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Antimicrobial Effect of *Filipendula ulmaria* Plant Extract Against Selected Foodborne Pathogenic and Spoilage Bacteria in Laboratory Media, Fish Flesh and Fish Roe Product

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Summary

Water-methanol extract from *Filipendula ulmaria* contains a variety of phenolic compounds, such as caffeic, *p*-coumaric and vanillic acid, myricetin, *etc*, which demonstrate antibacterial activity. Monitoring this activity in the broth using absorbance measurements showed that species of the Enterobacteriaceae family were more resistant than other Gram-negative and Gram-positive bacteria tested. Acidic environment enhanced the antibacterial activity of *Filipendula ulmaria* extract when it was tested against *Salmonella* Enteritidis PT4 and *Listeria monocytogenes* Scott A. The efficacy of *Filipendula ulmaria* extract against selected foodborne psychrotrophic bacteria was also tested using solid laboratory media and low incubation temperatures for better simulation of food preservation conditions. Higher concentrations of the extract, compared to minimum inhibitory concentration determined in the broth, were needed for satisfactory inhibition of spoilage bacteria. Potential use of *Filipendula ulmaria* extract as natural food preservative was also examined against natural spoilage flora and inoculated pathogenic bacteria on fish flesh and fish roe product (tarama salad). No significant differences of viable populations of spoilage or pathogenic bacteria were found between the treated samples and controls. Further trials of *Filipendula ulmaria* extract should be carried out in acidic foods with low fat and protein content, supplemented with additional adjuncts, in order to explore its potential as effective natural food antimicrobial agent.

Key words: natural antimicrobials, *Filipendula ulmaria*, antimicrobial activity, fish, spoilage, pathogens

Introduction

Consumers nowadays demand minimally preserved foods for maximum nutrient retention, without the addition of chemical preservatives. On the other hand, foods need to be safe, with prolonged shelf-life (*1*). An increasing interest in the use of natural antimicrobials as food preservatives has been recorded. Natural preparations from plants which contain phenolic compounds exhibit antimicrobial activity (*2,3*). Numerous essential oils, and extracts from plants have been tested for their antimicrobial properties against various food-borne microorgan-

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isms (*3–5*). Antimicrobials from plants can be used as an alternative to chemical preservatives in order to satisfy consumers' demand for safe, convenient and wholesome food (*2,6*). However, their potential as food preservatives has not been fully exploited yet.

Water-methanol extract from *Filipendula ulmaria* plant tissues contains a plethora of phenolic compounds that exhibit antimicrobial activity (*7*). The plant, commonly known as meadowsweet, belongs to Rosaceae family. The stems are about 1 to 2 meters tall, with dark green leaves on the upper side and whitish and downy underneath. The flowers are creamy-white, clustered close together in handsome irregularly-branched cymes, having a very strong, sweet smell.

Bacteria represent the main food safety hazards and also the main spoilage of animal origin foods such as fish and fish products. Enteric pathogens like *Salmonella* sp. and psychrotrophic bacteria such as *Listeria monocytogenes* are some of the potential microbiological hazards of fish and fish products (*8*). On the other hand, heterotrophic bacteria such as *Pseudomonas* sp., *Shewanella putrefaciens*, lactic acid bacteria, *etc*. are the main cause of fish and seafood spoilage (*9*). Inhibition and/or inactivation of such bacteria improves dramatically the safety and extends the shelf-life of fish and seafood.

The antimicrobial activity depends on the factors like temperature, pH, level of target microbial population, *etc*. (*10*). However, a very important factor that affects the fate of microorganisms in foods is the structure of the food matrix. Immobilized bacterial cells on solid surfaces behave differently in terms of growth rate and survival, hence liquid laboratory media are not suitable for the simulation of real food conditions (*11–13*). It is crucial to test the antimicrobial activity of potential food preservatives under more realistic conditions, like a solid substrate and/or a real food system.

The aim of this study is to evaluate the antibacterial efficacy of *F. ulmaria* extract, initially in liquid and solid laboratory media and subsequently in real seafood in order to evaluate its antibacterial potency under realistic conditions and draw conclusions about its usefulness as food preservative.

Materials and Methods

Plant extract preparation

Samples of *F. ulmaria* were collected from Mount Immitos in Attica, Greece, at 350 m altitude in May 2005. Samples (leaves and flowers) were dried in the air (at 25 °C in the dark) and used within 3 months of collection. The extraction method used for dried samples, the identification and quantification of phenolic compounds from *F. ulmaria* using reversed-phase high-performance liquid chromatography (RP-HPLC) were reported in a previous work (*7*).

