Antimicrobial potential of *Ericaria selaginoides* extracts against *Listeria monocytogenes* in "mató", a Catalan fresh cheese

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Abstract

The objective of this work was to assess the antimicrobial effect of crude extracts and non-polar and mid-polar subfractions of the brown macroalga *Ericaria selaginoides*, using a mid-polarity extraction medium. The activity was evaluated through challenge testing against *Listeria monocytogenes* in Catalan "mató" (fresh cheese) selected as a food matrix. Three concentrations were tested, representing $2 \times$, $4 \times$ and $10 \times$ Minimum Bactericidal Concentration values obtained for crude extract in previous in vitro assays. *Ericaria selaginoides* extracts inhibited *L. monocytogenes* growth in a dose-dependent manner. Intermediate concentrations of the three extracts were able to notably reduce the growth rate of the pathogen. Highest concentration of crude extract and non-polar subfraction were able to completely inhibit the growth of *L. monocytogenes* during the 10 days of storage at 8 °C. Moreover, a slight listericidal effect was observed in presence of the non-polar subfraction, reducing the *L. monocytogenes* level by ca. 3 log after 10 days of refrigerated storage. These results prove the potential of *E. selaginoides* extracts as a source of valuable antimicrobial compounds that can be used as natural food ingredients to develop safer fresh dairy food.

Keywords Phaophyceae · *Ericaria selaginoides* · Antimicrobial activity · Foodborne pathogens · *Listeria monocytogenes* · Challenge test

Introduction

Microbial food safety is crucial in the supply chains of food manufacturers in order to assure consumer protection from potential health risks as well as to reduce food losses, which can have a great impact in global economy (Hussain and Dawson 2013; Petruzzi et al. 2017) *Listeria monocytogenes*, the causative agent of listeriosis, is reported as the fifth most prevalent zoonotic foodborne disease in Europe and, although the number of cases is relatively low, it shows the highest hospitalisation and case fatality rates ($> 90\%$ and 14–30% respectively, depending on the year and the region)

(Desai et al. 2019; EFSA-ECDC 2021). It can be especially deleterious in neonates, pregnant women, and immuno-compromised or elderly people since it can cross intestinal, blood–brain and feto-placental barriers causing spontaneous abortion or severe infections such as septicaemia or meningitis (Lecuit 2005).

Listeria monocytogenes represents a noticeable threat in food industry as it can contaminate products during post-processing operations due to its ability to form biofilms in inert surfaces of food processing environment. It is usually found in a wide range of products, including raw vegetables, raw milk, raw meat, soft cheese, fish, and poultry, although the main source of human exposure are minimally processed and/or ready-to-eat products (RTE) as this pathogen has high tolerance to stressful pH conditions, freezing, drying, and heating, and can grow under refrigeration (Thakur et al. 2018). In this regard, a quantitative modelling suggests that more than 90% of invasive listeriosis is caused by consumption of RTE containing > 2000 colony forming units per gram (cfu g^{-1}) and that one-third of cases are related to growth at the consumer phase (EFSA Biohaz Panel (EFSA Panel on Biological Hazards) et al. 2018).

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Europe is a major player in global cheese industry and has the highest level of per capita of cheese consumption (Statista Research Department 2021). Among dairy products, soft cheeses are one of the most common causes of listeriosis, being detected at 0.5% in EU during 2020 and recognized by EFSA as a RTE product of risk for *L. monocytogenes* growth (EFSA-ECDC 2021). In this case, problems are mainly related to post-pasteurization contamination of milk, since they constitute a very suitable food matrices for the pathogen growth because of its physicochemical characteristics (pH 5.8–6.8, a_w 0.94–0.99, 0.1–1.2% NaCl) (Vrdoljak et al. 2016).

