sistema za vnos železa močno zmanjša (Asakura in sod., 2007; Miller in sod., 2009; Püning in sod., 2021). Tudi geni, ki so pomembni za stresni odziv (*cstA*, *pph, dcuA, hrcA*) so se manj izražali, kar ima lahko za posledico zmanjšano filmotvornost bakterij *C. jejuni* (Drozd in sod., 2014; Kim in sod., 2021; Rasmussen in sod., 2013). Pridobljeni podatki transkriptomske analize nam dajejo vpogled v celoten prepis genov, ki jih v trenutku tretiranja z eteričnem oljem sivke vrste *L. angustifolia* bakterije *C. jejuni*

izražajo ter nam odpirajo dodatne vidike in možnosti za raziskovanje novih tarč, ki bi nam pomagale pri nadzorovanju tega patogena v živilski industriji.

Vodilno vprašanje te naloge je bilo ali je zmanjšanje filmotvornosti bakterij *C. jejuni* posledica vpliva rastlinskih pripravkov sivke na sistem medcelične komunikacije teh bakterij. Zanimalo nas je tudi ali lahko z zaviranjem medcelične komunikacije vplivamo na filmotvornost bakterij *C. jejuni*. Da bi odgovorili na ta vprašanja, smo najprej optimizirali metodo preučevanja medcelične komunikacije bakterij *C. jejuni*. Za preučevanje medcelične komunikacije s signalno molekulo AI-2 se običajno uporabljajo posredne metode oz. poročevalski sevi, najpogosteje *V. harveyi* BB170 (Zhao in sod., 2018). *Vibrio harveyi* BB170 je mutanta LuxN$^-$, kar pomeni, da ne zaznava signalne molekule AHL. Proizvaja in zaznava signalne molekule AI-2 (Bassler in sod., 1997). Ker *V. harveyi* BB170 proizvaja in zaznava lastne signalne molekule AI-2, je velika možnost, da ob uporabi tega seva pri preučevanju medcelične komunikacije bakterij *C. jejuni* dobimo lažno pozitiven rezultat. Zato je bilo nujno poiskati optimalni sev, s katerim bomo lahko zaznavali signale molekule AI-2, ki jih proizvaja *C. jejuni*, in nam tako omogoči kvantifikacijo signalnih molekul. V naši raziskavi smo testirali štiri različne poročevalske seve *V. harveyi,* med katerimi se je *V. harveyi* MM30 pokazal kot najbolj občutljiv sev za zaznavanje signalnih molekul AI-2, ki jih proizvaja *C. jejuni*. *Vibrio harveyi* MM30 je mutanta LuxS$^-$, kar pomeni, da ne proizvaja signalne molekule AI-2, jih pa zaznava. Proizvaja in zaznava tudi signalne molekule AHL, za katere smo pokazali, da morajo biti prisotne, če želimo, da se poročevalski sev *V. harveyi* MM30 močno odzove na signalne molekule AI-2. Z matematičnim modeliranjem bioluminiscenčnega odziva poročevalskega seva *V. havreyi* MM30 po dodatku izrabljenega gojišča bakterij *C. jejuni* smo uspeli kvantificirati signalne molekule AI-2. Točnost kvantifikacije signalnih molekul AI-2 s poročevalskim sevom smo preverili tudi z direktno, analitsko metodo HPLC-FLD, ki smo jo za bakterije *C. jejuni* prvič vpeljali. Pokazali smo, da je poročevalski sev *V. harveyi* MM30 bolj občutljiv kot analitska metoda HPLC-FLD in točen, saj statistično značilne razlike med pridobljenimi meritvami nismo zaznali.

Rezultati tega doktorskega dela kažejo, da signalna molekula AI-2 predstavlja stranski produkt izražanja gena *luxS*, ki je sicer pomemben za centralni metabolizem bakterij *C. jejuni*, kar smo potrdili tudi v nedavni raziskavi (Ramić in sod., 2022). Delecija gena *luxS* pri bakteriji *C. jejuni* spremeni izražanje kar 765 genov v primerjavi z divjim tipom, od katerih je večina vključena v biološke procese, kot so Krebsov cikel, metabolizem piruvata, dušika in tiamina, ter sintezo lipopolisaharidov (Ramić in sod., 2022). Za signalno molekulo AI-2, ki jo proizvajajo bakterije *C. jejuni,* ne moremo trditi, da je del mehanizma zaznavanja kvoruma (angl. *Quorum Sensing*), saj se koncentracija AI-2 pri bakterijah *C. jejuni* linearno povečuje s povečanjem koncentracije celic, medtem ko pri zaznavanju kvoruma pride do t.i. »odziva stikala« (angl. *Switch-Like Response*) (Hense in Schuster, 2015). Dodatno smo opazili, da obstaja bistvena razlika v koncentraciji signalnih molekul AI-2 pri enaki celični gostoti bakterij *C. jejuni* v dveh različnih gojiščih, kar dodatno nakazuje, da signalne molekule AI-2 pri bakterijah *C. jejuni* niso komunikacijske molekule zaznavanja kvoruma.

Prav tako, ni poznan receptor za signalne molekule AI-2 pri bakterijah *C. jejuni*. Bioinformacijska analiza, ko so jo izvedli Parkhill in sod. (2000) ni pokazala, da obstajajo v genomu bakterij *C. jejuni* znani analogi receptorjev signalnih molekul AI-2. Glede na to, da je signalna molekula AI-2 medvrstna komunikacijska signalna molekula, je možno, da bakterije *C. jejuni* s svojimi signalnimi molekulami AI-2 spodbujajo filmotvornost drugih bakterij, ki potem proizvajajo zunajcelični matriks ter s tem zaščitijo kampilobaktra in mu omogočajo preživetje. Pokazano je bilo, da veliko število človeških oralnih komenzalnih bakterij proizvaja signalne molekule AI-2, ki so potrebne za tvorbo mešanega biofilma in razvoja zobnih oblog (Kikuchi in sod., 2005; McNab in sod., 2003; Rickard in sod., 2008). McNab in sod. (2003) so pokazali, da je signalna molekula AI-2 pomembna pri tvorbi mešanih biofilmov dveh pogostih oralnih bakterij, *Porphyromonas gingivalis* in *Streptococcus gordonii*. Med temi bakterijami se mešani biofilm ni razvil na polistirenski površini, ko sta obe vrsti bili brez gena *luxS*, ki je potreben za sintezo signalne molekule AI- 2. Ko so mutante gojili skupaj s heterolognim sevom divjega tipa, ki je vseboval funkcionalni gen *luxS*, se je mešani biofilm tvoril. Laganenka in Sourjik (2018) sta pokazala, da signalne molekule AI-2, ki jih proizvajajo bakterije *Enterococcus faecalis,* spodbujajo kolektivno obnašanje bakterij *E. coli* pri nizki celični gostoti. To posledično vodi k izboljšani avtoagregaciji bakterij *E. coli*, kot tudi h koagregaciji med temi dvemi vrstami. Prav tako sta Laganenka in Sourjik (2018) pokazala, da je preživetje bakterij *E. faecalis* in *E. coli* v stresnih razmerah boljše znotraj mešanega biofilma. Nove raziskave bi bilo potrebno usmeriti na mešane biofilme med kampilobaktrom in drugimi bakterijami, ki jih v živilski verigi pogostokrat zaznavamo ter preučiti vpliv medvrstne komunikacijske signalne molekule AI-2, ki jih proizvajajo bakterije *C. jejuni* na tvorbo mešanega biofilma.