Microbiological media and chemicals

The microbiological media were supplied by LAB M (Lancashire, UK), apart from cetrimide fucidin cephalosporin (CFC), streptomycin sulphate-thallus acetate-cycloheximide (actidione) (STAA), cefsulodin irgasan novobiocin (CIN) and *Aeromonas* agar base (RYAN) with ampicillin agar, which were supplied by Oxoid (Basingstoke, Hampshire, UK). Iron agar (IA) was made from its basic materials containing in g/L: peptone 20, meat extract 3.0, yeast extract 3.0, ferric citrate 3.0, sodium thiosulphate 0.3, NaCl 5, L-cysteine 0.6, agar 14, at pH adjusted to 7.4. All chemicals were provided by Sigma-Aldrich (Steinheim, Germany).

Microorganisms

The extract of *F. ulmaria* was tested against a panel of pathogenic and spoilage microorganisms, including non-toxinogenic *Escherichia coli* 0157:H7 NCTC 12900, *Salmonella* Enteritidis PT4, *Listeria monocytogenes* Scott A, *Staphylococcus aureus* ATCC 6538, *Pseudomonas fragi* ATCC 4973, *Shewanella putrefaciens* ATCC 8071, *Yersinia enterocolitica* CITY 844, *Aeromonas caviae* 27994 INCO and *Brochothrix thermosphacta* BR1. All microorganisms were taken from the culture collection of Laboratory of Microbiology and Biotechnology of Foods, Food Science and Technology Department, Agricultural University of Athens, Greece.

Microorganisms were frozen in bead vials (Protect; Technical Service Consultants Ltd, Heywood, Lancashire, UK). Resuscitation of bacterial strains was carried out in 10 mL of tryptone soy broth supplemented with 0.5 % yeast extract (TSBYE), incubated overnight at 37 °C for *E. coli* and *S.* Enteritidis, at 30 °C for *L. monocytogenes*, *S. aureus*, *Y. enterocolitica* and *A. caviae*, and at 25 °C for *P. fragi*, *S. putrefaciens* and *B. thermosphacta*. Resuscitated cultures were diluted tenfold in sterile ¼ strength Ringer's solution for the inoculation of 10 mL of TSBYE to yield an initial suspension of approx. 10 to 100 CFU/mL. All broths were then incubated statically at the aforementioned temperatures for each microorganism, for 20–24 h to guarantee that all cells were in the stationary phase before use in the experiments. The suspensions were centrifuged at 3000×*g* for 10 min, the pellets were washed with sterile ¼ strength Ringer's solution, centrifuged again and resuspended in sterile ¼ strength Ringer's solution. Appropriate number of decimal dilutions in sterile ¼ strength Ringer's solution was carried out to inoculate the liquid or solid test media and the real food systems with the initial populations of the tested bacteria.

Antimicrobial activity and MIC determination

Minimum inhibitory concentration (MIC) was determined by applying the broth microdilution method and monitoring the bacterial growth using absorbance measurement at 610 nm ($A_{610\ nm}$). The growth medium was TSBYE adjusted with HCl to pH=6.5. Methanol-water (60:40) solvent and plant extract both sterilized by filtration using 0.22-μm syringe filters (Minisart, NML, Sartorius, Göttingen, Germany) were added to sterile TSBYE to give a concentration of 0 and 25 % by volume, respectively. Appropriate volumes of these two growth media were pipetted into sterile microtitre plate wells to give a volume of 200 μL. Subsequently, 50 μL of growth medium inoculated with the test bacteria were added to each microtitre plate well. Hence, various concentrations of plant extract in growth medium from 0 to 20 % (by volume) were prepared with an initial bacterial popu-

lation of approx. 10^4 CFU/mL. Blanks with the addition of 50 μL of growth medium without bacterial cells were also prepared to avoid misleading results due to the oxidation of phenolics. The microtitre plates were incubated for 48 h at 37 °C for *E. coli* and *S.* Enteritidis, 30 °C for *L. monocytogenes*, *S. aureus*, *Y. enterocolitica* and *A. caviae* and at 25 °C for *P. fragi*, *S. putrefaciens* and *B. thermosphacta*. $A_{610\ nm}$ measurements were done every 4 h using Anthos HTII microtitre plate reader (Anthos Labtec Instruments, Salzburg, Austria).