Food safety regulations concerning *L. monocytogenes* in RTE products differ between countries. In Europe (EC 2005), microbiological criteria of a maximum of 100 cfu g^{-1} during the shelf-life of the product is stablished, whereas in other countries such as USA, a zero-tolerance policy is enforced (FSIS 2003a, b). Control measures based on risk approach could be implemented, either minimizing the occurrence of the pathogen in raw materials, reducing the levels by applying lethality or post-lethality treatment (PLT) and/or preventing its increase through the use of antimicrobial agents (AMA) or other processes.

Traditionally, synthetic additives have been used as preservatives in order to control microbial pathogens and extend the shelf-life of food. However, consumer demands for more "natural" products free of synthetic additives is rising and this tendency is gaining force in food industry, which is experiencing a shift towards the development of more sustainable, safer, and healthier food supplies (Bondi et al. 2017). Thus, this current scenario has encouraged the search for new and natural preservatives to be applied in bio-based preservation strategies.

Macroalgae have been traditionally consumed in Asian countries for centuries due to their high nutritional attributes while in Western countries their interest lies mainly in hydrocolloids, e.g., thickening, and gelling agents such as alginate, carrageenan, and agar; and their use as additives to animal feed and fertilizers (Kilinç et al. 2013). However, recently seaweed biomass production has increased exponentially (Araújo et al. 2021) due to their great potential uses as well as their value in bioactive compounds with application in multiple areas such as pharmaceutical, cosmetic and food industries (Leandro et al. 2020). More than 15,000 primary and secondary compounds have been isolated from brown macroalgae (Phaeophyceae), specially from the families Sargassaceae and Dictyotaceae, which constitute the main producers with more than 1100 secondary metabolites described in literature, mainly from the genera *Cystoseira*, *Fucus* and *Sargassum* (Blunt et al. 2011; Bouzidi et al. 2019). Interest has been focused on chemical structures typical from this group such as phlorotannins (phenolic compounds), fucoidans (sulphated polysaccharides) or fucoxanthin (carotenoid) due to their multiple described bioactivities: antioxidant, antibacterial, antifungal, anticancer, anti-inflammatory, antiallergic, among others (Andrade et al. 2013; Michalak and Chojnacka 2015; Martínez et al. 2021).

Macroalgae could constitute a great alternative as a source of potential compounds as natural biopreservatives in food industry due to their antimicrobial potential widely reported in literature (Cabral et al. 2021). Besides, their characteristic technological properties allow their incorporation in food matrices, improving their nutritional composition, their quality, their health-related benefits, as well as its shelf-life (Roohinejad et al. 2017).

The aim of this work was to evaluate the antimicrobial potential of extracts from the brown alga *Ericaria selaginoides* against the foodborne pathogen *L. monocytogenes* artificially inoculated in a Catalan fresh cheese ("mató") chosen as food matrix. From the best of our knowledge, it is the first time that derived extracts from *E. selaginoides* have been assessed in a food matrix to improve the safety through the inhibition of the growth of relevant pathogens.

Material and methods

Macroalgae collection

The marine macroalga *Ericaria selaginoides* (Linnaeus) Molinari-Novoa and Guiry 2020 (Ochrophyta, Phaeophyceae, Fucales, Sargassaceae) (formerly *Carpodesmia tamariscifolia* (Hudson) Orellana & Sansón and *Cystoseira tamariscifolia* (Hudson) Papenfuss) was collected on rocky substrata in the lower intertidal and upper subtidal zones (< 1 m depth) in March 2020 in Comillas (Cantabria region, Spain) within the geographical coordinates 43° 23′N / 4° 17′W.

Apical and median parts of the thallus from adult specimens were selected and carefully washed with sterile seawater to remove sediments and epibionts. Then samples were wrapped in sterile cloths moistened with seawater and kept under darkness, humid atmosphere (> 84%) and cool conditions (< 15 °C) into expanded polystyrene boxes (EPS) to preserve algae until transport to our laboratory at IRTA (Monells, Spain). Samples were kept at -80 ℃ until their processing. In the laboratory, algal biomass was ground and stored at -80 °C under vacuum and darkness conditions until extract preparation.