Vpliv rastlinskih pripravkov sivke na medcelično komunikacijo bakterij *C. jejuni* smo najprej želeli preveriti posredno preko bioluminiscenčnega odziva poročevalskega seva *V. harveyi* MM30 na izrabljeno gojišče bakterij *C. jejuni*, ki so bile gojene brez ali z rastlinskimi pripravki sivke vrst *L. angustifolia* in *L. x intermedia* v subinhibitornih koncentracijah. Pokazali smo, da se je bioluminiscenčni odziv poročevalskega seva *V. harveyi* MM30 po dodatku izrabljenega gojišča bakterij *C. jejuni*, ki so bile gojene z rastlinskimi pripravki sivke, zmanjšal v primerjavi z bioluminiscenčnim odzivom poročevalskega seva *V. harveyi* MM30 po dodatku izrabljenega gojišča bakterij *C. jejuni*, ki so bile gojene brez dodatka rastlinskih pripravkov sivke. Opazili smo, da je bil bioluminiscenčni odziv poročevalskega seva *V. harveyi* MM30 na izrabljeno gojišče bakterij *C. jejuni*, ki so bile gojene z etanolnimi izvlečki iz cvetov in odpadnega materiala sivke vrste *L. x intermedia,* manjši v primerjavi z bioluminiscenčnim odzivom na izrabljeno gojišče bakterij *C. jejuni*, ki so bile gojene z eteričnimi olji sivke vrste *L. x intermedia*. To je lahko posledica vezave signalnih molekul AI-2 na oborine v vzorcih etanolnih izvlečkov, ki so bile odstranjene skupaj z bakterijami med filtracijo. Podoben rezultat so dobili Bezek in sod. (2016), ki so pokazali, da etanolni izvlečki rastline *E. ruticarpa* zmanjšujejo bioluminiscenčni odziv poročevalskega seva *V. harveyi* za približno 90 %. Šimunović in sod. (2020) so pokazali, da eterično olje sivke vrste

L. hybrida zmanjšuje odziv poročevalskega seva *V. harveyi* za približno 66 %, kar je primerljivo z učinkom testiranih eteričnih olj sivke vrste *L. angustifolia* in *L. x intermedia.* Pri tem ni jasno ali rastlinski pripravki sivke vplivajo na koncentracijo signalnih molekul AI-2, izražanje gena *luxS* pri bakteriji *C. jejuni* ali celo na receptor poročevalskega seva, saj je mehanizem delovanja rastlinskih pripravkov na medcelično komunikacijo bakterij lahko različen in vključuje vpliv na zmanjšanje izražanja receptorskih beljakovin, zaviranje proizvodnje signalnih molekul, razgradnjo signalnih molekul kot tudi mimikrijo signalnih molekul (Kalia, 2013; Machado in sod., 2020).

V okviru te doktorske naloge nas je zanimalo, ali rastlinski pripravki sivke zmanjšujejo koncentracijo signalnih molekul AI-2 v izrabljenem gojišču bakterij *C. jejuni,* zato smo točno koncentracijo signalnih molekul AI-2 določili z metodo HPLC-FLD. Ugotovili smo, da ni bilo statistično značilne razlike med koncentracijo signalnih molekul AI-2 v izrabljenem gojišču bakterij *C. jejuni*, ki so bile gojene brez ali z dodatkom rastlinskih pripravkov sivke (eterično olje, etanolna izvlečka) in čistih spojin (linalol in linalil acetat). Ta rezultat ne potrjuje prve hipoteze, ki pravi, da bodo rastlinski pripravki sivke in izbrane čiste spojine v subinhibitornih koncentracijah zmanjšale koncentracijo signalnih molekul AI-2 v izrabljenem gojišču bakterij *C. jejuni*. Ker prve hipoteze nismo potrdili, ne moremo potrditi tudi druge, tretje in četrte hipoteze te doktorske naloge, saj so vse vezane na znižanje koncentracije signalnih molekul AI-2, kot posledica vpliva rastlinskih pripravkov. Rezultati transkriptomske analize in rezultati, pridobljeni z metodo rt-PCR, kažejo na to, da eterično olje sivke kot tudi čisti spojini linalol in linalil acetat statistično značilno ne spreminjajo izražanje gena *luxS* pri bakterij *C. jejuni*, kar je lahko razlog, da se tudi koncentracija signalnih molekul AI-2 ni značilno spremenila v izrabljenih gojiščih bakterij *C. jejuni*. Skladno s tem so Wagle in sod. (2019) v svoji raziskavi pokazali, da čiste spojine *trans*-cinamaldehid in karvakrol celo zvišata izražanje gena *luxS*, medtem ko eugenol ne vpliva na spremembo izražanja gena *luxS* pri bakteriji *C. jejuni*.

Signalna molekula AI-2 je medvrstna komunikacijska signalna molekula, ki lahko modulira filmotvornost različnih bakterij (Luo in sod., 2022). Opazili smo, da je bil bioluminiscenčni odziv poročevalskega seva *V. harveyi* MM30 manjši po dodatku izrabljenega gojišča bakterij *C. jejuni*, ki so bile gojene z rastlinskimi pripravki sivke v primerjavi z bioluminiscenčnim odzivom na izrabljeno gojišče bakterij *C. jejuni*, ki so bile gojene brez rastlinskih pripravkov sivke. To nam nakazuje, da je lahko tarča rastlinskih pripravkov receptorska beljakovina pri poročevalskem sevu, ampak za potrditev tega so potrebni dodatni poskusi. Rezultati sicer nakazujejo na to, da bi rastlinske pripravke lahko uporabljali za obvladovanje medvrstne komunikacije ter s tem zmanjšali filmotvornost, saj smo opazili zmerno in pozitivno korelacijo med zmanjšanjem odziva poročevalskega seva na izrabljeno gojišče bakterij *C. jejuni*, ki so bile gojene z dodatkom rastlinskih pripravkov sivke v subinhibitornih koncentracijah in zmanjšanjem pritrjevanja bakterij *C. jejuni* na abiotske površine. Podobno so zaznali Šimunović in sod. (2020), ki so tudi potrdili pozitivno korelacijo med zmanjšanjem odziva poročevalskega seva na izrabljeno gojišče bakterij *C. jejuni*, ki so bile

gojene z dodatkom rastlinskih pripravkov v subinhibitornih koncentracijah in zmanjšanjem pritrjevanja bakterij *C. jejuni* na abiotske površine. Dodatno smo ugotovili, da obstaja zmerna in pozitivna korelacija med zmanjšanjem pritrjevanja in nadaljnjo filmotvornostjo bakterij *C. jejuni*. To podpirajo tudi rezultati transkriptomske analize, kjer smo opazili, da eterično olje sivke vrste *L. angustifolia* vpliva na izražanje različnih bioloških procesov, vpletenih v filmotvornost bakterij *C. jejuni*. Transkriptomska analiza nam je odprla možnosti iskanja novih tarč, ki bodo pomagale pri zmanjševanju filmotvornosti in tveganja, ki jo bakterije *C. jejuni* predstavljajo za živilsko verigo in javno zdravstvo. Zmanjšanje medvrstne komunikacije med bakterijami v kombinaciji z dodatnimi tarčami, kot so npr. geni, vključeni v gibljivost in filmotvornost, sistem vnosa železa ali stresni odziv predstavljajo zanimive in obetavne tarče za obvladovanje biofilma bakterij *C. jejuni* v celotni živilski verigi.

3.2 SKLEPI

Bakterije *Campylobacter jejuni* so ubikvitarne bakterije, ki že desetletja predstavljajo izrazito nevarnost za živilsko industrijo kot tudi za javno zdravstvo. Zmožne so filmotvornosti na abiotskih in biotskih površinah, kar jim omogoča boljše preživetje in razširjenost v celotni živilski verigi. Nujno je poiskati alternativne učinkovine, ki bodo pomagale pri odstranjevanju kampilobaktrov iz živilske verige. V tej doktorski nalogi smo bili osredotočeni na optimizacijo metode gojenja biofilmov bakterij *C. jejuni* na abiotskih površinah; razvoj, optimizacijo in nadgradnjo posredne metode zaznavanja in kvantifikacije signalnih molekul AI-2, ki jih proizvajajo bakterije *C. jejuni*; ter na iskanje protibakterijskega in protifilmotvornega učinka rastlinskih pripravkov sivke vrst *L. angustifolia* in *L. x intermedia* proti bakteriji *C. jejuni*. Prišli smo do naslednjih ugotovitev:

- *Vibrio harveyi* MM30 je optimalen sev za preučevanje medcelične komunikacije bakterij *C. jejuni*, saj je njegov odziv na signalne molekule AI-2, ki jih proizvaja *C. jejuni* najmočnejši in najbolj občutljiv v primerjavi z odzivom ostalih poročevalskih sevov *V. harveyi*.
- Signalne molekule AI-2, ki jih proizvajajo bakterije *C. jejuni,* je možno zaznati in kvantificirati z direktno metodo, HPLC-FLD.
- Koncentracijo signalnih molekul AI-2, ki jih proizvajajo bakterije *C. jejuni,* je možno natančno in točno kvantificirati z uporabo poročevalskega seva *V. harveyi* MM30 in pri tem doseči 100-krat večjo občutljivost v primerjavi z metodo HPLC-FLD.
- Naraščanje koncentracije signalnih molekul AI-2 je linearno z naraščanjem koncentracije celic bakterij *C. jejuni* in odvisno od vrste gojišča, zaradi česar sklepamo, da je signalna molekula AI-2 pri bakterijah *C. jejuni* stranski produkt centralnega metabolizma, v katerega je vključen gen *luxS*.
- Bakterije *C. jejuni* proizvajajo največ biomase na interfazi trdna površina/tekočina.

