UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Saša PISKERNIK

PROTIMIKROBNA UČINKOVITOST RASTLINSKIH IZVLEČKOV *in vitro* IN V IZBRANIH ŽIVILIH

DOKTORSKA DISERTACIJA

ANTIMICROBIAL EFFICIENCY OF PLANT EXTRACTS *in vitro* AND IN SELECTED FOODS

DOCTORAL DISSERTATION

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AI Rastlinski izvlečki predstavljajo alternativo sintetičnim protimikrobnim snovem, ker v določenih razmerah lahko učinkovito inhibirajo patogene bakterije in kvarjenje hrane. Za določanje protimikrobne učinkovitosti izvlečkov obstajajo različne metode, prav tako lahko dobljene rezultate različno ovrednotimo. To pomeni, da rezultate posameznih študij težko primerjamo med seboj. V eksperimentih smo testirali različne metode za določanje protimikrobne učinkovitosti (metoda difuzije z diski, metoda razredčevanja v trdnem in tekočem gojišču, metoda razredčevanja v mikrotitrski ploščici). Med uporabljenimi metodami je bila metoda razredčevanja v mikrotitrski ploščici najbolj primerna. Zato smo s to metodo določili vrednosti MIK različnim izvlečkom, med katerimi so bili najbolj učinkoviti tisti, pri katerih je prevladovala karnozolna kislina. Izvlečki rožmarina so imeli boljši učinek na grampozitivne bakterije, kot na gramnegativne bakterije. Protimikrobni učinek izvlečkov rožmarina smo nadalje določili v živilskih modelih in v živilih. Na delovanje izvlečkov vplivajo različni dejavniki. Delež beljakovin in maščob v živilu zmanjša učinkovitost izvlečkov rožmarina. Protimikrobni učinek izvlečka rožmarina na bakterije vrste *Campylobacter jejuni* v piščančjem mesu v kombinaciji z zamrzovanjem je znižal število bakterij za 2 log enoti. Določili smo tudi protimikrobni učinek dveh izvlečkov rožmarina na vegetativne celice in spore bakterij vrste *Alicyclobacillus acidoterrestris* v jabolčnem soku. Oba izvlečka v vrednostih MIK inhibirata rast vegetativnih celic, hkrati pa ne vplivata na senzorične lastnosti jabolčnega soka. Vrednosti MIK niso vplivale na spore, vendar so pri tako nizkih koncentracijah spore lahko vzklile v vegetativne celice, na katere je potem inhibitorno deloval izvleček rožmarina. Vsi rezultati kažejo, da so izvlečki rožmarina lahko alternativni način konzerviranja različnih živil, vendar je pred njihovo uporabo v živilih potrebno preveriti čim več dejavnikov, ki lahko vplivajo na njihovo protimikrobno učinkovitost.

KEY WORDS DOCUMENTATION

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CX antimicrobials/plant extracts/rosemary extract/antimicrobial activity/minimal inhibitory concentration/growth inhibition/Gram-positive bacteria/Gram-negative bacteria/pathogens/spoilage bacteria/*in vitro*/chicken meat juice/chicken meat/foods/apple juice
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AA SMOLE MOŽINA, Sonja (supervisor)
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AB Plant extracts represent an alternative to synthetic antimicrobials, as under certain conditions, plant extracts can effectively inhibit the growth of pathogenic and spoilage bacteria. Several different methods for determination of such antibacterial activities are available, although there are different ways to interpret the results. This can thus make it difficult to compare data obtained across different studies. Therefore, we tested here the methods of agar disk diffusion and dilution, and broth microdilution and macrodilution. Among these, broth microdilution was the most suitable. We then used the broth microdilution method to determine the MICs of a range of selected plant extracts. Rosemary extracts were the most effective, and in particular those containing carnosic acid, with selectivity shown against Gram-positive bacteria, rather than Gram-negative bacteria. The antimicrobial activities of rosemary extracts were determined in different food models and foods, where different factors can affect the activities. Protein and fat in food had negative effects on the antimicrobial activities of these rosemary extracts. We also determined the antimicrobial activities of rosemary extracts on *Campylobacter jejuni* in chicken meat. A combination of the V40 rosemary extract and pre-freezing of the chicken meat reduced the *C. jejuni* cell numbers by more than 2 log units. The antimicrobial activities of rosemary extracts against vegetative cells and spores of *Alicyclobacillus acidoterrestris* in apple juice were also determined, as well as on the sensory properties of the apple juice. The *in-vitro* MICs of the rosemary extracts did not change the sensory properties of the apple juice, although they also had no effects the *A. acidoterrestris* spores. Thus, the spores can germinate and form vegetative cells even in the presence of these rosemary extracts. However, the rosemary extracts then inhibited *A. acidoterrestris* cell growth and reduced their vegetative cell numbers. These data indicate that rosemary extracts represent an alternative method for food conservation. Before their use in foods, however, the factors that affect the antimicrobial activities of the extracts should be specifically tested.

KAZALO VSEBINE

KAZALO ZNANSTVENIH DEL

KAZALO PRILOG

OKRAJŠAVE IN SIMBOLI

A15	izvleček rožmarina Aquarox15, Vitiva d.o.o.
A40	izvleček rožmarina Aquarox40, Vitiva d.o.o.
ATP	adenozin trifosfat
ATTC	Ameriška zbirka tipskih kultur (ang. American Type Culture Collection)
BAT	tekoče gojišče *Bacillus acidoterrestris* (ang. *Bacillus acidoterrestris* broth)
BPW	puferirana peptonska voda (ang. Buffered Peptone Water)
BSAC	Britansko združenje za protimikrobno kemoterapijo (ang. British Society for Antimicrobial Chemoterapy)
CLSI	Inštitut za klinične in laboratorijske standarde (ang. Clinical and Laboratory Standard Institute
cfu	colony forming units
DMSO	dimetil sulfoksid
EUCAST	Evropski komite za določanje protimikrobne učinkovitosti (ang. European Committee on Antimicrobial Susceptibility Testing)
EFSA	Evropska agencija za varnost hrane (ang. European Food Safety Authority)
GRAS	splošno priznano kot varno (ang. Generaly Recognized as Safe)
I18	izvleček rožmarina Inolens18, Vitiva d.o.o.
INT	2-p-jodofenil-3-p-nitrofenil-5-fenil tetrazolijev klorid
MBK	minimalna baktericidna koncentracija
MHB	Mueller Hinton bujon (ang. Mueller Hinton broth)
MIK	minimalna inhibitorna koncentracija (ang. minimal inhibitory concentration)
NCTC	Nacionalna zbirka tipskih kultur (ang. National Collection of Type Cultures)
TSP	trinatrijev fosfat (ang. trisodium phosphate)
TTC	2,3,5-trifenil tetrazolijev klorid
UVHVVR	Uprava Republike Slovenije za varno hrano, veterinarstvo in varstvo rastlin
V15	izvleček rožmarina Vivox15, Vitiva d.o.o.
V20	izvleček rožmarina Vivox20, Vitiva d.o.o.
V40	izvleček rožmarina Vivox40, Vitiva d.o.o.
V70	izvleček rožmarina Vivox70, Vitiva d.o.o.

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

1.1 PREGLED OBJAV

1.1.1 Predstavitev izbranih patogenih bakterij in kvarljivcev

Bolezni oz. okužbe, povzročene s hrano, predstavljajo velik javnozdravstveni problem. Okužbe s hrano oz. črevesne nalezljive bolezni lahko povzročajo bakterije, virusi ali paraziti (Newell in sod., 2010). Med okužbami, povzročenimi s hrano, predstavljajo zelo pomemben delež zoonoze, ker jih je težko zajeziti ravno zaradi vira okužb (Todd, 2014). Zoonoze so okužbe, ki se prenašajo iz živali na človeka preko direktnega kontakta ali preko kontaminiranih živil (Christou, 2011). Med glavnimi povzročitelji okužb s kontaminiranimi živili so že vrsto let bakterije rodov *Campylobacter* in *Salmonella* ter bakterije vrste *Escherichia coli*. Zaradi resnosti okužb so velik problem tudi bakterije vrste *Listeria monocytogenes* (Newell in sod., 2010). Med pomembne patogene bakterije, ki se prenašajo s hrano, štejemo tudi bakterije vrst *Bacillus cereus* in *Staphylococcus aureus* (Todd, 2014).

Glede na poročilo Evropske agencije za varno hrano (EFSA) in Uprave RS za varno hrano, veterinarstvo in varstvo rastlin (UVHVVR) za leto 2013 je v Evropski uniji in Sloveniji med zoonozami najbolj pogosta kampilobakterioza, ki jo povzročajo bakterije rodu *Campylobacter*, v večini primerov so to bakterije vrst *C. jejuni* in *C. coli*. Bakterije so bile v večini primerov izolirane iz mesa in kože brojlerjev (EFSA, 2015; UVHVVR, 2014). Bakterije rodu *Campylobacter* so spiralno zavite, gramnegativne bakterije. Za rast potrebujejo mikroaerofilno atmosfero s 5 % koncentracijo kisika in temperaturo 42 °C ter so občutljive na nizke vrednosti pH. Zaradi teh lastnosti se bakterije zunaj gostitelja oz. na hrani med predelavo ali skladiščenjem težko razmnožujejo. Kljub temu, da so bakterije termofilne, so občutljive na višje temperature, kot so npr. pasterizacija ali različni drugi postopki termične obdelave (Ganan in sod., 2012; Park, 2002).

V letu 2013 so bile druge najpogostejše okužbe z živili v Evropski uniji in Sloveniji povezane z bakterijami rodu *Salmonella*. Prijavljenih primerov je bilo glede na prejšnje leto manj, prav tako je v zadnjih 5 letih opazno upadanje primerov salmoneloze v članicah Evropske unije. V Evropski uniji in tudi v Sloveniji med prijavljenimi primeri prevladujejo serovari *S*. Enteritidis, *S*. Thypimurium in *S*. Infantis. Najbolj pogost vir izoliranih bakterij je bilo piščančje meso, glavni vzrok za izbruhe okužb s salmonelami pa so bila jajca in jajčni izdelki. V letu 2013 je bilo v Evropski uniji največ prijavljenih izbruhov, povzročenih s salmonelami (EFSA, 2015; UVHVVR, 2014). Bakterije rodu *Salmonella* so gramnegativne, fakultativno anaerobne gibljive palčke, ki ne tvorijo spor. Z okužbami s salmonelami so povezana živila živalskega izvora, npr. perutnina, govedina, jajca, mleko in izdelki iz teh živil (Ray in Bhunia, 2008).

V letu 2013 je bilo v Evropski uniji in Sloveniji povečano število primerov obolenj, ki jih povzročajo bakterije vrste *E. coli*, ki tvorijo verotoksin. Najbolj pogosto izoliran serotip je bil *E. coli* O157, in sicer iz mesa prežvekovalcev (EFSA, 2015; UVHVVR, 2014). Bakterije vrste *E. coli* so gramnegativne, fakultativno anaerobne, gibljive palčke in ne tvorijo spor. Predstavljajo del normalne mikroflore prebavnega trakta ljudi ter toplokrvnih

živali in ptičev. Patogenih sevov vrste *E. coli* je več, med njimi so najpomembnejše enterotoksigene *E. coli* (ETEC), enteropatogene *E. coli* (EPEC), enteroinvazivne *E. coli* (EIEC) in enterohemoragične *E. coli* (EHEC). Med enterohemoragičnimi je najbolj pogost serotip *E. coli* O157:H7 (Ray in Bhunia, 2008).

V porastu je tudi število prijavljenih primerov obolenj z bakterijami vrste *Listeria monocytogenes*. V primerjavi s prijavljenimi primeri kampilobakterioz in salmoneloz je število prijavljenih listerioz majhno, vendar je smrtnost pri teh obolenjih večja (EFSA, 2015; UVHVVR, 2014). Bakterije vrste *L. monocytogenes* so grampozitivne, fakultativno anaerobne bakterije, ki ne tvorijo spor in so v različnih naravnih okoljih pogosto prisotne (Allen in sod., 2016). Rastejo v širokem temperaturnem območju, med -1,5 °C in 45 °C (Lado in Yousef, 2007) in v različnih živilih, kot so npr. mleko in mlečni izdelki, meso in mesni izdelki, morska hrana in tudi listnata zelenjava (Gandhi in Chikindas, 2007). Po predvidevanjih je kar 99 % vseh primerov listerioze pri ljudeh posledica uživanja kontaminirane hrane. Večinoma so to predpripravljene jedi, kot so npr. razne salame, mlečni izdelki, prekajene ribe in morska hrana. Kljub temu, da so bakterije vrste *L. monocytogenes* načeloma občutljive na delovanje antibiotikov in ostalih konvencionalnih protimikrobnih snovi, se vseeno pojavljajo primeri odpornih sevov, predvsem izoliranih iz živil oz. živilsko-predelovalne industrije (Allen in sod., 2016).

Okužbe s hrano, ki jih povzročajo bakterije vrste *Staphylococcus aureus*, štejemo med intoksikacije, ki so posledica delovanja enterotoksinov. Njihova pogostnost v zadnjih letih upada, verjetno zaradi boljših higienskih ukrepov, s katerimi omejimo kontaminacijo in rast teh bakterij. Bakterije vrste *S. aureus* so grampozitivni koki. V živilih bakterije rastejo in tvorijo toksine, ne da bi spremenile senzorične lastnosti živil, kot so npr. meso in mesni izdelki, solate, solatni prelivi in siri (Ray in Bhunia, 2008).

Bakterije vrste *Bacillus cereus* so grampozitivne, fakultativno anaerobne gibljive palčke in tvorijo endospore. Vegetativne celice so občutljive na višje temperature, kot npr. pri postopku pasterizacije, medtem ko spore preživijo temperature, ki se običajno uporabljajo pri pripravi hrane. Bakterije vrste *B. cereus* povzročajo toksikoinfekcije, saj tvorijo toksine, med katerimi sta najbolj pomembna emetični in diarealni toksin. Veliko živil lahko vsebuje majhno število spor, ki pa ob zaužitju ne povzročajo težav. Pri vzklitju spor in namnožitvi vegetativnih celic pa le-te lahko povzročijo obolenje. Z okužbami so povezana različna živila, med njimi zelenjava, solate, meso, omake ter riž (Ray in Bhunia, 2008).

Poleg varnosti hrane, na katero lahko vplivajo patogene bakterije, je pomembna kakovost hrane. Na kakovost hrane vplivajo različni dejavniki, ki povzročajo kvar, tako kemijski, fizikalni in tudi mikrobiološki. Med njimi je pomemben mikrobiološki kvar, zaradi katerega kljub številnim poznanim načinom konzerviranja hrane propadejo velike količine hrane. Kvarjenje hrane se lahko pojavi že pri surovinah, med predelavo, skladiščenjem ali distribucijo (Gram in sod., 2002; Holley in Patel, 2005; Tajkarimi in sod., 2010). Konzerviranje živil je bilo za obstoj in preživetje človeka nujno že od nekdaj, prvi postopki so vključevali sušenje, soljenje, segrevanje in fermentacije (Gram in sod., 2002). Poleg teh metod se za podaljševanje obstojnosti in za zagotavljanje varnosti živil zelo veliko uporablja shranjevanje pri nizkih temperaturah, zamrzovanje, pasterizacija, omejevanje

dostopnosti hranil, dodatek sintetičnih protimikrobnih snovi ter v novejšem času pakiranje v modificirani in kontrolirani atmosferi. Vseeno pa različni ukrepi niso dovolj in mikrobiološki kvar in prisotnost patogenih mikroorganizmov še vedno ostajata problem (Holley in Patel, 2005; Negi, 2012; Tajkarimi in sod. 2010).

Med kvarljivci so pomembne bakterije rodu *Alicyclobacillus*, ki povzročajo kvar predvsem različnih sadnih pijač, med katerimi je največ primerov kvara v jabolčnem soku. Bakterije rodu *Alicyclobacillus* so aerobne, termofilne, acidofilne grampozitivne bakterije, ki tvorijo endospore. Pomembna značilnost je prisotnost ω-alicikličnih maščobnih kislin, ki predstavljajo večji del maščobnih kislin v celični membrani. Kljub temu obstaja nekaj vrst, ki nimajo take maščobnokislinske sestave (Smit in sod., 2011). Bakterije rodu *Alicyclobacillus* predstavljajo problem predvsem zaradi acidofilnih lastnosti in tvorbe spor, saj so zaradi tega bolj odporne na toplotno obdelavo, kot je npr. pasterizacija. Prav tako lahko preživijo v kislem okolju in povzročajo kvar pasteriziranih sadnih sokov. Kvar sadnih pijač oz. sokov se kaže predvsem kot spremenjena aroma, brez tvorbe plina, lahko pride do tvorbe usedline oz. pojava motnosti. Kljub tem spremembam pa običajno ne pride do večjih sprememb v prehranski vrednosti izdelka (Chang in Kang, 2004).

1.1.2 Naravne protimikrobne snovi

Protimikrobne snovi se uporabljajo za konzerviranje hrane oz. za kontrolo rasti kvarljivcev hrane, ki vplivajo na kakovost hrane, saj s svojimi metabolnimi produkti ali encimi poslabšajo vonj, okus, teksturo in barvo živila. Po drugi strani se uporabljajo za preprečevanje oz. kontrolo rasti patogenih bakterij, ki povzročajo zastrupitve s hrano, s čimer se zagotovi varnost živil (Negi, 2012; Tajkarimi in sod., 2010).

Glede na izvor protimikrobne snovi lahko delimo na tradicionalna oz. naravna in sintetična. Kot tradicionalna lahko imenujemo protimikrobne snovi, ki so v uporabi že dolgo časa in jih večina držav dovoljuje za konzerviranje hrane. Nekatere sintetične protimikrobne snovi lahko najdemo tudi v naravi, med njimi so npr. benzojska, citronska in jabolčna kislina. Prenekateri postopki za zagotavljanje mikrobiološke varnosti živil lahko poslabšajo senzorične lastnosti teh živil in s tem posledično zmanjšajo sprejemljivost pri potrošnikih. Želja današnjih potrošnikov so čim manj obdelana živila in živilski izdelki ter minimalna uporaba sicer dovoljenih sintetičnih aditivov. Pri vsem tem pa mora ostati zagotovljena varnost živila. Zaradi možnih negativnih stranskih učinkov sintetičnih aditivov na zdravje, se v zadnjem času pojavlja trend uporabe naravnih protimikrobnih snovi (Negi, 2012). Naravne protimikrobne snovi lahko glede na izvor delimo na rastlinske (npr. izvlečki, eterična olja), živalske (npr. hitozan, lizocim) in mikrobne (npr. bakteriocini) (Tiwari in sod., 2009). Med rastlinske protimikrobne snovi prištevamo začimbe in zelišča ter njihove izvlečke in eterična olja. Začimbe in zelišča se tradicionalno uporabljajo za izboljšanje okusa, vonja in barve živil. Poleg tega pa imajo lahko tudi protimikrobno in antioksidativno delovanje in se z njihovo uporabo lahko podaljša rok trajanja živil (Gyawali in Ibrahim, 2014; Negi, 2012; Tajkarimi in sod., 2010). Velik problem predstavlja tudi odpornost bakterij na protimikrobna zdravila, saj se v zadnjem času pojavlja vedno več primerov na različne antibiotike odpornih bakterij (EFSA, 2012; UVHVVR, 2014). Rastlinski izvlečki in njihove posamezne spojine lahko na odporne seve

bakterij vplivajo ne samo s protimikrobnim delovanjem, temveč tudi tako, da vplivajo na mehanizme odpornosti teh bakterij (Friedman, 2015).

Potrošniki so vedno bolj ozaveščeni in si želijo naravnih sestavin hrane, zato je pomembno iskanje novih alternativnih protimikrobnih snovi. Na tem mestu naravne protimikrobne snovi lahko pomagajo, saj rastline same proizvajajo številne protimikrobne metabolite, ki jih uporabljajo za obrambo pred škodljivimi vplivi mikroorganizmov iz okolja. Ljudje so rastline uporabljali za zdravljenje različnih bolezni in okužb že davno pred odkritjem in uporabo antibiotikov. Sestavine rastlinskega izvora so tudi danes prisotne v številnih zdravilih, saj so kemijsko zelo raznolike in zato zelo primeren in uporaben vir spojin. Spojine, prisotne v rastlinskih izvlečkih, se po strukturi razlikujejo od antibiotikov in imajo zato drugačen mehanizem delovanja (Gibbons, 2008).

V medicinske namene se uporablja med 14 in 28 % višjih rastlin, kar pomeni, da je veliko rastlinskih vrst še neraziskanih. Sekundarni metaboliti rastlin so tisti, ki so odgovorni za različne pozitivne učinke rastlinskih izvlečkov, naj bo to protimikrobno ali pa antioksidativno delovanje. Pri rastlinskih izvlečkih gre običajno za mešanico različnih spojin z različnim delovanjem in zato protimikrobno delovanje ni povezano samo z eno spojino, ampak običajno s kombinacijo teh sekundarnih metabolitov (Ncube in sod., 2008). Protimikrobni učinek je običajno boljši, če kombiniramo več protimikrobnih snovi, kot pa če uporabimo samo eno, saj določenih mikroorganizmov ne moremo inhibirati z običajno oz. dovolj nizko koncentracijo snovi (Negi, 2012). Protimikrobne spojine v rastlinskih izvlečkih ali kombinacijah različnih izvlečkov lahko delujejo sinergistično, antagonistično ali aditivno. Rastlinske izvlečke lahko kombiniramo tudi z antibiotiki ali kemoterapevtiki in na ta način dosežemo sinergistični učinek (Wagner in Ulrich-Merzenich, 2009). Primer so eksperimenti *in vitro* s kombinacijami nekaterih antibiotikov ter ksantohumula in lupulona, sestavinama izvlečka hmelja, ki so pokazale skupni učinek (Natarajan in sod., 2008) ter npr. kombinacija izvlečka origana in brusnic (Apostolidis in sod., 2008). Kombinacije rastlinskih izvlečkov z nizinom so lahko prav tako učinkovite in lahko bistveno izboljšajo delovanje izvlečkov, kot se je pokazalo v primeru izvlečka vednozelenega gornika. Izvleček sam ni imel protimikrobnega učinka na preiskovane bakterije, v kombinaciji z nizinom pa se je učinkovitost izvlečka na grampozitivne bakterije izboljšala (Dykes in sod., 2003). Zanimiv primer je tudi kombiniranje nizina z eteričnim oljem timijana, ki na grampozitivne bakterije vrste *L. monocytogenes* deluje sinergistično. Na gramnegativne bakterije vrste *E. coli* O157:H7 je taka kombinacija delovala aditivno (Solomakos in sod., 2008a, 2008b).

Veliko raziskav je bilo opravljenih na izvlečkih iz že poznanih in široko uporabljenih začimb in zelišč, kot so npr. izvlečki rožmarina, origana, žajblja, nageljnovih žbic, zelene, popra, lovorovih listov (Muñoz in sod., 2009; Witkowska in sod., 2013). Zanimivi pa so tudi izvlečki in eterična olja iz manj poznanih rastlin, kot so npr. *Garcinia quaesita*, *Alpinia galanga*, *Eucalyptus staigerana*, *Tasmania lanceolata* (Weerakkody in sod., 2010) in gorskega jetičnika (*Veronica montana*) (Stojković in sod., 2013) ali pa polifenolne spojine iz olivnega olja in kakava (Bubonja-Sonje in sod., 2011). Med poznanimi začimbami sta dobro raziskana tudi izvleček in eterično olje rožmarina. Rožmarin (*Rosmarinus officinalis* L.) je široko uporabljena začimba. Listi rožmarina se uporabljajo ali sveži, ali pa posušeni tako v kulinarične, kot tudi v medicinske namene, predvsem v

ljudski medicini (Ribeiro-Santos in sod., 2015). V Evropski uniji je izvleček rožmarina klasificiran kot aditiv s številko E392. Pod to številko spadajo samo deodorizirani izvlečki, ki vsebujejo karnozolno kislino in karnozol. Poleg Evropske unije, imajo podobno klasifikacijo še na Japonskem in Kitajskem, medtem ko v ostalih državah izvleček rožmarina ni zaveden kot aditiv za živila. Karnozolna kislina naj bi bila tudi najbolj pomembna učinkovina izvlečka rožmarina in odgovorna za njegovo protimikrobno in antioksidativno delovanje (Birtić in sod., 2015), različne študije so dokazale njegovo protimikrobno in antioksidativno delovanje *in vitro* (Đilas in sod., 2012; Lemos in sod., 2015; Weerakkody in sod., 2010).

Med proizvodnjo živil, predvsem sadja, nastaja velika količina stranskih oz. odpadnih produktov, kot so npr. tropine, semena, olupki, kaše in luščine. Ti produkti so lahko dober vir mineralov, organskih kislin in fenolnih spojin s protimikrobnim delovanjem. Koncentracija teh spojin je v odpadnih produktih lahko večja, kot pa v uporabnih delih sadja in zelenjave. Z uporabo odpadnih produktov in njihovih fenolnih spojin lahko na ekonomsko bolj ugoden način zagotovimo oz. izboljšamo varnost živil (Gyawali in Ibrahim, 2014). Dober primer tega so stranski produkti pri pridelavi vina, kot so kožice in pečke (Friedman, 2014; Katalinić in sod., 2010).

Večina izvlečkov, ki bi bili lahko uporabni v živilih, je v človeški prehrani uporabna že na tisoče let. Kljub temu pa za vse te izvlečke običajno ni toksikoloških informacij glede npr. priporočenega dnevnega vnosa. Take in tudi druge toksikološke informacije glede izvlečkov pa težko pridobimo, saj izvlečki niso standardizirani, sestava posameznih izvlečkov se razlikuje in je odvisna od sorte, geografske lege, uporabljenega dela rastline, starosti rastline, pogojev rasti, metode ekstrakcije ali sušenja, priprave, pakiranja in shranjevanja (Negi, 2012).

Spojine rastlinskega izvora imajo tudi svoje pomanjkljivosti, saj tržno niso dovolj zanimive oz. donosne in se večja farmacevtska podjetja raje ukvarjajo s proizvodnjo donosnejših zdravil (Gibbons, 2008). Ena od večjih pomanjkljivosti so visoke koncentracije, pri katerih se pokaže protimikrobna učinkovitost. Protimikrobno delovanje tudi ni osredotočeno samo na eno tarčo, saj spojine rastlinskega izvora delujejo na različna mesta v celici in na membrani. Običajno se rastlinske izvlečke smatra za neškodljive in netoksične produkte, ne glede na uporabljeno koncentracijo. Vseeno je pri takem predvidevanju potrebna previdnost, saj imajo tudi rastline in njihovi izvlečki lahko škodljive stranske učinke (Radulović in sod., 2013).

Med naravne protimikrobne snovi spadajo tudi različne spojine, ki jih tvorijo mikroorganizmi. Mlečnokislinske bakterije tvorijo peptide, imenovane bakteriocini, ki se lahko uporabljajo za zagotavljanje varnosti živil. Med bakteriocini je najbolj raziskan nizin, ki je tudi edini odobren za uporabo kot protimikroben dodatek v živilih. Ima širok spekter delovanja in je učinkovit proti grampozitivnim bakterijam in bakterijskim sporam, ki jih tvorijo bakterije rodov *Bacillus* in *Clostridium*. Spore so na delovanje nizina bolj občutljive kot vegetativne celice (Deegan in sod., 2006).

1.1.3 Mehanizmi protimikrobnega delovanja rastlinskih izvlečkov

Rastlinski izvlečki imajo širok spekter uporabe v živilski industriji, nekateri izvlečki imajo status GRAS (ang. Generally recognized as safe). Poleg protimikrobnega delovanja imajo rastlinski izvlečki tudi antioksidativno in antimutageno delovanje ter inhibirajo oksidacijo maščob v živilih. Za protimikrobno delovanje izvlečkov je odgovorno veliko število različnih komponent, vendar pa mehanizem delovanja polifenolnih komponent še ni točno poznan (Negi, 2012). Protimikrobne spojine rastlinskega izvora so v večini sekundarni metaboliti rastlin, pretežno so to fenoli in derivati fenolov – fenolne kisline, kinoni, saponini, flavonoidi, tanini, kumarini, terpenoidi in alkaloidi. Razlike v strukturi in kemijski sestavi teh spojin pomenijo tudi razliko v protimikrobnem delovanju. Hidroksilna skupina v fenolnih spojinah naj bi bila odgovorna za inhibitorno delovanje, saj lahko ta skupina vpliva na celično membrano bakterij in poruši strukturo membrane in tako sproži iztekanje celične vsebine. Hidroksilne skupine naj bi se tudi vezale na aktivne dele encimov in vplivale na metabolizem mikroorganizmov. Pomembno je tudi mesto hidroksilne skupine in mesto dvojnih vezi, ki prav tako vpliva na učinkovitost (Gyawali in Ibrahim, 2014). Protimikrobna učinkovitost fenolnih spojin (in ostalih protimikrobnih sekundarnih metabolitov) običajno narašča z njihovo naraščajočo lipofilnostjo (Radulović in sod., 2013). Prav tako so praviloma gramnegativne bakterije manj občutljive od grampozitivnih zaradi lipopolisaharidne zunanje membrane, ki omeji prehajanje hidrofobnih spojin (Davidson in sod., 2015; Negi, 2012; Tajkarimi in sod., 2010).

Protimikrobni učinek fenolnih spojin je odvisen tudi od koncentracije, saj pri nizkih koncentracijah fenolne spojine vplivajo na delovanje encimov, predvsem tistih, ki so vezani na produkcijo energije. Pri visokih koncentracijah pa fenolne spojine povzročijo denaturacijo beljakovin. Lahko motijo funkcije membrane, kot so npr. elektronski transport, privzemanje hranil, sinteza proteinov in nukleinskih kislin ter encimska aktivnost (Tiwari in sod., 2009).

Mehanizem delovanja bakteriocina nizina je bolj poznan in raziskan. Nizin ima širok spekter delovanja na grampozitivne bakterije. Na vegetativne celice deluje tako, da tvori pore v citoplazmatski membrani in tako poruši pH ravnovesje. Pride do iztekanja ionov in hidrolize ATP ter smrti celice (Deegan in sod., 2006). Na gramnegativne bakterije nizin nima učinka, saj njihova zunanja membrana prepreči dostop nizina in s tem njegovo delovanje na citoplazmatsko membrano (Thomas in Delves-Broughton, 2005). Bakterijske spore aliciklobacilov so na delovanje nizina bolj občutljive kot vegetativne celice, vendar je delovanje nizina odvisno od vrednosti pH medija in seveda od vrste medija oz. sadnega soka (Yamazaki in sod., 2000).

1.1.4 Metode določanja protimikrobne učinkovitosti

Rezultati različnih študij protimikrobnega delovanja rastlinskih izvlečkov so med sabo težko primerljivi, saj na dobljene rezultate vpliva več dejavnikov. Na učinkovitost izbranega izvlečka vplivajo okoljski in podnebni dejavniki med rastjo rastline ter različne metode ekstrakcije, različna laboratorijska gojišča za gojenje preiskovanih mikroorganizmov, velikost začetnega števila mikroorganizmov, uporaba različnih emulgatorjev in seveda izbira metode za določanje protimikrobne učinkovitosti ter

interpretacija rezultatov. Rezultate protimikrobne učinkovitosti lahko podamo kot minimalno inhibitorno koncentracijo (MIK) ali kot minimalno baktericidno koncentracijo (MBK), kjer pa obstaja več različnih definicij (Burt, 2004; Tajkarimi in sod., 2010). Za ustrezno in s tem pravilno določanje protimikrobne učinkovitosti so potrebne ustrezne metode. Za zmanjšanje vpliva različnih metod testiranja je zato potrebna standardizacija postopkov testiranja (Das in sod., 2010).