Effect of pH

The effect of pH on *F. ulmaria* extract efficacy was tested against the Gram-positive *L. monocytogenes* Scott A and the Gram-negative pathogen *S.* Enteritidis PT4. The selection of these two microorganisms was based on their importance and their resistance to low pH. Volumes of TSBYE, with or without plant extract at concentrations equal to MICs of the tested organism were adjusted to pH=7 and 4. Half of the TSBYE aliquots were adjusted using HCl and the other half using lactic acid. The broths were inoculated in duplicates with approx. 10^5 CFU/mL of the tested microorganism and incubated at 37 and 30 °C for 12 h for *S.* Enteritidis PT4 and *L. monocytogenes* Scott A, respectively. The population changes were recorded using spread plate technique on duplicate tryptone soy agar supplemented with 0.5 % yeast extract (TSAYE), after incubation for 48 h at 37 and 30 °C for *S.* Enteritidis PT4 and *L. monocytogenes* Scott A, respectively.

Application in solid laboratory media

Plates with TSAYE adjusted with HCl to pH=6.5, supplemented with 0.1 % (by mass per volume) glucose were used as solid model food matrices. Methanol-water (60:40) solvent and *F. ulmaria* extract were sterilized by filtration through 0.22-μm syringe filters (Minisart, NML) and added to sterile molten TSAYE to give the appropriate concentration equivalent to 0.0, 1.0 and 2.5 times the MIC for each of the tested microorganisms. Glucose sterilized by filtration through 0.22-μm syringe filters was also added. Portions of 20 mL of the molten agar medium were poured into Petri dishes and allowed to cool. Suspensions of 0.1 mL of *L. monocytogenes* Scott A, *A. caviae* 27994 INCO and *Y. enterocolitica* CITY 844 were spread onto agar plates to give an initial population of 10^3 to $5{\cdot}10^3$ CFU/g. The selection of these three pathogens was based on their ability to grow at refrigeration temperatures. The plates were sealed using parafilm to prevent desiccation and stored at 5 °C for 30 days. A mass of 1 g of solid agar substrate was taken aseptically in duplicates and homogenised into 9 mL of maximum recovery diluent (MRD-NaCl and bacteriological peptone 0.85 and 0.1 % (by mass per volume) respectively) by vortexing for 1 min. The population changes were monitored by spread plate technique using duplicate Petri dishes with TSAYE after 48 h of incubation at 30 °C. The population changes were fitted using the Baranyi equation (*14*). DMFIT software (Institute of Food Research, Reading, UK) was used for fitting and estimation of kinetic parameters (specific growth rate, lag phase duration and maximum population density).

Application in fish flesh and tarama salad

Fresh aquacultured gilthead sea bream (*Sparus aurata*) was purchased from the local supermarket and brought directly to the laboratory. The fish were immediately filleted aseptically. The fillets were cut in approx. 20-gramme pieces and placed into sterile Petri dishes. A mass of 1 kg of home-made tarama salad was prepared with 0.35 kg of cod roe, 0.35 kg of boiled potatoes, 200 of mL olive oil and 100 g of onion. All the ingredients were added together and homogenised in a blender for 5 min. An adequate amount of lemon juice was added to adjust the pH down to 4.5. Portions of 20 g of tarama salad were transferred into sterile Petri dishes.

A volume of 2 mL of methanol-water (60:40) solvent or *F. ulmaria* extract was added to each 20 g of fillet and to each 20 g of tarama salad portion to give a concentration of 0 or 10 % (by volume per mass). Resuscitated bacterial suspensions washed with sterile ¼ strength Ringer's solution were used for inoculation. *L. monocytogenes* Scott A was used to inoculate the fish fillets, while tarama salad portions were inoculated with *S.* Enteritidis PT4, *L. monocytogenes* Scott A, *Y. enterocolitica* CITY 844 or *A. caviae* 27994 INCO. Only *L. monocytogenes* Scott A from psychrotrophic pathogens was selected to inoculate fish fillets, since it was the only pathogen that could be counted using selective media without interfering with the background microbiota. The initial concentration of *L. monocytogenes* Scott A on fish fillets was adjusted to 10^4 CFU/g, while the initial concentrations of pathogens on tarama salad portions were adjusted to approx. 10^6 CFU/g. Both fish fillets and tarama salad portions were stored at 5 °C.

Duplicate portions of 10 g of fish flesh were taken aseptically every 24 h for 7 days and transferred to stomacher bags with 90 mL of MRD and homogenized for 60 s with a stomacher (Lab Blender 400, Seward Medical, London, UK), while duplicate portions of 1 g of tarama salad were taken aseptically every 48 h for 12 days and homogenised in 9 mL of MRD by vortexing for 1 min.

The pathogens were enumerated on: (*i*) PALCAM agar after 48 h of incubation at 30 °C for *L. monocytogenes* Scott A, (*ii*) xylose lysine deoxycholate (XLD) agar after 24 h of incubation at 37 °C for *S.* Enteritidis PT4, (*iii*) CIN agar after 24 h of incubation at 30 °C for *Y. enterocolitica* CITY 844, and (*iv*) RYAN agar after 48 h of incubation at 30 °C for *A. caviae* 27994 INCO.