- Rastlinski pripravki sivke vrst *L. angustifolia* ter izbrane čiste spojine v subinhibitornih koncentracijah zmanjšujejo gibljivost bakterij *C. jejuni* na poltrdnem gojišču.
- Rastlinski pripravki sivke vrst *L. angustifolia* in *L. x intermedia* ter izbrane čiste spojine v subinhibitornih koncentracijah zmanjšujejo pritrjevanje in filmotvornost bakterij *C. jejuni* na abiotske površine.
- Rastlinski pripravki sivke vrste *L. angustifolia* uspešno odstranjujejo zrel biofilm bakterij *C. jejuni* z abiotske površine.
- Rastlinski pripravki sivke vrste *L. angustifolia* in *L. x intermedia* ter izbrane čiste spojine v subinhibitornih koncentracijah zmanjšujejo bioluminiscenčni odziv poročevalskega seva *V. harveyi* MM30 na signalno molekulo AI-2 v izrabljenem gojišču bakterij *C. jejuni*.
- Rastlinski pripravki sivke vrste *L. angustifolia* ter izbrane čiste spojine (linalol in linalil acetat) v subinhibitornih koncentracijah ne zmanjšujejo koncentracije signalnih molekul AI-2 v izrabljenem gojišču bakterij *C. jejuni*.
- Eterično olje sivke vrste *L. angustifolia*, linalol in linalil acetat v subinhibitornih koncentracijah ne vplivajo na izražanje gena *luxS* pri bakterijah *C. jejuni*.
- Rastlinski pripravki sivke vrst *L. angustifolia* in *L. x intermedia* (eterična olja ter etanolni izvlečki iz cvetov in odpadnega materiala) imajo močnejši učinek na zmanjšanje bioluminiscenčnega odziva poročevalskega seva *V. harveyi* na izrabljeno gojišče bakterij *C. jejuni*, ki so bile gojene z rastlinskimi pripravki sivke, gibljivost, pritrjevanje in filmotvornost bakterij *C. jejuni* v primerjavi s čistimi spojinami.
- Obstaja pozitivna in zmerna korelacija med zmanjšanjem bioluminiscenčnega odziva poročevalskega seva *V. harveyi* na izrabljeno gojišče bakterij *C. jejuni*, ki so bile gojene z rastlinskimi pripravki sivke ter zmanjšanjem pritrjevanja na abiotske površine po tretiranju z rastlinskimi pripravki sivke vrste *L. angustifolia* in *L. x intermedia*.
- Obstaja pozitivna in zmerna korelacija med zmanjšanjem pritrjevanja in zmanjšanjem filmotvornosti bakterij *C. jejuni* na abiotske površine po tretiranju z rastlinskimi pripravki sivke vrste *L. x intermedia.*

4 POVZETEK (SUMMARY)

4.1 POVZETEK

Bakterije *C. jejuni* so vodeče povzročiteljice črevesnih okužb ljudi v EU že od leta 2005. Izrazito so razširjene v celotni živilski verigi zaradi svojih filmotvornih lastnosti, v kar štejemo tudi gibljivost, pritrjevanje in medcelično komunikacijo. Biofilmi omogočajo kampilobaktrom navzkrižno kontaminacijo različnih abiotskih površin in prehranskih izdelkov, kar povečuje tveganje za okužbo. Čeprav obstajajo različni načini obvladovanja bakterijskih biofilmov v živilski verigi, noben ni popolnoma zanesljiv in učinkovit. Zato je nujno potrebno poiskati nove načine obvladovanja bakterijskih biofilmov v živilski verigi, ki so lahko kurativni in preventivni. S kurativnimi načini odstranjujemo že obstoječe biofilme, medtem ko s preventivnimi načini preprečujemo filmotvornost. Poleg tega je potrebno poiskati alternativne pripravke, ki bi lahko pomagali v boju proti biofilmom, ki so vir patogenih in rezistentnih sevov.

V tej doktorski nalogi smo testirali različne pripravke sivk vrst *L. angustifolia* in *L. x intermedia* za obvladovanje biofilma bakterij *C. jejuni* na fiziološki in molekularni ravni. Iz cvetov sivk vrst *L. angustifolia* in *L. x intermedia* smo pripravili eterična olja in etanolne izvlečke. Iz odpadnega materiala, ki preostane po destilaciji eteričnega olja sivke, smo tudi pripravili etanolne izvlečke. Za vse pripravke smo naredili kemijsko karakterizacijo z GC-MS in LC-MS, katera nam je dala vpogled v kemijski profil pripravljenega materiala. Glede na kemijski profil eteričnih olj in etanolnih izvlečkov smo izbrali glavne čiste spojine (linalol, linalil acetat, kamfor, borneol, 1,8-cineol, *p*-kumarna kislina, *trans*-ferulna kislina), ki smo jih naprej uporabili v bioloških poskusih. Z obvladovanjem biofilma bakterij *C. jejuni* smo se spoprijeli na dva načina in sicer preko odstranjevanja zrelega biofilma in preko preprečevanja filmotvornosti. Za preprečevanje filmotvornosti smo uporabili tarče, ki so za filmotvornost bakterij *C. jejuni* izrednega pomena, in to so gibljivost, pritrjevanje in medcelična komunikacija. Glavni cilj te naloge je bil pokazati, če je mogoče biofilm bakterij *C. jejuni* obvladovati z zaviranjem medcelične komunikacije.

Najprej smo optimizirali metodo gojenja in preučevanja biofilmov bakterij *C. jejuni* na abiotski površini. Biofilme bakterij *C. jejuni* smo preučevali na interfazi trdna površina/tekočina, kjer imajo kampilobaktri optimalne pogoje za svojo rast in razvoj (optimalna koncentracija O_2). Za preučevanje smo uporabili konfokalno vrstično lasersko mikroskopijo, ki nam je omogočila vpogled v prostorsko strukturo biofilma, kot tudi ovrednotenje živosti celic ob uporabi metode barvanja živo/mrtvo (angl. *LIVE/DEAD*). Zrele biofilme bakterij *C. jejuni* smo odstranjevali z rastlinskimi pripravki sivke vrste *L. angustifolia* (eterično olje, etanolni izvleček iz cvetov in etanolni izvleček iz odpadnega materiala) ter ugotovili, da vsi pripravki odstranjujejo zrel biofilm bakterij *C. jejuni*. Med testiranimi pripravki je najboljši učinek na odstranjevanje zrelega biofilma bakterij *C. jejuni*

imelo eterično olje sivke vrste *L. angustifolia*, kar je lahko posledica vsebnosti številnih sekundarnih metabolitov.

Za preprečevanje filmotvornosti bakterij *C. jejuni* smo uporabili različne pripravke sivk vrst *L. angustifolia* in *L. x intermedia* v subinhibitornih koncentracijah. Glavne tarče za preprečevanje filmotvornosti so bile gibljivost, pritrjevanje in medcelična komunikacija bakterij *C. jejuni*. Da bi preverili vpliv na medcelično komunikacijo, smo določili koncentracijo signalnih molekul v izrabljenem gojišču bakterij *C. jejuni* ter izražanje gena *luxS* pri bakterijah *C. jejuni* po tretiranju z rastlinskimi pripravki sivke. Za preučevanje medcelične komunikacije bakterij *C. jejuni* smo najprej morali razviti, optimizirati in nadgraditi metodo preučevanja. Najprej smo vpeljali metodo HPLC-FLD, s katero smo lahko izmerili koncetracijo signalnih molekul AI-2, ki jih proizvajajo bakterije *C. jejuni.* Za namen preučevanja izredno nizkih koncentracij signalnih molekul AI-2 smo v nadaljevanju razvili biosenzorsko metodo. Testirali smo različne poročevalske seve *V. harveyi*, med katerimi se je *V. harveyi* MM30 pokazal kot najbolj občutljiv sev za preučevanje medcelične komunikacije bakterij *C. jejuni*. Z matematičnem modeliranjem nam je uspelo tudi določiti koncentracijo signalnih molekul AI-2 v izrabljenem gojišču bakterij *C. jejuni* ob uporabi novo razvite biosenzorske metode. Pridobljene rezultate smo potrdili z direktno, analitsko metodo HPLC-FLD in ugotovili, da je novo razvita biosenzorska metoda dovolj natančna in 100-krat bolj občutljiva v primerjavi z direktno, analitsko metodo HPLC-FLD.