Za določanje občutljivosti bakterij *in vitro* na tradicionalne antibiotike obstaja več protokolov oz. priporočil. S tem se ukvarjajo različne inštitucije, npr. CLSI - Inštitut za klinične in laboratorijske standarde, BSAC - Britansko združenje za protimikrobno kemoterapijo in EUCAST - Evropski komite za določanje protimikrobne učinkovitosti. Vse priporočene metode se lahko za uporabo oz. določanje protimikrobne učinkovitosti rastlinskih izvlečkov ustrezno prilagodijo (Tan in Lim, 2015; Wiegand in sod., 2008).

Metode za določanje protimikrobne učinkovitosti lahko razdelimo na metode difuzije in metode razredčevanja. Pri metodah difuzije so najbolj pogosto uporabljene metoda difuzije v trdnem gojišču z diski in z luknjicami (Ncube in sod., 2008). Metoda difuzije z diski je ena od prvih in tudi najbolj uporabljenih metod za določanje protimikrobne učinkovitosti. Razlog za tako množično uporabo je enostavna in ekonomsko ugodna izvedba. Primerna je predvsem za polarne spojine, medtem ko nepolarne spojine težko prehajajo in tako lahko dobimo lažno negativne rezultate. Prav tako ne moremo povezati koncentracije izbranega protimikrobnega izvlečka z premerom con okrog diskov (Tan in Lim, 2015). Metoda difuzije z diski ali luknjicami je kvalitativen test, primerna za hiter in enostaven pregled učinkovitosti rastlinskih izvlečkov (Burt in Reinders, 2003; Hammer in sod., 1999; Tan in Lim, 2015) in je še danes v uporabi za določanje protimikrobne učinkovitosti naravnih protimikrobnih snovi (Abdollahzadeh in sod., 2014; Djenane in sod., 2011; Tan in Lim, 2015).

Metode razredčevanja delimo glede na lastnosti uporabljenega gojišča. Pri metodi razredčevanja v trdnem gojišču preiskovan izvleček oz. spojino zmešamo z ustreznim trdnim gojiščem ter dodamo izbrano kulturo. S to metodo lahko določimo minimalno inhibitorno koncentracijo kot najnižjo koncentracijo, pri kateri ni bilo vidne rasti na gojišču (Cos in sod., 2006). Pri metodi razredčevanja v tekočem gojišču prav tako preiskovan izvleček zmešamo z ustreznim tekočim gojiščem in dodamo izbrano kulturo. Rezultat lahko ovrednotimo z merjenjem absorbance ali metodo štetja kolonij na trdnem gojišču (Burt, 2004).

Primerna metoda za kvantitativno določanje učinkovitosti izvlečkov je metoda razredčevanja v tekočem gojišču, izvedena v miktoritrski ploščici – metoda razredčevanja v mikrotitrski ploščici. Metoda v zadnjem času pridobiva na široki uporabnosti, saj s to metodo lahko določimo tako vrednosti MIK, kot tudi vrednosti MBK. Kljub temu ima tudi ta metoda določene pomanjkljivosti, predvsem lahko težavo povzročajo nepolarne spojine in izvlečki. Ta problem se lahko reši z uporabo različnih topil, kot sta metanol ali DMSO (dimetil sulfoksid). Kljub temu lahko določeni izvlečki tvorijo oborino (Tan in Lim, 2015). Vrednosti MIK pri metodi razredčevanja v mikrotitrski ploščici ovrednotimo na več načinov, npr. z vizualnim pregledom in določanjem vrednosti MIK na podlagi motnosti (Djenane in sod., 2011; Weerakkody in sod., 2010), lahko tudi s pregledom vzorcev pod

mikroskopom (Stojković in sod., 2013) ter z merjenjem absorbance pri različnih valovnih dolžinah (Othman in sod., 2011; Rivas in sod., 2010; Techathuvanan in sod., 2014). Zaradi obarvanosti izvlečkov in prisotnosti oborin je tako določanje vrednosti MIK lahko netočno. V ta namen se za odčitavanje rezultatov uporablja indikatorje (Tan in Lim, 2015; Valgas in sod., 2007). Za določanje metabolne aktivnosti se kot indikator lahko uporablja tetrazolijeve soli (Eloff, 1998; Palaniappan in Holley, 2010; Valgas in sod., 2007) ali resazurin (Mann in Markham, 1998; Sarker in sod., 2007). Pri določanju protimikrobnega učinka izvlečkov je pomembno spremljanje kinetike preživetja oz. odmiranja bakterij, v odvisnosti od časa, kjer določimo tudi hitrost in trajanje protimikrobnega učinka. V ta namen uporabimo metodo razredčevanja v tekočem gojišču, kjer učinek izvlečkov ob določenih časovnih intervalih določimo z merjenjem absorbance ali metodo štetja kolonij na trdnem gojišču (Burt, 2004).

1.1.5 Uporaba naravnih protimikrobnih snovi v živilih

Primarno se začimbe in zelišča dodaja hrani za izboljšanje okusa, arome in vonja. Zaradi svojega protimikrobnega delovanja pa lahko podaljšajo obstojnost živil. Pri izbiri protimikrobnih snovi je potrebno upoštevati skladnost dodatka z živilom, njegovo varnost ter načine konzerviranja in obstojnost dodanih snovi pri teh razmerah (Holley in Patel, 2005). Na delovanje rastlinskih protimikrobnih snovi vplivajo njihove kemijske lastnosti, kot so npr. hidrofobnost, topnost, hlapnost. Na učinkovitost v živilih bistveno vplivata vrednost pH in polarnost protimikrobne snovi. Poleg tega so pomembne še intrinzične lastnosti živil, kot so vrednost pH, vsebnost maščob, beljakovin, vode, antioksidantov, konzervansov ter fizikalna struktura živila, in ekstrinzične lastnosti, kot je npr. temperatura skladiščenja. Običajno se za doseganje protimikrobnega učinka v živilih porabi več eteričnega olja oz. rastlinskega izvlečka, kot v laboratorijskih gojiščih (v primerjavi s poskusi *in vitro*) (Negi, 2012; Tajkarimi in sod., 2010). Razlog za to je dejstvo, da so hidrofobna eterična olja topna v maščobnem delu živila. Bakterije se nahajajo v hidrofilnem delu živila in je zaradi take porazdelitve možnost kontakta med eteričnim oljem oz. protimikrobno snovjo in bakterijskimi celicami zmanjšana (Corbo in sod., 2009). Rastlinski izvlečki in eterična olja so bolj učinkovita v sadju in zelenjavi, saj taka živila vsebujejo manj maščob in imajo nižjo vrednost pH (Tajkarimi in sod., 2010).

Na stabilnost rastlinskih protimikrobnih snovi vpliva toplotna obdelava kot je sterilizacija, pasterizacija in sušenje. Kljub temu pa niso vse spojine enako občutljive na toplotno obdelavo, saj je pri nekaterih opazno boljše delovanje po toplotni obdelavi oz. nastajajo pri tem nove spojine, ki ohranijo ali pa celo izboljšajo delovanje izvlečkov. Toplotna obstojnost rastlinskih protimikrobnih snovi je odvisna tudi od vrste živila, ki smo ga želeli obdelati z naravnimi izvlečki. Če so v živilskem matriksu že prisotne druge polifenolne spojine in antioksidanti, lahko le-te pomagajo in ohranijo stabilnost in delovanje dodanih izvlečkov (Negi, 2012).

Testiranja v živilih zahtevajo drugačno obravnavo, saj so odvisna od nekaterih dejavnikov, ki jih v testiranjih *in vitro* ne zajamemo ali obravnavamo. Težave oz. dejavniki, zaradi katerih je uporaba naravnih protimikrobnih snovi omejena, so značilen in izrazit vonj in okus, kar je še posebej izrazito pri visokih koncentracijah. Problem lahko predstavlja tudi

topnost teh snovi v živilskih matriksih in njihova cena. Zato je del do sedaj raziskanih izvlečkov, ki so zares uporabni v živilih, majhen (Gyawali in Ibrahim, 2014; Tajkarimi in sod., 2010).

Rast in razmnoževanje mikroorganizmov v hrani je odvisno od različnih dejavnikov. Intrinzični dejavniki, ki vplivajo na mikroorganizme, se navezujejo na karakteristike živila. Med te dejavnike prištevamo dostopnost hranil, naravno prisotne ali dodane protimikrobne snovi, dostopnost vode oz. vrednost a_w in pH, struktura živila. Ekstrinzični dejavniki so dejavniki iz okolja, ki vplivajo na rast mikroorganizmov. Med temi so pomembni način pakiranja in atmosfera, temperatura in razmere skladiščenja (IFT/FDA, 2003).

Pri izbiri protimikrobnih snovi je potrebno upoštevati te dejavnike in tudi samo sestavo živila. Pomembna je vsebnost beljakovin, maščob in ogljikovih hidratov v živilu, ki mu dodajamo rastlinske izvlečke oz. eterična olja (Davidson in sod., 2015). Učinkovitost naravnih protimikrobnih snovi lahko preverimo najprej v modelnih živilih in šele nato naprej v živilih. Tako lažje ocenimo učinkovitost protimikrobnih snovi in predvidimo, kakšna bo učinkovitost v živilu (Gutierrez in sod., 2009).

Izvlečki rožmarina so pokazali dober protimikroben učinek na bakterije vrste *L. monocytogenes* v modelu brokolija (Muñoz in sod., 2009), bakterije vrst *E. coli, L. monocytogenes*, in *S.* Enteritidis v listnati zelenjavi (de Medeiros Barbosa in sod., 2016) ter na skupno število aerobnih bakterij v piščančjih hrenovkah (Rižnar in sod., 2006) in ribjem fileju (Gao in sod., 2014). Kombinacija izvlečka rožmarina in nizina je bistveno podaljšala rok trajanja ribjih filejev, hranjenih pri 4 °C, saj je zavrla rast bakterijske mikrobiote filejev (Gao in sod., 2014).

1.1.6 Vpliv naravnih protimikrobnih snovi na senzorične lastnosti živila

Ena glavnih pomanjkljivosti uporabe naravnih protimikrobnih snovi je njihova visoka koncentracija, ki jo je potrebno dodati v živila, da bi dosegli želeni protimikrobni učinek. Rastlinski izvlečki in eterična olja lahko spremenijo okus in vonj živil. Ta sprememba je običajno negativna in nezaželena, v nekaterih primerih pa lahko tudi pozitivna in s tem zaželena (Davidson in sod., 2015). Primer je raziskava vpliva eteričnega olja timijana v mletem ribjem mesu. Eterično olje timijana je bilo senzorično nesprejemljivo v koncentracijah, ki so imele protimikrobni učinek na bakterije vrste *L. monocytogenes* v mletem ribjem mesu. Nasprotno pa senzorično sprejemljiva koncentracija na bakterije v mletem ribjem mesu ni imela dobrega protimikrobnega učinka (Abdollahzadeh in sod., 2014). Dodatek naravne protimikrobne snovi lahko celo izboljša senzorične lastnosti živila. Primer je dodatek izvlečka rožmarina in nizina, ki sta v kombinaciji izboljšala senzorične lastnosti ribjih filejev (Gao in sod., 2014). Podoben primer je eterično olje origana, dodano v mleto ovčje meso. Vzorci z dodatkom 0,6 % in 0,9 % eteričnega olja origana so bili senzorično sprejemljivi. Koncentracija 0,6 % eteričnega olja origana je celo izboljšala senzorične lastnosti ovčjega mesa v primerjavi s kontrolo oz. z dodatkom višje koncentracije eteričnega olja origana (Govaris in sod., 2010).

1.2 NAMEN RAZISKAV IN HIPOTEZE

1.2.1 Namen raziskav

Namen raziskav je bil:

- Ovrednotiti protimikrobne učinkovitosti različnih rastlinskih izvlečkov na izbrane grampozitivne bakterije (*S. aureus*, *B. cereus*, *L. monocytogenes*, *A. acidoterrestris*, *A. hesperidum* in *A. cycloheptanicus*) in gramnegativne bakterije (*Salmonella* Infantis, *E. coli* O157:H7, *C. jejuni*, *C. coli*).
- Določiti najbolj primerno metodo ter uporabo ustreznih indikatorjev metabolne aktivnosti za določanje vrednosti MIK izbranih rastlinskih izvlečkov ter med izbranimi izvlečki določiti najbolj učinkovite.
- Določiti protimikrobno učinkovitost izvlečkov rožmarina na grampozitivne bakterije vrste *L. monocytogenes* in gramnegativne bakterije vrste *E. coli* v izbranih modelih živil, ter določili kako na protimikroben učinek vplivajo različni dejavniki, kot so delež živila, vrsta bakterij in začetno število bakterij, temperatura inkubacije ter vrsta izvlečka.
- Preučiti kako na preživelost izbranih bakterij vrste *C. jejuni* vpliva izvleček rožmarina in dodatek bakteriocina nizina v gojišču, piščančjem mesnem soku (živilski model) in piščančjem mesu v kombinaciji z nižjo temperaturo inkubacije (8 °C) in/ali kratkotrajnim predhodnim zamrzovanjem.
- Preučiti učinek izvlečkov rožmarina na senzorične lastnosti jabolčnega soka in določiti učinek izvlečkov rožmarina na rast vegetativnih celic in spor bakterij vrste *A. acidoterrestris* v gojišču, modelnem jabolčnem soku in jabolčnem soku.

1.2.2 Hipoteze

Postavili smo naslednje hipoteze:

Hipoteza 1
Kvantitativno inhibicijo mikrobne rasti izraža vrednost MIK (minimalna inhibitorna koncentracija), vendar je odvisna od uporabljene metode in ne odraža kinetike protimikrobnega delovanja.

Hipoteza 2
Boljši bo učinek rastlinskih izvlečkov na grampozitivne, kot na gramnegativne bakterije, vendar je to odvisno od izvlečka (rastlinske vrste in načina ekstrakcije).

Hipoteza 3
Protimikrobno delovanje je odvisno od medija, zato bo drugačno v razmerah *in vitro* (laboratorijskem mediju), kot v izbranih živilih (npr. mesnem soku, piščančjem mesu in jabolčnem soku).

2 ZNANSTVENA DELA

2.1 VREDNOTENJE METOD DIFUZIJE IN RAZREDČEVANJA ZA DOLOČANJE PROTIMIKROBNE UČINKOVITOSTI RASTLINSKIH IZVLEČKOV

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V raziskavi smo določili protimikrobno učinkovitost izbranih rastlinskih izvlečkov, mešanic izvlečkov in posameznih kislin. Za določanje vrednosti MIK rastlinskih izvlečkov smo pri eksperimentih uporabili metodo difuzije z diski, metodo razredčevanja v trdnem gojišču, metodo razredčevanja v tekočem gojišču in metodo razredčevanja v mikrotitrski ploščici. Med grampozitivnimi bakterijami smo izbrali bakterije vrst *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus* in med gramnegativnimi bakterije vrste *Escherichia coli* O157:H7, *Salmonella* Infantis, *Campylobacter jejuni*, *Campylobacter coli*. Metoda difuzije v trdnem gojišču z diski se je izkazala za uporabno samo kot presejalna metoda, ki je primerna za uporabo pred kvantitativnim določanjem vrednosti MIK z metodami razredčevanja. Za določanje protimikrobne učinkovitosti je bila najbolj učinkovita metoda razredčevanja v mikrotitrski ploščici. Tetrazolijevi soli TTC (2,3,5-trifenil tetrazolijev klorid) in INT (2-p-jodofenil-3-p-nitrofenil-5-fenil tetrazolijev klorid) smo uporabili kot indikatorja živosti aerobnih bakterij, medtem ko smo za določanje živosti mikroaerofilnih bakterij rodu *Campylobacter* uporabili reagent BacTiter-Glo. S krivuljami preživetja smo ob dodatku rastlinskih izvlečkov spremljali kinetiko inaktivacije bakterij. Tako smo vrednosti MIK, dobljene z metodo razredčevanja v mikrotitrski ploščici, potrdili kot tiste koncentracije rastlinskih izvlečkov, ki so inhibirale rast preiskovanih bakterij. Grampozitivne bakterije so bile najbolj občutljive na delovanje rastlinskih izvlečkov, med gramnegativnimi bakterijami so bile bakterije rodu *Campylobacter* po občutljivosti podobne grampozitivnim bakterijam. Ostale testirane gramnegativne bakterije pa so bile na delovanje rastlinskih izvlečkov bolj odporne.

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Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts

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ABSTRACT

The aim of this study was to evaluate diffusion and dilution methods for determining the antibacterial activity of plant extracts and their mixtures. Several methods for measurement of the minimal inhibitory concentration (MIC) of a plant extract are available, but there is no standard procedure as there is for antibiotics. We tested different plant extracts, their mixtures and phenolic acids on selected gram-positive (*Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes*) and gram-negative bacteria (*Escherichia coli* O157:H7, *Salmonella* Infantis, *Campylobacter jejuni*, *Campylobacter coli*) with the disk diffusion, agar dilution, broth microdilution and macrodilution methods. The disk diffusion method was appropriate only as a preliminary screening test prior to quantitative MIC determination with dilution methods. A comparison of the results for MIC obtained by agar dilution and broth microdilution was possible only for gram-positive bacteria, and indicated the latter as the most accurate way of assessing the antimicrobial effect. The microdilution method with TTC (2,3,5-triphenyl tetrazolium chloride) or INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) to indicate the viability of aerobic bacteria was found to be the best alternative approach, while only ATP determination was appropriate for microaerophilic *Campylobacter* spp. Using survival curves the kinetics of bacterial inactivation on plant extract exposure was followed for 24 h and in this way the MIC values determined by the microdilution method were confirmed as the concentrations of extracts that inhibited bacterial growth. We suggest evaluation of the antibacterial activity of plant extracts using the broth microdilution method as a fast screening method for MIC determination and the macrodilution method at selected MIC values to confirm bacterial inactivation. *Campylobacter* spp. showed a similar sensitivity to plant extracts as the tested gram-positive bacteria, but *S.* Infantis and *E. coli* O157:H7 were more resistant.

1. Introduction

Just as evaluation of biological activity is essential for the assessment of susceptibility to antibiotics, it is also necessary for screening new antimicrobials (ESCMID, 2000). Natural products, either pure compounds or standardized plant extracts, provide unlimited opportunities for novel and suitable additives and drug treatments because of their unmatched range of chemical diversity (Cos et al., 2006; O'Bryan et al., 2008). Several methods are currently available to detect their antimicrobial activity and since not all of them are based on the same principles, the results obtained are influenced not only by the method selected, but also by the microorganisms used, and by the extraction method or the degree of solubility of each test-compound (Valgas et al., 2007; Tripoli et al., 2007).

Antimicrobial test systems should ideally be simple, rapid, reproducible, inexpensive and maximize sample throughput in order to cope with a varied number of extracts and fractions (Hostettman et al., 1997). The CLSI (NCCLS, 2003a,b) has standardized the agar dilution method for quantitative determination of antibiotics. Broth dilution methods for inhibitory determination are also recommended by CLSI (NCCLS, 2003c), using different principles to assess microbial growth or its inhibition. Colorimetric methods could represent an alternative approach, using tetrazolium salts as indicators, since bacteria convert them to coloured formazan derivatives that can be quantified (Johnson et al., 1985; Ellof, 1998; Grare et al., 2008). While they are all good indicators of bacterial growth, difficulties arising because of autofluorescence, salt reduction and the antioxidant properties of plant products, especially for XTT (3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis (4-methoxy-6-nitro)benzene-sulfonic acid hydrate), TTC (2,3,5-triphenyl tetrazolium chloride) and resazurin, make them less suitable indicators for MIC assay (Carson et al., 1995; Mann and Markham, 1998; Rahman et al., 2004).

Several antimicrobial compounds are extracted from easily available sources, such as agricultural and horticultural crops (e.g.

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grapevine, citrus, hops, berries, tea leaves etc.), or medicinal plants such as pine, sage, rosemary, and many others (Natarajan et al., 2008; Sudjana et al., 2009). The aims of this study were: (i) to evaluate the antibacterial activity of plant extracts as the minimal inhibitory concentration (MIC) by diffusion and dilution methods for gram-positive (*Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*) and gram-negative bacteria (*Salmonella* Infantis, *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Campylobacter coli*); (ii) to discuss and suggest some recommendations for the use of the most appropriate methods and indicators for MIC determination by the microdilution method and (iii) to use several extracts from the *Lamiaceae* family (rosemary, sage) or of other origin (grape, olive, citrus, hop, and tea) with different active compounds to examine the suitability of the proposed method of testing natural antimicrobials.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Seven bacterial strains, namely *B. cereus* WSBC 10530 (clinical isolate), *S. aureus* ATCC 25923 (clinical isolate), *L. monocytogenes* ŽM58 (IHM, Würzburg, Germany), *S.* Infantis ŽM9 (poultry meat isolate), *E. coli* O157:H7 ŽMJ 129 (clinical isolate), *C. jejuni* ATCC 33560 (bovine faeces isolate) and *C. coli* ATCC 33559 (pig faeces isolate), were used for antibacterial testing. The cultivation/assay medium for *L. monocytogenes* and *E. coli* O157:H7 was Tryptone Soy Broth or Agar (TSB, TSA, Oxoid, Hampshire, UK), for *B. cereus*, *S. aureus* and *S.* Infantis it was Müeller Hinton Broth or Agar (MHB, MHA, Oxoid, Hampshire, UK), while for *C. jejuni* and *C. coli* defibrinated horse blood 5% (Oxoid, Hampshire, UK) was added to MHB or MHA. Bacterial cultures for antimicrobial testing were prepared by picking colony from 24-h-old TSA/MHA plates and it was suspended in an appropriate medium (5 mL). Cultures were grown aerobically for 20 h and continuously shaken at 100 rpm at 37 °C, while *Campylobacter* spp. were grown microaerobically at 42 °C. For antibacterial activity assays 1 mL of each culture was diluted with TSB or MHB medium to 10^3–10^6 CFU/mL.

2.2. Plant extracts and pure phenolic acids

The present study included commercial rosemary extract formulations with carnosic acid (V15, V20, V40, V70) or rosmarinic acid (A15, A40) as the main active phenolic compound, grape seed, olive leaves, green tea and sage extracts and extract mixtures of 1:1 V40/A40, V40/grape seed extract, V40/citrus extract, V40/olive leaves extract, and V40/hop extract (Vitiva d.d., Markovci, Slovenia). Lyophilized plant extracts were dissolved in absolute ethanol to prepare stock solutions and were further diluted in appropriate media to working solution. The activity of carnosic and rosmarinic acids (Chromadex, Santa Ana, CA) was also tested.

2.3. Minimal inhibitory concentration (MIC) determination

2.3.1. Disk diffusion method

For the disk diffusion assay (NARMS, 2002) 1 mL of each bacterial suspension was uniformly spread on a solid growth medium in a Petri dish. Four sterile paper disks (6 mm in diameter; Becton, Dickinson & Co.) were placed on the surface of each agar plate and were impregnated with 10 µL of the diluted plant extract. Plates were incubated for 24 h under appropriate cultivation conditions. Antibacterial activity as MIC was determined as the lowest concentration of plant extract or pure phenolic acid which produced an inhibition zone around a disk following the 24-h incubation (Valgas et al., 2007). Disks impregnated with sterile distilled water and ethanol served as negative controls and a disk with an antibiotic (oxytetracycline or nalidixic acid, Sigma-Aldrich GmbH, Steinheim, Germany) served as a positive control. Replicas at each concentration were performed.

2.3.2. Agar dilution method

For the agar dilution method two-fold serial dilutions of plant extracts or pure phenolic acids were made in molten MHA or TSA medium cooled down to 45 °C to obtain the desired final concentrations. Bacterial suspensions (0.1 mL with 10^5–10^6 CFU/mL) were then inoculated on solid TSA (*L. monocytogenes* and *E. coli* O157:H7) or MHA (*B. cereus*, *S. aureus* and *S.* Infantis) or MHA medium with 5% defibrinated horse blood (*C. jejuni* and *C. coli*). Agar plates were incubated aerobically at 37 °C for 48 h for all tested bacterial culture except for *Campylobacter* spp. that were grown microaerobically at 42 °C for 48 h. The MIC was defined as the lowest concentration of plant extract or pure phenolic compound in solid media where no growth was observed after 24 or 48 h (Weckesser et al., 2007). Negative controls included ethanol in amounts corresponding to the highest quantity present in the agar dilution assay. Inoculated agar plates without added plant extract served as positive controls.

2.3.3. Broth microdilution method

For the broth microdilution test 50 µL of each bacterial suspension in suitable growth medium was added to the wells of a sterile 96-well microtitre plate already containing 50 µL of two-fold serially diluted plant extract or pure phenolic acid in proper growth medium. The final volume in each well was 100 µL. Control wells were prepared with culture medium, bacterial suspension only, plant extracts only and ethanol in amounts corresponding to the highest quantity present. The contents of each well were mixed on a microplate shaker (Eppendorf, Hamburg, Germany) at 900 rpm for 1 min prior to incubation for 24 h in the cultivation conditions described above. The MIC was the lowest concentration where no viability was observed after 24 h on the basis of metabolic activity (Mourey and Canillac, 2002). To indicate respiratory activity the presence of colour was determined after adding 10 µL/well of INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride, Sigma) or TTC (2,3,5-triphenyl tetrazolium chloride, Sigma) dissolved in water (INT 2 mg/mL, TTC 20 mg/mL) and incubated under appropriate cultivation conditions for 30 min in the dark (Elliof, 1998). To determine the ATP activity the bioluminescence signal was measured by a Microplate Reader (Tecan, Mannedorf/Zurich, Switzerland) after adding 100 µL/well of BacTiter-Glo™ reagent (Promega, Madison, USA) and after 5 min incubation in the dark (Klančnik et al., 2009). Positive controls were wells with a bacterial suspension in an appropriate growth medium and a bacterial suspension in an appropriate growth medium with ethanol in amounts corresponding to the highest quantity present in the broth microdilution assay. Negative controls were wells with growth medium and plant extract or pure phenolic acid. All measurements of MIC values were repeated in triplicate.

2.3.4. Kinetics of inactivation using the broth macrodilution method

The plant extracts and pure phenolic acids were added to 5 mL of growth medium to give final concentrations in accordance with the results obtained by the agar diffusion, agar dilution and broth microdilution methods. The diluted bacterial cultures (1 mL with 10^5–10^6 CFU/mL beforehand diluted in growth medium) were inoculated in growth media already containing desired concentration of plant extract, shaken and incubated as described above. Bacterial growth was followed by taking samples at 0, 3, 6, 9 and 24 h and plating on cultivation media after serial sample dilutions. The bacterial number was calculated after incubation of the plates for 24 h and colony counting. Positive controls were performed in the same way, except without adding the plant extract. The MIC was defined as the lowest concentration of plant extract resulting in a significant decrease (>90%) in inoculum viability after 24 h of incubation (Burt, 2004). All experiments were independently repeated three or more times and the mean log CFU/mL as well as the standard deviations was calculated.

Table 1
Antimicrobial activity of plant extracts and pure phenolic acids expressed as MIC (mg/mL) determined by the disk diffusion and agar dilution methods.

Plant extract/pure phenolic acid	MIC (mg/mL)									
	Bacillus cereus WSBC 10530		*Staphylococcus aureus* ATCC 25923		*Salmonella* Infantis ŽM9		*Campylobacter jejuni* ATCC 33560		*Campylobacter coli* ATCC 33559	
	Disk diffusion	Agar dilution	Disk diffusion	Agar dilution	Disk diffusion	Agar dilution	Disk diffusion	Agar dilution	Disk diffusion	Agar dilution
V15	0.625	0.156	5.0	0.625	>100.0	9.0	40.0	8.0	40.0	8.0
V20	0.625	0.156	2.5	0.625	100.0	9.0	40.0	7.0	>40.0	7.0
V40	0.313	0.078	0.625	0.156	100.0	9.0	20.0	7.0	20.0	7.0
V70	0.313	0.078	0.625	0.078	100.0	8.0	10.0	5.0	10.0	5.0
A15	20.0	5.0	20.0	5.0	100.0	9.0	>40.0	10.0	>40.0	10.0
A40	5.0	1.25	20.0	5.0	100.0	8.0	>40.0	5.0	>40.0	10.0
Carnosic acid	ND	0.156	ND	0.156	ND	5.0	ND	5.0	ND	5.0
Rosmarinic acid	8.0	6.0	ND	10.0	ND	1.25	ND	1.25	ND	1.25
Grape seed extract	10.0	1.25	>40.0	10.0	100.0	10.0	>40.0	2.5	>40.0	2.5
Olive leaves extract	40.0	20.0	>40.0	40.0	100.0	20.0	>40.0	10.0	>40.0	10.0
Green tea extract	20.0	10.0	10.0	1.25	100.0	20.0	>40.0	5.0	>40.0	10.0
Sage extract	1.25	0.313	1.25	0.078	ND	ND	40.0	5.0	ND	5.0
V40/A40	0.625	0.156	2.5	0.313	100.0	10.0	>40.0	2.5	>40.0	5.0
V40/grape seed	0.625	0.156	2.5	0.313	100.0	10.0	>40.0	2.5	>40.0	2.5
V40/citrus extract	0.625	0.156	1.25	0.625	100.0	20.0	>40.0	10.0	>40.0	10.0
V40/olive leaves	0.625	0.156	2.5	0.313	100.0	20.0	20.0	10.0	20.0	10.0
V40/hop extract	0.156	0.020	0.625	0.020	100.0	10.0	ND	5.0	20.0	2.5

ND not determined.

3. Results

3.1. Evaluation of MIC values determined by different methods

MIC values of plant extracts and pure phenolic acids were determined as an evaluation of their antimicrobial activity against selected food-borne pathogenic bacteria.

Using the disk diffusion and agar dilution methods we tested the ability of bacteria to produce visible growth when certain concentration of plant extract or pure phenolic acid was added. MIC values as determined by the disk diffusion method ranged mostly from 0.313 to 40.0 mg/mL for gram-positive bacteria, indicating *B. cereus* as the most susceptible bacteria, while *Campylobacter* spp. and *S.* Infantis were more resistant because their growth was inhibited only in the range of 10.0–100.0 mg/mL (Table 1). Using the agar dilution method we determined the lowest concentration of plant extract and phenolic acid expressed as an MIC value that, under the assay conditions, inhibited visible growth of the bacteria investigated (Table 1). In general MIC values obtained by the agar dilution method were 3–20 times lower than MIC values obtained by the disk diffusion method, irrespective of the plant extract tested.

A good correlation in the range of sensitivity was obtained for antibacterial activity measured by the agar dilution and broth dilution methods for gram-positive bacteria such as *B. cereus* and *S. aureus* (Table 2) but not for gram-negative bacteria where a lower concentration of plant extract was sufficient for growth inhibition by the broth dilution method (Table 3).