The following spoilage microorganisms of fish were enumerated: (*i*) *Pseudomonas* sp., on cetrimide fucidin cephalosporin (CFC) agar, after incubation at 25 °C for 48 h, (*ii*) *B. thermosphacta*, on streptomycin sulphate-thallus acetate-cycloheximide (actidione) (STAA) agar, after incubation at 25 ºC for 72 h, (*iii*) yeasts/moulds on rose bengal chloramphenicol (RBC) agar, incubated at 25 °C for 72 h, (*iv*) total viable counts on plate count agar (PCA), incubated at 25 °C for 48 h, (*v*) lactic acid bacteria, on de Mann-Rogosa-Sharpe (MRS) agar, incubated at 25 °C for 72 h, (*vi*) Enterobacteriaceae, on violet red bile glucose agar (VRBGA), incubated at 37 °C for 24 h, and (*vii*) H_2S-producing bacteria (*S. putrefaciens*) on iron agar (IA), incubated at 25 °C for 48 h.

The tarama salad background flora such as lactic acid bacteria and yeasts/moulds were enumerated. *Pseudomonas* sp. was also counted in samples inoculated with *L. monocytogenes*, *Y. enterocolitica* and *S.* Enteritidis. Enterobacteriaceae were enumerated in samples inoculated with *L. monocytogenes* and *A. caviae* only. The pH of the first dilution was also measured using a pH meter.

Results and Discussion

Antimicrobial activity

The antimicrobial activity of *F. ulmaria* extract was evaluated by monitoring the $A_{610\ nm}$ changes. The $A_{610\ nm}$ curves were derived taking into account the $A_{610\ nm}$ values taken from cell suspension and blanks. The minimum turbidity value that was recorded as growth was the $A_{610\ nm}$ value of 0.05. The antimicrobial activity of *F. ulmaria* extract against the tested bacteria was quantified as percent inhibition. Percent inhibition of a specific plant extract concentration after specific time of incubation was defined as:

$$\frac{A_{610\ nm}\text{ of sample with plant extract} - 0.05}{A_{610\ nm}\text{ of sample with 0 \% plant extract} - 0.05} \qquad /1/$$

The percent inhibition of various concentrations of *F. ulmaria* extract in TSBYE at pH=6.5, against the tested microorganisms is shown in Table 1. The minimum plant extract concentration that gave 100 % inhibition, after 48 h of incubation, was recorded as MIC (Table 1).

Species of Enterobacteriaceae family were found to be the most resistant compared to other Gram-negative and Gram-positive bacteria. *E. coli* 0157:H7 NCTC 12900, *S.* Enteritidis PT4 and *Y. enterocolitica* CITY 84 were the most resistant with MIC values of 14, 10 and 8 % (by volume), respectively, followed by *S. aureus* ATCC 6538 (7 %), *L. monocytogenes* Scott A and *B. thermosphacta* BR1 (4 %), *P. fragi* ATCC 4973 (3 %) and finally *A. caviae* 27994 INCO and *S. putrefaciens* ATCC 8071 (2 %). Resistance of Gram-negative bacteria against a variety of antimicrobial compounds might be due to the permeability barrier and functions of the outer membrane (*15,16*). However, more strains of the tested species need to be used to draw precise inferences about the susceptibility of species to antimicrobial compounds from *F. ulmaria* extract.

The prepared plant extract that was used in this work contains (in mg/L): hydroxytyrosol (also known as dihydroxyphenylethanol) 78, gallic acid 49, caffeic acid 135, *p*-coumaric acid 139, vanillic acid 185, ferulic acid 169, myricetin 520 and (+)-catechin 19 (*7*). It has been documented that some of these compounds exhibit antimicrobial activity against various strains of *L. monocytogenes*, *Pseudomonas* sp., *S. aureus*, *E. coli*, *etc.* (*4,17,18*). The MICs of *F. ulmaria* extract were much higher compared to the MICs of various essential oils from plants. Plant essential oils have MIC values below 1 % (by volume) and sometimes as low as 0.01 % (by volume) against the common foodborne bacterial pathogens (*3,19*). Higher antimicrobial activity of essential oils compared to our extract might be due to the high content of compounds such as thymol, carvacrol, eugenol, cinnamaldehyde, cinnamic acid, *etc.*, which have demonstrated pronounced antimicrobial activity (*20,21*), compared to the lower antimicrobial activity that exhibited by compounds such as caffeic, coumaric, ferulic acid, *etc.* (*17*).