Pripravki sivk vrst *L. angustifolia* in *L. x intermedia* so se pokazali kot bolj učinkoviti pri zmanjševanju gibljivosti, pritrjevanja in filmotvornosti na abiotske površine v primerjavi s čistimi spojinami, ki jih v teh pripravkih najdemo. To je lahko posledica sinergističnega učinka čistih spojih v pripravkih sivke. Zanimivo je, da med testiranimi pripravki sivke (eterična olja, etanolni izvlečki iz cvetov in etanolni izvlečki iz odpadnega materiala) ni bilo bistvene razlike med učinkom na gibljivost, pritrjevanje in filmotvornost bakterij *C. jejuni* na abiotske površine, kar kaže na uporabnost odpadnega materiala. Bioluminiscenčni odziv poročevalskega seva *V. harveyi* MM30 na izrabljeno gojišče bakterij *C. jejuni*, ki so bile gojene z dodatkom pripravkov sivk, je bil prav tako manjši v primerjavi z bioluminiscenčnim odzivom na izrabljeno gojišče bakterij *C. jejuni*, ki so bile gojene s čistimi spojinami. Ta rezultat spet nakazuje na sinergističen učinek čistih spojin v pripravkih sivke. Med koncentracijo signalnih molekul AI-2 v izrabljenih gojiščih bakterij *C. jejuni*, ki so bile gojene brez ali s pripravki sivke (eterično olje in etanola izvlečka) in čistimi spojinami (linaol in linalil acetat) nismo zaznali statistično značilne razlike. Prav tako eterično olje sivke, linalol in linalil acetat niso pokazali učinka na izražanje gena *luxS*, ki je odgovoren za sintezo signalnih molekul AI-2.

Dodatno smo ugotovili, da je signalna molekula AI-2 verjetno pri bakterijah *C. jejuni* prej stranski produkt centralnega metabolizma, kot signalna molekula AI-2, ki je del mehanizma zaznavanja kvoruma. Koncentracija AI-2 pri bakterijah *C. jejuni* se linearno povečuje s povečanjem koncentracije celic, medtem ko pri zaznavanju kvoruma pride do t.i. »odziva

stikala«. Prav tako je bila koncetracija signalnih molekul AI-2 odvisna od sestave gojišča, čeprav je bila koncetracija celic enaka. Narejena transkriptomska analiza nam je omogočila vpogled v celoten prepis genov, ki se v trenutku tretiranja z eteričnim oljem sivke pri bakteriji *C. jejuni* izražajo in nam je podala nove tarče, ki se lahko uporabljajo v boju proti filmotvornosti bakterij *C. jejuni*. Te vključujejo gene, pomembne za mehanizem gibljivosti in filmotvornosti, sistem vnosa železa in stresni odziv bakterij *C. jejuni*.

Signalna molekula AI-2 je medvrstna komunikacijska signalna molekula ter obstaja velika možnost, da bakterije *C. jejuni* s svojimi signalnimi molekulami AI-2 spodbujajo filmotvornost drugih bakterij, kar sproži proizvodnjo zunajceličnega matriksa in s tem zaščito kampilobaktra, kar mu omogoča preživetje v živilski verigi. Pripravki sivke ne zmanjšujejo koncentracije signalnih molekul AI-2 in ne zmanšujejo izražanja gena *luxS*, ampak vplivajo na bioluminiscenčni odziv poročevalskega seva *V. harveyi* MM30 po dodatku izrabljenega gojišča bakterij *C. jejuni*, ki so bile gojene s pripravki sivke. To nakazuje na to, da pripravki sivke vplivajo na medvrstno komunikacijo. Določili smo zmerno in pozitivno korelacijo med zmanjšanjem bioluminiscenčnega odziva poročevalskega seva *V. harveyi* MM30 po dodatku izrabljenega gojišča bakterij *C. jejuni*, ki so bile gojene s pripravki sivke in zmanjšanjem pritrjevanja bakterij *C. jejuni* na abiotske površine. Določili smo tudi zmerno in pozitivno korelacijo med zmanjšanjem pritrjevanja bakterij *C. jejuni* na abiotske površine in zmanjšanjem nadaljnje filmotvornosti teh bakterij. Zmanjšanje medvrstne komunikacije med bakterijami v kombinaciji z dodatnimi tarčami, kot so npr. geni, vključeni v gibljivost in filmotvornost, sistem vnosa železa ali stresni odziv, predstavljajo zanimive tarče za obvladovanje biofilma bakterij *C. jejuni* v celotni živilski verigi.

4.2 SUMMARY

Campylobacter jejuni has been the leading cause of human gastrointestinal infections in the EU since 2005. These bacteria are widespread throughout the food production chain due to their biofilm forming properties, which also include motility, attachment and intercellular communication. Biofilms allow *Campylobacter* to cross-contaminate various abiotic surfaces and food products, increasing the risk of infection. Although there are various methods of controlling bacterial biofilms in the food production chain, none of them are completely reliable and effective. Therefore, it is urgently necessary to find new ways of controlling bacterial biofilms in the food prodution chain, which can be both curative and preventive. Curative methods remove already existing biofilms, while preventive methods prevent biofilm formation. In addition, it is necessary to find alternative preparations that could help in the fight against biofilms, which are the source of pathogenic and resistant strains.

In this doctoral thesis, we tested different preparations of lavender species, *L. angustifolia* and *L. x intermedia*, for controlling the biofilm of *C. jejuni* at the physiological and molecular

level. Essential oils and ethanol extracts were prepared from lavender flowers of *L. angustifolia* and *L. x intermedia* species. Ethanol extracts were also prepared from the waste material remaining after the distillation of lavender essential oil. For all preparations, we performed chemical characterization with GC-MS and LC-MS, which gave us an insight into the chemical profile of the prepared material. Based on the chemical profile of the essential oils and ethanol extracts, we selected the main pure compounds (linalool, linalyl acetate, camphor, borneol, 1,8-cineole, *p*-coumaric acid, *trans*-ferulic acid), which we further used in biological experiments. Controlling the biofilm of *C. jejuni* was tackled in two ways, namely by removing the mature biofilm and by preventing biofilm formation. To prevent biofilm formation, we used targets that are extremely important for the biofilm formation of *C. jejuni*, namely motility, attachment and intercellular communication. The main goal of this work was to show if the biofilm of *C. jejuni* can be controlled by inhibiting intercellular communication.

First, we optimized the method of culturing and studying biofilms of *C. jejuni* on an abiotic surface. Biofilms of *C. jejuni* were studied at the solid/liquid interface, where *Campylobacter* have optimal conditions for their growth and development (optimal O_2 concentration). For the study, we used confocal laser scanning microscopy, which enabled us to gain an insight into the spatial structure of the biofilm as well as to evaluate the vitality of the cells using the LIVE/DEAD staining method. Mature biofilms of *C. jejuni* were removed with plant preparations of lavender species *L. angustifolia* (essential oil, ethanol extract from flowers and ethanol extract from waste material), and we found that all preparations remove mature biofilm of *C. jejuni*. Among the tested preparations, the essential oil of lavender had the best effect on removing the mature biofilm of *C. jejuni*, which may be due to the content of many secondary metabolites.

To prevent biofilm formation of *C. jejuni*, we used different preparations of lavender species *L. angustifolia* and *L. x intermedia* in subinhibitory concentrations. The main targets for preventing biofilm formation were motility, attachment and intercellular communication of *C. jejuni*. In order to check the influence on intercellular communication, we determined the concentration of AI-2 signalling molecules in the spent medium of *C. jejuni* and the expression of the *luxS* gene in *C. jejuni* after the treatment with lavender preparations. To study the intercellular communication of *C. jejuni*, we first had to develop, optimize and upgrade the study method. First, we introduced the HPLC-FLD method with which we could measure the concentration of AI-2 signaling molecules of *C. jejuni*. In the following, we developed a biosensor method for the purpose of studying extremely low concentrations of AI-2 signalling molecules. We tested different *V. harveyi* reporter strains, among which *V. harveyi* MM30 proved to be the most sensitive strain for studying the intercellular communication of *C. jejuni*. Using mathematical modeling for biosensor method, we also managed to determine the concentration of AI-2 signalling molecules in the spent medium of *C. jejuni*. The obtained results were confirmed by the direct, analytical method, HPLC-

FLD, and we found that our developed biosensor method is sufficiently accurate and 100 times more sensitive compared to the direct, analytical method, HPLC-FLD.