MIC values determined for gram-positive bacteria by the broth macrodilution method were, with few exceptions (V70, A15 and carnosic acid/*B. cereus*; V15 and rosmarinic acid/*S. aureus*; V40/*L. monocytogenes*), the same as MIC values determined by the broth microdilution method (Table 2). More exceptions were found for gram-negative strains of *S.* Infantis and *Campylobacter* spp. (Table 3).

In the study, we also evaluated different indicators for quantification of microbial growth in the broth microdilution assay, which

Table 2
Antimicrobial activity of plant extracts and pure phenolic acids expressed as MIC (mg/mL) determined by the broth macrodilution and microdilution methods for gram-positive bacteria.

Plant extract/pure phenolic acid	MIC (mg/mL)											
	Bacillus cereus WSBC 10530				*Staphylococcus aureus* ATCC 25923				*Listeria monocytogenes* ŽM58			
	Macrodilution	Microdilution			Macrodilution	Microdilution			Macrodilution	Microdilution MIC		
		TTC	INT	ATP		TTC	INT	ATP		TTC	INT	ATP
V15	0.156	0.156	0.313	0.156	0.156	0.313	0.313	0.156	ND	0.313	0.313	0.313
V20	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156	ND	0.156	0.156	0.156
V40	0.078	0.078	0.078	0.078	0.078	0.156	0.078	0.078	0.313	0.156	0.156	0.078
V70	0.078	0.039	0.039	0.039	0.039	0.039	0.039	0.039	0.156	0.156	0.039	0.039
A15	2.5	1.25	2.5	2.5	5.0	5.0	5.0	5.0	ND	10.0	10.0	10.0
A40	1.25	1.25	1.25	1.25	5.0	5.0	5.0	5.0	ND	5.0	5.0	2.5
Carnosic acid	0.078	0.156	0.156	0.078	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156
Rosmarinic acid	5.0	5.0	5.0	5.0	2.5	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Grape seed extract	1.25	1.25	1.25	1.25	ND	1.25	1.25	1.25	ND	ND	1.25	ND
Olive leaves extract	5.0	5.0	5.0	5.0	ND	5.0	5.0	5.0	ND	10.0	5.0	10.0
Green tea extract	0.156	0.156	0.156	0.156	ND	0.156	0.156	0.156	ND	0.156	0.156	0.156
Sage extract	0.313	0.313	0.156	0.156	ND	0.313	0.313	0.078	0.156	0.156	0.156	0.156
V40/A40	0.156	0.156	0.156	0.156	ND	0.313	0.313	0.156	0.313	0.313	0.313	0.313
V40/grape seed	0.156	0.156	0.156	0.156	ND	0.313	0.313	0.156	0.156	0.156	0.156	0.156
V40/citrus extract	ND	0.156	0.156	0.156	ND	0.313	0.313	0.313	ND	0.313	0.313	0.313
V40/olive leaves	0.156	0.156	0.156	0.156	ND	0.313	0.313	0.313	ND	0.156	0.156	0.156
V40/hop extract	0.156	0.156	0.156	0.156	0.313	0.313	0.313	0.313	0.156	0.156	0.156	0.156

ND not determined.

Table 3
Antimicrobial activity of plant extracts and pure phenolic acids expressed as MIC (mg/mL) determined by the broth macrodilution and microdilution methods for gram-negative bacteria.

Plant extract/pure phenolic acid	MIC (mg/mL)													
	Salmonella Infantis ŽM9				*Escherichia coli* O157:H7				*Campylobacter jejuni* ATCC 33560			*Campylobacter coli* ATCC 33559		
	Macrodilution	Microdilution			Macrodilution	Microdilution			Macrodilution	Microdilution		Macrodilution	Microdilution	
		TTC	INT	ATP		TTC	INT	ATP		TTC/INT	ATP		TTC/INT	ATP
V15	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	1.25	–	1.25	1.25	–	0.625
V20	5.0	2.5	2.5	5.0	5.0	5.0	5.0	5.0	0.625	–	0.625	0.625	–	0.313
V40	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	0.156	–	0.156	0.313	–	0.156
V70	2.5	2.5	2.5	2.5	5.0	5.0	2.5	2.5	0.078	–	0.078	0.078	–	0.078
A15	2.5	10.0	2.5	2.5	ND	10.0	10.0	10.0	2.5	–	2.5	2.5	–	1.25
A40	2.5	10.0	2.5	2.5	ND	5.0	2.5	5.0	2.5	–	2.5	2.5	–	0.625
Carnosic acid	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	0.156	–	0.156	0.156	–	5.0
Rosmarinic acid	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	1.25	–	1.25	1.25	–	2.5
Grape seed extract	ND	5.0	5.0	5.0	ND	2.5	2.5	2.5	ND	–	2.5	ND	–	2.5
Olive leaves extract	ND	5.0	5.0	5.0	ND	5.0	5.0	5.0	ND	–	5.0	ND	–	5.0
Green tea extract	ND	1.25	0.625	0.625	ND	1.25	1.25	1.25	ND	–	0.625	ND	–	0.625
Sage extract	ND	10.0	10.0	10.0	ND	2.5	2.5	2.5	ND	–	0.625	0.625	–	0.625
V40/A40	1.25	1.25	1.25	1.25	2.5	2.5	2.5	2.5	0.156	–	0.313	0.156	–	0.156
V40/grape seed	1.25	1.25	1.25	1.25	2.5	1.25	1.25	1.25	ND	–	0.313	ND	–	0.156
V40/citrus extract	ND	1.25	2.5	2.5	ND	2.5	2.5	2.5	ND	–	0.313	ND	–	0.156
V40/olive leaves	ND	1.25	1.25	1.25	ND	2.5	2.5	2.5	ND	–	0.313	ND	–	0.156
V40/hop extract	1.25	1.25	1.25	2.5	2.5	2.5	2.5	2.5	0.156	–	0.156	0.156	–	0.156

ND not determined.
'–' not achieved.

could represent an alternative approach. The majority of bacteria tested in this study reduced TTC and INT and gave clearly defined endpoints (Tables 2 and 3). In most cases MIC values were the same irrespective of the use of TTC or INT. The broth microdilution method with TTC or INT used as an indicator for metabolic activity was not suitable for *C. jejuni* and *C. coli* which are microaerophilic with lower reduction kinetics. For *Campylobacter* spp. the microdilution method based on measurement of the metabolic indicator ATP was successfully used (Table 3). When ATP was used as a viability indicator other non-*Campylobacter* strains also gave results comparable to TTC or INT. The results suggest that the microdilution method in combination with ATP measurement offers a suitable alternative to currently used methods.

As evident in Tables 1 and 2, the broth microdilution method is more sensitive than screening agar methods and consequently most appropriate for a rapid quantitative determination of the antimicrobial activity of plant extracts. Using the microdilution method the antibacterial activity expressed as MIC was at the same or at lower concentrations compared to the other dilution methods. Following the kinetics at the concentration predetermined as the MIC by the microdilution method, the effect was visible as multiplication inhibition and consequently no additional growth in 24 h. Thus, the use of microdilution is preferable since the most accurate range of antibacterial activity was detected and the results correlated with characterization of the growth inhibitory efficiency of the plant extracts used, assessed from survival curves. The microdilution method is more economical of time and resources and is well suited to screening many combinations of different bacteria and plant extracts.

The growth, survival and death curves for *B. cereus*, *S. aureus*, *C. jejuni* and *S.* Infantis at various concentrations of plant extracts are shown in Figs. 1 and 2. Following the kinetics of inactivation in broth media over 24 h of incubation, growth was inhibited at concentrations lower than these shown by the disk diffusion and agar dilution methods for gram-negative bacteria (Table 2, Fig. 2). The examples shown in Figs. 1 and 2 for gram-positive and gram-negative bacteria clearly demonstrate that MIC values determined by the microdilution methods for V40/hop extract were really the concentrations of extracts that inhibited bacterial growth. As the tested concentrations of extracts were high already initial level of bacteria was different.

3.2. Antibacterial activity of plant extracts

The tested plant extracts differ in their origin and consequently in the content of phenolic acids. Plant extracts with carnosic acid as the major component of total phenolic content (V15, V20, V40, V70) were more effective than plant extracts with rosmarinic acid (A15, A40). In addition, the mixtures between V40 and A40, grape seed, citrus extract, olive leaves or hop showed an activity similar to that detected for the V40 extract and pure carnosic acid. Grape, olive and green tea extracts were much less efficient and sage more effective against all tested bacteria. Though all plant extracts showed antimicrobial activity, the response for each bacterium tested was different. From the results, it is obvious that the assayed plant extracts have different modes of action and exhibited stronger biological activity against gram-positive bacteria. The best antibacterial activity, examined by the broth microdilution method, was seen against *B. cereus* and the

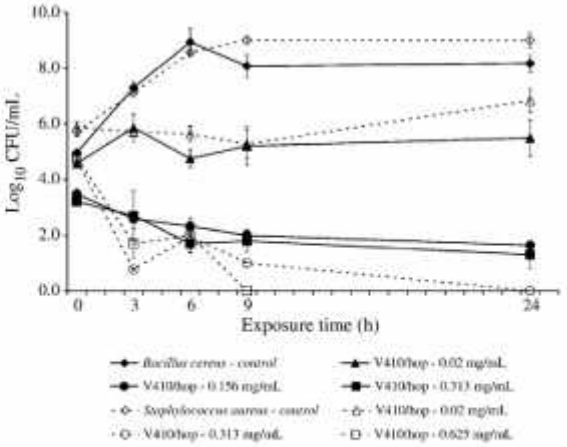

Fig. 1. *Bacillus cereus* and *Staphylococcus aureus* growth, survival and death curves on exposure to rosemary extract mixture V40:hop. Each point represents the log of the mean ± SD (standard deviation) CFU/mL.

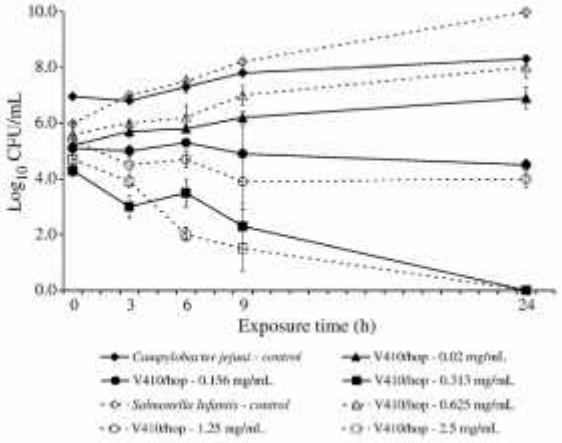

Fig. 2. *Campylobacter jejuni* and *Salmonella* Infantis growth, survival and death curves on exposure to rosemary extract mixture V40:hop. Each point represents the log of the mean ± SD (standard deviation) CFU/mL.

lowest activity was against *S.* Infantis. Interestingly, the results of the broth microdilution test developed with *C. jejuni* and *C. coli* also indicated that *Campylobacter* spp. are more sensitive gram-negative bacteria compared to *S.* Infantis and *E. coli* O157:H7, which proved to be more resistant to plant extract treatment.

4. Discussion

Antimicrobial agents are chemical compounds present in, or added to, foods that retard microbial growth or cause microbial death. In the last decades, there has been particular interest in the use of abundant naturally occurring antimicrobials (herbs, spices and plants) (Burt, 2004). The ability to compare the results for antimicrobial plant compounds or extracts from different studies is limited because of differences in the methodologies used and different definitions of MIC (Burt, 2004). As the results from different studies need to be comparable, we examined the antimicrobial activity of plant extracts and their mixtures by the diffusion and dilution methods against gram-positive and gram-negative bacteria in order to propose a uniform procedure of testing plant extract antibacterial activity, prior to their *in vivo* application.

4.1. Meaning of MIC values determined by different methods

Diffusion methods are attractive because of their simplicity and low cost, but they are, like all agar-based methods, labour and time-intensive. On the basis of the higher MIC values obtained when the disk diffusion method was used in our experiments, it appears that this diffusion method could not always be a reliable method for screening the antimicrobial activity of plant extracts. As previously reported, the absence of an inhibition zone did not necessarily mean the compound was inactive, especially for less polar compounds, which diffuse more slowly into the culture medium (Moreno et al., 2006). The diffusion assay is not suited to natural antimicrobial compounds that are scarcely soluble or insoluble in water and thus their hydrophobic nature prevents uniform diffusion through the agar media (Mann and Markham, 1998).

For quantitative determination of antibacterial activity the agar dilution method is more appropriate, where antibacterial activity of plant extracts was shown at lower concentrations compared to the disk diffusion method. The agar dilution and broth microdilution methods produced comparable results and a good level of agreement only for gram-positive bacteria. Our evaluation included colorimetric determination using TTC or INT, and ATP determination by bioluminescence measurement. Tetrazolium salts have previously been used in the broth microdilution method to enhance the detection of bacterial growth in several studies (Johnson et al., 1985; Al-Bayati, 2008). Reduction results in an easily identified colour change occurring at a cell density meaningful for MIC testing. Reduction occurs due to its function as an artificial terminal electron acceptor in respiration. Tetrazolium salts are not appropriate for microaerophilic campylobacters since they indicate the respiratory activity. *Campylobacter* growth or its inhibition by different plant extracts or essential oils is usually measured by diffusion methods or following the growth kinetics of inactivation (Friedman et al., 2002; Fisher and Phillips, 2006). Our results showed that the broth microdilution method with ATP measurement is a rapid and accurate way of testing antimicrobial efficiency for all the tested bacteria, including campylobacters. As the microdilution method by TTC or INT produced comparable results and cost may restrict the use of ATP indicator, we suggest using INT for quantitative antimicrobial determination for normal growing bacterial strains and ATP for microaerophilic species like *Campylobacter* spp. This method may be an acceptable alternative for quantitative determination of bacterial susceptibility to plant extracts (Ellof, 1998; Muraina et al., 2009).

4.2. Antibacterial activity of plant extracts

All the tested plant extracts (10), phenolic acids (2) and extract mixtures (5) had antibacterial activity that was determined against different food-borne bacteria. A relationship between antimicrobial activity and chemical composition was previously demonstrated (Klančnik et al., 2009; Katalinić et al., 2010). Rosemary (*Rosmarinus officinalis* L.) extract is widely used as a rich source of phenolic compounds with high antimicrobial activity, associated with carnosic and rosmarinic acid (Campo et al., 2000; Petersen and Simmonds, 2003; Moreno et al., 2006). As reported, carnosic acid and carnosol, rosmanol and ferruginol are also responsible for the biological activity of sage (*Salvia* sp.) along with the phenolic rosmarinic and salvianolic acids (Markowski, 2008). The results in Tables 1–3 show sage extract to be very effective. However, grape, olive and green tea extracts were much less efficient against all the tested bacteria. We can conclude that plant extracts efficiently inhibit the growth of gram-positive bacteria and *Campylobacter*, especially commercial extracts and mixtures containing carnosic acid as the main phenolic component. MIC values of sage were in the range similar to those detected for extracts containing carnosic acid. Pure rosmarinic acid was much less efficient against gram-positive bacteria and campylobacters than carnosic acid. The main reason for the differences in bacterial susceptibility could be the outer membrane surrounding the cell wall in gram-negative bacteria, which restricts diffusion of compounds through its lipopolysaccharide covering, as previously reported (Vaara, 1992). In addition, the periplasmatic space contains enzymes which are capable of breaking down foreign molecules introduced from the outside (Vaara, 1992). *Campylobacter* spp. are known to be untypical in terms of their ecological features, sensitive to different environmental conditions with unknown different mechanisms and regulation pathways (Park, 2002). In this context, *Campylobacter* spp. appear to be more sensitive than other gram-negative bacteria i.e. *S.* Infantis and *E. coli* O157:H7.

As several methods for measurement of the MIC value of plant extracts are available but there is no standard procedure, and since the results from numerous studies need to be comparable, we can suggest evaluation of the antibacterial activity of a plant extract using the microdilution method as a fast screening method for MIC determination and the macrodilution method at selected MIC concentrations to confirm bacterial inactivation. Only limited data are presently available on the antimicrobial activity of plant extracts in food systems and this indicates continuation of our study and the need for further work.

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2.2 PREISKOVANJE NEKATERIH DEJAVNIKOV, KI VPLIVAJO NA PROTIMIKROBNO UČINKOVITOST IZVLEČKOV ROŽMARINA V ŽIVILSKIH MODELIH Z METODO RAZREDČEVANJA V MIKROTITRSKI PLOŠČICI

Klančnik A, Piskernik S., Smole Možina S., Gašperlin L., Jeršek B. 2011. Investigation of some factors affecting the antibacterial activity of rosemary extracts in food models by a food microdilution method. International Journal of Food Science and Technology, 46: 413–420.

Določili smo učinkovitost izvlečkov rožmarina V40 (RosmE1) in V70 (RosmE2) na bakterije vrst *Listeria monocytogenes* in *Escherichia coli* in sicer v živilskih modelih (50 % delež živila in 50 delež % gojišča). Za pripravo živilskih modelov smo uporabili mesne, zelenjavne in mlečne izdelke. Želeli smo preveriti nekatere dejavnike, ki lahko vplivajo na vrednost MIK. Med temi dejavniki so vrsta ter velikost začetnega števila bakterij, vrsta ter delež živila in temperatura inkubacije. Za določanje vrednosti MIK smo prilagodili metodo razredčevanja v mikrotitrski ploščici, tako da smo pripravili 50 % modele izbranih živil. Protimikrobna učinkovitost izvlečkov rožmarina je bila v modelih iz mlečnih in mesnih živil slabša, kot v laboratorijskih gojiščih. Bakterije vrste *L. monocytogenes* so bile na delovanje izvlečkov rožmarina bolj občutljive, saj smo določili nižje vrednosti MIK, kot pri bakterijah vrste *E. coli*. V laboratorijskem gojišču smo pri nižjem začetnem številu bakterij vrste *E. coli* določili nižje vrednosti MIK. Nasprotno pa smo pri bakterijah vrste *L. monocytogenes* določili višje vrednosti MIK. V živilskih modelih smo pri obeh vrstah bakterij določili enake ali višje vrednosti MIK kot v laboratorijskem gojišču. Metoda razredčevanja v mikrotitrski ploščici se je izkazala za enostavno, hitro, ponovljivo in poceni metodo, ki jo lahko prilagodimo tako, da določimo protimikroben učinek rastlinskih izvlečkov tudi v živilskih modelih.

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Original article

Investigation of some factors affecting the antibacterial activity of rosemary extracts in food models by a food microdilution method

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Summary The activity of rosemary phenolic extracts against *Listeria monocytogenes* and *Escherichia coli* was evaluated in 50% food models of meat, vegetable and dairy products in relation to some factors that can affect MIC (minimal inhibitory concentration) values (inoculum level, proportion of food, temperature) using a new food microdilution method. It was shown that the interactions of meat and milk components with plant extracts reduced the antibacterial effectiveness of rosemary extracts. MIC values for *L. monocytogenes* were lower than for *E. coli* in all tested conditions. A lower inoculation level caused a decrease in MIC values for *E. coli* but an increase in MIC values for *L. monocytogenes* in control media. In food models, MIC values were higher or equal to MIC values in control media regardless of bacterial type. We showed that the food microdilution method represents a simple, rapid, reproducible and inexpensive method for testing the antimicrobial efficiency of plant extracts in food systems.

Keywords Antimicrobial activity, food microdilution method, food models, minimal inhibitory concentration, plant extracts.

Introduction

For the assurance of food safety, nowadays consumers are increasingly demanding food products with natural alternatives to chemical additives, but with increased safety, quality and shelf-life. Antimicrobials are used in food to control natural spoilage processes (food preservation) and to prevent or control the growth of micro-organisms, including pathogenic micro-organisms (food safety) (Corbo *et al.*, 2009; Tajkarimi *et al.*, 2010). However, despite the wide range of phenolic compounds available as potential antimicrobials, relatively few are reported to be suitable for practical use in particular food products. To enable the more extensive use of natural antimicrobials as preservatives, there is a need to examine their efficacy and functionality in the models of food systems and real food conditions. Only limited data are available for different foods and treatments (Owen & Palombo, 2007; Gutierrez *et al.*, 2009; Hayes *et al.*, 2010; Santas *et al.*, 2010).

The antimicrobial activities of plant extracts in foods are influenced by many factors, such as pH, a_w, target microorganisms, food components, types of food constituents, the presence of other preservatives or certain enzymes, processing and storage temperature, storage atmosphere, partition coefficients, as well as the interactions between these factors (Anonymous, 2003; Burt, 2004; Becerril *et al.*, 2007; Solomakos *et al.*, 2008; Gutierrez *et al.*, 2009). The effect of foods on the antibacterial activity of plant extracts has been investigated. The presence of food components may change the solubility and phase distribution parameters of antibacterial constituents or interfere with the plant extract and alter its antimicrobial efficiency. For example, fat in full cream milk provides protection for bacterial cells, but carbohydrates in foods do not appear to protect bacteria from the action of essential oils and plant extracts (Fisher & Phillips, 2006; Owen & Palombo, 2007; Chollet *et al.*, 2008; Kotzekidou *et al.*, 2008; de Oliveira *et al.*, 2010; Tajkarimi *et al.*, 2010). In addition, the antibacterial effect of a plant extract in food may be decreased because there is greater availability of nutrients than in laboratory media and bacteria may repair damaged cells faster (Burt, 2004; Gutierrez *et al.*, 2009). Other effects on the antimicrobial activity of plant extracts, such as processing and/or storage temperature or inoculum level, have not been studied so extensively. Rivas *et al.* (2010) found that in general the antimicrobial activity of carvacrol or thymol against *E. coli* O157:H7 increased with decreasing storage temperature, water activity, pH and inoculum level in the medium.

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For example, by decreasing the pH values at all other specified conditions, the enhanced antimicrobial effect can be attributed to the direct effect of pH or to the better dissolution of the antimicrobial compounds in the lipid phase of the bacterial membrane (Juven *et al.*, 1994; Koutsoumanis *et al.*, 1999; Basti *et al.*, 2007). Growth of *L. monocytogenes* in a broccoli model system with rosemary extract stored at 8 °C was inhibited, with reductions in growth rate and an increase of the lag phase being achieved over 30 days of storage (Muñoz *et al.*, 2009). Other studies of Apostolidis *et al.* (2008) showed that the level of initial inoculum of *L. monocytogenes* on cooked ground beef systems was important for the inhibitory effect of phenolic extracts of oregano and cranberry at 4 °C. When the inoculum level was in the range of 4–5 log CFU mL^{-1} (colony forming unit), the degree of inhibition was lower than when initial inoculum levels were of 3–4 log CFU mL^{-1}. Also application of essential oils in the vapour phase showed an increased antimicrobial effect over application of the same essential oils in solid media and thus potential for the use of antimicrobial packaging with less active components of natural antimicrobials (Goñi *et al.*, 2009). Recently, modern techniques such as liposome encapsulation of bioactive plant compounds were introduced for stabilisation, protection against deterioration, prevention of volatility losses and interactions with other ingredients and for possible improvement of antioxidative and antimicrobial activities (Liolios *et al.*, 2009; Wang *et al.*, 2009).

To study the effectiveness of plant extracts in different foods and to assess the influence of different factors in food processing, growth inhibition curves are normally used to determine the concentration of a plant extract that has antimicrobial activity (Burt, 2004; Gutierrez *et al.*, 2009; Muñoz *et al.*, 2009; Rivas *et al.*, 2010). However, in the absence of a well-defined microbiological model, it is usually difficult to evaluate the efficiency of alternative antimicrobials in food or food models and the influence of different factors on antimicrobial activity. For these reasons, there is a need to develop fast screening methods for the evaluation of antimicrobial activity in several food systems.

The effectiveness of rosemary extracts (RosmE1 and RosmE2) as potential food preservatives, in particular their activity against foodborne gram-positive *Listeria monocytogenes* and gram-negative *Escherichia coli*, was examined in the current study. Their antimicrobial effects were evaluated in food models containing 50% of either of meat (minced meat, patè), vegetable (cauliflower, potato) and dairy products (curds) in BPW (buffered peptone water). The main purpose of our work was to evaluate selected factors that can affect determination of the antimicrobial activity of rosemary plant extracts using a food microdilution method. Besides the effect of the bacterium and food proportion, the effects of inoculum level in combination with incubation temperature on the antibacterial activity of two commercial rosemary extracts (RosmE1 and RosmE2) were also evaluated.

Materials and methods

Bacterial strains and growth conditions

Listeria monocytogenes ŽM58 (IHM, Würzburg, Germany) and *Escherichia coli* ŽM 370 (ATCC 11229, USA) were used for antibacterial testing. Bacterial cultures for antimicrobial testing were grown in 5 mL of Müller Hinton Broth (MHB, Oxoid, CM0405, Hampshire, UK) aerobically for 20 h, continuously shaken at 1.7 s^{-1} at 37 °C. For preparation of lower and higher inoculum levels, 1 mL of each culture was diluted with one-quarter-strength buffered peptone water (BPW, Oxoid, CM0509, Hampshire, UK), and 150 μL of this diluted culture was added to either 10 mL of MHB or one-quarter-strength BPW containing 50% of food to obtain 10^6–10^7 and 10^3–10^4 CFU mL^{-1}, respectively.

Rosemary extracts

This study included commercial rosemary extract formulations with 40% (RosmE1) and 70% (RosmE2) of carnosic acid as the main active phenolic compound (Vitiva d.d., Markovci, Slovenia). The contents of total phenolic compounds were determined for RosmE1 as 198 mg mL^{-1} and for RosmE2 as 438 mg mL^{-1} (Klančnik *et al.*, 2009). Lyophilised rosemary extracts were dissolved in absolute ethanol to prepare stock solutions (40 mg mL^{-1}) and were further diluted in MHB or in one-quarter-strength BPW to prepare working solutions.

Preparation of food samples and food microdilution method

Minced meat, patè, curds, frozen cauliflower and frozen potato were bought on the retail market. To prepare food models, 50 g of each food was added to 50 mL of one-quarter-strength BPW in a blender bag and homogenised in a laboratory blender (Stomacher, Seward 400, UK) for 2 min at normal power. All suspensions were autoclaved at 121 °C for 15 min prior to use to eliminate microbial contamination. The suspensions were then homogenised with an ultra-turrax apparatus (Ultra-Turrax T25, Janke & Kunkel, Staufen, Germany) in sterile glass bottles.

The broth microdilution and food microdilution methods were used for measuring minimal inhibitory concentrations (MIC) in MHB or in one-quarter-strength BPW with 50% food under different conditions.

Fifty microlitres of each bacterial suspension in MHB or in one-quarter-strength BPW with 50% food was added to the wells of a sterile 96-well microtitre plate already containing 50 μL of twofold serially diluted plant extract in an appropriate media. The final volume in each well was 100 μL. Control wells were prepared with culture medium (sterility control), plant extract in 50 μL of MHB (negative control), bacterial suspension in 50 μL of MHB (positive control) and ethanol in amounts corresponding to the highest quantity present (from 12.5%) (positive control to prove that there was no bacterial growth inhibition by ethanol). The contents of each well were mixed on a microplate shaker (Eppendorf, Hamburg, Germany) at 1.7 s^{-1} for 1 min prior to incubation for 24 h at 37 °C. The MIC was defined as the lowest concentration where no viability was observed after 24 h on the basis of metabolic activity. To indicate respiratory activity, the presence of a purple colour was determined visually after adding 10 μL/well of INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride, Sigma) dissolved in water (2 mg mL^{-1}) and incubated at 37 °C for 30 min in the dark (Klančnik *et al.*, 2010). All measurements of MIC values were repeated in triplicate.

Design of experiment

The design of the experiment with variations in factors that can affect minimal inhibitory concentration of the rosemary extracts (inoculum level, proportion of food, temperature) for *L. monocytogenes* and *E. coli* is presented in Figure 1.

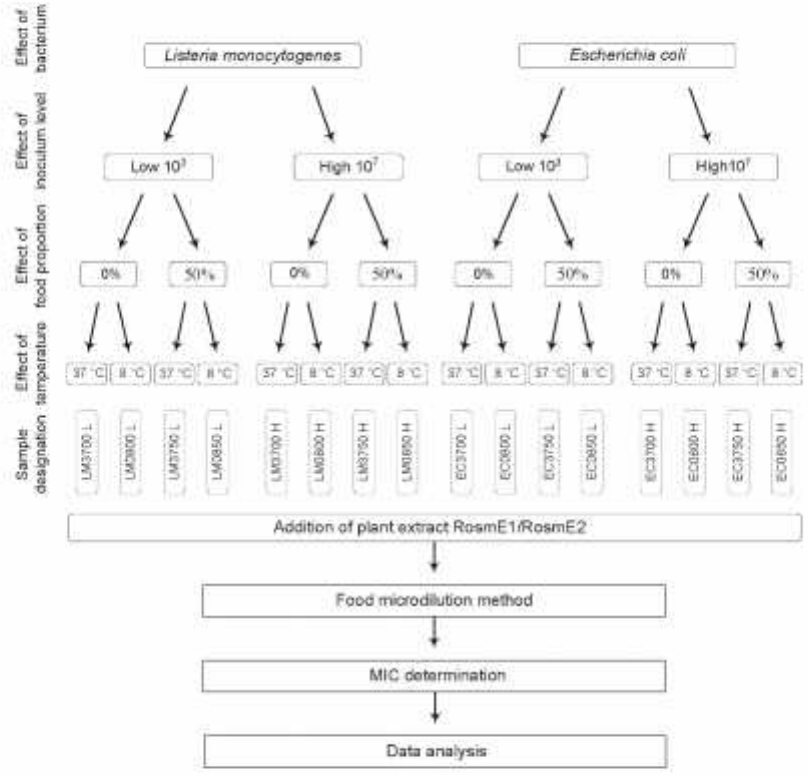

Figure 1 Design of experiment performed with each selected food (cauliflower, potatoes, minced meat, paté, curds) to evaluate the effects of type of bacteria, inoculum level, proportion of food in medium, temperature and type of food on MIC determination.

Data analysis

The experimental data for MIC were evaluated statistically using the SAS/STAT program (SAS Software, 1999; SAS Institute Inc., Cary, NC, USA). The basic statistical parameters were calculated by the MEANS procedure. The data were tested for normal distributions and analysed using the general linear model (GLM) procedure. The statistical model included the main effect of food (cauliflower, potatoes, minced meat, paté, curds) and interaction between the type of bacteria, temperature and the proportion of food in the medium (groups: EC0800, EC0850, EC3700, EC3750, LM0800, LM0850, LM3700 and LM3750). The means for experimental groups were obtained using Duncan's procedure and compared at the 5% probability level.

Results

We changed the inocula (type, level), growth medium (proportion of food, type of food) and incubation temperature to evaluate their effects on the efficacy of rosemary phenolic extracts. Combinations were screened against *Escherichia coli* and *Listeria monocytogenes* using the broth and food microdilution methods. The statistically evaluated MIC values obtained for RosmE1 and RosmE2 in MHB and meat (minced meat, paté), vegetable (cauliflower, potato) and dairy product (curds) model media using lower levels of inoculum of *L. monocytogenes* and *E. coli* are presented in Tables 1 and 2 and using higher levels of inoculum for both bacteria in Tables 3 and 4. Data analysis showed that the main effect of food (cauliflower, potatoes, minced meat, paté, curds) and interactions between the type of bacteria, temperature and proportion of food in the medium on MIC values were significant at less than the 0.01% level. Thus, the results were analysed and presented as shown in Tables 1–4.