Seaweed extraction procedure

Crude extracts

Crude algae extract was prepared according to Rubiño et al. (2022a) using a mid-polarity extraction medium composed

of a mixture of hexane–isopropanol-water in proportion 10:80:10 (v:v:v). Briefly, pooled supernatants (3000 xg, 10 min, 4 ℃) of three consecutive extractions performed at room temperature for 60 min were evaporated to dryness under vacuum at room temperature (Thermo Scientific Savant SpeedVac SPD120 Vacuum; Thermo Fisher Scientific, USA). The dried extract (5.48% fresh algae) was collected and then kept at -20 °C until further use (Section Preparation of extracts). Because of safety concern regarding to the use of some organic solvents to produce macroalgae extracts with possible further applications for food, in this study the current legislation was taken into consideration (2009/32/EC) (EC 2009) and food-grade solvents were selected.

Two-phase extraction procedure

Crude extract was also subjected to further purification by liquid–liquid separation. The crude extract was transferred into a separating funnel and hexane (VWR Chemicals, USA) was added in proportion 1:1 (v:v). The mixture was thoroughly shaken for 3 min and kept aside for layer separation. After clear phase separation, the top (non-polar) and bottom (mid-polar) phases were collected. The non-polar subfraction was evaporated to dryness at room temperature under vacuum and the mid-polar subfraction was lyophilized. The weight of dried subfractions was collected and then kept at -20 °C until further use (Section Preparation of extracts).

Experimental design of the challenge test

Strains and culture conditions

The selected target bacterium, *Listeria monocytogenes* CTC1011 (serotype 1/2c) was a strain isolated from food that belongs to the IRTA-Food Safety and Functionality Program's culture collection.

The recommendations given in the technical guidance document for conducting shelf-life studies on *L. monocytogenes* in RTE products (EURL Lm 2021) were followed to obtain early stationary phase and cold adapted culture. Stocks of *L. monocytogenes* were kept at -80 ℃ in 20% glycerol as cryoprotectant. The bacterial culture was performed using BHI medium (Brain Heart Infusion broth, Oxoid Thermo Fisher Scientific, USA) in two consecutive steps; first an inoculum was incubated 18 h at 37 ℃ and then 72 h at 8 ℃, until ca. 10^9 cfu mL^{-1}. The physiological state of the cold adapted cells simulates the chilled real conditions usually found in food industry for RTE products.

Preparation of extracts

Prior to testing the antimicrobial activity of crude extract and the two subfractions (non-polar and mid-polar) in the fresh-cheese model, the crude extract and the non-polar subfraction were resuspended under sterile conditions in a mixture of water-glycerol-Tween 80 in proportion 80:10:10 (v:v:v), whereas the mid-polar subfraction was dissolved in sterile water.

The antimicrobial activity of extracts was previously assessed in vitro by means of agar-disk diffusion and microdilution plate assays (Rubiño et al. 2022b). The obtained Minimum Bactericidal Concentration (MBC) (6.54 mg mL^{-1}) for the crude extract against *L. monocytogenes* was used as reference concentration to calculate the concentration of extract applied in the food matrix. In particular, three different concentrations were tested corresponding to $2\times$, $4\times$ and $10\times$ the MBC for the crude extract. " The MBC values for subfractions showed a mean of 0.44 mg mL-1 and 4.29 mg mL-1 for the non-polar and mid-polar, respectively. The amount to be tested was calculated according to the proportion they represent in the crude extract: 70% and 30% for non-polar and mid-polar subfractions, respectively. Thus, final extract concentrations used were: 14, 28 and 70 mg g^{-1} for crude extracts; 5, 10 and 20 mg g^{-1} for the non-polar subfraction and 10, 25 and 50 mg g^{-1} for the mid-polar subfraction.