Preparations of lavender species *L. angustifolia* and *L. x intermedia* have shown to be more effective in reducing motility, attachment and biofilm formation on abiotic surfaces compared to the pure compounds found in these preparations. This may be due to the synergistic effect of the pure compounds in the lavender preparations. It is interesting that among the tested preparations of lavender (essential oils, ethanol extracts from flowers and ethanol extracts from waste material) there was no significant difference between the effect on the motility, attachment and biofilm formation of *C. jejuni* on abiotic surfaces, which confirms the good effect of the waste material as well. The bioluminescence response of the reporter strain *V. harveyi* MM30 to the spent medium of *C. jejuni* cultured with the addition of lavender preparations was also lower compared to the bioluminescence response to the spent medium of *C. jejuni* cultured with pure compounds. This result again suggests a synergistic effect of pure compounds in lavender preparations. We did not detect any statistically significant difference between the concentration of AI-2 signal molecules in the spent media of *C. jejuni*, which were grown without or with lavender preparations (essential oil and ethanol extract) and pure compounds (linaol and linalyl acetate). Likewise, lavender essential oil, linalool and linalyl acetate did not show effect on the expression of the *luxS* gene, which is responsible for the synthesis of AI-2 signalling molecules.

Additionally, we found that the AI-2 signalling molecule in *C. jejuni* is probably a by-product of central metabolism, and we cannot directly claim that the AI-2 signalling molecule is part of the quorum sensing mechanism, since the concentration of AI-2 increases linearly with increasing cell concentration, while in quorum sensing it increases like "switch response". Also, the concentration of AI-2 signalling molecules depended on the composition of the culture medium, although the concentration of the cells was the same. The transcriptomic analysis gave us the insight into the entire transcript of genes that are expressed at the time of treatment with lavender essential oil in *C. jejuni* and gave us new targets that can be used in the fight against the biofilm formation of *C. jejuni*. These include genes important for the mechanism of motility and biofilm formation, the iron uptake system and the stress response of *C. jejuni*.

The AI-2 signalling molecule is an interspecies communication signalling molecule, and there is a great possibility that AI-2 signalling molecules produced by *C. jejuni* stimulate the biofilm formation of other bacteria, which then produce an extracellular matrix and thereby protect *Campylobacter* and enable them to survive in the food chain. Lavender preparations do not reduce the concentration of AI-2 signalling molecules and do not decrease the expression of the *luxS* gene, but they affect the bioluminescence response of the reporter strain *V. harveyi* MM30 after the addition of the spent medium of *C. jejuni* that were grown with lavender preparations. This suggests that lavender preparations affect interspecies communication. We determined a moderate and positive correlation between the reduction

of the bioluminescence response of the reporter strain *V. harveyi* MM30 after the addition of the spent medium of *C. jejuni* grown with lavender preparations and the reduction of the attachment of *C. jejuni* to abiotic surface. We also determined a moderate and positive correlation between the reduction of the attachment of *C. jejuni* to abiotic surface and the reduction of further biofilm formation of these bacteria. Reduction of interspecies communication between bacteria in combination with additional targets such as genes involved in motility and biofilm formation, the iron uptake system or the stress response represent interesting targets for managing the biofilm of *C. jejuni* throughout the whole food chain.

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Abisado R. G., Benomar S., Klaus J. R., Dandekar A. A., Chandler J. R. 2018. Bacterial quorum sensing and microbial community interactions. mBio, 9, 3: e02331-17, doi:10.1128/MBIO.02331-17: 13 str.

Adler L., Alter T., Sharbati S., Gölz G. 2015. The signalling molecule autoinducer-2 is not internalised in *Campylobacter jejuni*. Berliner und Münchener tierärztliche Wochenschrift, 128, 3-4: 111–116

Afonso A. C., Sousa M., Simões L. C., Simões M. 2022. Phytochemicals against drug-resistant bacterial biofilms and use of green extraction solvents to increase their bioactivity. Advances in Experimental Medicine and Biology, doi:10.1007/5584_2022_723: 18 str. (v tisku)

Agunos A., Waddell L., Léger D., Taboada E. 2014. A systematic review characterizing on-farm sources of *Campylobacter* spp. for broiler chickens. PLOS ONE, 9, 8: e104905, doi:10.1371/JOURNAL.PONE.0104905: 20 str.

Alara O. R., Abdurahman N. H., Ukaegbu C. I. 2021. Extraction of phenolic compounds: A review. Current Research in Food Science, 4: 200–214

Ampomah-Wireko M., Luo C., Cao Y., Wang H., Nininahazwe L., Wu C. 2021. Chemical probe of AHL modulators on quorum sensing in Gram-negative bacteria and as antiproliferative agents: A review. European Journal of Medicinal Chemistry, 226: 113864, doi:10.1016/J.EJMECH.2021.113864: 25 str.

Angane M., Swift S., Huang K., Butts C. A., Quek S. Y. 2022. Essential oils and their major components: An updated review on antimicrobial activities, mechanism of action and their potential application in the food industry. Foods, 11, 3: 464, doi:10.3390/FOODS11030464: 26 str.

Antunes L. C. M., Ferreira R. B. R. 2009. Intercellular communication in bacteria. Critical Reviews in Microbiology, 35, 2: 69–80

Aseev L. V., Koledinskaya L. S., Boni I. V. 2016. Regulation of ribosomal protein operons rplM-rpsI, rpmB-rpmG, and rplU-rpmA at the transcriptional and translational levels. Journal of Bacteriology, 198, 18: 2494–2502

Azeredo J., Azevedo N. F., Briandet R., Cerca N., Coenye T., Costa A. R., Desvaux M., Di Bonaventura G., Hébraud M., Jaglic Z., Kačániová M., Knøchel S., Lourenço A., Mergulhão F., Meyer R. L., Nychas G., Simões M., Tresse O., Sternberg C. 2016. Critical review on biofilm methods. Critical Reviews in Microbiology, 43, 3: 313–351

Bassler B. L., Greenberg E. P., Stevens A. M. 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. Journal of Bacteriology, 179, 12: 4043–4045

Bezek K., Kurinčič M., Knauder E., Klančnik A., Raspor P., Bucar F., Smole Možina S. 2016. Attenuation of adhesion, biofilm formation and quorum sensing of *Campylobacter jejuni* by *Euodia ruticarpa*. Phytotherapy Research, 30, 9: 1527–1532

Borges A., Meireles A., Mergulhão F., Melo L., Simões M. 2020. Biofilm control with

enzymes. V: Recent trends in biofilm science and technology. Simoes M., Borges A., Chaves Simoes L. (ur.). Amsterdam, Academic Press: 249–271

Boyer M., Wisniewski-Dyé F. 2009. Cell–cell signalling in bacteria: not simply a matter of quorum. FEMS Microbiology Ecology, 70, 1: 1–19

Bridier A., Sanchez-Vizuete P., Guilbaud M., Piard J. C., Naïtali M., Briandet R. 2015. Biofilm-associated persistence of food-borne pathogens. Food Microbiology, 45: 167–178

Camilli A., Bassler B. L. 2006. Bacterial small-molecule signaling pathways. Science, 311, 5764: 1113-1116

Carrascosa C., Raheem D., Ramos F., Saraiva A., Raposo A. 2021. Microbial biofilms in the food industry-A comprehensive review. International Journal of Environmental Research and Public Health, 18, 4: 2014, doi:10.3390/ijerph18042014: 31 str.

Castillo S., Heredia N., Arechiga-Carvajal E., García S. 2014. Citrus extracts as inhibitors of quorum sensing, biofilm formation and motility of *Campylobacter jejuni*. Food Biotechnology, 28, 2: 106–122

Cawthraw S. A., Newell D. G. 2010. Investigation of the presence and protective effects of maternal antibodies against *Campylobacter jejuni* in chickens. Avian Diseases, 54, 1: 86–93

Chassagne F., Samarakoon T., Porras G., Lyles J. T., Dettweiler M., Marquez L., Salam A. M., Shabih S., Raschid Farrokhi D., Quave C. L., Echeverria J., Joy McGaw L., Urzua A. 2021. A systematic review of plants with antibacterial activities: A taxonomic and phylogenetic perspective. Frontiers in Pharmacology, 11: 586548, doi:10.3389/fphar.2020.586548: 29 str.