Table 1. Inhibitory effect of various concentrations of *Filipendula ulmaria* extract in TSBYE at pH=6.5, against the tested pathogenic and spoilage bacteria

Microorganism	φ(plant extract)/%	percent inhibition			
		t(incubation)/h			
		12	24	36	48
Listeria monocytogenes Scott A	2	n.d.	90.0	71.1	66.8
	3	n.d.	96.6	87.2	85.5
	4	n.d.	100.0	100.0	100.0
Salmonella Enteritidis PT4	6	94.1	72.5	80.1	76.1
	8	100.0	100.0	72.7	73.2
	10	100.0	100.0	100.0	100.0
Escherichia coli 0157:H7	6	100.0	44.3	23.3	0.0
	10	100.0	88.4	55.7	18.1
	14	100.0	100.0	100.0	100.0
Yersinia enterocolitica	2	100.0	51.5	26.0	0.0
	5	100.0	93.3	62.1	3.4
	8	100.0	100.0	100.0	100.0
Staphylococcus aureus	3	n.d.	89.7	20.9	20.4
	5	n.d.	96.7	27.8	17.2
	7	n.d.	100.0	100.0	100.0
Aeromonas caviae	0.5	n.d.	45.6	0.0	0.0
	1	n.d.	76.8	64.7	46.3
	2	n.d.	100.0	100.0	100.0
Pseudomonas fragi	1	0.0	0.0	0.0	0.0
	2	100.0	94.4	60.1	50.2
	3	100.0	100.0	100.0	100.0
Shewanella putrefaciens	0.5	100.0	14.7	2.9	0.0
	1	100.0	64.7	62.0	44.4
	2	100.0	100.0	100.0	100.0
Brochothrix thermosphacta	2	n.d.	100.0	71.1	63.6
	3	n.d.	100.0	100.0	75.6
	4	n.d.	100.0	100.0	100.0

n.d.=growth was not detected ($A_{610\ nm}$ of control was not above the value of 0.05)

Effect of pH on antimicrobial activity

Plant extract was found to be far more active at acidic pH than neutral, especially in the presence of 100 mM of lactic acid. In the case of *L. monocytogenes* Scott A, the bacteriostatic concentration of 4 % (by volume) at pH=7.0 was bactericidal at acidic pH=4 (Figs. 1a and b). Without plant extract the population of *L. monocytogenes* Scott A in TSBYE (pH=4) did not change after 12 h, while in the presence of 4 % (by volume) plant extract the population dropped from 5.5 down to 1.3 log CFU/mL after 6 h of incubation at 30 °C (Fig. 1b). At pH=4 in the presence of 100 mM of lactic acid, the population of *L. monocytogenes* Scott A dropped faster in the treatment with 4 % (by volume) plant extract, presumably due to the additional antimicrobial effect of lactate (Fig. 1c). The pre-

sence of lactic acid at neutral pH did not affect *L. monocytogenes* Scott A population, while in the presence of plant extract only a slight decrease of about 0.5 log CFU/mL was observed after 12 h of incubation at 30 °C (Fig. 1d).

At pH=4 with HCl as acidulant, the presence of 10 % (by volume) plant extract inhibited but did not cause any population reduction of *S*. Enteritidis PT4 at least for 12 h of incubation (Fig. 2a). The same was observed at pH=7 (Fig. 2b). *S*. Enteritidis PT4 population declined fast when lactic acid was present, but the inactivation rate was higher in the presence of 10 % (by volume) plant extract. The population dropped below detection limit of 10 CFU/mL after 4 h of incubation at 37 °C (Fig. 2c). This can be attributed to the fact that organic acids can cause sublethal injury to Gram-negative bacteria (*22*) and render them more sensitive to various antimicrobial compounds. Particularly lactic acid is capable of disrupting outer membrane of Gram-negative bacteria and sensitizing *S. enterica* ser. Typhimurium to phenolic compounds (*23,24*). On the other hand, the presence of lactic acid at neutral pH did not affect bacterial population (Fig. 2d).

It seems that the combined effect of low pH with the phenolic compounds increases the antibacterial efficacy of plant extract. The increased efficacy can be attributed to: (*i*) increased antimicrobial activity of phenolic compounds at acidic pH (*5,17*), (*ii*) their stability at low pH values (*25*), and (*iii*) the acid stress imposed on bacterial cells.