Table 1 MIC values determined for RosmE1 at lower inoculation level of *L. monocytogenes* and *Escherichia coli*

	MIC (mg mL^{-1})					
Group/food	Cauliflower	Potatoes	Meat	Paté	Curds	P_{food}
EC0800 L	0.31^{c}	0.31^{c}	0.31^{c}	0.31^{d}	0.31^{b}	–
EC0850 L	1.25^{xy}	1.25^{xy}	5.00^{xw}	5.00^{xw}	1.25^{xy}	<0.0001
EC3700 L	1.25^{a}	1.25^{a}	1.25^{b}	1.25^{c}	1.25^{a}	–
EC3750 L	0.63^{xy}	0.63^{xy}	1.25^{xy}	2.5^{xx}	1.25^{xy}	<0.0001
LM0800 L	0.08^{e}	0.08^{d}	0.08^{d}	0.08^{e}	0.08^{c}	–
LM0850 L	0.63^{xy}	0.63^{xy}	1.25^{xy}	5.00^{xw}	1.25^{xy}	<0.0001
LM3700 L	0.02^{f}	0.02^{e}	0.02^{e}	0.02^{f}	0.02^{d}	–
LM3750 L	0.16^{xy}	0.08^{xz}	0.02^{yzw}	0.08^{xz}	1.25^{xx}	<0.0001
P_{group}	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

Level of significance: highly statistically significant: $P \leq 0.001$. Groups with a different superscript within column (^{a,b,c,d,e,f}) differ significantly ($P \leq 0.05$). Foods with a different superscript within a row (^{x,y,z,w}) differ significantly ($P \leq 0.05$).
Legend: EC: *E. coli*; LM: *Listeria monocytogenes*; 08: 8 °C; 37: 37 °C; 00: no food in medium; 50: 50% of food in medium; L: low inoculum of 10^3 CFU mL^{-1}.

Table 2 MIC values determined for RosmE2 at lower inoculation level of *Listeria monocytogenes* and *Escherichia coli*

	MIC (mg mL^{-1})					
Group/food	Cauliflower	Potatoes	Meat	Paté	Curds	P_{food}
EC0800 L	0.31^{c}	0.31^{c}	0.31^{d}	0.31^{d}	0.31^{c}	–
EC0850 L	0.31^{by}	0.63^{xy}	2.50^{xw}	2.50^{xw}	0.63^{by}	<0.0001
EC3700 L	0.63^{a}	0.63^{a}	0.63^{c}	0.63^{c}	0.63^{b}	–
EC3750 L	0.63^{xy}	0.63^{xy}	1.25^{xy}	2.5^{xx}	1.25^{xy}	<0.0001
LM0800 L	0.08^{d}	0.08^{d}	0.08^{e}	0.08^{e}	0.08^{d}	–
LM0850 L	0.31^{by}	0.31^{by}	0.63^{xy}	1.25^{bx}	0.63^{by}	<0.0001
LM3700 L	0.01^{d}	0.01^{f}	0.01^{g}	0.01^{g}	0.01^{e}	
LM3750 L	0.08^{by}	0.04^{xy}	0.04^{by}	0.04^{by}	0.63^{bx}	<0.0001
P_{group}	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

Level of significance: highly statistically significant: $P \leq 0.001$. Groups with a different superscript within column (^{a,b,c,d,e,f}) differ significantly ($P \leq 0.05$). Foods with a different superscript within a row (^{x,y,z,w}) differ significantly ($P \leq 0.05$).
Legend: See Table 1.

Effect of food proportion on MIC values

The results show significant influence of a 50% food proportion in the medium on MIC values with both rosemary phenolic extracts used. Their antimicrobial activity decreased in media with food components, regardless of a whether a low or high level of bacterial inoculum was used. Some exceptions were found, mostly for *E. coli* at 37 °C in media with cauliflower and potatoes (Tables 1 and 3) where MIC values decreased in media with added food or remained the same (meat and curds in Table 1, meat, paté and curds in Table 3). A few exceptions were also found for *E. coli* at 8 °C in media with cauliflower and potatoes (Tables 2, 3 and 4) where MIC values were the same irrespective of added food. Only one exception was found in the case of *L. monocytogenes* at 37 °C in the meat models (Table 1), where MIC values were the same irrespective of food addition.

Effect of type of food on MIC values

When *E. coli* and *L. monocytogenes* were exposed to rosemary extracts in food models, it was observed that the MIC values were significantly higher in the range of 16-fold to 156-fold than those obtained in the MHB control media. Interestingly, the efficacy of natural alternative antimicrobials appears to be reduced by certain food components. However, more significant

Table 3 MIC values determined for RosmE1 at higher inoculation level of *L. monocytogenes* and *Escherichia coli*

	MIC (mg mL^{-1})					
Group/food	Cauliflower	Potatoes	Meat	Paté	Curds	P_{food}
EC0800 H	1.25^{b}	1.25^{D}	1.25^{c}	1.25^{c}	1.25^{b}	–
EC0850 H	1.25^{by}	1.25^{Dz}	10.00^{ax}	5.00^{ay}	1.25^{bz}	<0.0001
EC3700 H	2.50^{a}	2.50^{A}	2.50^{b}	2.50^{b}	2.50^{a}	–
EC3750 H	1.25^{by}	1.25^{Dy}	2.50^{bx}	2.50^{bx}	2.50^{ax}	<0.0001
LM0800 H	0.02^{d}	0.02^{f}	0.02^{e}	0.02^{e}	0.02^{c}	–
LM0850 H	1.25^{by}	1.25^{Dy}	1.25^{cy}	5.00^{ax}	1.25^{by}	<0.0001
LM3700 H	0.02^{d}	0.02^{f}	0.02^{e}	0.02^{e}	0.02^{c}	–
LM3750 H	0.16^{cy}	0.16^{Ey}	0.31^{dy}	0.31^{dy}	1.25^{bx}	<0.0001
P_{group}	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

Level of significance: highly statistically significant: $P \leq 0.001$. Groups with a different superscript within column (^{a,b,c,d,e,f}) differ significantly ($P \leq 0.05$). Foods with a different superscript within a row (^{x,y,z}) differ significantly ($P \leq 0.05$).
Legend: EC: *Escherichia coli*; LM: *Listeria monocytogenes*; 08: 8 °C; 37: 37 °C; 00: no food in medium; 50: 50% of food in medium; H: high inoculum of 10^7 CFU mL^{-1}.

Table 4 MIC values determined for RosmE2 at higher inoculation level of *Listeria monocytogenes* and *Escherichia coli*

	MIC (mg mL^{-1})					
Group/food	Cauliflower	Potatoes	Meat	Paté	Curds	P_{food}
EC0800 H	0.63^{b}	0.63^{D}	0.63^{c}	0.63^{d}	0.63^{b}	–
EC0850 H	0.63^{bx}	0.63^{Dx}	10.00^{ax}	5.00^{ay}	1.25^{az}	<0.0001
EC3700 H	0.16^{c}	0.16^{E}	0.16^{d}	0.16^{f}	0.16^{c}	–
EC3750 H	1.25^{ay}	1.25^{Dy}	2.50^{bx}	2.50^{bx}	1.25^{ay}	<0.0001
LM0800 H	0.02^{d}	0.02^{f}	0.02^{f}	0.02^{g}	0.02^{d}	–
LM0850 H	0.63^{by}	0.63^{Dy}	0.63^{cy}	1.25^{cx}	0.63^{by}	<0.0001
LM3700 H	0.02^{d}	0.02^{f}	0.02^{f}	0.02^{g}	0.02^{d}	–
LM3750 H	0.16^{cy}	0.08^{Ey}	0.31^{dy}	0.31^{dy}	1.25^{ax}	<0.0001
P_{group}	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

Level of significance: highly statistically significant: $P \leq 0.001$. Groups with a different superscript within column (^{a,b,c,d,e,f}) differ significantly ($P \leq 0.05$). Foods with a different superscript within a row (^{x,y,z}) differ significantly ($P \leq 0.05$).
Legend: See Table 3.

were differences in rosemary ethanolic extract efficiency when applied to different food models containing higher levels of a nutrient component, such as protein in the meat model, starch in vegetables and fat in the dairy product. *E. coli* incubated at 8 °C were more susceptible to RosmE1 and RosmE2 in the presence of cauliflower, potatoes and curds than in minced meat and paté. MIC values of RosmE1 in curds were comparable to those observed in the vegetable media (Table 3). The same effect was observed when RosmE2 was used in food models. Different types of food showed that MIC values for *E. coli* at 37 °C were lower in cauliflower and potato models at the lower inoculation level (Tables 1 and 2) than in curds, meat and paté models. For the higher inoculation level, MIC values in vegetable and curds models were the same and again lower than in meat models (Table 4). The effect of different foods on MIC values determined for *L. monocytogenes* was in general comparable to the effect on MIC values established for *E. coli*. At 8 °C and the lower inoculation level, MIC values in cauliflower and potato models were lower than MIC values in the other tested food models (Tables 1 and 2), but for the higher inoculation level MIC values in cauliflower, potato and curds models were lower than MIC values in meat models. An effect of the type of food on MIC values determined for *L. monocytogenes* at 37 °C was found, but only in curds broth were they higher than MIC values in other food models, irrespective of the type of extract and inoculation level.

Effect of inoculum level on MIC values

The level of inoculum affected MIC values; in media without food, they were lower at the lower inoculation level than at the higher inoculation level for *E. coli* at 8 °C and 37 °C. For *L. monocytogenes* in MHB, we found equal MIC values at 37 °C for RosmE1 irrespective of inoculum level and lower MIC values for the lower inoculum level for RosmE2, while lower MIC values for the higher inoculum size at 8 °C were found for both rosemary extracts (Tables 1–4). In food models, the influence of inoculum level was determined for *E. coli* at 8 °C in the meat model for RosmE1 and in cauliflower, meat, paté and curds models for RosmE2 and also at 37 °C in vegetable media, meat and curds for RosmE1 and in vegetable media and meat for RosmE2; in all cases, MIC values were higher for the higher inoculum level (Tables 1 and 3). We can conclude that when *E. coli* were exposed to either rosemary extract at 37 °C, the inoculum level more significantly affected MIC than the type of food model. The MIC values determined for *L. monocytogenes* in food models at both tested temperatures were lower in cauliflower and potatoes for the lower level of inoculum, but in meat, paté and curds the values remained the same (Tables 2 and 4). Similarly as for *E. coli*, for *L. monocytogenes*, we can conclude that the level of inoculum had a greater influence on MIC values than the type of food.

Effect of temperature on MIC values

The MIC values of rosemary extracts were also affected by the temperature of incubation. On testing their antimicrobial activity in media without food at 8 °C, MIC values were higher than at 37 °C for *L. monocytogenes* and just the opposite for *E. coli*. When antimicrobial activity was tested in food models at the lower

inoculation level, MIC values were in most cases higher at the lower temperature for both bacteria (Tables 1 and 2). Exceptions were observed for both bacteria in the curds model (Table 1), in curds for *L. monocytogenes* (Table 2) and in potato for *E. coli* (Table 2) where MIC values were equal, irrespective of temperature. There were also two exceptions where MIC values determined for *E. coli* in potato and curds models were lower at 8 °C than at 37 °C (Table 2). The effect of temperature on MIC values determined at the higher inoculation level in food models was also statistically significant, and for meat and paté models at the lower temperature of 8 °C, MIC values were higher than MIC values at 37 °C (Tables 3 and 4). There was no effect of temperature on MIC values when they were determined in cauliflower and potato models for *E. coli* and in the curds model for *L. monocytogenes* (Table 3). The opposite effect of temperature on MIC values was determined in 4 cases, for *E. coli* in the curds model (Table 3), cauliflower and potato models (Table 4) and for *L. monocytogenes* in the curds model (Table 4). In these cases, MIC values were lower at 8 °C than at 37 °C.

Effect of type of bacteria on MIC values

Listeria monocytogenes was more sensitive than *E. coli* in all tested conditions. There were some exceptions, like the curds model, where the MIC values obtained were equal for *E. coli* and *L. monocytogenes* regardless of extract and temperature (Tables 1–4).

Effect of type of extract on MIC values

The tested rosemary extracts differed in their relative amounts of carnosic acid. Therefore, a relationship between antimicrobial activities could also be demonstrated. MIC values determined for RosmE2 were lower than or equal to MIC values for RosmE1, irrespective of all tested variations. More significant differences in efficiency were not obvious, as in both extracts the major component of the total phenolic content was carnosic acid (Klančnik *et al.*, 2009).

Discussion

Natural substances should be tested for their antimicrobial properties in food and then afterwards could be used as additives in foods to assure their quality, safety or/and extended shelf-life. Moreover, consumer demand for more convenient, minimally processed and natural food is ever increasing. Therefore, the safety and effectiveness of minimal processing in practice is ensured by multifactor chemical and physical treatment of food in conjunction with other preservation techniques (Anonymous, 2003; Manas & Pagan, 2005). As a great need exists to verify the efficiency of alternative antimicrobials during different treatments and in different food products, we examined the *in vivo* application of natural products in food models for selected gram-positive and gram-negative bacteria that can be found in a wide variety of raw and processed foods, namely milk and dairy products, various meats or meat products and vegetables (McClure, 2000; Gandhi & Chikindas, 2007). Another aspect of the investigation was focused on the methodology to propose a uniform procedure for testing the antibacterial activity of plant extracts, including multifactor processing conditions.

Several studies have demonstrated that the interaction of food components with alternative antimicrobials might result in decreased antimicrobial activity (Chollet *et al.*, 2008; Gutierrez *et al.*, 2008, 2009; Zhang *et al.*, 2009). In our study, rosemary phenolic extracts were also more effective against bacteria *in vitro* than when added to food models, as shown by increased MIC values in food models in comparison with MIC values determined in media without food. This reduction in efficacy might represent a limitation on their use as antimicrobial agents in foods, because the addition of high concentrations is likely to impart a certain flavour to foods. But the wide potential application of rosemary extracts and its major constituents in food products supports their use (Burt, 2004). As reported, the greater availability of nutrients in foods compared to laboratory media may enable bacteria to repair damaged cells faster (Burt, 2004). As most foods are mainly composed of carbohydrates, fats and proteins, it is important to analyse the influence of these components on the antimicrobial activity of any potential antimicrobial compound. For this reason, we selected vegetables, meat and a dairy product. In our study, MIC values were higher in high-protein and high-fat meat and dairy products than those in vegetable products. For a more detailed analysis, different level of fat, protein and starch should be tested, but we already can conclude that the efficacy of natural alternative antimicrobials may be reduced by food components, mainly protein and fat content.

Besides food components, extrinsic determinants such as temperature or the characteristics of bacteria can also affect bacterial sensitivity (Burt, 2004; Glass & Johnson, 2004; Klančnik *et al.*, 2009). In this study, we confirmed our previous results – that the rosemary extracts were much more active against gram-positive than against gram-negative bacteria (Klančnik *et al.*, 2010). In addition, in this study, we obtained similar results; MIC values were lower for gram-positive *L. monocytogenes* than for gram-negative *E. coli* for the selected plant extracts in all tested conditions. The reason for the different sensitivity of gram-positive and gram-negative bacteria could be ascribed to the structural differences between these microorganisms. Gram-negative bacteria have an additional outer membrane carrying structural

lipopolysaccharide (LPS) components, making the cell wall more impermeable to antimicrobials. Gram-positive bacteria should be more susceptible, having only an outer peptidoglycan layer that is not an effective permeability barrier (Scherrer & Gerhardt, 1971).

Research priorities and future trends in the application of natural antimicrobials are currently being focused on the impact of food product formulation and thus its intrinsic parameters, as well as extrinsic storage parameters, on the design of efficient food preservation systems (Tiwari *et al.*, 2009). Summarising our results, MIC values in media without food were higher at 8 °C than at 37 °C for *L. monocytogenes*, and MIC values were lower at 8 °C than at 37 °C for *E. coli*. Interestingly, MIC values in foods at the lower inoculation level were in most cases higher at lower temperature for both bacteria. No effect of temperature on MIC values was observed when they were determined in cauliflower and potato models for *E. coli* and in curds for *L. monocytogenes*. Thus, differences in temperature may exert an important influence on bacterial ability to actively metabolise and grow. As reported, the temperature range that permits the growth of psychrotrophic *L. monocytogenes* is between −1.5 and 45 °C (Lado & Yousef, 2007; Zhang *et al.*, 2007), while *E. coli* cells are not able to multiply and grow at temperatures lower than 7 °C (Nannapaneni *et al.*, 2008). In this study, regardless of some exceptions, the effective use of combinations of rosemary extracts with realistic (low) storage temperatures in food models indicated the protection of food products against *L. monocytogenes* and *E. coli*.

As evident from variation in the rosemary plant extracts used and in the level of inoculum, comparative analyses are questionable because of the various methodologies used in different conditions in antimicrobial susceptibility testing. MIC values for RosmE2 were lower or equal to MIC values for RosmE1. As previously reported, the antimicrobial activity depended on the chemical nature of the phenolic compounds in the extracts more than on their concentrations (Klančnik *et al.*, 2009, 2010). MIC values were lower for the lower inoculum level than MIC values determined for the higher inoculum level for *E. coli*, but MIC values were higher for the lower inoculum level for *L. monocytogenes* when they were determined in media without food. MIC values determined in food models were higher for the higher inoculum level for *E. coli* and higher or equal for *L. monocytogenes*. The effect of inoculum level should be determined according to the type of bacteria and their interactions with other parameters that are specific to a particular food.

We can summarise that the MIC of an antimicrobial compound is affected by different parameters such as the nature of the test organism used, the inoculum level, temperature and the composition of the medium. As the food microdilution method using INT produced reproducible results, we suggest this method is suitable for the quantitative determination of antimicrobial effects for aerobic normally growing bacterial strains. This method may be an acceptable alternative to the more expensive and time-consuming classical quantitative determination of bacterial susceptibility to plant extracts in food models using the kinetics of inactivation in the broth macrodilution method. This latter method can be suggested for final confirmation of the antimicrobial effect of a plant extract in a particular food.

Conclusion

Some factors that are important in determining the effectiveness of natural antimicrobials in foods were considered. Commercial rosemary phenolic extracts RosmE1 and RosmE2 were more effective against gram-positive *L. monocytogenes* than against gram-negative *E. coli* and in MHB and in food models. Furthermore, both extracts were more effective against bacteria when applied to food products containing high carbohydrate levels, but lower levels of fats or proteins. Rosemary extracts could be more effective at lower microbial contamination levels, although the type of microorganism seems to have an influence. However, RosmE1 and RosmE2 can be used alone or in combination with the chilling of food products.

Comparison of published *in vivo* data is difficult. Variations related to food product formulations, microorganisms present and their contamination levels, and the environmental conditions contribute to differences in results. An additional problem is extreme variability in the composition of plant phenolic extracts, because of their natural origin and different extraction methods. Finally, the results of nonstandardised antibacterial tests are not comparable. According to our results studies performed in food model, media are necessary prior to further application in food matrices, rather than those obtained in laboratory media. Using the food microdilution method, we evaluated some factors that alone or in combination can affect the efficiency of natural antimicrobials and concluded that the method can be applied as a simple, rapid, reproducible and inexpensive technique in a variety of food models.

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2.3 ZMANJŠANJE ŠTEVILA BAKTERIJ VRSTE *Campylobacter jejuni* Z NARAVNIMI PROTIMIKROBNIMI SNOVMI V RAZMERAH, PODOBNIH PIŠČANČJEMU MESU

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Preverili smo protimikroben učinek izvlečkov rožmarina in bakteriocina nizina na bakterije vrste *Campylobacter jejuni* v različnih razmerah. Za določanje protimikrobne učinkovitosti smo uporabili metodo razredčevanja v mikrotitrski ploščici ter metodo razredčevanja v tekočem gojišču za določanje kinetike rasti in odmiranja. Na koncu smo učinkovitost preverili še na koščkih piščančjega mesa. Učinkovitost smo preverili pri nižji temperaturi inkubacije, z ali brez predhodnega kratkotrajnega zamrzovanja. Potrdili smo, da ima izvleček rožmarina boljši učinek v gojišču, kot pa v piščančjem mesnem soku, ter da nižja temperatura inkubacije podaljša čas preživelosti bakterij vrste *C. jejuni* v piščančjem mesnem soku. Največji protimikrobni učinek je imel izvleček rožmarina, medtem ko nizin sam ni imel protimikrobnega učinka na bakterije vrste *C. jejuni*. Prav tako nismo določili protimikrobnega učinka kombinacije nizina in izvlečka rožmarina. Kombinacija predhodnega zamrzovanja in izvlečka rožmarina je imela sinergističen učinek tako v laboratorijskem gojišču, kot tudi v mesnem modelu.

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Reduction of *Campylobacter jejuni* by natural antimicrobials in chicken meat-related conditions

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ABSTRACT

Nowadays it is essential to test new preservation and decontamination procedures using naturally occurring chemicals against important pathogenic bacteria in meat. We tested the antimicrobial effect of rosemary extracts and the bacteriocin nisin against *Campylobacter jejuni* at a low storage temperature (8 °C) with or without short-term pre-freezing. The antimicrobial effect of rosemary extract was four times greater in laboratory media than in chicken meat juice. Furthermore, low temperature storage conditions prolonged the survival of *C. jejuni* in chicken meat juice. Nisin, with an approximately 1.0 log reduction was neither effective alone nor in combination with the extract. Pre-freezing with plant extract addition proved to be effective treatment by more than 3.0 log reduction in 48 h. The results in chicken meat food model again showed the synergistic effect of freezing and plant extract antimicrobial activity. As the combination of pre-freezing and plant extract treatment reduced the cell number by more than 2.0 log reduction, studies should be conducted to further evaluate this promising treatment for *Campylobacter* reduction in the poultry meat supply.

1. Introduction

Campylobacteriosis is the world's leading bacterial food-borne illness and the most frequently reported zoonosis in humans (EFSA, 2009; Westrell et al., 2009; Smole, Kurinčič, Klančnik, & Mavri, 2010). To reduce the incidence of human *Campylobacter* infections, different approaches have been used to reduce human exposure to *Campylobacter* from broiler meat, the main vehicle of human infection (Corry & Atabay, 2001; Humphrey, O'Brien, & Madsen, 2007). According to recent research (Moran, Scates, & Madden, 2009; Smole Možina, Kurinčič, Kramar, Ursič, & Katalinić, 2009; Suzuki & Yamamoto, 2009) and reports of official monitoring systems (EFSA, 2009), as high as 90–100% of raw chicken meat in retail markets may be contaminated by *Campylobacter*. Several quantitative risk assessments for *Campylobacter* in broiler meat have been developed recently and these conclude that the most effective intervention measures aim at reducing the *Campylobacter* number, rather than reducing the prevalence (Nauta et al., 2009). Current food safety approaches for reduction of *Campylobacter* contamination of retail meat and poultry products include many pre- and post-harvest practices, such as implementation of biosecurity measures at the farm or reduction of *Campylobacter* counts through physical and/or chemical decontamination of meat (Loretz, Stephan, & Zweifel, 2010; Nørrung & Buncic, 2008).Studying physical decontamination of broiler carcasses at slaughter, Boysen and Rosenquist (2009) showed that none of the physical decontamination techniques performed in the slaughter house with naturally contaminated broiler chickens was as effective as freezing. In addition to physical treatments, several recent studies evaluated the effectiveness of chemical compounds in reducing *Campylobacter* on chicken carcasses or related food models such as skin and chicken meat samples (Del Río, Panizo-Morán, Prieto, Alonso-Calleja, & Capita, 2007; Del Río, Gonzales de Caso, Prieto, Alonso-Calleja, & Capita, 2008; Riedel, Brøndsted, Rosenquist, Nygaard Haxgart, & Christensen, 2009). These analyses emphasized that the chemistry of the decontamination agent, treatment time as well as the food matrix affected the outcome in an unpredictable manner and, therefore, further studies are needed to evaluate the reduction effectiveness of specific chemicals (Riedel et al., 2009).

Beside microbial reduction capacity, additional criteria, such as consumer acceptance, human health aspects, development of antimicrobial resistance and environmental safety have to be considered in implementation of chemical decontamination of chicken meat (Riedel et al., 2009). Decontamination treatments

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also must be considered as part of an integral food safety system (Loretz et al., 2010). In this respect it is important that consumers prefer naturally occurring agents, since 'green' consumerism and use of plant extracts is becoming more and more popular (Burt, 2004). Therefore, currently there is a great demand to screen for suitable natural decontaminants and alternative treatment methods (Chouliara, Karatapanis, Savvaidis, & Kontominas, 2007).

For the assurance of food safety nowadays consumers are demanding food products with natural alternatives to chemical additives, and but with increased safety, quality and shelf-life. Antimicrobials are used in food to control natural spoilage processes and to prevent or control the growth of microorganisms, including pathogenic microorganisms (Tajkarimi, Ibrahim, & Cliver, 2010). Much attention has been focused on extracts from herbs and spices which have been traditionally used to improve the sensory, odour or pigment characteristics and extend the shelf-life of foods (Gibbons, 2008). Beside this, it was demonstrated that nisin with GRAS (generally recognized as safe) status did not possess antimicrobial activity against Gram-negative *Escherichia coli* in minced beef, but in combination with essential oil of thyme showed an additive effect (Solomakos, Govaris, Koidis, & Botsoglou, 2008a). Interestingly low temperature and also food material decreases the sensitivity of *Arcobacter*, phylogenetically related to *Campylobacter*, to nisin in broth (Cervenka, 2007; Fisher, Rowe,& Phillips, 2007; Phillips & Duggan, 2002). Rosemary (*Rosmarinus officinalis* L.), a member of the Lamiaceae family, is used as a functional food, and several extract formulations of rosemary have been proven to have antioxidant and also anti-*Campylobacter* activity (Klančnik, Guzej, Kolar, Abramovič, & Smole Možina, 2009). Interestingly, campylobacters have been confirmed to be more sensitive to rosemary extracts than other gram-negative bacteria. Although the exact mechanism behind this stress sensitivity towards natural substances remains to be elucidated, it may be speculated that it depends on the lack of stress adaptive responses in *Campylobacter* present in other gram-negative food-borne bacteria (Klančnik, Guzej, Jamnik et al., 2009; Klančnik et al., 2009).

In this study the antimicrobial activity of selected rosemary extract formulations on *Campylobacter jejuni* in different environmental conditions was examined. The combined effects of the extract and nisin with low (8 °C) storage temperature and/or freezing on the survival of *C. jejuni* in laboratory media and imitated food environment chicken meat juice were monitored. Finally, the activity of selected extract concentrations on *Campylobacter* in a chicken meat model at 8 °C and in combination with short-term pre-freezing was also evaluated.

2. Materials and methods

2.1. Bacterial strains and growth conditions

In the study, the reference strains *C. jejuni* ATCC33560 and *C. jejuni* NCTC11168 as well as poultry meat and human clinical isolate, *C. jejuni* K49/4 and *C. jejuni* 375-06 respectively, were used. The strains were stored in Brain Heart Broth (Merck, 1.10493.0500, Darmstadt, Germany) containing glycerol and lysed horse blood (Oxoid, SR048C, Hampshire, UK) at −80 °C and sub-cultured on Columbia agar base (Oxoid, CM0331, Hampshire, UK) at 42 °C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2).

2.2. Plant extracts formulations and nisin

Lyophilized rosemary extract formulations (V40, V70, A40 and 118, supplied by Vitiva d.d., Markovci, Slovenia) were used in the study. They were dissolved in 96% ethanol to prepare stock solutions at a concentration of 10.0 mg/mL and further diluted in Müller Hinton Broth (MHB, Oxoid, CM0405, Hampshire, UK) to a working solution of 1.25 mg/mL or lower. Nisin (Sigma Aldrich, N5764-25G, St. Louis, USA) stock solutions were prepared in 0.02 M HCl at a concentration of 25.0 mg/mL.

2.3. Testing the effect of chilling temperature and/or short pre-freezing

For determination of the impact of cooling in stress response analysis, the cells were exposed to the cooling temperature either in MHB or chicken meat juice and chilled to 8 ± 1 °C in a microaerophilic atmosphere. Replicate samples were removed from storage each day for 7 days and the surviving campylobacters enumerated. Non-stressed bacterial culture, taken at the same time as the stressed culture, served as the control.

Experiments to test the combined effect of freezing, nisin and plant extract were performed in chicken meat juice. Selected concentrations of plant extract in MHB and initial inoculum in the chicken meat juice at approximately 10^3 CFU/mL were prepared. These samples, with or without nisin added, were frozen to −20 °C and held at −20 °C ± 1 °C in the freezer for 24 h. After 24 h of freezing, samples were thawed and sampled immediately, then stored at 8 °C under microaerophilic conditions. A control sample without added plant extract was also prepared.

2.4. Antibacterial efficiency testing

Minimum inhibitory concentrations (MIC) were determined using the broth microdilution method and calculated from the lowest concentration where no metabolic activity was observed after 24 h on the basis of the absence of a bioluminescence signal, as described by Klančnik et al. (2009) and Klančnik, Piskernik, Jeršek, and Smole Možina (2010). Control wells were prepared with culture medium, bacterial suspension only, plant extracts only and ethanol in amounts corresponding to the highest quantity present. All measurements of MIC values were performed in triplicate and the most representative values were used.

The kinetics of inhibition and/or inactivation was evaluated using the broth macrodilution method as described by Klančnik et al. (2009, 2010). No plant extracts were added to the control sample. Bacterial survival was followed by taking samples at different intervals (0, 4, 24, 48, 72, 96, 120, 168 h) and plating onto modified Abeyta-Hunt-Bark (mAHB, Oxoid, Hampshire, UK) agar plates, which were prepared as described by Rosenquist, Sommer, Nielsen, and Christensen (2006). The MIC was the lowest concentration that inhibited bacterial growth and the minimal bactericidal concentration (MBC) was the lowest concentration which resulted in non-detectable cells after 24 h of incubation. All experiments included two replicants and were independently repeated three or more times and the mean log CFU/mL as well as standard deviations were calculated.

For both micro- and macrodilution methods tested plant extract concentrations were in the range from 1.25 mg/mL to 0.02 mg/mL. For inocula preparation the strain was incubated for 20 h in MHB and for antibacterial activity assays 1.0 mL of each, appropriately diluted (in MHB) to cca. 10^6 CFU/mL, was used. To test the effect of different inocula, i.e. initial concentrations of campylobacters in the tested samples, three inoculum levels were used: approx.10^7 CFU/mL as high, 10^5 CFU/mL as medium and 10^3 CFU/mL as low level of *C. jejuni* culture.

Table 1
Minimal inhibitory concentrations (MICs) of four rosemary extract formulations against different *C. jejuni* strains. A combination of V40 and poultry meat isolate *C. jejuni* K49/4 was selected for further testing.