Costa Vasconcelos Alves A. M., Salles de Brito E. H., Glauber Peixoto Ferreira F., Jales de Hollanda Celestino J. 2020. Methods for studying microbial biofilm. International Journal of Development Research, 10, 12: 43100-43104

Ćirić A. D., Petrović J. D., Glamočlija J. M., Smiljković M. S., Nikolić M. M., Stojković D. S., Soković M. D. 2019. Natural products as biofilm formation antagonists and regulators of quorum sensing functions: A comprehensive review update and future trends. South African Journal of Botany, 120: 65–80

Das K., Tiwari R. K. S., Shrivastava D. K. 2010. Techniques for evaluation of medicinal plant products as antimicrobial agents: Current methods and future trends. Journal of Medicinal Plants Research, 4, 2: 104–111

Delle Side D., Giuffreda E., Tredici S. M., Talà A., Pennetta C., Alifano P. 2015. Quorum sensing: Complexity in the bacterial world. Chaos, Solitons and Fractals, 81: 551–555

Diggle S. P., Griffin A. S., Campbell G. S., West S. A. 2007. Cooperation and conflict in quorum-sensing bacterial populations. Nature, 450, 7168: 411–414

Dogsa I., Oslizlo A, Stefanic P., Mandic-Mulec I. 2014. Social interactions and biofilm formation in *Bacillus subtilis*. Food Technology and Biotechnology, 52, 2: 149-157

Dorman H. J. D., Deans S. G. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology, 88, 2: 308–316

Drozd M., Chandrashekhar K., Rajashekara G. 2014. Polyphosphate-mediated modulation of *Campylobacter jejuni* biofilm growth and stability. Virulence, 5, 6: 680–690

Duarte A., Luís Â., Oleastro M., Domingues F. C. 2016. Antioxidant properties of coriander essential oil and linalool and their potential to control *Campylobacter* spp. Food Control, 61: 115–122

Eberhard A., Burlingame A. L., Eberhard C., Kenyon G. L., Nealson K. H., Oppenheimer N. J. 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry, 20, 9: 2444–2449

EFSA/ECDC. 2021. The European Union One Health 2020 Zoonoses Report. EFSA Journal, 19, 12: 6971, doi:10.2903/j.efsa.2021.6971: 324 str.

El-Tarabily K. A., El-Saadony M. T., Alagawany M., Arif M., Batiha G. E., Khafaga A. F., Elwan H. A. M., Elnesr S. S., Abd El-Hack M. 2021. Using essential oils to overcome bacterial biofilm formation and their antimicrobial resistance. Saudi Journal of Biological Sciences, 28, 9: 5145–5156

Elvers K.T., Park S.F. 2002. Quorum sensing in *Campylobacter jejuni*: detection of a *luxS* encoded signalling molecule. Microbiology, 148, 5: 1475–1481

Erega A., Stefanic P., Dogsa I., Danevčič T., Simunovic K., Klančnik A., Možina S. S., Mandic-Mulec I.. 2021. Bacillaene mediates the inhibitory effect of *Bacillus subtilis* on *Campylobacter jejuni* biofilms. Applied and Environmental Microbiology, 87, 12: e02955-20, doi:10.1128/AEM.02955-20: 14 str.

Facciolà A., Riso R., Avventuroso E., Visalli G., Delia S. A., Laganà P. 2017. *Campylobacter*: from microbiology to prevention. Journal of Preventive Medicine and Hygiene, 58: 79–92

Federle M. J. 2009. Autoinducer-2-based chemical communication in bacteria: complexities of interspecies signaling. Contributions to Microbiology, 16: 18–32

Feng J., Ma L., Nie J., Konkel M. E., Lu X. 2018. Environmental stress-induced bacterial lysis and extracellular DNA release contribute to *Campylobacter jejuni* biofilm formation. Applied and Environmental Microbiology, 84, 5: e02068-17, doi:10.1128/AEM.02068-17: 18 str.

Ferriol-González C., Domingo-Calap P. 2020. Phages for biofilm removal. Antibiotics, 9, 5: 268, doi:10.3390/ANTIBIOTICS9050268: 16 str.

Flemming H.-C., Wingender J., Szewzyk U., Steinberg P., Rice S. A., Kjelleberg S. 2016. Biofilms: an emergent form of bacterial life. Nature Reviews Microbiology, 14: 563–575

Flemming H.-C., Wuertz S. 2019. Bacteria and archaea on Earth and their abundance in biofilm. Nature Reviews Microbiology, 17: 247–260

Franklin M. J., Chang C., Akiyama T., Bothner B. 2015. New technologies for studying biofilms. Microbiology Spectrum, 3, 4: MB-0016-2014, doi:10.1128/microbiolspec.MB-0016-2014: 23 str.

Galié S., García-Gutiérrez C., Miguélez E. M., Villar C. J., Lombó F. 2018. Biofilms in the food industry: Health aspects and control methods. Frontiers in Microbiology, 9: 898,

doi:10.3389/fmicb.2018.00898: 18 str.

Gill C., Bahrndorff S., Lowenberger C. 2017. *Campylobacter jejuni* in *Musca domestica*: An examination of survival and transmission potential in light of the innate immune responses of the house flies. Insect Science, 24, 4: 584–598

Gölz G., Kittler S., Malakauskas M., Alter T. 2018. Survival of *Campylobacter* in the food chain and the environment. Current Clinical Microbiology Reports, 5, 2: 126–134

Guerry P. 2007. *Campylobacter* flagella: not just for motility. Trends in Microbiology, 15, 10: 456–461

Guglielmi P., Pontecorvi V., Rotondi G. 2020. Natural compounds and extracts as novel antimicrobial agents. Expert Opinion on Therapeutic Patents, 30, 12: 949–962

Gutman J., Walker S. L., Freger V., Herzberg M. 2013. Bacterial attachment and viscoelasticity: Physicochemical and motility effects analyzed using quartz crystal microbalance with dissipation (QCM-D). Environmental Science and Technology, 47, 1: 398–404

Hanzelka B. L., Greenberg E. P. 1996. Quorum sensing in *Vibrio fischeri*: evidence that S-adenosylmethionine is the amino acid substrate for autoinducer synthesis. Journal of Bacteriology, 178, 17: 5291–5294

Hassan M. M., Lawan I. A., Fan X., Guo Y., Yu Y., Jin X., Qiu J., Guan X., Tianpei H. 2020. Beyond risk: Bacterial biofilms and their regulating approaches. Froniters in Microbiology, 11: 928, doi:10.3389/fmicb.2020.00928: 20 str.

Hense B. A., Schuster M. 2015. Core principles of bacterial autoinducer systems. Microbiology and Molecular Biology Reviews, 79, 1: 153–169

Holmes K., Tavender T. J., Winzer K., Wells J. M., Hardie K. R. 2009. AI-2 does not function as a quorum sensing molecule in *Campylobacter jejuni* during exponential growth *in vitro.* BMC Microbiology, 9: 214, doi: 10.1186/1471-2180-9-214: 11 str.

Hympanova M., Terlep S., Markova A., Prchal L., Dogsa I., Pulkrabkova L., Benkova M., Marek J., Stopar D. 2020. The antibacterial effects of new N-alkylpyridinium salts on planktonic and biofilm bacteria. Frontiers in Microbiology, 11: 573951, doi:10.3389/FMICB.2020.573951: 12 str.

Igwaran A., Okoh A. I. 2019. Human campylobacteriosis: A public health concern of global importance. Heliyon, 5, 11: e02814, doi:10.1016/J.HELIYON.2019.E02814: 14 str.

Indikova I., Humphrey T. J., Hilbert F. 2015. Survival with a helping hand: *Campylobacter* and microbiota. Frontiers in Microbiology, 6: 1266, doi: 10.3389/FMICB.2015.01266: 6 str.

Freeman J.A., Bassler B. L. 1999. A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. Molecular Microbiology, 31, 2: 665–677

Jamwal K., Bhattacharya S., Puri S. 2018. Plant growth regulator mediated consequences of secondary metabolites in medicinal plants. Journal of Applied Research on Medicinal and Aromatic Plants, 9: 26–38

Jeon B., Itoh K., Misawa N., Ryu S. 2003. Effects of quorum sensing on *flaA* transcription

and autoagglutination in *Campylobacter jejuni*. Microbiology and Immunology, 47, 11: 833–839

Kalia V. C. 2013. Quorum sensing inhibitors: An overview. Biotechnology Advances, 31, 2: 224–245

Kassem I. I., Chandrashekhar K., Rajashekara G. 2013. Of energy and survival incognito: A relationship between viable but non-culturable cells formation and inorganic polyphosphate and formate metabolism in *Campylobacter jejuni*. Frontiers in Microbiology, 4: 183, doi:10.3389/FMICB.2013.00183: 8 str.

Khameneh B., Iranshahy M., Soheili V., Fazly Bazzaz B. S. 2019. Review on plant antimicrobials: a mechanistic viewpoint. Antimicrobial Resistance and Infection Control, 8: 118, doi: 10.1186/s13756-019-0559-6: 28 str.