Effect of plant extract in solid laboratory media

F. ulmaria extract affected the growth of psychrotrophic bacteria such as *L. monocytogenes* Scott A, *Y. enterocolitica* CITY 844 and *A. caviae* 27994 INCO in solid laboratory media. The effect of plant extract on specific growth rate, duration of lag phase and maximum population density of the tested microorganisms are summarized in Table 2. The addition of plant extract delayed the growth of microorganisms in a concentration-dependent manner: the higher the concentration, the higher the growth inhibition. For example, specific growth rates of *L. monocytogenes* Scott A, *A. caviae* 27994 INCO and *Y. enterocolitica* CITY 844 without the addition of plant extract were 0.49, 0.51 and 0.7 day^{-1} respectively, while in the presence of plant extract at the concentration equal to their MICs, the specific growth rates were reduced down to 0.21, 0.25, and 0.2 day^{-1} respectively. At concentrations of 2.5 times of their MICs, the specific growth rates were further reduced down to 0.06, 0.18 and 0.08 day^{-1} for *L. monocytogenes* Scott A, *A. caviae* 27994 INCO and *Y. enterocolitica* CITY 844, respectively. It can be noticed that sufficient inhibition of the tested bacteria was observed at higher concentrations, indicating that under more realistic testing conditions, higher concentrations of plant extract antimicrobials are required.

Lag phases were affected only at higher plant extract concentrations. Lag phase durations of *L. monocytogenes* Scott A, *A. caviae* 27994 INCO and *Y. enterocolitica* CITY 844 without the addition of plant extract were 7.4, 3.9 and 3.7 day respectively, while in the presence of plant extract at concentration equal to 2.5 times their MICs, the lag phase durations were increased to 7.8, 7.7 and 4.3 day, respectively (Table 2). Maximum population densities were also affected by the presence of plant extract. *L. monocytogenes* Scott A maximum population density was 9.1 log CFU/g, while in the presence of plant extract at concentration equal to its MICs (4 % by volume) it was 7.0 log CFU/g (Table 2).

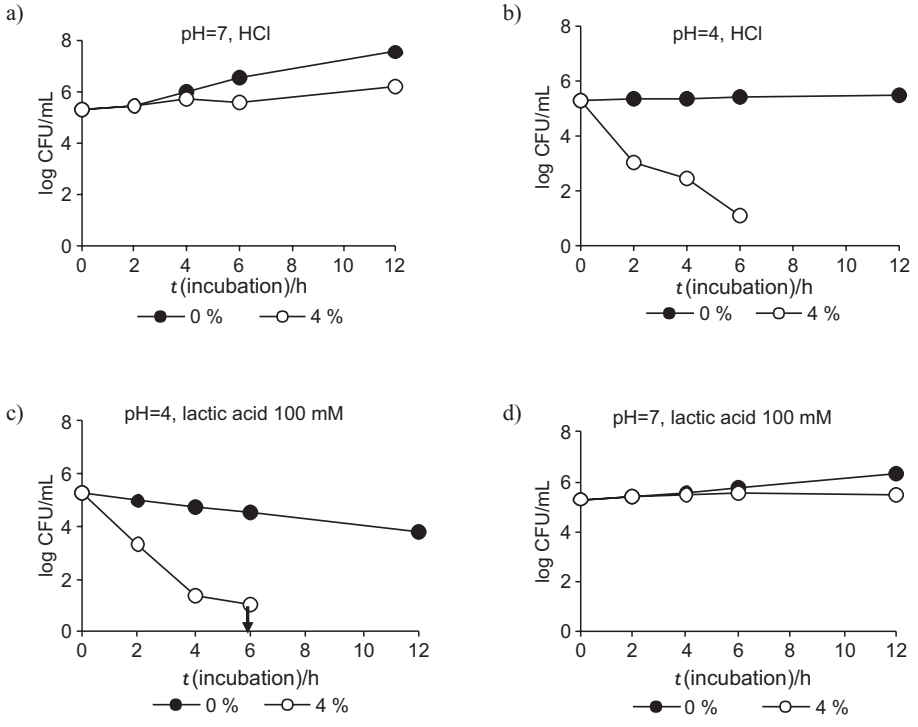

Fig. 1. Population changes of *Listeria monocytogenes* Scott A in TSBYE at pH=7 (a, d) and pH=4 (b, c) using HCl (a, b) and lactic acid (c, d) as acidulants in the absence (●) or presence (○) of 4 % (by volume) plant extract, during incubation at 30 °C. Each curve point is the mean value of duplicate measurements. The arrow (↓) indicates that the values were below enumeration detection limit