Fresh cheese model: Preparation, inoculum, and storage conditions

A pasteurised cow's milk fresh cheese typical from Catalonia called "mató" commercially available at local retailers, was selected as a food matrix. The labelled cheese composition was 10% fat (including 6.9% saturated fat), 8.3% protein, 3.1% carbohydrates (being 1.3% free sugars), 0.1% NaCl, 250 mg (100 g)$^{-1}$ calcium (31% Nutrient Reference Values (NRV)), 165 mg (100 g)$^{-1}$ phosphorous (23% NRV). Fresh cheese at the early stage of its shelf-life was used for the assays. Two independent trials using two batches of fresh cheese and newly dissolved crude extract and subfractions were performed on different days. Fresh cheese samples were prepared under aseptic environment into a biosafety cabinet (Scanlaf Mars Pro, Labogene, Denmark) in miniaturised format in 12-well plates (Fig. 1).

Three gram of fresh cheese was weighed in each individual well and each concentration of prepared extract was added (0.45 v/w) in two wells (duplicate) into the food matrix. Afterwards, each well was inoculated (1% v/w) with 10^4 cfu g^{-1} of the *L. monocytogenes* culture and gently mixed with a sterile stick until complete homogenization. Two wells without any extract but inoculated with
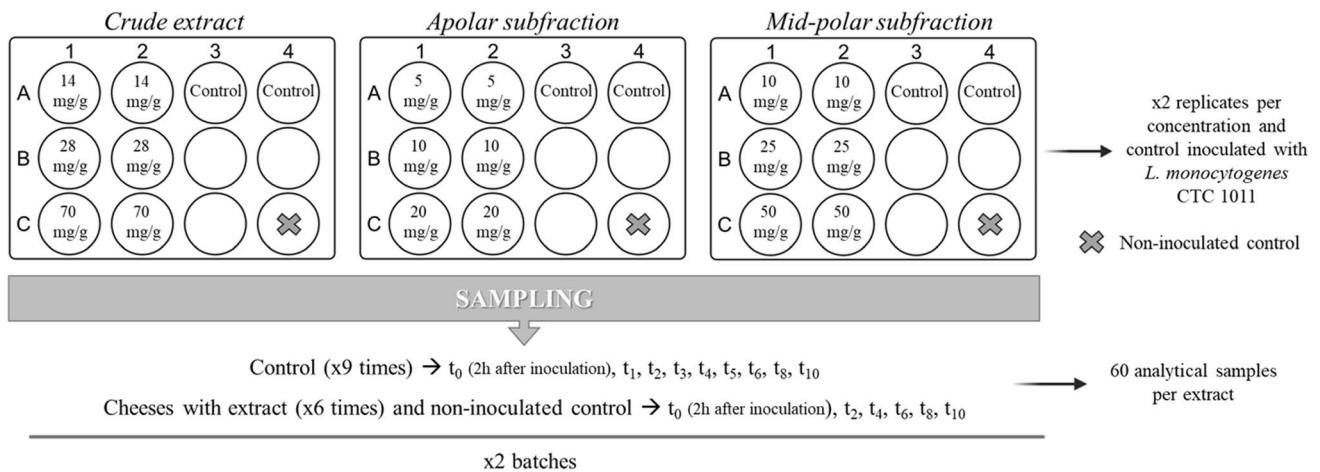
Fig. 1 Challenge test experimental design

L. monocytogenes were used as control. One well without extracts nor *L. monocytogenes* inoculum was also included in the assay to verify the hygienic conditions of handling and storage (i.e., non-inoculated control).

The microtiter plates were covered and sealed in poly-amide/polyethylene (PA/PE) bags (oxygen permeability of 50 cm^3 m^{-2} per 24 h and low water vapor permeability of 2.8 g m^{-2} per 24 h) (Sistemvac, Estudi Graf S.A., Girona, Spain). Sealed 12-well microtiter plates were stored for 10 days at 8 °C. Temperature was constantly recorded by real-time wireless LABGUARD system (BioMerieux, Marcy l'Etoile, France).