Khan U. A., Rahman H., Niaz Z., Qasim M., Khan J., Tayyaba, Rehman B. 2013. Antibacterial activity of some medicinal plants against selectedhuman pathogenic bacteria. European Journal of Microbiology and Immunology, 3, 4: 272-274

Kikuchi T., Mizunoe Y., Takade A., Naito S., Yoshida S. I. 2005. Curli fibers are required for development of biofilm architecture in *Escherichia coli* K-12 and enhance bacterial adherence to human uroepithelial cells. Microbiology and Immunology, 49, 9: 875–884

Kim S. H., Chelliah R., Ramakrishnan S. R., Perumal A. S., Bang W. S., Rubab M., Daliri E. B. M., Barathikannan K., Elahi F., Park E., Jo H. Y., Hwang S. B., Oh D. H. 2021. Review on stress tolerance in *Campylobacter jejuni*. Frontiers in Cellular and Infection Microbiology, 10: 596570, doi:10.3389/FCIMB.2020.596570: 17 str.

Klančnik A., Šimunović K., Sterniša M., Ramić D., Smole Možina S., Bucar F. 2021. Anti-adhesion activity of phytochemicals to prevent *Campylobacter jejuni* biofilm formation on abiotic surfaces. Phytochemistry Reviews, 20, 1: 55–84

Kreling V., Falcone F. H., Kehrenberg C., Hensel A. 2020. *Campylobacter* sp.: Pathogenicity factors and prevention methods—new molecular targets for innovative antivirulence drugs? Applied Microbiology and Biotechnology, 104, 24: 10409–10436

Kreuder A. J., Ruddell B., Mou K., Hassall A., Zhang Q., Plummera P. J. 2020. Small noncoding RNA cjnc110 influences motility, autoagglutination, AI-2 localization, hydrogen peroxide sensitivity, and chicken colonization in *Campylobacter jejuni*. Infection and Immunity, 88, 7: e00245-20, doi:10.1128/IAI.00245-20: 26 str.

Laganenka L., Sourjik V. 2018. Autoinducer 2 dependent *Escherichia coli* biofilm formation is enhanced in a dual-species coculture. Applied and Environmental Microbiology, 84, 5: e02638-17, doi:10.1128/AEM.02638-17: 15 str.

Li Z., Nair S. K. 2012. Quorum sensing: How bacteria can coordinate activity and synchronize their response to external signals? Protein Science, 21, 10: 1403-1417

Lilley B. N., Bassler B. L. 2000. Regulation of quorum sensing in *Vibrio harveyi* by LuxO and Sigma-54. Molecular Microbiology, 36, 4: 940–954

Lin J., Overbye Michel L., Zhang Q. 2002. CmeABC Functions as a multidrug efflux System in *Campylobacter jejuni*. Antimicrobial Agents and Chemotherapy, 46, 7: 2124-2131

Luo A., Wang F., Sun D., Liu X., Xin B. 2022. Formation, development, and cross-species

interactions in biofilms. Frontiers in Microbiology, 12: 757327, doi:10.3389/FMICB.2021.757327: 12 str.

Machado I., Silva L. R., Giaouris E. D., G., Melo L. F, Simões M. 2020. Quorum sensing in food spoilage and natural-based strategies for its inhibition. Food Research International, 127: 108754, doi:10.1016/J.FOODRES.2019.108754: 12 str.

Mavri A., Ribič U., Smole Možina S. 2016. The biocide and antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli*. V: Emerging and traditional technologies for safe, healthy and quality food. Nedović V., Raspor P., Lević J., Tumbas Šaponjac V., Barbosa-Cánovas G. (ur.). Cham, Springer: 269–283

Mavri A., Smole Možina S. 2013. Development of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* adapted to biocides. International Journal of Food Microbiology, 160, 3: 304–312

McNab R., Ford S. K., El-Sabaeny A., Barbieri B., Cook G. S., Lamont R. J. 2003. LuxS-based signaling in *Streptococcus gordonii*: Autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. Journal of Bacteriology, 185, 1: 274–284

Michie K. L., Cornforth D. M., Whiteley M. 2016. Bacterial tweets and podcasts #signaling#eavesdropping#microbialfightclub. Molecular and Biochemical Parasitology, 208, 1: 41–48

Miller C. E., Williams P. H., Ketley J. M. 2009. Pumping iron: Mechanisms for iron uptake by *Campylobacter.* Microbiology, 155, 10: 3157–3165

Mishra R., Kumari Panda A., De Mandal S., Shakeel M., Singh Bisht S., Khan J. 2020. Natural anti-biofilm agents: Strategies to control biofilm-forming pathogens. Frontiers in Microbiology, 11: 566325, doi:10.3389/fmicb.2020.566325: 23 str.

Mou K. T., Plummer P. J. 2016. The impact of the LuxS mutation on phenotypic expression of factors critical for *Campylobacter jejuni* colonization. Veterinary Microbiology, 192: 43–51

Naito M., Frirdich E., Fields J. A., Pryjma M., Li J., Cameron A., Gilbert M., Thompson S. A., Gaynor E. C. 2010. Effects of sequential *Campylobacter jejuni* 81-176 lipooligosaccharide core truncations on biofilm formation, stress survival, and pathogenesis. Journal of Bacteriology, 192, 8: 2182–2192

Negi P. S. 2012. Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. International Journal of Food Microbiology, 156, 1: 7–17

On S. L. W. 2001. Taxonomy of *Campylobacter*, *Arcobacter*, *Helicobacter* and related bacteria: Current status, future prospects and immediate concerns. Journal of Applied Microbiology, 90, 30: 1-14

Papenfort P., Bassler B.L. 2016. Quorum sensing signal-response systems in Gram-negative bacteria. Nature Reviews Microbiology, 14, 9: 576–588

Parkhill J., Wren B. W., Mungall K., Ketley J. M., Churcher C., Basham D., Chillingworth T., Davies R. M., Feltwell T., Holroyd S., Jagles K., Karlyshev A. V., Moule S., Pallen

M. J., Penn C. W., Quail M. A., Rajandream M. A., Rutherford K. M., van Vliet A. H., Whitehead S., Varrell B. G. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature, 403, 6770: 665-668

Parte A. C., Sardà Carbasse J., Meier-Kolthoff J. P., Reimer L. C., Göker M. 2020. List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. International Journal of Systematic and Evolutionary Microbiology, 70: 5607–5612

Pereira C. S., Thompson J. A, Xavier K. B. 2013. AI-2-mediated signalling in bacteria. FEMS Microbiology Reviews, 37, 2: 156–181

Pfaffl M. W. 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research, 29, 9: e45, doi:10.1093/NAR/29.9.E45: 6 str.

Pisoschi A. M., Pop A., Georgescu C., Turcuş V., Olah N. K., Mathe E. 2018. An overview of natural antimicrobials role in food. European Journal of Medicinal Chemistry, 143: 922–935

Plummer P. J. 2012. LuxS and quorum-sensing in *Campylobacter*. Frontiers in Cellular and Infection Microbiology, 2: 22, doi:10.3389/FCIMB.2012.00022: 9 str.

Plummer P., Sahin O., Burrough E., Sippy R., Mou K., Rabenold J., Yaeger M., Zhang Q. 2012. Critical role of LuxS in the virulence of *Campylobacter jejuni* in a guinea pig model of abortion. Infection and Immunity, 80, 2: 585, doi:10.1128/IAI.05766-11: 9 str.

Powell L. F., Lawes J. R., Clifton-Hadley F. A., Rodgers J., Harris K., Evans S. J. 2012. The prevalence of *Campylobacter* spp. in broiler flocks and on broiler carcases, and the risks associated with highly contaminated carcases. Epidemiology and Infection, 140, 12: 2233–2246

Püning C., Su Y., Lu X., Gölz G. 2021. Molecular mechanisms of *Campylobacter* biofilm formation and quorum sensing. Current Topics in Microbiology and Immunology, 431: 293–319

Quiñones B., Miller W. G., Bates A. H., Mandrell R. E. 2009. Autoinducer-2 production in *Campylobacter jejuni* contributes to chicken colonization. Applied and Environmental Microbiology, 75, 1: 281–285

Ramić D., Dogša I., Smole Možina S. 2021. Obvladovanje biofilma bakterij *Campylobacter jejuni* z zaviranjem medcelične komunikacije. V: Mikrobiološke ideje na prepihu: zbornik prispevkov. Stopar D. (ur.). Ljubljana, Biotehniška fakulteta, Oddelek za živilstvo: 63-74

Ramić D., Jug B., Šimunović K., Tušek Žnidarič M., Kunej U., Toplak N., Kovač M., Fournier M., Jamnik P., Smole Možina S., Klančnik A. 2022. The role of *luxS* in *Campylobacter jejuni* beyond intercellular signaling. Microbiology Spectrum (v recenziji)

Rasmussen J. J., Vegge C. S., Frøkiær H., Howlett R. M., Krogfelt K. A., Kelly D. J., Ingmer H. 2013. *Campylobacter jejuni* carbon starvation protein A (CstA) is involved in peptide utilization, motility and agglutination, and has a role in stimulation of dendritic cells. Journal of Medical Microbiology, 62, 8: 1135–1143

Reeser R. J., Medler R. T., Billington S. J., Jost B. H., Joens L. A. 2007. Characterization of *Campylobacter jejuni* biofilms under defined growth conditions. Applied and Environmental Microbiology, 73, 6: 1908–1913

Relucenti M., Familiari G., Donfrancesco O., Taurino M., Li X., Chen R., Artini M., Papa R., Selan L. 2021. Microscopy methods for biofilm imaging: Focus on SEM and VP-SEM pros and cons. Biology, 10, 1: 51, doi:10.3390/biology10010051: 17 str.