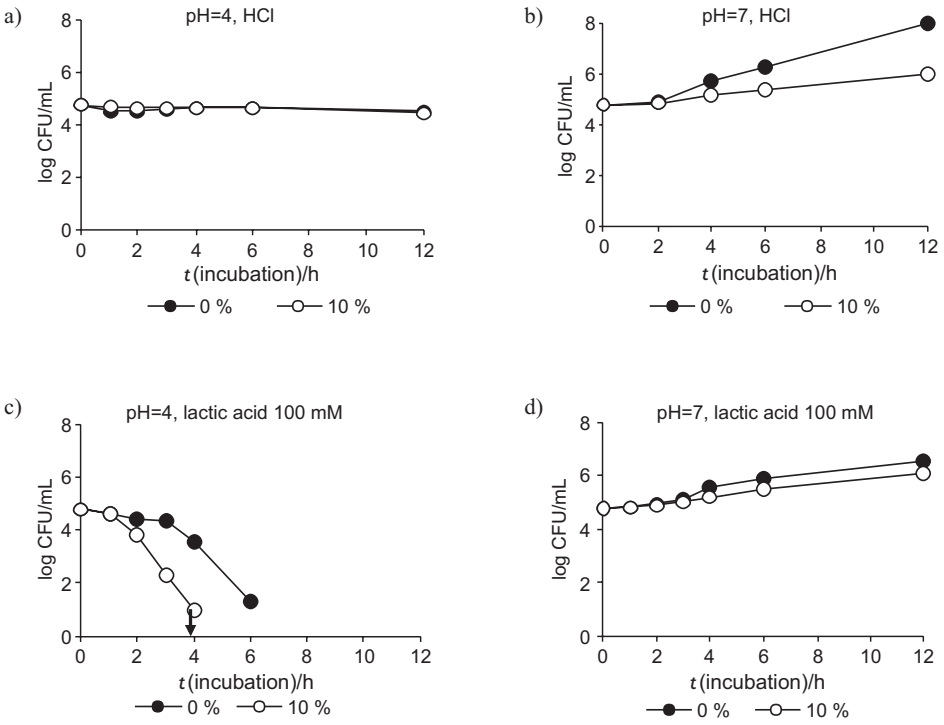

Fig. 2. Population changes of *Salmonella* Enteritidis PT4 in TSBYE at pH=4 (a, c) and at pH=7 (b, d) using HCl (a, b) and lactic acid (c, d) as acidulants in the absence (●) or presence (○) of 10 % (by volume) plant extract, during incubation at 37 °C. Each curve point is the mean value of duplicate measurements. The arrow (↓) indicates that the values were below enumeration detection limit

Determination of MIC using broths and optimum incubation temperatures for short period of time (usually 24 or 48 h) gives an indication of the antimicrobial potency of the tested compound. Study of bacterial growth on solid substrates is more suitable for food preservative challenge tests. In such cases higher concentrations of food antimicrobials are required to demonstrate satisfactory inhibition. The reduced effect of plant extract in solid substrates may be due to a number of factors. Growth of immobilized microbial cells in solid substrates varies from those in planktonic liquid cultures, since bacterial colonies in solid matrices are constrained (*13*). Nutrient availability in some cases is diminished due to diffusion limitation compared to planktonic cells (*11*). Similarly, the antimicrobial compounds might have limited access to bacterial colonies. The effect of most of the intrinsic factors differs against planktonic and immobilised cells. Water activity, pH, oxygenation, composition, structural and mechanical properties of the substrate affect in various ways the bacterial growth (*26–28*). These interactions are usually complicated and sometimes controversial, but in most cases the efficacy of antimicrobial preservatives is diminished.

Effect of plant extract in fish flesh and tarama salad

The addition of 10 % (volume per mass) plant extract to fish flesh did not affect at all the populations of

Table 2. Kinetic parameters derived by applying Baranyi equation (*14*) using DMFIT software for the tested microorganisms grown on solid model food system

Microorganism	φ(plant extract)	Specific growth rate	Reduction	Lag phase	Extension	Maximum population	Standard error of fit	R^2
	%	day^{-1}	%	day	%	log CFU/g		
	0	0.49		7.4		9.1	0.160	0.99
L. monocytogenes	4 (MIC)	0.21	57.1	7.2	0.0	7.0	0.154	0.99
	10 (2.5·MIC)	0.06	87.7	7.8	5.4	*	0.152	0.92
	0	0.51		3.9		9.1	0.147	0.99
A. caviae	2 (MIC)	0.25	50.9	3.7	0.0	8.4	0.184	0.99
	5 (2.5·MIC)	0.18	64.7	7.7	97.4	6.6	0.105	0.99
	0	0.70		3.7		9.5	0.254	0.99
Y. enterocolitica	8 (MIC)	0.20	71.4	3.4	0.0	*	0.323	0.97
	20 (2.5·MIC)	0.08	88.6	4.3	16.2	*	0.101	0.98