Sampling scheme and analytical determinations

Variations in pH (Crison Instruments, Spain) and water activity (a$_w$) by an Aqualab system (Ferrer Lab, Spain) were tested in representative sample of fresh-cheese model. The initial microbiological status of miniaturized cheeses was controlled by Enterobacteriaceae counts using the TEMPO system (Biomerieux, Marcy-l'Étoile, Francia) and total aerobic counts were also measured initially and at subsequent sampling times.

Microbial analyses were performed after 2 h of fresh cheese sample preparation being stored at 8 °C (t$_0$) and almost every day in controls (t$_1$, t$_2$, t$_3$, t$_4$, t$_5$, t$_6$, t$_8$, t$_{10}$), whereas samples with seaweed extracts were assayed every 2 days (t$_2$, t$_4$, t$_6$, t$_8$, t$_{10}$) during storage under refrigeration at 8 °C.

Two gram of sample was weighed, diluted 1:10 in 0.1% Bacto Peptone physiological saline solution (Difco Laboratories, USA) with 0.85% NaCl and mixed thoroughly. Then, appropriate tenfold serial dilutions were performed in saline solution and spread onto CHROMagar Listeria plates (CHROMagar, France). Plate counting was performed after incubation at 37 °C after 48 h, with the detection limit set at 4 cfu g^{-1}.

Growth curve fitting and statistical analysis

To estimate the growth kinetic parameters of *L. monocytogenes*, the primary growth model of Baranyi and Roberts (1994) was fitted to the log count data of each experiment using DMFit Add-in for MS-Excel (downloaded from ComBase portal, www.combase.cc). The goodness of fit was assessed by means of root-mean-square deviation (RMSE) and adjusted determined coefficient (R$^2_{adj}$).

The growth rate values allowed quantitative characterisation of the effect of the seaweed extract on the safe shelf-life of refrigerated cheese. From the food safety point of view, the growth rate values determine the time needed to reach the critical pathogen concentration of 100 cfu g^{-1}, as the maximum level allowed by the microbiological criteria established by the Regulation (CE) 2073/2005 (EC 2005). A hypothetical initial contamination of 1 cfu g^{-1} was assumed as a conservative but reasonably foreseeable scenario (Ross 2010; Jofré et al. 2019). The "time to 2-log increase" was used as an indicator of the safe shelf-life (SSL, Hereu et al. 2014) of the product and was compared in relative terms (RSSL, expressed in fold-increase) with control cheese without extract to quantify the impact of the seaweed extract. The growth potential of *L. monocytogenes* was calculated by subtracting the initial log count to the maximum population density.

The effect of the concentration on the growth rate (μ) was statistically analysed by one-way analysis of variance (ANOVA), following post-hoc multiple pairwise comparison Tukey–Kramer test. The dose-dependent effect was analysed by comparison of linear regression slopes.

Results

### Effect of seaweed crude extract on L. monocytogenes behaviour in fresh cheese

The effect of crude extracts over *L. monocytogenes* growth was tested using a traditional Catalan fresh cheese called "mató" as a food matrix. The extract addition into the food product had minimal effect on the a_w (0.989 ± 0.002) and pH (6.76 ± 0.02) values. Total aerobic microorganisms and Enterobacteriaceae were under the limit of detection (< 10 cfu g^{-1} and < 100 cfu g^{-1}, respectively) in the commercial product, and no colonies were counted in control samples without pathogen inoculation nor addition of extract during the storage time.

Figure 2 shows the behaviour of *L. monocytogenes* inoculated in fresh cheese with three increasing concentrations of *E. selaginoides* crude extract in comparison with the control fresh cheese.