Rickard A. H., Campagna S. R., Kolenbrander P. E. 2008. Autoinducer-2 is produced in saliva-fed flow conditions relevant to natural oral biofilms. Journal of Applied Microbiology, 105, 6: 2096–2103

Roy R., Tiwari M., Donelli G., Tiwari V. 2018. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. Virulence, 9, 1: 522–554

Rozman V., Bogovič Matijašić B., Smole Možina S. 2018. Antimicrobial resistance of common zoonotic bacteria in the food chain: An emerging threat. V: Antimicrobial resistance - a global threat. Kumar Y. (ur.). London, IntechOpen, doi:10.5772/INTECHOPEN.80782: 20 str.

Rukambile E., Sintchenko V., Muscatello G., Kock R., Alders R. 2019. Infection, colonization and shedding of *Campylobacter* and *Salmonella* in animals and their contribution to human disease: A review. Zoonoses and Public Health, 66, 6: 562–578

Rutherford S. T., Bassler B. L. 2012. Bacterial quorum sensing: Its role in virulence and possibilities for its control. Cold Spring Harbor Perspectives in Medicine, 2, 11: a012427, doi:10.1101/CSHPERSPECT.A012427: 25 str.

Saleem M., Nazir M., Ali M. S., Hussain H., Lee Y. S., Riaz N., Jabbar A. 2009. Antimicrobial natural products: an update on future antibiotic drug candidates. Natural Products Report, 27: 238-254

Satpathy S., Sen S. K., Pattanaik S., Raut S. 2016. Review on bacterial biofilm: An universal cause of contamination. Biocatalysis and Agricultural Biotechnology, 7: 56–66

Silva J., Leite D., Fernandes M., Mena C., Gibbs P. A., Teixeira P. 2011. *Campylobacter* spp. As a foodborne pathogen: A review. Frontiers in Microbiology, 2: 200, doi:10.3389/FMICB.2011.00200: 12 str.

Stefanic P., Kraigher B., Lyons N. A., Kolter R., Mandic-Mulec I. 2015. Kin discrimination between sympatric *Bacillus subtilis* isolates. PNAS, 112, 45: 14042–14047

Steindler L., Venturi V. 2007. Detection of quorum-sensing N-acyl homoserine lactone signal molecules by bacterial biosensors. FEMS Microbiology Letters, 266, 1: 1–9

Surette M. G., Miller M. B., Bassler B. L. 1999. Quorum sensing in *Escherichia coli, Salmonella typhimurium*, and *Vibrio harveyi*: A new family of genes responsible for autoinducer production. PNAS, 96, 4: 1639–1644

Šikić Pogačar M., Klančnik A., Bucar F., Langerholc T., Smole Možina S. 2016. Anti-adhesion activity of thyme (*Thymus vulgaris* L.) extract, thyme post-distillation waste, and olive (*Olea europea* L.) leaf extract against *Campylobacter jejuni* on polystyrene and intestine epithelial cells. Journal of the Science of Food and Agriculture, 96, 8:

2723–2730

Šimunović K., Ramić D., Xu C., Smole Možina S. 2020. Modulation of *Campylobacter jejuni* motility, adhesion to polystyrene surfaces, and invasion of int407 cells by quorum-sensing inhibition. Microorganisms, 8, 1: 104, doi:10.3390/microorganisms8010104: 14 str.

Šimunović K., Stefanic P., Klančnik A., Erega A., Mandic Mulec I., Smole Možina S. 2022. *Bacillus subtilis* PS-216 antagonistic activities against *Campylobacter jejuni* NCTC 11168 are modulated by temperature, oxygen, and growth medium. Microorganisms, 10, 2: 289, doi:10.3390/MICROORGANISMS10020289: 14 str.

Teh A. H. T., Lee S. M., Dykes G. A. 2019. Association of some *Campylobacter jejuni* with *Pseudomonas aeruginosa* biofilms increases attachment under conditions mimicking those in the environment. PLOS ONE, 14, 4: e0215275, doi:10.1371/JOURNAL.PONE.0215275: 15 str.

Teh K. H., Flint S., French N. 2010. Biofilm formation by *Campylobacter jejuni* in controlled mixed-microbial populations. International Journal of Food Microbiology, 143, 3: 118–124

Terlep S., Hympanova M., Dogsa I., Pajk F., Stopar D. 2022. Photoacoustic removal of *Enterococcus faecalis* biofilms from titanium surface with an Er:YAG laser using super short pulses. Lasers in Medical Science, 37, 1: 381–390

Teunis P. F. M., Falkenhorst G., Ang C. W., Strid M. A., De Valk H., Sadkowska-Todys M., Zota L., Kuusi M., Rota M. C., Simonsen J. B., Mølbak K., Van Duynhoven Y. T. H. P., Van Pelt W. 2013. *Campylobacter* seroconversion rates in selected countries in the European Union. Epidemiology and Infection, 141, 10: 2051–2057

Tram G., Day C. J., Korolik V. 2020. Bridging the gap: A role for *Campylobacter jejuni* biofilms. Microorganisms, 8, 3: 452, doi:10.3390/microorganisms8030452: 11 str.

Turan N. B., Chormey D. S., Büyükpınar Ç., Engin G. O., Bakirdere S. 2017. Quorum sensing: Little talks for an effective bacterial coordination. Trends in Analytical Chemistry, 91: 1–11

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology, 3, 7: research0034.1, doi:10.1186/GB-2002-3-7-RESEARCH0034: 12 str.

Vaou N., Stavropoulou E., Voidarou C., Tsigalou C., Bezirtzoglou E. 2021. Towards advances in medicinal plant antimicrobial activity: A review study on challenges and future perspectives. Microorganisms, 9, 10: 2041, doi:0.3390/microorganisms9102041: 28 str.

Wagle B. R., Upadhyay A., Upadhyaya I., Shrestha S., Arsi K., Liyanage R., Venkitanarayanan K., Donoghue D. J., Donoghue A. M. 2019. Trans-cinnamaldehyde, eugenol and carvacrol reduce *Campylobacter jejuni* biofilms and modulate expression of select genes and proteins. Frontiers in Microbiology, 10: 1837, doi:10.3389/fmicb.2019.01837: 16 str.

Waters C. M., Bassler B. L. 2006. The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. Genes and Development, 20: 2754-2767

Whiteley M., Diggle S. P., Greenberg E. P. 2017. Bacterial quorum sensing: the progress and promise of an emerging research area. Nature, 551: 313–320

Xavier J. B., Kim W., Foster, K. R. 2011. A molecular mechanism that stabilizes cooperative secretions in *Pseudomonas aeruginosa*. Molecular Microbiology, 79, 1: 166–179

Xie F., Xiao P., Chen D., Xu L., Zhang B. 2012. miRDeepFinder: A miRNA analysis tool for deep sequencing of plant small RNAs. Plant Molecular Biology, 80, 1: 75–84

Zhao J, Quan C., Jin L, Chen M. 2018. Production, detection and application perspectives of quorum sensing autoinducer-2 in bacteria. Journal of Biotechnology, 268: 53–60

Zhong X., Wu Q., Zhang J., Ma Z., Wang J., Nie X., Ding Y., Xue L., Chen M., Wu S., Wei X., Zhang Y. 2020. *Campylobacter jejuni* biofilm formation under aerobic conditions and inhibition by ZnO nanoparticles. Frontiers in Microbiology, 11: 207, doi:10.3389/FMICB.2020.00207: 6 str.

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PRILOGE

Priloga A: Dovoljenje založnika revije »Applied and Environmnetal Microbiology« za objavo članka z naslovom »Antibiofilm potential of Lavandula preparations against *Campylobacter jejuni*«

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Priloga B: Pokritost steklene površine (po globini) z biofilmom bakterij *C. jejuni* NCTC 11168 (Ramić in sod., 2021)