Plant extract	MIC (mg/mL)			
	C. jejuni ATCC 33560	*C. jejuni* K49/4	*C. jejuni* NCTC 11168	*C. jejuni* 375–06
V40	0.16 ± 0.05	0.16 ± 0.05	0.08 ± 0.02	0.08 ± 0.02
V70	0.08 ± 0.02	0.16 ± 0.05	0.16 ± 0.05	0.16 ± 0.05
A40	0.31 ± 0.10	0.16 ± 0.08	0.16 ± 0.08	0.31 ± 0.10
118	0.16 ± 0.05	0.31 ± 0.18	0.31 ± 0.18	0.31 ± 0.18

2.5. Food models

2.5.1. Chicken meat juice

For the preparation of chicken meat juice, as a well imitated food environment, commercially frozen chickens without giblets were placed in a container, unwrapped and thawed at room temperature. Afterwards the collected chicken meat juice was centrifuged (10,000 rpm for 10 min), sterilized (through a 0.45 μm filter) and stored at −20 °C as previously described (Riedel et al., 2009). Before use, tubes containing chicken meat juice were thawed at room temperature for 1 h (Birk, Ingmer, Andersen, Jørgensen, & Brøndsted, 2004).

2.5.2. Chicken meat model

Meat samples were prepared as described by Riedel et al. (2009), but with some modifications. Chicken meat pieces were carved from commercially bought chicken fillets. Since Campylobacter-free chicken fillets were not available, we added an additional control, which was just a carved piece of chicken meat. No culture or extract was added to that piece. It served only for the purpose of monitoring the possible presence of campylobacters.

Inoculum for food model analysis was prepared as described by Riedel et al. (2009) with final concentration of approx. 10^8 CFU/mL in the 5 mL of chicken meat juice which resulted in 5.5×10^4 CFU/mL on the chicken meat sample.

Samples were dip-treated and controls were prepared consisting of chicken meat samples which were just inoculated. Treatment of the meat samples was performed using 50.0 mL of appropriate extract concentration (0.20 mg/mL in the dipping solution) and dipping of meat samples for 1 min. Samples were then divided into 6 sets: 3 sets of samples were frozen for 24 h at −20 °C, followed by thawing and immediate quantification of one set of samples. The two remaining sets were further stored at 8 °C for 24 h, one set of samples under microaerophilic and one set under aerobic conditions. Of the remaining 3 sets of samples, two sets were stored at 8 °C for 24 h under either microaerophilic or aerobic conditions. One set of samples was quantified immediately after treatment. As controls, sterile water and 10% trisodium phosphate (TSP, 04278 Riedel, Sigma–Aldrich, Germany) in the dipping solution were used. Sterile water was used to determine the effect of the dipping procedure for 1 min (Riedel et al., 2009).

Quantification of *C. jejuni* in meat rinse was performed as described by Riedel et al. (2009), but with some modifications. When 10-fold serial dilutions were made, we spread plated 0.1 mL of the appropriate dilution onto mAHB.

3. Results and discussion

3.1. Selection of plant extracts for further testing

To identify the rosemary extract that was the most efficient in reducing the number of *Campylobacter*, MICs were determined for four rosemary extract formulations, which were selected previously from a larger set of plant phenolic extracts, pure species formulations or their mixtures (Klančnik et al., 2009, 2010; Klančnik, Piskernik, Lipoglavšek, & Smole Možina, 2009) against reference *C. jejuni* ATCC33560, NCTC 11168, as well as human clinical isolate (375-06) and retail chicken meat isolate (K49/4). The results showed that oil-soluble V40 and V70 with carnosic acid as the main antimicrobially active phenolic compound (Klančnik et al., 2009) were the most effective extracts against all strains tested and V40 was selected for further determination of antimicrobial activity. Because the sensitivity of the four strains was not significantly different (Table 1), *C. jejuni* K49/4 isolated from fresh retail chicken meat (Zorman & Smole Možina, 2002) was chosen for further testing.

3.2. Growth inhibition/cell reduction in MHB

First, the growth inhibition/survival of *C. jejuni* in laboratory media at 42 °C under microaerophilic conditions at different initial cell concentrations were monitored. A difference in the efficacy of the extract was observed when a low initial level of cells (10^3 CFU/mL) was tested. At the concentration 0.08 mg/mL V40 did not prevent a slight increase in population of cca. 1.0 log unit (ten-fold), but higher concentrations reduced the population under the detectable level after 24 h (Table 2). In a similar way the efficiency of extracts at chilling incubation temperature, 8 ± 1 °C in MHB was tested. The results are presented in Fig. 1.

At a low initial level of cells (10^3 CFU/mL) a concentration at 0.31 mg/mL of V40 immediately reduced the population by approx. 1.0 log unit (ten times), and under the detectable level in 24 h. The other two concentrations (0.16 mg/mL and 0.08 mg/mL) achieved the same result in 48 h, but the control culture also started to decrease at the same rate. When an initial level of cells of 10^7 CFU/mL was used, the extract addition did not show any significant antimicrobial effect. The number of cells decreased slowly and reached an undetectable level after 7 days (Fig. 1). In comparison to the efficiency in growth inhibition at 42 °C, there was no visible effect at the chilling temperature.

Table 2
Growth inhibition/cell reduction of *C. jejuni* 49/4 in MHB (42 °C for 24 h) at different V40 concentrations and three inoculation levels (approx. 10^7 is high CFU/mL; 10^5 is medium CFU/mL; 10^3 is low CFU/mL). Detection limit was 10^2 CFU/mL.

MHB, 42 °C/Plant extract V 40	High inoculation level (log CFU/mL)		Medium inoculation level (log CFU/mL)		Low inoculation level (log CFU/mL)	
	$t = 0$ h	$t = 24$ h	$t = 0$ h	$t = 24$ h	$t = 0$ h	$t = 24$ h
Control *C. jejuni*	6.51 ± 0.03	8.11 ± 0.16	4.73 ± 0.09	9.04 ± 0.16	3.29 ± 0.06	7.80 ± 0.19
0.08 mg/mL	5.81 ± 0.11	8.15 ± 0.23	4.65 ± 0.04	7.54 ± 0.27	3.12 ± 0.13	4.47 ± 0.30
0.16 mg/mL	5.81 ± 0.09	5.80 ± 0.26	4.72 ± 0.06	4.69 ± 0.19	3.03 ± 0.11	2.89 ± 0.22
0.31 mg/mL	5.81 ± 0.05	ND	4.57 ± 0.14	ND	2.22 ± 0.16	ND

ND not detected.

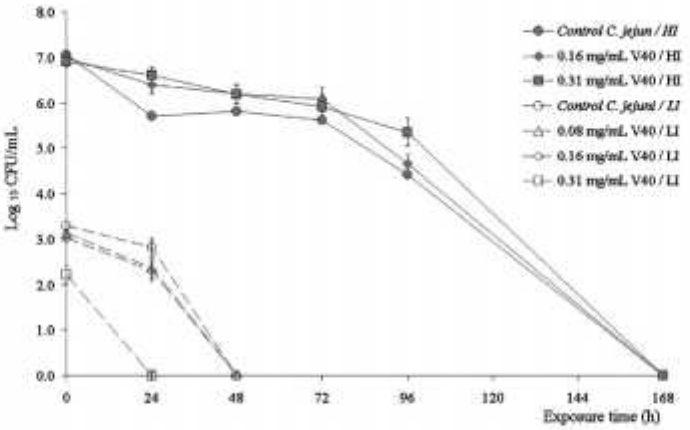

Fig. 1. Survival curves of C. jejuni 49/4 in MHB (at 8 °C for 7 days) at different V40 concentrations and two inoculation levels (approx. 10^7 is high CFU/mL - HI; 10^3 is low CFU/mL - LI).

3.3. Growth inhibition/cell reduction in chicken meat juice

The test to determine MICs was performed at 42 °C under microaerophilic conditions in either MHB or chicken meat juice using the microdilution method. Our results show that the extract was more efficient in laboratory media (0.16 mg/mL) than in chicken meat juice (0.63 mg/mL). Because chicken meat juice is the fluid that is produced when frozen chickens are thawed and it resembles the composition of meat (Birk et al., 2004), the activity of the plant extract may be diminished due to reaction with food components such as lipids, proteins, and carbohydrates. Previous studies have also shown that plant extracts and essential oils are more effective when tested *in vitro* than when evaluated in real foods (Burt, 2004). Therefore, our study was mainly focused on the activity of the plant extract in chicken meat juice that resembles the environment of *Campylobacter* in meat.

The tested extract concentrations had no effect on growth inhibition of *C. jejuni* in chicken meat juice at the high initial cell concentration (10^7 CFU/mL). At medium and low initial cell concentrations the highest extract concentration used (0.31 mg/mL) was efficient in *Campylobacter* reduction as no colonies could be detected (Table 3).

When the test in chicken meat juice was performed at the chilling temperature and two initial cell concentrations, similar results were achieved, as shown in Table 3. Efficient cell reduction was obtained only at high extract concentration and low cell inoculum, and only after 120 h of storage (Fig. 2.).

3.4. Combined conditions for Campylobacter reduction: short-term freezing and plant extract addition

As presented above, plant phenolic extract V40 was not efficient in quick reduction of *C. jejuni* in realistic conditions for meat storage, as chicken meat juice had a protective impact on *C. jejuni* at low temperature. So we continued by testing for possible synergistic activity of an acceptable concentration of V40 (0.20 mg/mL) with nisin and/or short-term freezing. Repeated freeze-thawing can destroy bacteria and it has been reported that strains of *Campylobacter* are sensitive to freezing and/or freeze-thawing (Archer, 2004). Very recently Boysen and Rosenquist (2009) confirmed freezing to be the most efficient physical treatment for reduction of *Campylobacter* counts in industrial conditions at slaughter. However, Bhaduri and Cottrell (2004) showed that *C. jejuni* can survive refrigeration and freezing and that these treatments alone are not enough. Another study showed that the oxidative stress sensitivity of *C. jejuni* depends on temperature and bacterial survival is better at low temperatures (Garenaux et al., 2008).

C. jejuni was monitored over a period of 4 days under the combined effect of V40 (0.20 mg/mL) and nisin (1000 IU) at 8 °C in chicken meat juice, under microaerophilic conditions (Fig. 3). The immediate effect of V40 and the combination of V40 and nisin was an approximately 1.0 log reduction. This reduction was not great enough and the results suggest that nisin had no antimicrobial effect against *C. jejuni*, not even when combined with rosemary extract. This reduction is not correlated with the increased susceptibility of bacteria after treatment with nisin as e.g. just published for *Listeria innocua* (Lehrke, Hernaez, Mugliaroli, von Staszewski, & Jagus, 2010). It has been reported that nisin alone cannot be used as an antimicrobial agent against Gram-negative bacteria (Solomakos, Govaris, Koidis, & Botsoglou, 2008b), but our results suggest that for *C. jejuni* this is also true in combination with the plant extract. Our findings are in agreement with the results obtained by Dykes, Amarowicz, and Pegg (2003). They observed no antimicrobial activity of nisin alone against *C. jejuni*, and also noticed a lack of

Table 3
Growth inhibition/cell reduction of C. jejuni 49/4 in chicken meat juice (42 °C for 24 h) at different V40 concentrations and three inoculation levels (approx.10^7 is high CFU/mL; 10^5 is medium CFU/mL; 10^3 is low CFU/mL). Detection limit was 10^2 CFU/mL.

Chicken meat juice, 42 °C/Plant extract V 40	High inoculation level (log CFU/mL)		Medium inoculation level (log CFU/mL)		Low inoculation level (log CFU/mL)	
	t = 0 h	t = 24 h	t = 0 h	t = 24 h	t = 0 h	t = 24 h
Control *C. jejuni*	6.93 ± 0.08	7.82 ± 0.29	4.68 ± 0.04	9.04 ± 0.18	3.19 ± 0.06	7.09 ± 0.21
0.08 mg/mL	6.80 ± 0.06	7.90 ± 0.18	4.77 ± 0.05	8.54 ± 0.33	3.12 ± 0.04	6.82 ± 0.19
0.16 mg/mL	6.61 ± 0.03	7.96 ± 0.09	4.76 ± 0.04	7.53 ± 0.42	3.27 ± 0.05	6.31 ± 0.32
0.31 mg/mL	6.41 ± 0.10	7.86 ± 0.21	4.67 ± 0.05	ND	3.12 ± 0.08	ND

ND not detected.

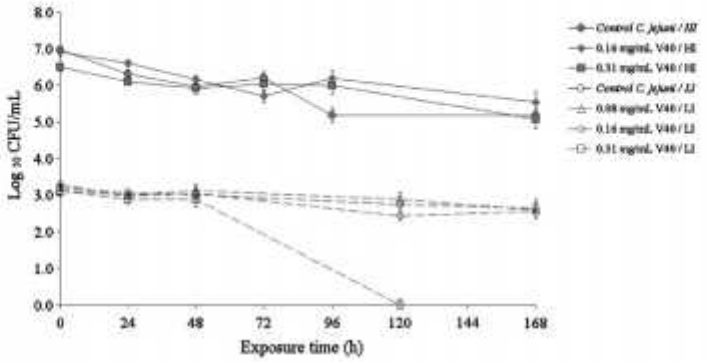

Fig. 2. Survival curves of *C. jejuni* 49/4 in chicken meat juice (at 8 °C for 7 days) at different V40 concentrations and two inoculation levels (approx. 10^7 is high CFU/mL – HI; 10^3 is low CFU/mL – LI).

antimicrobial activity of a nisin and plant extract mixture against *C. jejuni* and some other Gram-negative bacteria.

For testing the effect of freeze/thaw treatment, followed by incubation with the extract and/or nisin, the samples were kept at −20 °C for 24 h, followed by thawing, immediate sampling and subsequent incubation over a period of four days at 8 °C, under microaerophilic conditions. Neither the rosemary extract nor nisin showed any initial effect on *C. jejuni* cells. Again nisin demonstrated no antimicrobial activity, either alone or in combination with the extract. But 24 h-freezing had some effect on the survival of *C. jejuni* as the initial reduction of *C. jejuni* cells was 0.5 log, compared to the *C. jejuni* cells that were not frozen. Although the frozen control survived for (at least) 4 days, extract addition killed *C. jejuni* cells in 48 h. When no freezing was employed beforehand, *C. jejuni* cells treated with the extract survived for a longer period and were killed only after 96 h. The addition of nisin was not confirmed as useful, either alone or in combination with plant extract, but short-term freezing and plant extract synergistically reduced campylobacters under the detectable level (by more than 3.0 logs) in 48 h (Fig. 3).

3.5. Chicken meat model

On the basis of the above results, rosemary extract (at a concentration 0.20 mg/mL in the dipping solution) was chosen for the chicken meat model experiment. The effect of a storage temperature of 8 °C with or without 24 h pre-freezing on the efficacy of V40 on growth inhibition/cell reduction of *C. jejuni* was also assessed in this experiment. As controls, sterile water and 10% TSP dipping solution were used. Sterile water was used to determine the effect of the dipping procedure for 1 min (Riedel et al., 2009) and reduction was less than 0.5 logs. We used TSP as a control, since it is assigned as GRAS by the FDA and is approved for use in the broiler slaughter process in the USA (Capita, Alonso-Calleja, Garcia-Fernandez, & Moreno, 2002). The European Union (EU) has evaluated TSP for application as a decontamination agent on poultry carcasses, but currently chemical decontamination is not approved for use in the EU (SCVPH, 2003). TSP (10 ± 2%) is used as a processing aid in a 15 s treatment which results in approx. 2.0 log reduction of microbial contamination (Capita et al., 2002; Okolocha & Ellerbroek, 2005). In our experiment TSP also achieved an approximately 2.0 logs reduction compared to the non-treated sample (data not shown). All the samples were prepared in one experiment and divided into two groups. One group was dip treated for 1 min, followed by 24 h freezing at −20 °C. After thawing, one third of these samples were quantified immediately, the remaining two thirds were further incubated for 48 h at 8 °C, under microaerophilic or aerobic conditions (data not shown). The same was applied for the other group, only that these samples were not frozen beforehand. This provided the controls to examine and determine the effect of freezing on the survival of *C. jejuni*.

Although the selected extract concentration alone did not possess a significant antimicrobial effect, in combination with

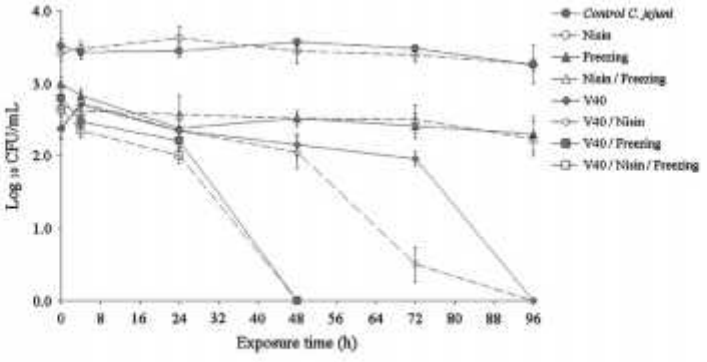

Fig. 3. Survival curves of *C. jejuni* 49/4 in chicken meat juice at 8 °C, plant extract V40 (0.20 mg/mL) and/or nisin (1000 IU) and/or pre-freezing (24 h at −20 °C).

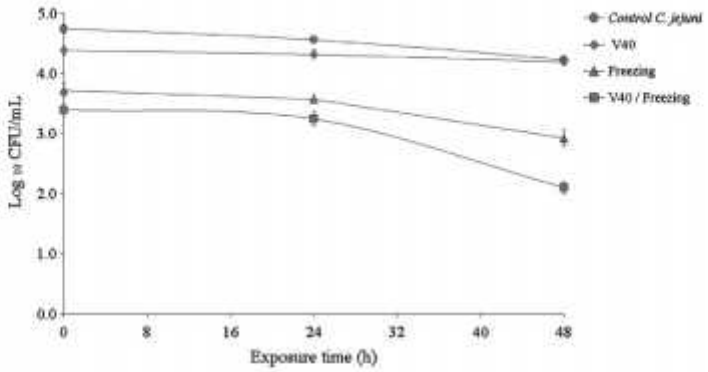

Fig. 4. Survival curves of *C. jejuni* 49/4 in chicken meat model at 8 °C, plant extract V40 (0.20 mg/mL) and pre-freezing (24 h at −20 °C).

short-term (24 h, Fig. 4) freezing an efficient average reduction of *C. jejuni* cells of more than 2.0 logs was achieved in 48 h. A similar effect (data not shown) was achieved when samples were incubated under aerobic conditions.

4. Conclusion

Numerous treatments have been implemented so far to reduce the risk of *Campylobacter* before final distribution of fresh chicken meat to retail outlets. However, campylobacteriosis still remains the most frequently reported bacterial food-borne zoonosis. Therefore, new treatments are needed to reduce this important pathogen on chicken meat. A reduction of *Campylobacter* by 2.0 logs on chicken meat might represent a reduction in *Camyplobacter* risk via chicken meat consumption, as estimated by several risk assessments (Lindqvist & Lindblad, 2008; Loretz et al., 2010; Nauta et al., 2009; Rosenquist, Nielsen, Sommer, Norrung, & Christensen, 2003). One possible method to achieve this reduction could be the application of physical and chemical treatments that reduce the *Campylobacter* concentration on meat. Two important aspects in application of these methods are the antimicrobial activity on the meat and acceptance of the treatment by the consumer. Naturally occurring antimicrobial agents were chosen as these may be accepted by the consumer. We combined these treatments with freezing as the most efficient physical treatment for reduction of *Campylobacter*. Freezing probably results in sensitisation of the bacterial cell membrane and thus increases the antimicrobial activity of natural compounds which are not otherwise antimicrobially efficient in meat storage conditions.

Only the application of an acceptable plant phenolic extract concentration combined with pre-freezing reduced campylobacters by more than 2.0 logs which are considered to be efficient in significant reduction of *Camyplobacter* risk via chicken meat consumption. A better understanding of how bacteria cope with stress conditions (e.g. resistance to antimicrobial compounds with different target sites in the cell, such as hydrophobic plant phenolic extracts and/or bacteriocins, i.e. nisin) on one hand, and adapt to a protective environment (e.g. chicken meat juice/meat components) on the other, will be critical in designing new (combined) intervention strategies and control methods for food safety management.

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2.4 KONTROLA VEGETATIVNIH CELIC IN SPOR BAKTERIJ RODU *Alicyclobacillus* V JABOLČNEM SOKU Z IZVLEČKI ROŽMARINA

Piskernik S., Klančnik A., Gašperlin L., Smole Možina S., Jeršek B. 2016. Control of *Alicyclobacillus* spp. vegetative cells and spores in apple juice with rosemary extracts. Food Control, 60: 205–214

Z metodo razredčevanja v mikrotitrski ploščici smo določili protimikrobni učinek izvlečkov rožmarina na izbrane seve bakterij vrst *A. acidoterrestris*, *A. hesperidum* in *A. cycloheptanicus* v laboratorijskem gojišču. Določili smo vpliv izbranih koncentracij izvlečkov rožmarina na senzorične lastnosti jabolčnega soka ter določili, da dodatek izvlečkov rožmarina v vrednostih MIK ni imel vpliva na barvo, vonj, okus ali motnost jabolčnega soka. S krivuljami preživetja smo spremljali kinetiko bakterijske rasti in ugotovili, da izvlečka rožmarina pri vrednostih MIK zmanjšata število vegetativnih celic tako v laboratorijskem gojišču, kot v jabolčnem soku. Preverili smo tudi učinek izvlečkov rožmarina na spore bakterij vrste *A. acidoterrestris* in določili indekse inhibicije. Vrednosti MIK na spore v laboratorijskem gojišču niso imele bistvenega učinka, v jabolčnem soku pa smo določili >15 % indeks inhibicije. Preverili smo še učinek 4-kratnih vrednosti MIK na bakterijske spore, kjer so rezultati pokazali večji indeks inhibicije v gojišču kot v jabolčnem soku. Izbrana izvlečka rožmarina pri vrednostih MIK nista učinkovala na spore, vendar pa so spore pri teh koncentracijah lahko vzklile. Na tako vzklite vegetativne celice sta izvlečka lahko učinkovala skupaj z nižjo vrednostjo pH soka in drugimi sestavinami jabolčnega soka.

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Control of *Alicyclobacillus* spp. vegetative cells and spores in apple juice with rosemary extracts

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ABSTRACT

In this study, we initially investigated the antimicrobial activities of two commercial rosemary extracts (V20, V40) against *Alicyclobacillus* strains, with the ultimate purpose to determine whether these can be used in apple juice for the control of growth of alicyclobacilli. Minimum inhibitory concentrations (MICs) were first determined, and put through sensory analysis. Addition of the rosemary extracts to apple juice at their MICs did not change the colour, odour, taste or opacity of the apple juice. Growth kinetics studies with these rosemary extracts indicated a reduction in vegetative cells for *Alicyclobacillus acidoterrestris*, *Alicyclobacillus hesperidum* and *Alicyclobacillus cycloheptanicus* in *Bacillus acidoterrestris* broth and in apple juice. Further studies with *A. acidoterrestris* spores showed that the MICs of these rosemary extracts had relatively low effects on spore numbers in *B. acidoterrestris* broth, but had a spore number inhibition index >15% in apple juice. A four-fold increase in the rosemary extract concentrations showed the opposite effects: greater reduction in spores in *B. acidoterrestris* broth (inhibition index, >60%) than in apple juice (inhibition index, <10%). These data indicate that at their MICs, the V20 and V40 rosemary extracts allow outgrowth of spores but reduce vegetative cells, acting together with the low pH or other particular constituents of the apple juice. Rosemary extracts applied at their MICs thus represent an alternative method for the control of *A. acidoterrestris* in apple juice.

1. Introduction

Alicyclobacilli are aerobic or facultative anaerobic and Gram-positive bacteria, and their general characteristics include thermophilic, acidophilic and endospore-forming properties. Their distinctive characteristics include ω-alicyclic fatty acids, which represent the majority of the fatty-acid composition of their cell membrane, although there are some species (such as *Alicyclobacillus pomorum*, *Alicyclobacillus contaminans*, *Alicyclobacillus macrosporangiidus*, *Alicyclobacillus pohliae* and *Alicyclobacillus feeroxydans*) that do not contain this type of fatty acids, but straight and/or branched chain saturated fatty acids (Smit, Cameron, Venter, & Witthuhn, 2011; Wisotzkey, Jurtshuk, Fox, Deinhard, & Poralla, 1992).

Alicyclobacilli are spoilage bacteria, and they represent a serious problem in the fruit-juice industry. This spoilage by alicyclobacilli is most common in apple juice, although other types of juices and iced teas are also susceptible to alicyclobacilli (Duong & Jensen, 2000; Walls & Chuyate, 2000). The main source of isolation of alicyclobacilli is soil and thermal acid springs, although they are also associated with various fruit surfaces and juices, acidic beverages, fruit-juice concentrates, dried hibiscus flowers, various types of herbal teas, iced teas and their ingredients (Smit et al., 2011; Walker & Phillips, 2008), and apple and pear flavouring (Oteiza, Soto, Ortiz Alvarenga, Sant'Ana, & Giannuzzi, 2014).

Alicyclobacilli represent a problem because of their spore-forming nature and their resistance to pasteurisation treatments (Steyn, Cameron, & Witthuhn, 2011). Due to the rise in 'green consumerism', natural antimicrobials have been gaining greater and greater significance (Tajkarimi, Ibrahim, & Cliver, 2010), and the use of natural antimicrobials against alicyclobacilli, such as bacteriocins, has been investigated (Grande et al., 2005; Komitopoulou, Boziaris, Davies, Delves-Broughton, & Adams, 1999; Pei, Yue, & Yuan, 2013). Among the natural antimicrobials that have shown activity against *Alicyclobacillus acidoterrestris*, these are cinammaldehyde, eugenol, limonene and lysozyme (Bevilacqua, Campaniello, Speranza, Sinigaglia, & Corbo, 2013; Bevilacqua, Ciuffreda, Sinigaglia, & Corbo, 2014; Bevilacqua, Corbo, & Sinigaglia, 2008a; Bevilacqua,

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Corbo, & Sinigaglia, 2008b; Bevilacqua, Corbo, & Sinigaglia, 2010). Here, cinnamaldehyde was shown to be more effective against alicyclobacilli than limonene, although combinations of such natural antimicrobials (e.g., cinnamaldehyde and eugenol, biocitro citrus extract and lemon extract) tend to be more effective than when these are tested individually. However, although herbs are used and appreciated for their flavour, there remains the problem of the sensory effects that such natural antimicrobials might have when used in food products. These effects have generally been less studied, but they should not be disregarded (Tajkarimi et al., 2010).

The initial aim of the present study was to investigate the antimicrobial activities of two commercial rosemary extracts (V20, V40) against *A. acidoterrestris*, *Alicyclobacillus hesperidum* and *Alicyclobacillus cycloheptanicus* strains. We then determined the influence of these two rosemary extracts across a wide concentration range on the sensory properties of apple juice, as the ultimate purpose was to determine whether these two rosemary extracts can be used in apple juice for the control of growth of alicyclobacilli. Furthermore, we investigated the antibacterial effects of the sensory-acceptable concentrations of these rosemary extracts on the growth of *A. acidoterrestris*, *A. hesperidum* and *A. cycloheptanicus* strains in 50% apple juice and apple juice. The activities of the selected rosemary extract concentrations were also studied in terms of their effects on the endospores of *A. acidoterrestris*.

2. Materials and methods

2.1. Rosemary extracts and apple juice

In the present study, two commercial rosemary extract formulations known as V20 and V40 were used (Vitiva d.d., Markovci, Slovenia). According to the manufacturer, the main active phenolic compound in these extract formulations is carnosic acid, which they document as representing 19.660% and 40.694% in V20 and V40, respectively. Stock solutions (10 mg/ml) of these extracts were prepared in absolute ethanol (Merck, Darmstadt, Germany), and further diluted in *Bacillus acidoterrestris* (BAT) broth (Döhler, Darmstadt, Germany), 50% apple juice, or apple juice as the working extract solutions. For the preparation of 50% apple juice, equal portions of commercial apple juice (Fructal d.o.o., Slovenia) and BAT broth were mixed aseptically in a sterile flask, with the commercially bought apple juice also used as obtained.

2.2. Strains of Alicyclobacillus

A. acidoterrestris ŽMJ184 (DSMZ 3922, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) was used in the study as the reference strain, along with two alicyclobacillus food isolates, isolated from sugar: *A. hesperidum* ŽMJ193 and *A. cycloheptanicus* ŽMJ197. These strains were stored at −80 °C in BAT broth with 20% glycerol.

2.3. Determination of antibacterial activities

2.3.1. Broth microdilution method

The broth microdilution method was used to determine the antimicrobial effects of the V20 and V40 rosemary extracts against *A. acidoterrestris*, *A. hesperidum* and *A. cycloheptanicus*. Experiments were carried out in BAT broth in 96-well microtitre plates (Nunc, Denmark), as described by Klancnik, Piskernik, Jersek, and Smole Možina (2010). Tested concentrations of both extracts were in the range from 125 μg/ml to 1 μg/ml. Inoculum was prepared by diluting the overnight culture to approximately 10^6 cfu/mL of which spores represented approximately 10^5 cfu/ml. The minimal inhibitory concentrations (MICs) were determined by the change in colour after the addition of 2 mg/ml 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium dye (Sigma–Aldrich, Steinheim, Germany). All of the experiments were carried out in triplicate.

2.3.2. Broth macrodilution method

The growth kinetics studies for the antibacterial activities of the V20 and V40 rosemary extracts were monitored over a period of 24 h, in BAT broth, 50% apple juice, and apple juice. The concentrations of the rosemary extracts used were based on the MICs for the reference strain (*A. acidoterrestris*) and for the two food isolates (*A. hesperidum*, *A. cycloheptanicus*), as determined with the broth microdilution method. The control samples consisted of BAT broth, 50% apple juice, or apple juice and the appropriate alicyclobacillus strain without the addition of the rosemary extracts. The experiments were carried out as previously described by Klancnik et al. (2010). To determine the bacterial growth, samples were taken at different times (i.e., 0, 4, 8, 24 h) and plated on BAT agar (Merck, Darmstadt, Germany). These plates were incubated for 3 days–5 days at 45 °C. Experiments were repeated three times, and the mean number of cells and standard deviations were calculated.

2.4. Sensory analysis

To evaluate the sensory qualities, a panel of four qualified and experienced panellists in the field of fruit juices was formed, with the sensory properties of coded (blinded) samples of apple juice tasted in a standard sensory laboratory. The same panel evaluated all of the samples. On the basis of a preliminary tasting, for the purpose of the present evaluation, the panel decided in favour of, and applied, an analytical-descriptive test (Golob, Jamnik, Bertoncelj, & Kropf, 2005). The analysis was performed by scoring the sensory attributes of apple juice samples according to a non-structured scale from 1 to 4 to 7 (i.e., 1-4-7). Here, a score of 4 points was considered optimal, while scores ≥4.5 indicated greater expression of a property (i.e., to excess; e.g., darker colour, better smell, better flavour), and those of ≤3.5 indicated lesser expression of a property (i.e., insufficient; e.g., paler colour, worst smell, worse flavour). The exception here was for turbidity, which was evaluated by scoring on a structured scale of 1–7 points, where a higher score indicated greater expression of a given property.

For the sensory evaluation, the apple juice samples were conditioned at room temperature (20 °C), and then offered to the panellists in separate glasses for the evaluation. To neutralise the taste between sampling, the panel used the central dough of white bread that was soaked in tepid lemon-flavoured water (1%).