The results of the growth kinetic parameters obtained from primary model fitting are presented in Table 1, together with the satisfactory goodness of fit indexes (i.e., $R^2_{adj} = 0.78$–0.99; SE = 0.05–0.37). The evaluation of the effect of purified subfractions of crude extract was undertaken in order to assess the chemical nature related to the antimicrobial activity against *L. monocytogenes*. To facilitate comparisons, equivalent amounts of purified subfractions based on the proportional content in crude extract of each subfraction (70% and 30%) were tested: 5, 10 and 20 mg g^{-1} for the non-polar subfraction and 10, 25 and 50 mg g^{-1} for the mid-polar subfraction.

Despite using concentrations 2×, 4× and 10× higher than MBC, no immediate listericidal effect was observed in any of the fresh cheeses, as little differences in the initial counts were observed after 2 h of inoculation. In control samples, average counts increased from 3.95 ± 0.14 to 8.06 ± 0.15 cfu g^{-1} after 10 days, with an average growth rate of 0.80 ± 0.06 days^{-1}. In general, the effects observed on the growth rate of *L. monocytogenes* when exposed at increasing concentrations of the crude extract showed a statistically significant (ANOVA: $F_{5,23} = 2.64$, $p < 0.001$), with a clear dose-dependent relationship as shown by the statistically slope ($p < 0.05$) of the linear trend between the square root of the growth rate and the concentration of extracts (Fig. 3).

Thus a slight effect on its growth rate was observed in cheeses supplemented at the lowest concentration (14 mg g^{-1}). However, with intermediate concentration (28 mg g^{-1}) growth rate halved, having a considerable impact in the safe shelf-life, which increased almost twice compared to the control cheese. The most remarkable effect was observed at the highest concentration of crude extract, since *L. monocytogenes* growth was completely inhibited during the 10 days of storage (growth potential < 0.5 log), therefore *L. monocytogenes* would not be relevant for setting the safe shelf-life of the product (EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards) et al. 2020).
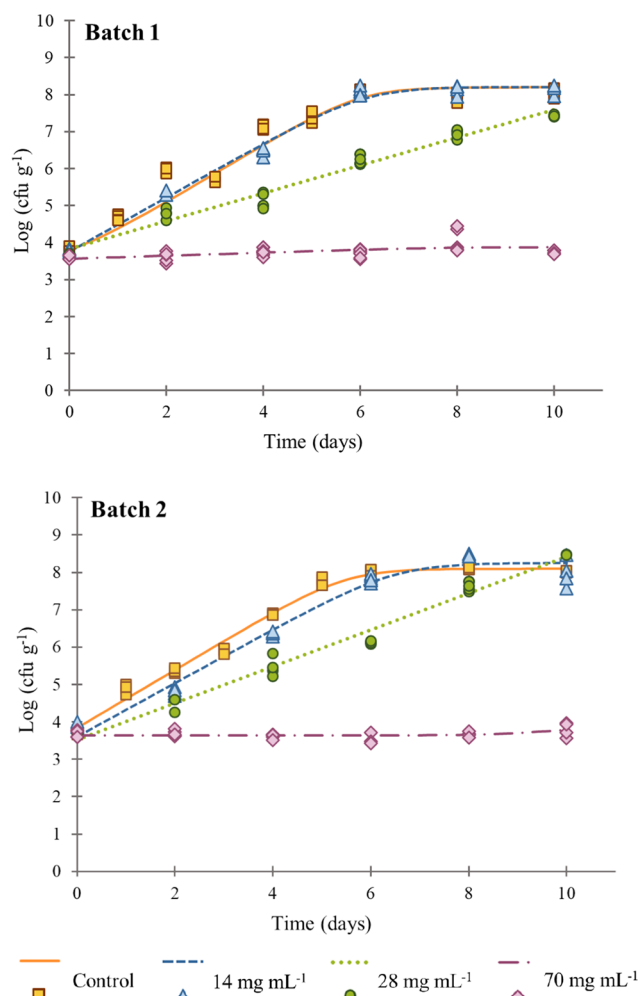
Fig. 2 Behaviour *of Listeria monocytogenes* inoculated in fresh cheeses stored at 8 °C in presence of *E. selaginoides* crude extract at three concentrations for the two independent batches performed. Symbols represent the experimental observed data and lines the fit of the primary growth model