Model food system supplemented with 0 %, minimum inhibitory concentration (MIC) and 2.5·MIC plant extract, stored at 5 °C
*Maximum population was not reached within the time interval that experiment lasted

spoilage microflora. Initial populations were quite high at the beginning of the experiment with total microbial flora at the level of 5 log CFU/g of fish flesh. Total microbial population reached the level of 7.9 log CFU/g within 3 days (72 h) of storage at 5 °C, which was also the point of organoleptic rejection of the fish flesh due to the off-odours. After 3 days of storage, the population of *Pseudomonas* sp. reached the level of 7.7 log CFU/g, while H_2S-producing bacteria reached the level of 5.1 log CFU/g, followed by *B. thermosphacta* at 4.8 log CFU/g. Enterobacteriaceae, lactic acid bacteria and yeast/moulds did not grow at levels above 4 log cycles at the time of spoilage. *L. monocytogenes* Scott A population was not affected at all by the presence of plant extract. Its population was not changed during 3 days of shelf-life of fish flesh stored at 5 °C supplemented or not with 10 % (volume per mass) plant extract. Growth occurred after the 4th day of storage, reaching the level of 5 log CFU/g after 8 days of storage at 5 °C (data not shown). *Pseudomonas* sp. and H_2S-producing bacteria (mainly *S. putrefaciens*) have been reported as the main spoilage microorganisms of fish from temperate waters stored at low temperatures (*9*). Our findings are in agreement with other results, regarding microbial spoilage of the Mediterranean aquaculture fish, such as gilthead sea bream and sea bass stored on air at low temperatures (*29*,*30*).

The presence of plant extract did not substantially affect bacterial populations in tarama salad. *L. monocytogenes* Scott A, *A. caviae* 27994 INCO and *Y. enterocolitica* CITY 844 populations were virtually unaffected in tarama salad with or without 10 % (volume per mass) plant extract after 12 days of storage at 5 °C. Lactic acid bacteria and yeast/moulds were the main spoilage flora that grew during the storage period, while *Pseudomonas* sp. were below the detection limit of 10^2 CFU/g and Enterobacteriaceae were as low as $1–2 \cdot 10^2$ CFU/g throughout the 12 days of storage (data not shown). Lactic acid bacteria population was a bit higher compared to yeast/moulds. Populations of both lactic acid bacteria and yeast/moulds were totally unaffected by the addition of 10 % (mass per volume) plant extract and both reached the level of $5 \cdot 10^7–10^8$ CFU/g by the end of storage period of 12 days at 5 °C. The pH of the product with or without plant extract was not changed after 12 days of storage at 5 °C.

Plant extract was inactive against natural spoilage flora and inoculated pathogens in tarama salad regardless of the lower pH value (pH=4.5) of this product compared to fish flesh (pH=6.2). Antimicrobial activity of *F. ulmaria* extract phenolics is negligible compared to other natural antimicrobial systems such as oregano essential oil or mixture of oregano and cranberry phenolics when applied in real food systems. Application of oregano essential oil in a concentration as low as 0.05 % (volume per mass) reduced the growth of *Photobacterium phosphoreum* and extended the shelf-life of cod fillets packed in modified atmosphere (*31*), while at the concentration of 0.5 % (volume per mass) it was able to reduce *S*. Enteritidis PT4 population in tarama salad stored at 5 °C (*32*). Application of oregano and cranberry phenolic mixture inhibited and inactivated *L. monocytogenes* when applied on cod fillets (*33*). Nevertheless, higher concentrations of natural extracts compared to those determined in broths are required to inhibit pathogens like *L. monocytogenes* in meat and dairy systems (*34*,*35*).

The diminished efficacy of *F. ulmaria* extract when applied in real food systems like fish flesh and fish roe salad may be due not only to its relatively weak inherent action but also to other factors. Real foods contain proteins, lipids, oil droplets, *etc*, which interfere with the antimicrobial action of phenolic compounds. Antimicrobial efficacy of essential oils is affected by food composition and microstructure (*27*). Food components such as proteins and fats may protect microorganisms, and antimicrobial molecules might be inactivated due to adsorption on food particles (*6*). Solubilisation of antimicrobials into the lipid fraction and reaction with fatty free radicals might also be responsible for their minimal antimicrobial action (*36*,*37*).

Conclusions

F. ulmaria plant extract showed antimicrobial activity in the broth at low pH, preferably in the presence of lactic acid and in agar food model system. Its antimicrobial activity was limited in the tested real food systems. Based on this study, the efficacy of *F. ulmaria* extract needs to be newly challenged in acidic foods with low fat and protein content. Cocktail of strains of various bacterial species also has to be used. The presence of lactic acid or any other factor that may impose stress on foodborne bacteria is worth testing in order to evaluate its usefulness as natural food antimicrobial.

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