The samples for the sensory analysis were prepared as follows. The V20 and V40 rosemary extracts were dissolved in ethanol at various stock concentrations. The apple juice samples were labelled alphabetically, as A to J. As detailed in Table 1, samples A to D had V20 added to final concentrations from 62.5 μg/ml to 7.8 μg/ml, respectively. Samples E to H had the same for the V40 extract, to final concentrations from 31.3 μg/ml to 3.9 μg/ml, respectively. Sample I was the control sample with added ethanol at 0.78 μg/ml, as the amount of ethanol that was present in samples A to H. Sample J was apple juice without any extract or ethanol additions.

2.5. Spore preparation and analysis

Spores of *A. acidoterrestris* were prepared using sporulation broth and agar medium as described by Silva, Gibbs, Vieira, and Silva (1999). Briefly, overnight cultures of *A. acidoterrestris* grown in sporulation broth at 45 °C were spread-plated onto sporulation agar plates and further incubated at 45 °C for 3 days. Then, 2 ml sterile water was added to each plate, the spores were removed from the surface, and spore suspensions from the various plates were

Table 1
Sensory attributes (means ± standard deviation) of the apple juice according to the different concentrations of the V20 and V40 rosemary extracts added.

Rosemary extract/sample	Sample code	Final concentration (μg/ml)	Sensory property scores			
			Colour (1–4–7)	Smell (1–4–7)	Flavour (1–4–7)	Turbidity (1–7)
V20	A	62.5	3.0 ± 0^{c}	2.5 ± 0^{d}	2.0 ± 0^{d}	3.0 ± 0^{b}
	B	31.3	3.6 ± 0.3^{b}	3.1 ± 0.3^{c}	2.6 ± 0.3^{c}	2.0 ± 0^{b}
	C	15.6	3.8 ± 0.3^{ab}	3.6 ± 0.3^{b}	3.4 ± 0.3^{a}	1.5 ± 0^{c}
	D	7.8	4.0 ± 0^{a}	4.0 ± 0^{a}	3.8 ± 0.3^{a}	1.0 ± 0^{d}
V40	E	31.3	3.3 ± 0.3^{c}	2.4 ± 0.3^{d}	1.6 ± 0.3^{e}	2.0 ± 0^{b}
	F	15.6	3.8 ± 0.3^{ab}	3.1 ± 0.3^{c}	2.4 ± 0.3^{c}	1.5 ± 0^{c}
	G	7.8	3.9 ± 0.3^{ab}	3.5 ± 0.4^{b}	3.0 ± 0.4^{b}	1.1 ± 0.3^{d}
	H	3.9	3.9 ± 0.3^{ab}	3.8 ± 0.3^{ab}	3.6 ± 0.3^{a}	1.0 ± 0^{d}
Ethanol	I	0.78 μl/ml	3.9 ± 0.3^{ab}	4.0 ± 0^{a}	3.8 ± 0.3^{a}	1.0 ± 0^{d}
Control	J	No addition	4.0 ± 0^{a}	4.0 ± 0^{a}	4.0 ± 0^{a}	1.0 ± 0^{d}

Data are means ± standard deviation (n = 4).
Means with different letters within columns (a, b, c, d, e) differ significantly ($p \leq 0.05$; significance of differences between different concentrations and V20 or V40 rosemary extract added). With a structured scale 1-4-7, a score of 4 points is optimal, a score of ≥4.5 represents improved attributes, and a score of ≤3.5 represents diminished attributes. With a structured scale of 1–7, a higher score represents an enhanced attribute (turbidity).

combined and centrifuged at 9500× g for 15 min at 4 °C. The pellet was resuspended in 50% (v/v) aqueous ethanol, and centrifuged after 60 min, as before. The supernatant was discarded, and the pellet was washed four times with sterile water, with centrifugation as before. The spore suspension in sterile water was kept at 4 °C for a minimum of 1 week before use. Before the experiments, the spore suspension was heat treated at 80 °C for 10 min to kill vegetative cells, and the total spore count was determined (Silva et al., 1998).

The effects of the rosemary extracts on *A. acidoterrestris* spores were evaluated using the broth macrodilution method. These experiments were carried out in BAT broth, 50% apple juice, and apple juice. The V20 and V40 rosemary extracts were tested at two concentrations, as the MICs and at a four-fold higher concentration, with a control sample without the addition of the rosemary extracts included. These samples were inoculated with the spore suspension and incubated at 45 °C, with the numbers of spores followed by taking samples at different times (i.e., 0, 4, 24, 48 h). Each sample at each time was heated to 80 °C for 10 min, to destroy the vegetative cells, and was then spread-plated onto BAT agar. These plates were incubated for 3 days–5 days at 45 °C. Experiments were repeated three times and the mean cell numbers and spore numbers were determined as described for the mean number of viable cells. Vegetative cell numbers were calculated as the differences in the cell numbers between the non-heated and heated samples at each given sampling time. The inhibition index was calculated as follows:

$$I.I. = \frac{(Nc - Ns)}{Nc} * 100, \quad (1)$$

where Nc is the number of spores (log cfu/ml) in the control sample, and Ns is number of spores (log cfu/ml) in the experimental samples (Bevilacqua et al., 2008a).

2.6. Data analysis

One-way analysis of variance (ANOVA) and Duncan's multiple range tests with Excel, version 2003 were used to determine the significance of the differences between control samples and samples treated with either V20 or V40 rosemary extracts. The differences were calculated for vegetative cell numbers and spore numbers after either 24 h or 48 h of incubation. The experimental data for sensory qualities and inhibition indices were evaluated statistically using the SAS/STAT programme (SAS Software, 1999). The basic statistical parameters were calculated using the MEANS procedure. The data obtained were tested for normal distributions and analysed using the general linear model procedure. The statistical model for the sensory attributes of the apple juice included the main effects of the treatment groups (i.e., rosemary extract and concentration used: A–J). The statistical model for the inhibition indices against the *A. acidoterrestris* spores included the main effects of the treatment groups (i.e., two rosemary extracts at two concentrations), the three types of media (i.e., BAT broth, 50% apple juice, apple juice), and the interaction for the treatment group × type of media. The least square means (referred to as means in the text) for the experimental groups were obtained using the relevant procedure, and they were compared at the 5% probability level.

3. Results

3.1. Antibacterial efficiency testing

The MICs for the V20 and V40 rosemary extracts were determined using the broth microdilution method, on the basis of the concentration where no metabolic activity was observed, as described by Klančnik et al. (2010). The results showed that both of these rosemary extracts that contained carnosic acid as the main antimicrobially active phenolic compound were effective against *A. acidoterrestris*, *A. hesperidum* and *A. cycloheptanicus*, with MICs of 7.8 μg/ml for V20, and 3.9 μg/ml for V40.

3.2. Sensory analysis

We then determined the influence of these added V20 and V40 rosemary extracts on the sensory properties of apple juice. The rosemary extracts were added over the range of doubling concentrations starting from their MICs, as the final concentration ranges of 7.8 μg/ml to 62.5 μg/ml for V20 (sample code A–D), and 3.9 μg/ml to 31.3 μg/ml for V40 (sample code E–H). An ethanol control sample was included as 0.78 μl/ml added to apple juice (sample code I), because the rosemary extracts were dissolved in ethanol prior to their addition. The no-addition control was apple juice with no rosemary extract or ethanol added (sample code J).

The sensory attributes of the apple juice samples were scored with a non-structured scale from 1 to 4 to 7, where a score of 4 points was regarded as optimal, scores of ≥4.5 indicated improved attributes, and scores of ≤3.5 indicated diminished attributes. With regards to the turbidity, a structured scale was used, with a score scale from 1 to 7 points, where a higher score indicated an enhanced turbidity.

As can be seen from the data in Table 1, the additions of the different concentrations of the V20 and V40 rosemary extracts to

apple juice had significant effects on the descriptors evaluated for the colour, smell, flavour and turbidity. At the MICs, as the concentrations of 7.8 μg/ml V20 and 3.9 μg/ml V40, these did not significantly affect the sensory qualities of the apple juice (Table 1, D, H; $p \geq 0.05$). However, the addition of the higher concentrations of the V20 and V40 rosemary extracts to apple juice (Table 1, A–C, E–G) significantly affected the sensory qualities, which were no longer acceptable: the colour became brighter, the smell and flavour were worse, and turbidity appeared. Although the colour changes were only significant for the highest concentrations of V20 and V40, the two-fold and four-fold increases in V20 and V40 showed significant worsening effects for smell, flavour and turbidity (Table 1).

3.3. Growth kinetics and growth inhibition of vegetative cells

The growth kinetics over the 24-h period in the laboratory BAT broth for the *A. acidoterrestris* reference strain and for the two food isolates of *A. hesperidum* and *A. cycloheptanicus* were comparable (Fig. 1). The addition of V20 and V40 at their MICs showed significant antimicrobial effects and growth inhibition of the vegetative

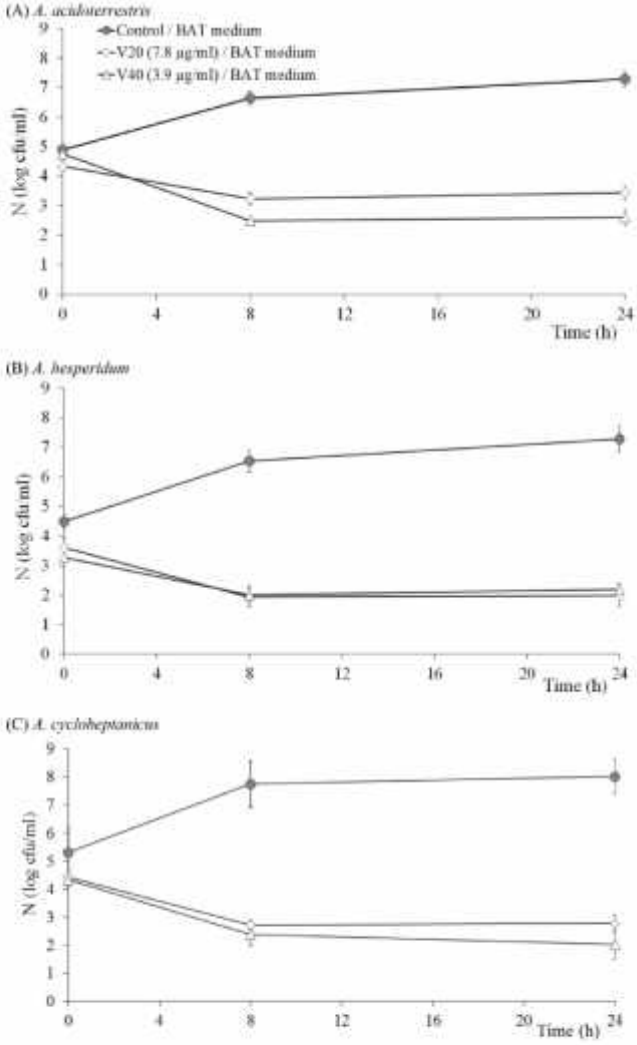

Fig. 1. Growth kinetics of *A. acidoterrestris* (A), *A. hesperidum* (B) and *A. cycloheptanicus* (C) in BAT broth without (control) and with added V20 (7.8 μg/ml) and V40 (3.9 μg/ml) rosemary extracts. Data are means ± standard deviations (n = 3).

cells of all three of these strains of alicyclobacilli. Both of these extracts showed the greatest effects during the first 8 h of incubation, with cell concentrations of 3.2 log cfu/ml and 2.5 log cfu/ml for V20 and V40, respectively (Fig. 1A). The antibacterial effects remained almost the same over the final 16 h of incubation, and reached cell concentrations of 3.4 log cfu/ml and 2.6 log cfu/ml for V20 and V40, respectively (Fig. 1A). Similar cell concentrations were confirmed with V20 and V40 for *A. hesperidum* and *A. cycloheptanicus* (Fig. 1B and C). This growth inhibition showed greater effects of V40 over V20 on these alicyclobacilli, except for *A. hesperidum*. There was a significant difference between mean cell numbers of the control sample and samples containing both of the extracts after 24 h of incubation ($p < 0.05$).

Previous studies have also shown that plant extracts can be more effective for inhibition of bacterial cell growth when tested *in vitro* than when evaluated in actual foods (Burt, 2004; Piskernik, Klančnik, Tandrup Riedel, Brøndsted, & Smole Možina, 2011). Therefore *A. acidoterrestris*, *A. hesperidum*, and *A. cycloheptanicus* were monitored over a period of 24 h under the treatments with the MICs of V20 (7.8 μg/ml) and V40 (3.9 μg/ml) in 50% apple juice and apple juice (Fig. 2). Here the alicyclobacillus growth was inhibited with both of these growth media, with final cell

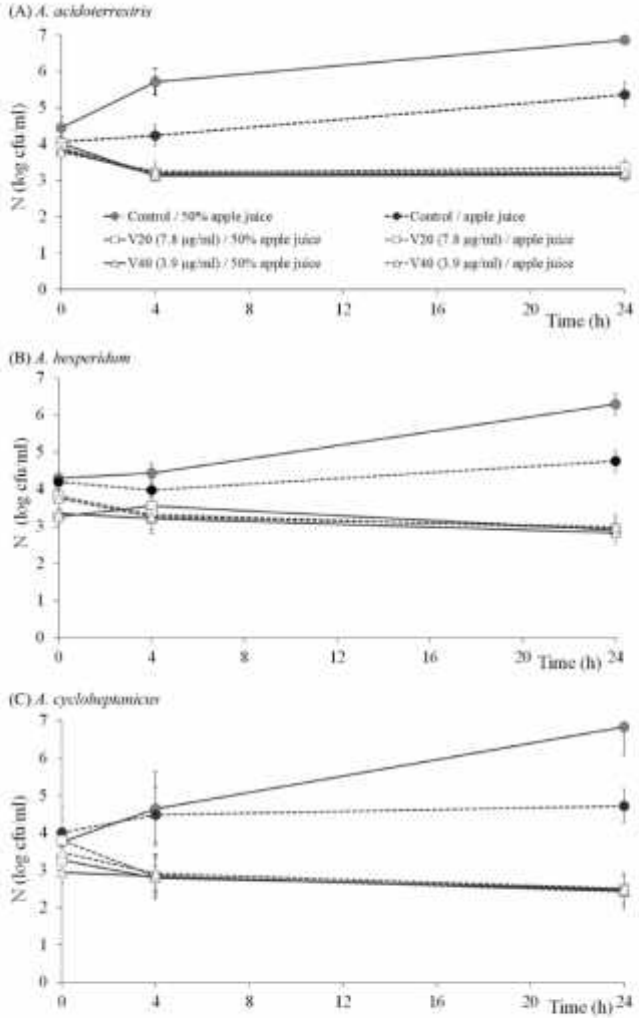

Fig. 2. Growth kinetics of *A. acidoterrestris* (A), *A. hesperidum* (B) and *A. cycloheptanicus* (C) in 50% apple juice and apple juice without (control) and with added V20 (7.8 μg/ml) and V40 (3.9 μg/ml) rosemary extracts. Data are means ± standard deviations ($n = 3$).

concentrations in 50% apple juice from 6.3 log cfu/ml to 6.9 log cfu/ml according to the different alicyclobacillus strains, and in apple juice from 4.7 log cfu/ml to 5.4 log cfu/ml for all three strains. Thus, significant growth inhibition was obtained at the MICs of both the V20 and V40 extracts, with the cell concentrations at around 3 log cfu/ml after 24 h in 50% apple juice and apple juice ($p < 0.05$).

Interestingly, with no additions made to the incubations, when all three of these alicyclobacilli strains were monitored in 50% apple juice and apple juice, the data showed lower growth kinetics when comparing to BAT broth, as seen from the differences in the cell concentrations for *A. acidoterrestris* after 24 h: 6.9 log cfu/ml in 50% apple juice, and 5.4 log cfu/ml in apple juice, in comparison to 7.3 log cfu/ml in BAT broth (Figs. 1A and 2A). The data for the growth kinetics of the other two strains, *A. hesperidum* and *A. cycloheptanicus* (Figs. 1B,C and 2B,C) confirm these differences in the vegetative cell numbers for the 50% apple juice and apple juice compared to BAT broth, of 1.5 log cfu/ml to 2.1 log cfu/ml.

3.4. Effects of rosemary extracts on Alicyclobacillus spores

After we determined that these rosemary extracts inhibit the growth of *Alicyclobacillus* vegetative cells also in 50% apple juice and apple juice, we tested the effects of the V20 and V40 rosemary extracts on the spores of the selected *A. acidoterrestris* strain. Here, again, we tested the sensory-acceptable MICs of the V20 (7.8 μg/ml) and V40 (3.9 μg/ml) extracts. Additionally, the four-fold higher concentrations of the rosemary extracts, of 31.3 μg/ml and 15.6 μg/ml, respectively, were tested, and these data serve as a comparison to determine in what way these extracts affect the spore numbers. The reason for the inclusion of the four-fold higher concentrations related to the nature of the spores. We expected that at their MICs, these rosemary extracts would not show sufficient activity against the spores, so we also tested these higher concentrations.

As the spore suspensions were heat treated before the experiments (see Section 2.5), there were no vegetative cells present in the initial inoculum. When the spore dynamics were monitored in BAT broth, under the control conditions (no addition of extracts), the number of spores initially decreased at 4 h (3.0 log cfu/ml), and then increased over the following 24 h (6.4 log cfu/ml), which was then maintained from 24 h to 48 h of incubation (6.5 log cfu/ml). With the addition of both concentrations of V20, the number of spores also initially decreased at the same rate over the first 4 h, but then remained low over the following 24 h (spore numbers, 2.0 log cfu/ml and 2.6 log cfu/ml for MIC and four-fold higher concentration, respectively; Fig. 3A). However, when monitored again after 48 h of incubation, V20 extract at the MIC was no longer effective, as the spore numbers increased to 6.5 log cfu/ml. In contrast, when the four-fold higher concentration of V20 was used, the spore numbers remained unchanged from 24 h to 48 h, at 2.5 log cfu/ml (Fig. 3A). For the number of vegetative cells, these increased during the first 4 h of incubation for the control sample (4.6 log cfu/ml) as well as for samples containing both concentrations of V20 extract (2.9 log cfu/ml and 3.0 log cfu/ml for MIC and four-fold higher concentration, respectively), which was due to outgrowth of spores. From this point, the kinetics of the vegetative cells for the control sample and both of the V20 extract concentrations were similar for the spore dynamics. The number of vegetative cells remained approximately the same over the following 24 h of incubation, but increased to 6.3 log cfu/ml at 48 h of incubation. V20 at its MIC was not effective enough, and the vegetative cell numbers increased to 6.1 log cfu/ml at 48 h of incubation. The four-fold higher V20 concentration maintained the vegetative cell numbers at 2.1 log cfu/ml (Fig. 3A). With both spore and vegetative cell numbers, significant differences were observed between the control sample and the samples treated with the four-fold higher concentration of V20 extract ($p < 0.05$).

The data for the spore numbers of these alicyclobacilli in the 50% apple juice and apple juice confirmed the effects on the vegetative cell growth kinetics, again indicating that these media are less favourable for the growth of these alicyclobacilli, compared to BAT broth. However, as can be seen from the data in Fig. 3B and C, neither 50% apple juice nor apple juice were optimal for spore germination. This can be seen from the lower spore numbers of the non-treated control *A. acidoterrestris* in 50% apple juice (5.2 log cfu/ml) and apple juice (4.6 log cfu/ml) over the full 48 h incubations. These final spore numbers were comparable to the initial spore numbers, which indicates that only low numbers of the spores germinated (Fig. 3B and C). There were significant differences in spore numbers between the control and treated samples in 50% apple juice ($p < 0.05$) and between the control sample and the sample with the MIC of V20 in apple juice ($p < 0.05$). For the vegetative cells, an outgrowth of spores was seen at 4 h of incubation (3.7 log cfu/ml in 50% apple juice, and 3.7 log cfu/ml in apple juice), but the vegetative cell numbers were maintained at approximately the same levels during the further incubations (4.7 log cfu/ml in 50% apple juice, and 4.1 log cfu/ml in apple juice) (Fig. 3B and C). Here, there was a significant difference between vegetative cell numbers of the control sample and the sample treated with the four-fold higher concentration of V20 extract in apple juice ($p < 0.05$). These data in Fig. 3B and C also show that the V20 rosemary extract was effective in 50% apple juice and apple juice, although its effects were delayed, as the conditions for spore germination were not optimal. Similar spore dynamics and vegetative cell kinetics were observed for the V40 extract (Fig. 4).

To more directly compare these effects of the addition of the two concentrations of the V20 and V40 rosemary extracts on the spore numbers of *A. acidoterrestris* obtained in BAT broth, 50% apple juice, and apple juice, the inhibition indices were calculated from the data for the spore numbers at the end of the incubations, as suggested by Bevilacqua et al. (2008a) (Table 2). To determine the effects of the media on spore numbers of *A. acidoterrestris*, the inhibition indices were compared with respect to growth medium, and to the V20 and V40 concentrations (Table 2). The highest index of inhibition was obtained in BAT broth at the four-fold higher concentration of V20 (61.0%) and at the four-fold higher concentration of V40 (65.4%), while it was lower in 50% apple juice (MIC of V20, 27.1%; four-fold higher concentration of V40, 19.2%) and in apple juice (MIC of V20, 16.3%; MIC of V40, 20.6%). However, in the comparisons of the inhibition indices obtained in all three of the media after 48 h of incubation, there was a difference between these in all three of the media for both the rosemary extracts and their concentrations.

To determine the effects of the rosemary extracts (V20, V40) and their concentrations (MIC, four-fold higher concentration) on spore numbers of *A. acidoterrestris*, the inhibition indices were compared with respect to the tested media (Table 2). After 48 h of inhibition, the inhibition indices were higher at the four-fold higher concentrations of V20 and V40 in BAT broth, while in 50% apple juice and apple juice this was not seen with V20 (the inhibition index was higher at the MIC than at the four-fold higher concentration), and also in apple juice, the inhibition index of V40 at its MIC was higher (20.6%) than at its four-fold higher concentration (9.2%).

4. Discussion

The food industry is increasingly focussed on alternative preservation methods that might replace traditional food preservation techniques. This is especially the case for the heat-resistant alicyclobacilli, which have numerous routes for contamination, including the raw materials, equipment, and water used in the

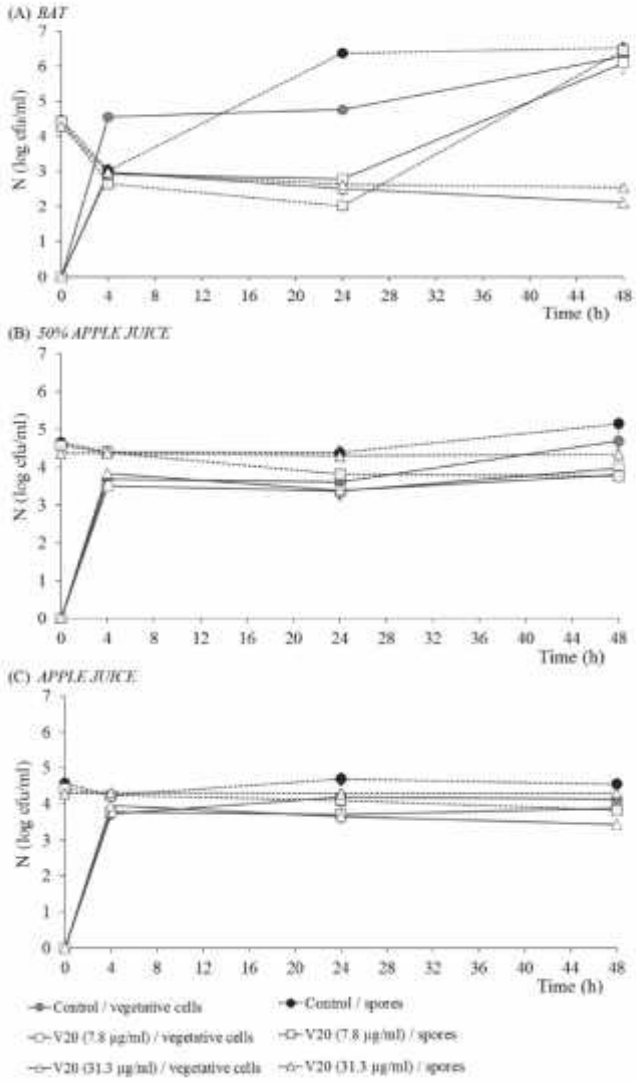

Fig. 3. Growth kinetics of *A. acidoterrestris* spores and vegetative cells without (control) and with V20 rosemary extract (7.8 µg/ml, 31.3 µg/ml) in BAT broth (A), 50% apple juice (B) and apple juice (C). Data are means ± standard deviations (n = 3).

processing. The control of alicyclobacilli vegetative cells and spores during fruit processing, and especially in fruit juice, is thus important for the prevention of further spoilage during storage and to avoid off-odours (Chang & Kang, 2004; Merle & Montville, 2014; Tianli, Jiangbo, & Yahong, 2014).

In the present study, we investigated the control of vegetative cells and spores of alicyclobacilli in apple juice using two rosemary extracts. To the best of our knowledge, only a few studies have investigated the antimicrobial activities of natural substances in apple juice or other juices, and also while including a sensory analysis (Bevilacqua et al., 2010; Giner et al., 2012; Tyagi, Gottardi, Malik, & Guerzoni, 2013). Indeed, to determine how useful these rosemary extracts might actually be, the sensory analysis gives us an important perspective. Here, we have confirmed that different concentrations and types of commercial rosemary extracts can have significant impact on the sensory qualities when added to

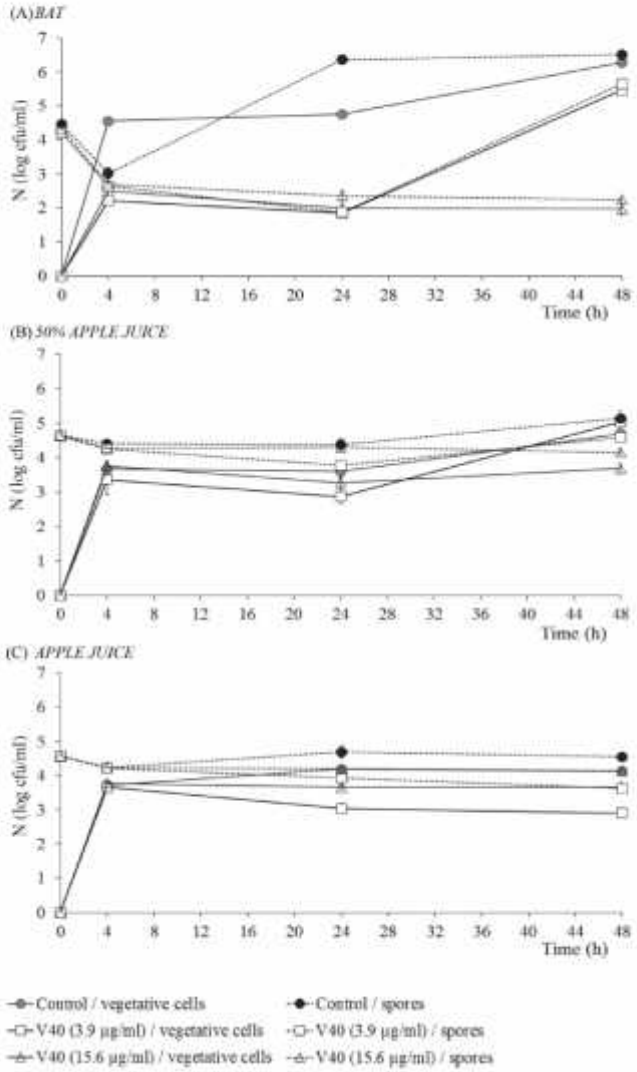

Fig. 4. Growth kinetics of *A. acidoterrestris* spores and vegetative cells without (control) and with V40 rosemary extract (7.8 μg/ml, 31.3 μg/ml) in BAT broth (A), 50% apple juice (B) and apple juice (C). Data are means ± standard deviations (n = 3).

apple juice. However, we demonstrated that at the MICs against *A. acidoterrestris*, *A. hesperidum* and *A. cycloheptanicus* of both the V20 (7.8 μg/ml) and V40 (3.9 μg/ml) rosemary extracts, there were no effects on the sensory characteristics of apple juice. These V20 and V40 concentrations were further tested for growth inhibition of vegetative cells and for reduction of spore numbers.

According to these MICs of the V20 and V40 rosemary extracts tested, which differ in their chemical compositions mainly in terms of their contents of carnosic acid (19.7%, 40.7%, respectively, according to the manufacturer), they are more effective on these alicyclobacillus strains compared to several Gram-negative bacteria, such as *Campylobacter jejuni* and *Campylobacter coli* (MIC range, 15.6 μg/ml to 62.5 μg/ml), and *Escherichia coli* and *Salmonella infantis* (MICs, >250 μg/ml), and also several Gram-positive bacteria, such as *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes* (MIC range, 7.8 μg/ml to 15.6 μg/ml) (Klančnik et al., 2010).

The data for inhibition of the growth kinetics of these alicyclobacilli showed better survival of *A. acidoterrestris*, *A. hesperidum* and

Table 2
Inhibition indices for the *A. acidoterrestris* spore numbers after the 48 h incubation in BAT broth, 50% apple juice, and apple juice for the V20 and V40 rosemary extracts.

Medium	Inhibition index (%)				
	V20 rosemary extract		V40 rosemary extract		p_C Value
	7.8 μg/ml (MIC)	31.3 μg/ml	3.9 μg/ml (MIC)	15.6 μg/ml	
BAT broth	0.8 ± 2.8Cc	61.0 ± 1.2Aa	13.1 ± 4.0ABb	65.4 ± 0.4Aa	<0.0001
50% apple juice	27.1 ± 1.2Aa	15.7 ± 0.0Bc	10.7 ± 0.8Bd	19.2 ± 0.5Bb	0.0001
Apple juice	16.3 ± 4.5Ba	5.7 ± 1.2Cb	20.6 ± 1.8Aa	9.2 ± 0.8Cb	0.014
p_M Value	0.008	<0.001	0.068	<0.0001	

Means with different letters within a row (a,b,c) differ significantly ($p \leq 0.05$, significance of differences between different extracts at different concentrations); means with different letters within a column (A, B, C) differ significantly ($p \leq 0.05$, significance of differences between BAT broth, 50% apple juice and apple juice). Data are means ± standard deviation ($n = 3$).

A. cycloheptanicus in the laboratory BAT broth, which indicates that a characteristic or constituent of apple juice and other factors contributes to this slower growth in apple juice. There are many factors that can affect the activity of plant extracts, such as pH and composition of the food, and the type and amount of antimicrobial used (Negi, 2012). As reported by Grande et al. (2005), different fruit juices also have different influences on the growth of alicyclobacilli, with better survival of their vegetative cells seen for pear juice than pineapple juice. Bevilacqua et al. (2010) also noted that lower amounts of natural antimicrobials were needed to inhibit growth of alicyclobacilli in apple juice than in their laboratory media, which was due to the apple juice used being a less favourable environment for their growth. However, in the present study, both of the rosemary extracts were efficient in preventing or controlling the growth of alicyclobacilli vegetative cells, both in the laboratory media and when added to 50% apple juice and apple juice.

When comparing the inhibition indices after 48 h of incubation, the four-fold higher concentrations of both extracts were effective in BAT broth, but in apple juice, the MICs were more effective than the four-fold higher concentrations of both extracts. As can be seen from the results for vegetative cell numbers, the outgrowth of spores was the greatest in BAT broth. Also, the effects of higher concentrations of both extracts were greater in BAT broth, as can be seen from the higher inhibition indices. The outgrowth of spores was weaker in both 50% apple juice and apple juice. The inhibition indices were lower in 50% apple juice and apple juice than in BAT broth, which is due to the influence of the apple juice on alicyclobacilli growth, which was weaker here than in BAT broth. Also, as shown by Bevilacqua et al. (2008b), a lower pH of the medium improved the activity of cinnamaldehyde, compared to a higher medium pH. As apple juice already has a lower pH (here, it was 3.71) than BAT broth (at pH 4.06), it appears that this also helps to explain the better efficiency of the extracts in the apple juice.

The highest inhibition index was achieved with the MIC values for both of the extracts tested after 48 h of incubation in apple juice. This is comparable to the reduction in spores in apple juice promoted by 80 μg/ml biocitro and lemon extracts in combination with mild heat treatment (Bevilacqua et al., 2013). However, our data show that the higher concentrations of the extracts, as those that were four-fold their MICs, makes it more difficult for spores to germinate. Bevilacqua et al. (2008b) showed that cinnamaldehyde causes a loss of germination ability according to its increasing concentrations. Our data show that when germination was inhibited, fewer vegetative cells manage to form, and therefore the extracts had lower effects on the spore numbers. The indirect effects of epigallocatechin gallate on vegetative cells of different strains of *Bacillus* after germination suggested that spores cannot adsorb these phenolic compounds (Shigemune et al., 2012). Molva and Baysal (2015) demonstrated that a grape-seed extract can cause changes to the cell structure and also inhibit the formation of spores of *A. acidoterrestris*, although their extract did not compromise the spores. They also did not see germination of the spores into vegetative cells in samples treated with their grape-seed extract, whereas both vegetative cells and spores were present in non-treated samples (Molva & Baysal, 2015).

Overall, the data in the present study show that more spores germinate at the lower concentrations of the extracts, and therefore there were greater effects of the extracts on the vegetative cells. Thus, the bioactivities of these rosemary extracts on the alicyclobacilli spores that will be present in apple juice clearly demonstrate their potential for use to reduce microbiological risk of a variety of fruit products.

5. Conclusions

These rosemary extracts (V20, V40) can be used as natural additives to apple juice, as they do not change the sensory qualities of the juice at the same concentrations that they inhibit vegetative cells of *A. acidoterrestris*, *A. hesperidum* and *A. cycloheptanicus*. Furthermore, in this study of the activities of these rosemary extracts against the spores of *A. acidoterrestris* in apple juice, we have shown that these low concentrations of extracts (i.e., at their MICs) effectively reduce the spore numbers. These rosemary extracts at these low concentrations did not show sporicidal effects, but they allowed germination of the spores and instead inhibit cell growth, thus reducing the vegetative cell numbers. The addition of these rosemary extracts to apple juice might thus be one of the promising alternatives in the control of alicyclobacilli, which represent important food-spoilage bacteria.

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3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

3.1.1 Primerjava metod za določanje protimikrobne učinkovitosti

V literaturi najdemo številne metode za določanje protimikrobne učinkovitosti rastlinskih izvlečkov. Poleg različnih metod, obstaja tudi več različnih definicij vrednosti MIK. Zaradi teh razlik je težko med sabo primerjati različne študije in njihove rezultate (Burt, 2004; Tajkarimi in sod., 2010). Z različnimi metodami smo preverili in določili protimikrobno učinkovitost različnih rastlinskih izvlečkov, mešanic rastlinskih izvlečkov ter posameznih kislin. Uporabili smo metodo difuzije v trdnem gojišču, metodo razredčevanja v trdnem in v tekočem gojišču. Metodo razredčevanja v tekočem gojišču smo uporabili za določanje kinetike rasti in odmiranja preiskovanih bakterij ob dodatku izvlečka ter za določanje vrednosti MIK izvlečkov z uporabo metode razredčevanja v tekočem gojišču v mikrotitrski ploščici. Želeli smo primerjati rezultate dobljene z različnimi metodami in glede na te rezultate podati priporočila za enoten postopek določanja protimikrobne učinkovitosti rastlinskih izvlečkov.

3.1.1.1 Primerjava rezultatov dobljenih z metodo difuzije in metodo razredčevanja v trdnem gojišču

Z metodo difuzije in razredčevanja v trdnem gojišču smo preverili različne koncentracije izbranih izvlečkov, mešanic izvlečkov in čistih fenolnih kislin. Vrednosti MIK določene z metodo difuzije so bile pri grampozitivnih bakterijah v razponu od 0,313 mg/ml do 40,0 mg/ml. Med grampozitivnimi bakterijami so bile na rastlinske izvlečke najbolj občutljive bakterije vrste *B. cereus*. Gramnegativne bakterije so bile na delovanje izvlečkov bolj odporne, vrednosti MIK so se gibale od 10,0 mg/ml do 100,0 mg/ml, med njimi pa so bile najbolj občutljive bakterije rodu *Campylobacter*. Pri primerjavi rezultatov dobljenih z metodo difuzije v trdnem gojišču in metodo razredčevanja v trdnem gojišču smo določili razlike v dobljenih vrednostih MIK pri vseh uporabljenih izvlečkih. Vrednosti MIK dobljene z metodo difuzije v trdnem gojišču so bile od 2 do 30 krat višje, kot vrednosti dobljene z metodo razredčevanja v trdnem gojišču.

Izvedba metode difuzije v trdnem gojišču z diski je enostavna in cenovno ugodna. Z uporabo te metode smo dobili višje vrednosti MIK, zato ta metoda ni ustrezna oz. primerna za preverjanje protimikrobnega učinka rastlinskih izvlečkov. Višje vrednosti MIK, dobljene z metodo difuzije z diski v primerjavi z ostalimi metodami, so določili tudi drugi avtorji (Bubonja-Sonje in sod., 2011; Djenane in sod., 2012). Pomanjkljivost metode difuzije z diski predstavlja tudi dejstvo, da pri nekaterih izvlečkih ne določimo vidnih inhibicijskih con, kar pa ne pomeni, da izvleček ni učinkovit. Na inhibicijske cone lahko vpliva topnost izbrane protimikrobne snovi in obseg difuzije. Izvlečki, ki so slabo topni v vodi težje prehajajo v gojišče, kar pomeni slabšo protimikrobno učinkovitost (Bubonja-Sonje in sod., 2011; Valgas in sod., 2007). Podobno na premer inhibicijske cone vpliva polarnost protimikrobne snovi, saj nepolarne oz. manj polarne spojine težko prehajajo v gojišče (Tan in Lim, 2015). Zato je med tema dvema metodama boljša izbira in uporaba metode razredčevanja v trdnem gojišču.

3.1.1.2 Primerjava rezultatov dobljenih z metodami razredčevanja

Pri primerjavi rezultatov dobljenih z metodo razredčevanja v trdnem in metodo razredčevanja v tekočem gojišču, smo za večino izvlečkov pri grampozitivnih bakterijah določili dobro povezavo oz. skladanje med dobljenimi vrednostmi MIK. Pri gramnegativnih bakterijah pa se rezultati teh dveh metod ne skladajo, saj dobimo nižje vrednosti MIK z metodo razredčevanja v tekočem gojišču.

Podobno je razvidno pri primerjavi rezultatov dobljenih z metodo razredčevanja v trdnem gojišču in metodo razredčevanja v mikrotitrski ploščici. Pri grampozitivnih bakterijah se rezultati skladajo za večino izvlečkov, pri gramnegativnih pa je razlik med obema metodama več. Vrednosti MIK dobljene z metodo razredčevanja v mikrotitrski ploščici so bile enake ali nižje, kot vrednosti MIK dobljene z ostalimi uporabljenimi metodami (metoda difuzije ter razredčevanje v trdnem oz. tekočem gojišču). Za grampozitivne bakterije se te vrednosti MIK gibljejo v razponu od 0,039 mg/ml do 10 mg/ml, za gramnegativne od 1,25 mg/ml do 10 mg/ml, medtem ko smo pri bakterijah rodu *Campylobacter* določili vrednosti MIK v razponu od 0,078 mg/ml do 2,5 mg/ml. Zaradi boljše občutljivosti je metoda razredčevanja v mikrotitski ploščici bolj primerna za hitro in enostavno kvantitativno določanje protimikrobne učinkovitosti rastlinskih izvlečkov.

Pri metodi razredčevanja v mikrotitrski ploščici smo za kvantifikacijo mikrobne rasti uporabili več različnih indikatorjev metabolne aktivnosti, in sicer tetrazolijevi soli TTC in INT ter reagent BacTiter-Glo za merjenje bioluminiscentnega signala. V večini primerov smo dobili jasne rezultate, prav tako nismo beležili bistvene razlike med vrednostmi MIK določenimi z indikatorjem TTC ali INT. Tetrazolijeve soli so uporabne za določanje protimikrobne učinkovitosti različnih izvlečkov (Al-Bayati 2008; Eloff, 1998; Palaniappan in Holley, 2010). Raztopina spojin TTC in INT je brezbarvna, obe spojini delujeta kot akceptorja elektronov, ki se ob prisotnosti metabolno aktivnih celic reducirata do obarvanega formazana (Eloff, 1998). Z uporabo tertrazolijevih soli lahko enostavno določimo metabolno aktivnost oz. dihanje pri aerobnih bakterijah, omejitev pa predstavljajo mikoraerofilne bakterije rodu *Campylobacter*. Zato je za te bakterije primerna uporaba reagenta BacTiter-Glo oz. določanje količine ATP z merjenjem bioluminiscentnega signala. Bioluminiscentni signal je sorazmeren količini ATP v vzorcu. Količina ATP pa je sorazmerna količini metabolno aktivnih celic v vzorcu (Promega, 2015). Določanje živosti in s tem metabolno aktivnih celic z reagentom BacTiter-Glo je bilo primerljivo z uporabo tetrazolijevih soli TTC in INT tudi pri ostalih testiranih bakterijah. Zato za določanje živosti pri aerobnih bakterijah priporočamo uporabo reagenta INT, saj so rezultati primerljivi z določanjem količine ATP, vendar je izvedba z INT cenejša in zato bolj primerna. Za določanje metabolno aktivnih celic bakterij rodu *Campylobacter* pa priporočamo uporabo reagenta BacTiter-Glo.

Kinetiko rasti bakterij ob prisotnosti različnih koncentracij izvlečkov smo določali z metodo razredčevanja v tekočem gojišču. Rezultati spremljanja kinetike rasti gramnegativnih in grampozitivnih bakterij pokažejo inhibicijo rasti pri koncentracijah nižjih od tistih, ki smo jih kot vrednosti MIK določili z metodami difuzije v trdnem gojišču z diski in metodo razredčevanja v trdnem gojišču. Prav tako rezultati spremljanja kinetike

rasti pokažejo in potrdijo, da vrednosti MIK določene z metodo razredčevanja v mikrotitrski ploščici inhibirajo razmnoževanje bakterijskih celic.

3.1.1.3 Protimikrobna učinkovitost rastlinskih izvlečkov v laboratorijskem gojišču

Preverili smo učinkovitost večih vrst rastlinskih izvlečkov, kombinacij izvlečkov in posameznih fenolnih kislin, ki so pogoste sestavine izbranih izvlečkov. Izvlečki se med seboj razlikujejo po izvoru, načinu ekstrakcije in po vsebnosti fenolnih kislin oz. posameznih komponent. Pri vseh testiranih izvlečkih oz. kislinah smo določili protimikrobni učinek. Pri tem smo določili razlike, saj smo pri izvlečkih s prevladujočo karnozolno kislino (V15, V20, V40, V70) določili boljšo učinkovitost, kot pri izvlečkih kjer je prevladovala rožmarinska kislina (A15, A40). Mešanice izvlečkov V40/A40, V40/grozdne pečke, V40/citrusi, V40/oljčni listi in V40/hmelj so bile po učinkovitosti podobne izvlečku V40 ali čisti karnozolni kislini. Prav tako smo pri izvlečku žajblja določili podobno učinkovitost, kot pri izvlečkih s karnozolno kislino. Pri ostalih izvlečkih (grozdne pečke, oljčni listi in zeleni čaj) smo določili slabšo učinkovitost, predvsem v primerjavi z izvlečki s karnozolno kislino. Tudi Sacco in sod. (2015) poročajo o boljši učinkovitosti izvlečkov z večjo vsebnostjo karnozolne kisline, v primerjavi z izvlečki z večjo vsebnostjo rožmarinske kisline. Rezultati potrjujejo druge študije, ki prav tako navajajo povezavo med protimikrobno učinkovitostjo in sestavo izvlečkov (Katalinić in sod., 2010; Klančnik in sod., 2009).

Izvlečki so imeli boljši učinek na grampozitivne bakterije ter bakterije rodu *Campylobacter*, kot pa na preostale testirane gramnegativne bakterije (*S.* Infantis in *E. coli*). Med preiskovanimi bakterijami so bile na delovanje izvlečkov najbolj občutljive bakterije vrste *B. cereus*, najbolj odporne pa *S.* Infantis. Razlike v odpornosti gramnegativnih bakterij na delovanje rastlinskih izvlečkov, so lahko zaradi sestave zunanje membrane, ki obdaja njihovo celično steno. Ta predstavlja dodatno zaščito in prepreči prehajanje spojin skozi lipopolisaharidni dvosloj (Vaara, 1992). Izjema so bakterije rodu *Campylobacter*, ki za rast potrebujejo posebne razmere, poleg tega pa so občutljive na različne vplive iz okolja (Park, 2002). Podobno so pokazali tudi Friedman in sod. (2002), ki so za bakterije vrste *C. jejuni* sicer določili večjo občutljivost na eterična olja in njihove sestavine, kot za bakterije vrst *L. monocytogenes*, *E. coli* in *S. enterica*. Občutljivost teh bakterij lahko izhaja iz zahtevnih razmer za rast (npr. mikroaerofilna atmosfera, obogatena gojišča). Lahko pa je vzrok tudi v sestavi zunanje membrane in celične stene bakterij vrste *C. jejuni* v primerjavi z ostalimi gramnegativnimi in tudi grampozitivnimi bakterijami (Friedman in sod., 2002).

Metodo razredčevanja v mikrotitrski ploščici oz. modifikacijo te metode smo uporabili tudi v vseh nadaljnjih eksperimentih. Z metodo razredčevanja v mikrotitrski ploščici smo v laboratorijskem gojišču določili vrednosti MIK izvlečkov V40, V70, A40 in I18 na seve bakterij vrste *C. jejuni* ATCC 33560, *C. jejuni* K49/4, *C. jejuni* NCTC 11168 in *C. jejuni* 375-06. Določene vrednosti MIK so bile v razponu od 0,08 mg/ml do 0,31 mg/ml. Kot indikator živosti smo uporabili reagent BacTiter-Glo. Na podlagi teh rezultatov smo potem izbrali izvleček V40 in sev *C. jejuni* K49/4 za nadaljnje eksperimente. Prav tako smo z metodo razredčevanja v mikrotitrski ploščici in indikatorjem INT določili protimikrobno

učinkovitost dveh izvlečkov rožmarina, V20 in V40 na bakterije vrst *A. acidoterrestris*, *A. hesperidum*, *A. cycloheptanicus*. Vrednosti MIK so bile za vse seve enake, in sicer za V20 7,8 µg/ml in za V40 3,9 µg/ml. Določene vrednosti MIK (7,8 µg/ml za V20 in 3,9 µg/ml za V40) kažejo, da sta oba izvlečka bolj učinkovita na izbrane seve aliciklobacilov, kot pa na ostale testirane gramnegativne in grampozitivne bakterije.

Poleg metode razredčevanja v mikrotitrski ploščici za določanje vrednosti MIK, smo uporabili še metodo razredčevanja v tekočem gojišču za določanje kinetike rasti oz. odmiranja preiskovanih bakterij ob dodatku izbranega izvlečka in na ta način dodatno preverili učinkovitost izvlečkov in potrdili vrednosti MIK.

3.1.2 Dejavniki, ki vplivajo na protimikrobno učinkovitost rastlinskih izvlečkov

Pomembno je, da učinkovitost naravnih protimikrobnih snovi določimo in ovrednotimo tudi v živilih oz. sistemih, ki vsebujejo parametre specifične za posamezno skupino živil. Živila so sestavljena predvsem iz beljakovin, maščob in ogljikovih hidratov, ki lahko medsebojno delujejo s protimikrobnimi snovmi in zmanjšajo njihovo učinkovitost (Davidson in sod., 2015). Zato smo želeli preveriti, kakšen vpliv ima dodatek različnih živil na vrednosti MIK izvlečkov rožmarina. Preverili smo delovanje izvlečkov rožmarina V40 in V70 na bakterije vrst *L. monocytogenes* in *E. coli*, ki ju najdemo v različnih živilih, predvsem mleku in mlečnih izdelkih, mesu in mesnih izdelkih ter v zelenjavi (Gandhi in Chikindas, 2007; McClure, 2000). V ta namen smo izbrali različna živila, in sicer mešano mleto meso, mesno pašteto, cvetačo, krompir in skuto. Pripravili smo modele živil s 50 % deležem posameznega živila in 50 % deležem ¼ razredčenega gojišča BPW. Za primerjavo smo uporabili laboratorijsko gojišče MHB. Vrednosti MIK smo določili z metodo razredčevanja v mikrotitrski ploščici v 50 % živilskem modelu in v gojišču MHB. Kot indikator metabolne aktivnosti smo uporabili INT. Poleg dveh različnih izvlečkov in različnih vrst bakterij, smo preverili tudi, kakšen vpliv na protimikrobno učinkovitost rastlinskih izvlečkov imata začetno število bakterijskih celic (višje začetno število celic ~10^7 cfu/ml in nižje začetno število celic ~10^3 cfu/ml) in temperatura inkubacije (8 °C in 37 °C). V literaturi najdemo številne študije protimikrobnega učinka naravnih protimikrobnih snovi v različnih živilskih modelih in živilih. Vse te rezultate je težje primerjati z našimi in tudi med sabo nasploh, saj se v raziskavah z modelom živil oz. samimi živili za določanje protimikrobnega učinka uporabljajo različne metode in tudi različni pogoji pri katerih se ta učinek preverja.

3.1.2.1 Vpliv deleža in vrste živila na vrednosti MIK

Že v samem gojišču, brez dodatka protimikrobne snovi, lahko na rast bakterij vplivajo različni dejavniki, kot so npr. temperatura, vrednost pH, aktivnost vode (Rivas in sod., 2010). Pri določanju učinkovitosti naravnih protimikrobnih snovi dodatek živila vpliva na učinkovitost. Naši rezultati kažejo, da sta bila oba rožmarinska izvlečka bolj učinkovita v laboratorijskem gojišču, kot v živilskih modelih, saj smo določili višje vrednosti MIK tam, kjer je bil gojišču dodan 50 % delež živila, ne glede na to, kakšno je bilo začetno število bakterijskih celic. Podobno poročajo tudi Djenane in sod. (2011, 2012), saj so za protimikroben učinek v mletem govejem mesu porabili dvakrat več eteričnih olj, kot pri

eksperimentih v laboratorijskem gojišču (Djenane in sod., 2011, 2012). Tudi v modelu brokolija je eterično olje rožmarina manj učinkovito, saj so za protimikrobni učinek potrebne višje koncentracije, kot pa v laboratorijskem gojišču (Muñoz in sod., 2009). V živilu z velikim deležem beljakovin je bila rast bakterij vrste *L. monocytogenes* boljša, prav tako pa je bil boljši tudi protimikroben učinek eteričnega olja origana in timijana. Na boljši učinek eteričnih olj vpliva tudi nižja vrednost pH. Prisotnost škroba oz. ogljikovih hidratov in sončničnega olja pa zmanjša učinkovitost delovanja eteričnih olj. Tudi v modelu solate je bila učinkovitost eteričnih olj boljša kot pa v laboratorijskem gojišču (Gutierrez in sod., 2008, 2009).

Tudi vrsta živila statistično značilno vpliva na vrednosti MIK obeh izbranih izvlečkov pri obeh vrstah testiranih bakterij. Dobljene vrednosti MIK so od 16 do 156 krat višje v prisotnosti 50 % deleža živila, kot v primerjavi z gojiščem MHB. Vseeno pa je razlika v delovanju izvlečkov rožmarina v prisotnosti izbranih živilskih modelov. V mesnih in mlečnih živilskih modelih z večjim deležem beljakovin in maščob smo določili višje vrednosti MIK, kot v zelenjavnih živilskih modelih in tako pokazali, da imajo komponente živil, predvsem beljakovine in maščobe vpliv na protimikrobno učinkovitost rastlinskih izvlečkov. Zhang in sod. (2009) prav tako poročajo, da se z večjo koncentracijo maščob in škroba veča tudi vrednost MIK monolavrina, medtem ko delež proteinov na vrednosti MIK ni imel vpliva. Vpliv živila in vrednosti pH na delovanje timola so potrdili tudi Shah in sod. (2012). Timol je imel protimikrobni učinek na bakterije vrst *E. coli* in *L. monocytogenes* v dveh izbranih živilih – jabolčnem vinu in mleku. Za inhibicijo rasti v mleku je bila potrebna 9 krat večja količina timola kot v jabolčnem vinu. de Madeiros Barbosa in sod. (2016) poročajo, da so bila izbrana eterična olja origana in timijana slabše učinkovita v sveži zelenjavi, kot pa v zelenjavnem bujonu.

3.1.2.2 Vpliv začetnega števila bakterij na vrednosti MIK

Začetno število bakterij je prav tako imelo vpliv na vrednosti MIK izbranih izvlečkov rožmarina. V gojišču MHB so bile pri bakterijah vrste *E. coli* vrednosti MIK nižje pri nižjem začetnem številu bakterij, ne glede na temperaturo inkubacije. Za bakterije vrste *L. monocytogenes* v gojišču MHB smo pri višji temperaturi inkubacije, ob dodatku izvlečka V40 določili enake vrednosti MIK, ne glede na začetno število bakterij. Ob dodatku izvlečka V70 in nižjem začetnem številu celic, so bile vrednosti MIK nižje, kot pri višjem začetnem številu celic. Pri nižji temperaturi inkubacije so bile vrednosti MIK pri obeh izvlečkih nižje pri višjem začetnem številu bakterij, kot pri nižjem začetnem številu.

V eksperimentih z živilskimi modeli smo pri višjem začetnem številu bakterij vrste *E. coli* določili višje vrednosti MIK. Pri bakterijah vrste *L. monocytogenes* pa smo določili višje ali enake vrednosti MIK. Začetno število bakterij je imelo vpliv tudi na protimikroben učinek timola in karvakrola na bakterije vrste *E. coli* O157:H7 v laboratorijskem gojišču. Obe spojini sta imeli boljši protimikrobni učinek pri nižjem začetnem številu bakterij (Rivas in sod., 2010). V modelu živila s kuhanim mletim govejim mesom je imelo začetno število bakterij vrste *L. monocytogenes* vpliv na protimikrobni učinek izvlečka origana in brusnic. Pri nižjem začetnem številu bakterij je bila inhibicija rasti večja, kot kadar je bilo uporabljeno višje začetno število bakterij (Apostolidis in sod., 2008). Rezultati tako za

bakterije vrste *E. coli*, kot *L. monocytogenes* kažejo, da ima začetno število bakterij, ne glede na uporabljen izvleček, večji vpliv na vrednosti MIK, kot vrsta živila.

3.1.2.3 Vpliv temperature na vrednosti MIK

Tudi temperatura inkubacije ima vpliv na vrednosti MIK testiranih izvlečkov. Primerjava vrednosti MIK obeh izvlečkov v gojišču MHB kaže, da so za bakterije vrste *E. coli* vrednosti MIK nižje pri 8 °C, in višje pri 37 °C. Nasprotno smo določili za bakterije vrste *L. monocytogenes*, saj so bile vrednosti MIK višje, ko so bili vzorci inkubirani pri 8 °C. Vrednosti MIK določene v živilskih modelih pri nižjem začetnem številu celic obeh vrst bakterij so bile v večini primerov višje pri 8 °C. Pri kombinacijah živilskega modela s cvetačo in krompirjem in bakterijami vrste *E. coli* ter živilskega modela s skuto in bakterijami vrste *L. monocytogenes* nismo določili vpliva temperature na vrednosti MIK. Bakterije se na spremembe temperature odzivajo različno, tem razlikam prilagodijo metabolizem in samo rast. Bakterije vrste *L. monocytogenes* so psihrotrofne in rastejo v temperaturnem območju od -1,5 do 45 °C (Lado in Yousef, 2007). Na drugi strani pa bakterije vrste *E. coli* pri temperaturah pod 10 °C ne rastejo (Ray in Bhunia, 2008). Zanimiv je primer uporabe vanilina v mleku proti bakterijam vrst *L. monocytogenes* in *E. coli*, kjer je bil vanilin bolj učinkovit pri 14 dnevni inkubaciji pri 7 °C, kot pri 35 °C. Avtorji to razliko pripisujejo stopnji nenasičenosti lipidov v membrani, kar povzroči motnje v membrani in s tem boljše delovanje vanilina (Cava-Roda in sod., 2012). Tudi protokatehujska kislina, kot glavna učinkovina izvlečka rastline gorski jetičnik (*Veronica montana* L.), je imela boljši protimikroben učinek na bakterije vrste *L. monocytogenes* v kajmaku pri 4 °C, kot pri 25 °C (Stojković in sod., 2013). Eterično olje timijana, nizin ter njuna kombinacija sta imela na bakterije vrst *L. monocytogenes* in *E. coli* O157:H7 v govejem mesu (Solomakos in sod., 2008a, 2008b) ter na bakterije vrste *S.* Enteritidis v ovčjem mesu (Govaris in sod., 2010) boljši učinek pri 10 °C, kot pa pri 4 °C.

3.1.2.4 Vpliv vrste bakterij na vrednosti MIK

Pri vseh testiranih razmerah so se za bolj občutljive izkazale bakterije vrste *L. monocytogenes*. Izjema predstavlja živilski model s skuto, kjer so bile določene vrednosti MIK enake za obe vrste bakterij. Rezultati tako potrjujejo predhodne rezultate, kjer smo ravno tako določili večjo občutljivost grampozitivnih bakterij. Podobno poročajo tudi Gutierrez in sod. (2009), ki so v eksperimentih določili boljši protimikrobni učinek eteričnih olj na grampozitivne bakterije, tako v gojišču, kot v modelu solate in govejem izvlečku.

Grampozitivne in gramnegativne bakterije se razlikujejo v občutljivosti oz. odpornosti na rastlinske izvlečke. Vzrok za to je lahko razlika v sami strukturi obeh skupin bakterij. Gramnegativne bakterije imajo bolj kompleksno sestavo, ki vključuje tudi zunanjo membrano. Ta vsebuje lipopolisaharide, ki predstavljajo še dodatno oviro. Zaradi tega je celična stena bolj zaščitena in hidrofobne protimikrobne spojine težje prehajajo skozi (Davidson in sod., 2015; Holley in Patel, 2005).

3.1.2.5 Vpliv vrste izvlečka na vrednosti MIK

Glavna razlika med uporabljenima izvlečkoma je predvsem vsebnost karnozolne kisline. Za izvleček V70, ki vsebuje več karnozolne kisline smo pri vseh testiranih kombinacijah določili nižje oz. enake vrednosti MIK, kot za izvleček V40. Kljub temu naši rezultati, nakazujejo, da lahko s kombinacijo rožmarinskih izvlečkov in nizkimi temperaturami skladiščenja inhibiramo rast bakterij vrst *L. monocytogenes* in *E. coli* v živilskih modelih.

3.1.3 Vpliv izvlečka rožmarina na bakterije vrste *C. jejuni*

3.1.3.1 Protimikrobna učinkovitost izvlečka V40 na bakterije vrste *C. jejuni* v gojišču in piščančjem mesnem soku

Bakterije vrste *C. jejuni* predstavljajo velik problem pri varnosti piščančjega mesa, zato smo poleg določanja protimikrobnega učinka izvlečka rožmarina na bakterije vrste *C. jejuni* v gojišču, določili tudi protimikroben učinek izvlečka v piščančjem mesnem soku. Piščančji mesni sok dobimo tako, da odtajamo zamrznjene piščance in zberemo izcejeno tekočino, ki je po sestavi podobna piščančjem mesu (Birk in sod., 2004). Za eksperimente smo ga izbrali, ker posnema naravno okolje kampilobaktrov na piščančjem mesu. Z metodo razredčevanja v mikrotitrski ploščici smo najprej preverili učinek izvlečka V40 na bakterije vrste *C. jejuni* K49/4 v gojišču MHB in piščančjem mesnem soku v mikroaerofilni atmosferi po 24 h pri 42 °C. Za določanje metabolne aktivnosti smo uporabili reagent BacTiter-Glo. Vrednosti MIK so bile nižje v gojišču (0,16 mg/ml), kot v piščančjem mesnem soku (0,63 mg/ml), kar je skladno z ostalimi rezultati, ki nakazujejo boljšo učinkovitost rastlinskih izvlečkov *in vitro*.

Z metodo razredčevanja v gojišču smo z določanjem kinetike rasti preverili učinkovitost treh različnih koncentracij izvlečka V40 pri treh različnih začetnih številih (višje ~10^7 cfu/ml, srednje ~10^5 cfu/ml, nižje ~10^3 cfu/ml) bakterij vrste *C. jejuni* K49/4 v gojišču MHB in v piščančjem mesnem soku. Vzorce smo inkubirali mikroaerofilno pri 42 °C in 8 °C. Pri optimalni temperaturi inkubacije je bila v gojišču MHB najbolj učinkovita najvišja uporabljena koncentracija (0,31 mg/ml), kjer je število celic po 24 h inkubaciji padlo pod mejo detekcije, ne glede na začetno število bakterij. V piščančjem mesnem soku je bila ta koncentracija učinkovita samo pri srednjem in nižjem začetnem številu bakterij in znižala število bakterij pod mejo detekcije.

Pri inkubaciji na 8 °C smo učinek izvlečka V40 preverjali pri dveh različnih začetnih številih bakterij (višje ~10^7 cfu/ml in nižje ~10^3 cfu/ml začetno število celic). V gojišču MHB pri višjem začetnem številu izvleček V40 ni imel učinka, število celic je padalo enako, kot pri kontroli. Pri nižjem začetnem številu je bila učinkovita samo najvišja koncentracija (0,31 mg/ml). Podobne rezultate so v gojišču ob dodatku izvlečka lupine kostanja dobili tudi Lee in sod. (2016). Rezultati v piščančjem mesnem soku nakazujejo podoben učinek, kot pri inkubacija na optimalni temperaturi. Izvleček V40 je bil učinkovit samo pri najvišji uporabjeni koncentraciji in pri nižjem začetnem številu celic, kjer je število celic po 120 h inkubacije padlo pod mejo detekcije. Pri ostalih koncentracijah in kontrolah je število celic počasi padalo, ni pa padlo pod mejo detekcije. Izvleček V40 v piščančjem mesnem soku pri nižji temperaturi ni imel učinka. Možen razlog je lahko

dejstvo, da ima pri nižjih temperaturah skladiščenja piščančji mesni sok zaščitni učinek na celice bakterij vrste *C. jejuni*. Tudi Birk in sod. (2004) so pokazali, da celice bakterij vrste *C. jejuni* NCTC 11168 pri 5 °C preživijo dlje v piščančjem mesnem soku, kot v gojišču. Prav tako so pokazali, da pri 5 °C bakterije preživijo dalj časa kot pri 10 °C. Sestavine piščančjega mesnega soka naj bi pripomogle pri podaljšanju preživelosti.

3.1.3.2 Kombinacija učinkov zamrzovanja in izvlečka V40 na bakterije vrste *C. jejuni* v piščančjem mesnem soku

Preverili smo možen sinergistični učinek izbrane koncentracije V40 (0,2 mg/ml) skupaj z nizinom (1000 IU) in/ali kratkotrajnim zamrzovanjem pri -20 °C v piščančjem mesnem soku. Zamrzovanje smo vključili, ker naj bi bile bakterije rodu *Campylobacter* občutljive na zamrzovanje ter kombinacijo zamrzovanja in tajanja (Archer, 2004). Na drugi strani imamo rezultate študije, ki nakazuje, da lahko bakterije vrste *C. jejuni* preživijo temperature hlajenja in zamrzovanja, tako da samo uporaba nizkih temperatur ni dovolj učinkovit ukrep (Bhaduri in Cottrell, 2004). Piščančji mesni sok v primerjavi z laboratorijskim gojiščem verjetno vsebuje več zaščitnih dejavnikov, ki lahko zaščitijo bakterijske celice med zamrzovanjem. Zato ima zamrzovanje na bakterije vrste *C. jejuni* večji učinek v gojišču in tako lahko dosežemo večjo zmanjšanje celic v gojišču, kot v piščančjem mesnem soku (Birk in sod., 2006).

Z metodo razredčevanja v piščančjem mesnem soku smo določili kinetiko rasti in tako preverili učinek izvlečka V40 (0,2 mg/ml) in nizina (1000 IU) ter njuno kombinacijo na bakterije vrste *C. jejuni* K49/4. Vzorce smo inkubirali 4 dni mikroaerofilno pri 8 °C. Takoj ob dodatku izvlečka V40 in kombinacije V40/nizin, se je število bakterij znižalo za približno 1 log enoto ter do konca inkubacije padlo pod mejo detekcije. Rezultati kažejo, da nizin sam ni imel učinka na bakterije vrste *C. jejuni* K49/4, kot tudi ne v kombinaciji z izvlečkom V40. Na gramnegativne bakterije vrste *E. coli* nizin sam po sebi ni pokazal protimikrobnega učinka, je bil pa učinkovit v kombinaciji z eteričnim oljem timijana in njuna kombinacija je imela aditiven protimikroben učinek (Solomakos in sod., 2008a). V našem primeru rezultati protimikrobnega delovanja kombinacije V40/nizin na bakterije vrste *C. jejuni* niso potrdili protimikrobnega učinka. Do podobnih zaključkov so prišli tudi Dykes in sod. (2003), ki prav tako niso potrdili protimikrobnega učinka samega nizina na bakterije vrste *C. jejuni*, kot tudi ne v kombinaciji z rastlinskim izvlečkom vednozelenega gornika (Dykes in sod., 2003). Predhodno 24 urno zamrzovanje je imelo učinek na bakterije, saj je že na začetku znižalo število bakterij za 0,5 log enote v primerjavi z bakterijami, ki niso bile predhodno zamrznjene. Bakterije so v predhodno zamrznjenem kontrolnem vzorcu preživele 4 dni inkubacije pri 8 °C, saj se njihovo število tekom eksperimenta ni spremenilo. Dodatek izvlečka V40 je v kombinaciji z zamrzovanjem znižal število bakterij pod mejo detekcije po 48 h inkubaciji pri 8 °C. V vzorcu, kjer je bil dodan samo izvleček V40, brez zamrzovanja pa se je število bakterij znižalo pod mejo detekcije po 96 h inkubacije. Dodatek nizina (tudi v kombinaciji z zamrzovanjem) ni imel protimikrobnega učinka. Kombinacija kratkega predhodnega zamrzovanja in izbrane koncentracije izvlečka V40 je pokazala sinergistično delovanje in znižala število bakterij pod mejo detekcije (kar predstavlja več kot 3 log enote).

3.1.3.2 Protimikrobna učinkovitost izvlečka V40 na baterije vrste *C. jejuni* na površini piščančjega mesa

Za ta del eksperimenta smo uporabili izvleček V40 in kombinacijo predhodnega zamrzovanja ter inkubacije pri 8 °C. Na koščke mesa smo nanesli bakterije vrste *C. jejuni* K49/4 in jih tretirali s pomakanjem v raztopino izvlečka V40. Del vzorcev smo predhodno za 24 h zamrznili. Kontrolni vzorec (namesto izvlečka rožmarina) je predstavljala tudi 10 % raztopina TSP (trinatrijev fosfat). TSP je v ZDA označen kot GRAS in je dovoljen za uporabo v procesu klanja brojlerjev (Capita in sod., 2002), medtem ko v Evropski uniji uporaba kemične dekontaminacije ni dovoljena (Purnell in sod., 2014). Pomakanje piščančjega mesa v 10 % raztopino TSP pomeni zmanjšanje mikrobne populacije mesa za približno 2 log enoti (Capita in sod., 2002), kar smo potrdili tudi v naših eksperimentih. Tako zmanjšanje števila bakterij rodu *Campylobacter* na piščančjem mesu lahko pomeni tudi zmanjšanje tveganja za zaužitje teh bakterij prek kontaminiranega mesa (Loretz in sod., 2010; Nauta in sod, 2009). Zmanjšanje bakterij vrste *C. jejuni* za 2 log enoti smo dosegli tudi v naših eksperimentih, vendar ne samo z dodatkom izvlečka V40, saj izbrana koncentracija izvlečka rožmarina ni imela takega protimikrobnega učinka. Skupaj s predhodnim zamrzovanjem in inkubacijo pri 8 °C pa smo dosegli zmanjšanje za več kot 2 log enoti v 48 h. Pri tem nismo določili bistvenih razlik med mikroaerofilno in aerobno inkubacijo vzorcev. Tudi Birk in sod. (2010) so s kombinacijo marinade in shranjevanja pri 4 °C znižali število bakterij vrste *C. jejuni* na piščančjih filejih za 1,2 log enote. Učinkovita je bila tudi aplikacija vinske kisline v kombinaciji s shranjevanjem na 4 °C, saj so na ta način znižali število bakterij vrste *C. jejuni* na koščkih piščančjega mesa za 2 log enoti.

3.1.4 Vpliv izvlečkov rožmarina na bakterije rodu *Alicyclobacillus* v jabolčnem soku

3.1.4.1 Vpliv izvlečkov rožmarina na senzorične lastnosti soka

Senzorična ustreznost oz. sprejemljivost rastlinskih izvlečkov je pomemben dejavnik pri določanju njihove uporabnosti (Davidson in sod., 2015). Kljub temu ni veliko študij delovanja naravnih protimikrobnih snovi, ki vključujejo tudi senzorično analizo (Bevilacqua in sod., 2010; Giner in sod., 2012; Tyagi in sod., 2013). Preverili smo, kakšen vpliv ima dodatek različnih koncentracij izvlečkov V20 in V40 na senzorične lastnosti jabolčnega soka. Potrdili smo, da imata oba izvlečka pri različnih koncentracijah lahko vpliv na senzorične lastnosti jabolčnega soka. Predvsem je bil vpliv izvlečkov V20 in V40 opazen pri višjih testiranih koncentracijah, saj je barva jabolčnega soka postala svetlejša, vonj in okus sta se poslabšala, sok pa je postal moten. Dokazali pa smo, da vrednosti MIK izvlečka V20 (7,8 µg/ml) in izvlečka V40 (3,9 µg/ml) nista imeli vpliva na senzorične lastnosti jabolčnega soka.

3.1.4.2 Vpliv izvlečkov rožmarina na vegetativne celice bakterij rodu *Alicyclobacillus*

Delovanje obeh izvlečkov rožmarina (V20 in V40) na bakterije vrst *A. acidoterrestris*, *A. hesperidum*, *A. cycloheptanicus* smo s spremljanjem kinetike rasti preverili tako v laboratorijskem gojišču BAT, kot tudi v modelu soka. Uporabili smo modelni jabolčni sok (50 % delež jabolčnega soka in 50 % delež gojišča) in jabolčni sok ter vrednosti MIK obeh

izvlečkov. Pri teh rezultatih je opazen vpliv dodatka živila na samo rast vegetativnih celic, saj je bila rast že v kontrolnih vzorcih inhibirana. To je opazno že v modelnem jabolčnem soku (kontrolni vzorec ob koncu inkubacije doseže ~10^6 cfu/ml) in tudi v jabolčnem soku (kontrolni vzorec ob koncu inkubacije doseže ~10^5 cfu/ml), v primerjavi s kontrolo v gojišču BAT (~10^7 cfu/ml ob koncu inkubacije). Na slabšo rast v jabolčnem soku lahko vplivajo lastnosti in specifične komponente soka, oz. sama vrsta soka, saj je bila npr. rast bakterij vrste *A. acidoterrestris* boljša v hruškovem, kot v ananasovem soku (Grande in sod., 2005). Na delovanje izvlečkov vplivajo različne sestavine živil, pH živila in tudi vrsta ter tudi količina protimikrobne snovi (Davidson in sod., 2015; Negi, 2012). Bevilacqua in sod. (2010) poročajo o vplivu medija na delovanje cinamaldehida in evgenola. Jabolčni sok naj bi bil v primerjavi z laboratorijskim gojiščem manj ugodno okolje za rast bakterij vrste *A. acidoterrestris* in za protimikroben učinek je bila potrebna manjša količina izbranih protimikrobnih snovi, kot v laboratorijskem gojišču (Bevilacqua in sod., 2010).

3.1.4.3 Vpliv izvlečkov rožmarina na spore bakterij vrste *A. acidoterrestris*

Dokazali smo, da oba izvlečka rožmarina (V20 in V40) protimikrobno učinkujeta na vegetativne celice izbranih sevov bakterij rodu *Alicyclobacillus*. Želeli smo določiti tudi njun učinek na spore teh bakterij. Za ta del eksperimenta smo uporabili suspenzijo spor bakterij vrste *A. acidoterrestris* in vrednosti MIK obeh izvlečkov. Predvidevali smo, da tako nizke koncentracije ne bodo učinkovale na spore, zato smo uporabili tudi štirikrat višje koncentracije tako izvlečka V20 (31,3 µg/ml), kot tudi izvlečka V40 (15,6 µg/ml). Učinek obeh izvlečkov rožmarina smo spremljali 48 h v gojišču BAT, modelnem jabolčnem soku in jabolčnem soku.

Pri kontrolnem vzorcu v gojišču BAT se je po prvih 4 h inkubacije število spor zmanjšalo in število vegetativnih celic povečalo, kar nakazuje na vzklitje spor. Število spor se je v času do 24 h povečalo in do konca inkubacije ostalo na približno enaki vrednosti. Ob dodatku obeh izvlečkov rožmarina se je število spor po 4 h inkubacije zmanjšalo, podobno kot pri kontrolnem vzorcu. Število spor je do 24 h inkubacije ostalo približno enako pri vseh koncentracijah izvlečkov. Po 48 h inkubacije pri vrednostih MIK se je število spor in vegetativnih celic povečalo, koncentraciji uporabljenih izvlečkov nista več učinkovali. Pri 4 krat višjih koncentracijah izvlečkov je število spor ostalo približno enako, kot po 4 h inkubacije. Pri vzorcih z dodanim izvlečkom rožmarina se je število vegetativnih celic v prvih 4 h inkubacije povečalo, spore so vzklile enako, kot pri kontrolnem vzorcu. Do konca inkubacije kinetika rasti vegetativnih celic poteka podobno kot dinamika spor.

V modelnem jabolčnem soku in jabolčnem soku je bila spet vidna slabša rast že pri kontrolnem vzorcu, kar nakazuje, da je sok manj ugodno gojišče za rast bakterij vrste *A. acidoterrestris* v primerjavi z rastjo v gojišču BAT. Modelni jabolčni sok in jabolčni sok nista primeren medij za vzklitje spor. Število spor na začetku in koncu inkubacije je primerljivo, kar nakazuje, da je vzklilo le majhno število spor. V prvih 4 h inkubacije se je število vegetativnih celic povečalo in ostalo v tem rangu do konca inkubacije. Protimikrobni učinki izvlečkov rožmarina so v jabolčnem soku vidni z zakasnitvijo, saj so opazni kasneje kot v laboratorijskem gojišču BAT, kar je povezano s tem, da sok ni idealno okolje za klitje spor.

3.1.4.4 Indeks inhibicije

Iz dobljenih rezultatov vpliva izvlečkov rožmarina na spore bakterij vrste *A. acidoterrestris* smo izračunali indekse inhibicije (enačba povzeta po Bevilacqua in sod., 2008a) in na ta način primerjali učinek obeh izvlečkov rožmarina. Indekse inhibicije smo primerjali ob koncu inkubacije, se pravi po 48 h. Višja koncentracija obeh izvlečkov rožmarina je bila najbolj učinkovita v gojišču BAT, indeks inhibicije za izvleček V20 je bil 61,0 % ter za izvleček V40 65,4 %. V jabolčnem soku je bila bolj učinkovita vrednost MIK obeh izvlečkov, kjer smo za izvleček V20 določili 16,3 % in za izvleček V40 20,6 % indeks inhibicije. Glede na rast vegetativnih celic, spore najlažje vzklijejo v gojišču BAT. Večji indeks inhibicije pri višjih koncentracijah obeh izvlečkov v gojišču BAT pomeni, da smo največji protimikrobni učinek določili pri teh koncentracijah obeh izvlečkov. Klitje spor v vegetativne celice je bilo slabše v modelnem jabolčnem soku in tudi v jabolčnem soku, saj so bili indeksi inhibicije nižji, kot v gojišču BAT. Zaradi vpliva jabolčnega soka na rast bakterij, je rast v modelnem jabolčnem soku in jabolčnem soku slabša kot v gojišču BAT. Boljšo učinkovitost izvlečkov v jabolčnem soku lahko pripišemo tudi razlikam v vrednosti pH. Jabolčni sok ima nižji pH (v našem primeru je bila vrednost pH 3,71), kot gojišče BAT (vrednost pH 4,01). Bevilacqua in sod. (2008b) so ugotovili, da nižje vrednosti pH gojišča oz. rastnega medija izboljšajo delovanje cinamaldehida v primerjavi z gojišči z višjimi vrednostmi pH. Protimikrobni učinek cinamaldehida je tudi povezan s koncentracijo. Nižje koncentracije cinamaldehida so povzročile reverzibilno inhibicijo klitja spor, pri višjih koncentracijah pa spore izgubijo sposobnost klitja (Bevilacqua in sod., 2008b). Naši rezultati kažejo, da pri višjih koncentracijah obeh izvlečkov rožmarina spore težje vzklijejo. Ker je bilo klitje spor v jabolčnem soku slabše, se je posledično tvorilo manj vegetativnih celic, zato sta imela izvlečka rožmarina slabši učinek na skupno število spor v vzorcu. Shigemune in sod. (2012) podobno poročajo o vplivu epigalokatehin galata na bakterije vrste *B. cereus*. Epigalokatehin galat na spore ne deluje oz. ne inhibira klitja, deluje pa na vegetativne celice. Epigalokatehin galat se na vegetativne celice adsorbira, medtem ko se na spore ne, verjetno zaradi različne strukture površin vegetativnih celic in spor (Shigemune in sod., 2012). Uporaba izvlečka grozdnih pečk je prav tako pokazala, da izvleček ne vpliva na same spore, povzroči pa spremembe v strukturi celic in inhibira nastanek spor bakterij vrste *A. acidoterrestris*. Prav tako avtorji niso določili vzklitja spor v vegetativne celice pri vzorcih tretiranih z izvlečkom grozdnih pečk. Pri kontrolnih vzorcih pa so bile vegetativne celice in spore prisotne (Molva in Baysal, 2015).

3.2 SKLEPI

- Minimalna inhibitorna koncentracija (MIK) rastlinskega izvlečka je merilo učinkovitosti protimikrobne aktivnosti. Za določitev vrednosti MIK rastlinskih izvlečkov je najbolj primerna metoda razredčevanja v mikrotitrski ploščici z uporabo INT ali določitvijo ATP, za določitev kinetike rasti pa metoda razredčevanja v tekočem gojišču s štetjem kolonij na trdnih gojiščih.

- Grampozitivne bakterije (bakterije rodov *Alicyclobacillus, Bacillus, Listeria, Staphylococcus*) so bolj občutljive na delovanje rastlinskih izvlečkov kot gramnegativne bakterije (bakterije rodov *Campylobacter, Escherichia, Salmonella*). Testirani izvlečki se razlikujejo glede na izvor in način ekstrakcije (največjo protimikrobno učinkovitost so imeli izvlečki rožmarina, v katerih je prevladovala karnozolna kislina in izvleček žajblja, manjšo pa izvlečki rožmarina, v katerih je prevladovala rožmarinska kislina ter izvlečki grozdnih pečk, oljčnih listov in zelenega čaja).

- Na protimikroben učinek rastlinskih izvlečkov vpliva več dejavnikov:
  - medij (vsi rastlinski izvlečki so bili bolj učinkoviti v laboratorijskih gojiščih kot v živilskih modelih in živilih);
  - sestava živila (rastlinski izvlečki so bili bolj učinkoviti v živilskih modelih, ki so vsebovali večji delež ogljikovih hidratov in manj maščob ali beljakovin);
  - število bakterij (pri večjem številu bakterij je za enako učinkovitost izvlečka potrebna večja količina izvlečka v laboratorijskem gojišču in v živilskih modelih) in njihova oblika (izvlečka rožmarina delujeta na vegetativne celice bakterij vrste *A. acidoterrestris* v jabolčnem soku, nimata pa učinka na spore; vendar pa iz spor vzklijejo vegetativne celice, na katere lahko izvlečka rožmarina učinkujeta);
  - temperatura (učinkovitost izvlečkov rožmarina je bila boljša pri nižji temperaturi, vendar je aktivnost odvisna tudi od preiskovanih bakterij);
  - kombinacija protimikrobnih dejavnikov (na bakterije vrste *C. jejuni* v piščančjem mesu ima največji protimikroben učinek kombinacija izvlečka rožmarina in zamrzovanja, medtem ko dodatek nizina k protimikrobnemu učinku ni pripomogel).

- Pri vrednotenju protimikrobne učinkovitosti rastlinskih izvlečkov je poleg že navedenih dejavnikov pomembna senzorična analiza živila, saj le-ta lahko ne glede na dobro protimikrobno učinkovitost omeji uporabo rastlinskih izvlečkov v živilih.

4 POVZETEK

4.1 POVZETEK

Mikroorganizmi so prisotni na vseh področjih živilsko predelovalne industrije, od pridelave do predelave ter skladiščenja živil. Povzročajo dva glavna problema, in sicer na eni strani patogene bakterije povzročajo okužbe s hrano, na drugi strani pa kvarljivci povzročajo kvar hrane. Zato se za kontrolo mikroorganizmov živilom dodaja protimikrobne snovi (Davidson in sod., 2015). Med možne vire protimikrobnih snovi lahko štejemo tudi rastline, ki vsebujejo številne spojine in so s tem pomemben vir novih biološko aktivnih molekul, ki imajo lahko tudi protimikrobno delovanje. Rastline, pri katerih poznamo protimikrobno delovanje, so lahko pomembne pri konzerviranju živil in s tem zagotavljanju varnosti (Negi, 2012).

Za določanje protimikrobne učinkovitosti rastlinskih izvlečkov in njihovih posameznih komponent obstajajo številne metode. Te so večinoma izpeljane in prilagojene iz postopkov določanja učinkovitosti antibiotikov in niso standardizirane za določanje učinkovitosti naravnih protimikrobnih snovi. Poleg tega obstaja več različnih načinov ovrednotenja rezultatov. Vse to pomeni, da rezultate težko primerjamo med seboj, zato potrebujemo enotno metodo, katere rezultati bi bili lažje primerljivi. Za določanje protimikrobnega učinka smo uporabili metodo difuzije v trdnem gojišču, metodo razredčevanja v trdnem ali tekočem gojišču in metodo razredčevanja v mikrotitrski ploščici. Metoda difuzije z diski ni primerna za določanje protimkrobnega učinka rastlinskih izvlečkov, saj smo dobili višje vrednosti MIK kot pri ostalih metodah. Glede na dobljene rezultate predlagamo uporabo metode razredčevanja v mikrotitrski ploščici. Ta metoda je učinkovita in relativno enostavna za izvedbo ter omogoča določitev vrednosti MIK in MBK. S to metodo lahko na hiter in enostaven način pregledamo in določimo protimikrobno učinkovitosti večjemu številu rastlinskih izvlečkov.

Izvlečki rožmarina so poznani kot bogat vir fenolnih spojin z dobrim protimikrobnim učinkom, ki se navezuje na karnozolno in rožmarinsko kislino (Sacco in sod., 2015). Poleg rožmarinskih izvlečkov, je dobro učinkovitost pokazal tudi izvleček žajblja, ki vsebuje karnozolno kislino, karnozol, rosmanol, feruginol in rožmarinsko kislino (Matkowski, 2008). Manj učinkoviti so bili izvlečki grozdnih pečk, oljčnih listov in zelenega čaja, ne glede na preiskovano bakterijo. Rožmarinska kislina je pokazala slabši učinek v primerjavi z karnozolno kislino, tako pri grampozitivnih bakterijah, kot tudi pri bakterijah rodu *Campylobacter*. Testirani izvlečki so bili bolj učinkoviti pri inhibiciji grampozitivnih kot gramnegativnih bakterij. Med gramnegativnimi bakterijami so izjema bakterije rodu *Campylobacter*, ki so po občutljivosti na delovanje izvlečkov primerljive z drugimi grampozitivnimi bakterijami. Med grampozitivnimi po občutljivosti izstopajo bakterije rodu *Alicyclobacillus*, ki so se izkazale za daleč najbolj občutljive.

Protimikroben učinek izvlečkov je potrebno določiti tudi v živilskih modelih in kasneje še v živilih, saj le na ta način lahko potrdimo njihovo učinkovitost in uporabnost. Na delovanje izvlečkov rožmarina in tudi ostalih naravnih protimikrobnih snovi lahko vplivajo različni dejavniki, med njimi delež in vrsta živila, temperatura shranjevanja, vrsta testiranih bakterij. Vpliv teh dejavnikov smo določili z metodo razredčevanja v mikrotitrski ploščici

v 50 % živilskih modelih. Izvlečka rožmarina V40 in V70 sta imela boljši učinek v živilih z večjim deležem ogljikovih hidratov. V živilih z večjim deležem maščob in beljakovin sta bila izvlečka manj učinkovita. Izvlečka sta imela večji učinek na grampozitivne bakterije vrste *Listeria monocytogenes*, kot na gramnegativne bakterije vrste *Escherichia coli*. Prav tako lahko za inhibicijo bakterij uporabimo oba izvlečka skupaj v kombinaciji z nižjo temperaturo shranjevanja, čeprav je delovanje v živilskih modelih pri nižji temperaturi odvisno od testirane vrste bakterij. Tudi pri določanju učinkovitosti izvlečkov v živilskih modelih ni standardiziranih metod, kar pomeni, da tudi take rezultate različnih študij težko primerjamo med seboj. Zato predlagamo uporabo metode razredčevanja v mikrotitrski ploščici v živilskih modelih za pregled učinkovitosti izvlečkov v različnih živilih in s tem preverjanje vplivov različnih dejavnikov na njihovo učinkovitost.

Naslednji korak pri določanju uporabnosti izbranega izvlečka rožmarina V40 je bil določanje njegove učinkovitost v živilu in sicer na piščančjem mesu. Za zmanjšanje števila bakterij vrste *Campylobacter jejuni* na svežih piščancih pred distribucijo v maloprodajo obstaja več metod oz. postopkov. Kljub vsemu ostajajo bakterije rodu *Campylobacter* in s tem kampilobakterioza med največkrat javljenimi zoonozami. Zato potrebujemo nove učinkovite postopke za zmanjšanje teh bakterij na piščančjem mesu. Bakterije vrste *C. jejuni* smo nanesli na kose piščančjega mesa in jih tretirali z izvlečkom rožmarina. Dokazali smo, da je uporaba izbrane koncentracije izvlečka, skupaj z zamrzovanjem, znižala število celic za 2 log enoti, kar posledično pomeni tudi zmanjšanje tveganja za okužbo s temi bakterijami prek okuženega piščančjega mesa.

Preverili smo še učinek izvlečkov rožmarina na bakterije rodu *Alicyclobacillus*, ki so eden izmed pomembnih kvarljivcev sadnih sokov in povzročajo veliko škode v industriji sadnih pijač. Preverili smo tudi, kakšen vpliv imata izvlečka rožmarina V20 in V40 na senzorične lastnosti jabolčnega soka. Naravne protimikrobne snovi v koncentracijah, ki imajo protimikrobni učinek, lahko vplivajo na senzorične lastnosti živila. Običajno je ta učinek negativen in tako živilo ni sprejemljivo za potrošnika. Oba izvlečka rožmarina v koncentracijah, ki inhibirajo rast vegetativnih celic izbranih sevov bakterij rodu *Alicyclobacillus* ne spremenita oz. ne vplivata na senzorične lastnosti jabolčnega soka. Pri vrednostih MIK oba izvlečka učinkovito zmanjšata število spor bakterij vrste *A. acidoterrestris* v jabolčnem soku. Pri vrednostih MIK sicer nimata sporocidnega učinka, vseeno pa pri teh koncentracijah lahko iz spor vzklijejo vegetativne celice, na katere lahko izvlečka potem učinkujeta inhibitorno. Tako lahko dodatek izvlečkov rožmarina v jabolčni sok predstavlja alternativen ukrep za kontrolo vegetativnih celic bakterij vrste *A. acidoterrestris*.

Rastlinski izvlečki so alternativen način konzerviranja živil in lahko kot snovi z protimikrobnim delovanjem veliko pripomorejo k varnosti živil. Kljub temu je pred njihovo uporabo v živilih potrebno preveriti več dejavnikov, ki lahko vplivajo na njihovo protimikrobno učinkovitost. Že ena sama 'negativna ocena' lahko namreč pomeni nesprejemljivost rastlinskega izvlečka v živilu.

4.2 SUMMARY

Microorganisms are present throughout the food industry, beginning at the farm level, and on through food processing and storage. Microorganisms pose two major concerns: on the one hand, there are pathogenic bacteria that cause foodborne diseases, and on the other hand, there are the spoilage microorganisms that cause food spoilage. Antimicrobials are therefore added to food to inhibit the growth of (or to kill) these microorganisms (Davidson et al., 2015). Plants can also be considered as antimicrobial agents, as they are known to contain many different compounds and to be important sources of biological molecules that have antimicrobial activities. Such plants, or extracts from such plants, can be used for the preservation of food and to ensure food safety (Negi, 2012).

Many different methods are available for the determination of antimicrobial activities of plant extracts or their components. Most of these are derived and adapted from methods for the determination of the antimicrobial activities of antibiotics, although they have not been standardized for use with natural antimicrobials. Also there are different ways of interpreting the data that can be obtained with these various methods, and this thus makes it difficult to compare data obtained from different studies in different laboratories. In the present study, we used the agar disk diffusion and dilution, and broth microdilution and macrodilution methods to determine the antimicrobial effects of selected plant extracts. The disk diffusion method is not suitable for the determination of the antimicrobial effects of plant extracts, as MIC values obtained with this method were the highest of these methods used. Overall, according to the results obtained with these methods, we recommend the use of the broth microdilution method to determine the antimicrobial effects of plant extracts. The microdilution method was the simplest and most effective method to determine the MICs of these plant extracts, and it is also an easy and rapid way to screen large quantities of plant extracts.

Rosemary extracts are known to be a rich source of phenolic compounds related to carnosic and rosmarinic acid that show antimicrobial activities (Sacco et al., 2015). Furthermore, a sage extract has also demonstrated good antimicrobial effects. The main components of this sage extract were carnosic acid, carnosol, rosmanol, ferruginol, and rosmarinic acid (Matkowski, 2008). Extracts of grape seeds, olive leaves, and green tea appear less effective, independent of the bacteria tested. Rosmarinic acid was less effective against Gram-positive bacteria and *Campylobacter* spp., than carnosic acid. The other extracts tested proved to be more efficient against Gram-positive than Gram-negative bacteria. *Campylobacter* spp. were the most susceptible among the Gram-negative bacteria, with comparable effects seen to those of Gram-positive bacteria. Among the Gram-positive bacteria, *Alicyclobacillus* spp. were by far the most susceptible.

To define the effectiveness and usefulness of any plant extract, it is important to determine its antimicrobial effects not only in laboratory media, but also in food models or systems, and in foods. Among the different factors that can affect the activities of these natural antimicrobials, and of rosemary extracts in particular, there are the form and the type of food and its storage temperature, and the type of bacteria used in the testing. The effects of these factors were determined using the broth microdilution method in food models that contained 50 % of the food model examined. The V40 and V70 rosemary extracts were

more effective in food models that contained high levels of carbohydrate, and less effective in food models that contained high levels of fat and protein. Both of these extracts showed greater effects against the Gram-positive *Listeria monocytogenes* than the Gram-negative *Escherichia coli*. These extracts can also be used in combination with low storage temperatures to inhibit growth of the bacteria, although these effects are also species specific.

The next phase of antimicrobial testing of the V40 rosemary extract was to determine its activity in food – in particular, on chicken meat. Although there are different methods available for the reduction of *Campylobacter jejuni* cells on fresh chicken meat before it is transported for retail sale, *Campylobacter* spp., and the associated campylobacteriosis, is still the most frequently reported foodborne illness. Therefore, there is the need for new and efficient treatments for the reduction of these bacteria in chicken meat. In the present study, we showed that a combination of rosemary extract and short-term pre-freezing of chicken meat can reduce *C. jejuni* populations by 2 log units. This level of reduction should also mean a reduction in the risk of *Campylobacter* infection through contaminated chicken meat.

We also determined the effects of the V20 and V40 rosemary extracts on *Alicyclobacillus* spp. These bacteria are an important spoilage microorganism that is of great concern in the fruit-juice industry. As natural antimicrobials can also have effects on the sensory properties of food when added at the levels required for their antimicrobial activities, we also determined the effects of these rosemary extracts on the sensory properties of apple juice. When these rosemary extracts are added at their *in-vitro* MICs, this did not change the sensory properties of the apple juice, and thus they can be used as natural additives in apple juice. At their MICs against *Alicyclobacillus acidoterrestris*, both of these extracts inhibited the growth of the vegetative cells, although they did not show sporicidal effects on the spores; however, they did reduce the spore numbers. Thus, although the addition of these extracts to apple juice at their MICs still allowed the spores to germinate, the growth of the vegetative cells that formed was inhibited. The addition of these rosemary extracts to apple juice thus represents an alternative strategy for the control of *A. acidoterrestris*.

Plant extracts can be considered as an alternative form of food preservation and can contribute to food safety based on their antimicrobial activities. Several factors can affect the antimicrobial activities of plant extracts in foods, and these factors need to be examined and defined before their use in any particular system. Indeed, with the important considerations of food safety, even one 'negative assessment' might render a plant extract as unacceptable for human use, and therefore not to be used in foods.

5 VIRI

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