Table 1 Summary of growth kinetic parameters obtained from primary model fitting to *L. monocytogenes* counts in fresh cheese during storage at 8 °C 10 days with or without one of three extracts of *E. selaginoides* at three different concentrations. Mean and standard deviation of two independent batches. The goodness of fit was assessed by R^2 adjusted coefficient and RMSE
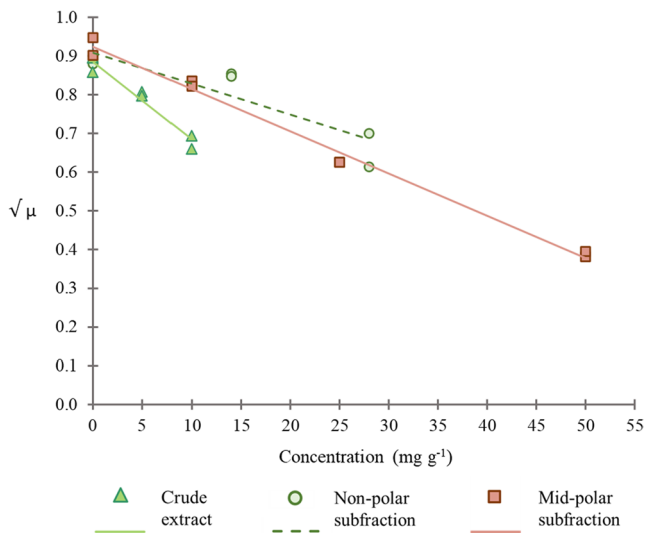
Extract	Assayed concentration (mg g^{-1})	Log N_0 (Log cfu g^{-1})[a]	μ (day^{-1})[a]	Log N_{max} (Log cfu g^{-1})[a]	Growth potential (Log)	Time to 2 log increase (days)	RSSL
		Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	
Control	0	3.95 ± 0.14	0.80 ± 0.06[A]	8.06 ± 0.15	4.11 ± 0.24	2.50 ± 0.17	-
Crude extract	14	3.69 ± 0.11	0.72 ± 0.02[AB]	8.23 ± 0.06	4.55 ± 0.14	2.77 ± 0.09	1.11
	28	3.68 ± 0.19	0.43 ± 0.07[BC]	8.02 ± 0.49*	4.35 ± 0.67	4.70 ± 0.72	1.88
	70	3.63 ± 0.07	NG	-	-	-	n.a
Non-polar subfraction	5	4.06 ± 0.31	0.65 ± 0.03[AB]	7.80 ± 0.11	3.74 ± 0.42	3.09 ± 0.12	1.24
	10	3.86 ± 0.30	0.46 ± 0.03[BC]	7.82 ± 0.16	3.96 ± 0.36	4.38 ± 0.28	1.75
	20	3.77 ± 0.14	NG	-	-	-	n.a
Mid-polar subfraction	10	3.91 ± 0.10	0.69 ± 0.02[AB]	8.20 ± 0.17	4.29 ± 0.21	2.89 ± 0.09	1.16
	25	3.85 ± 0.20	0.37 ± 0.01[B]	7.33 ± 0.41*	3.44 ± 0.32	5.44 ± 0.13	2.18
	50	3.94 ± 0.20	0.20 ± 0.05[CD]	5.41 ± 0.21	1.48 ± 0.04	10.60 ± 2.70	4.24

[a] Kinetic parameters—Log N_0: initial bacterial concentration; μ: growth rate; Log N_{max}: maximum population density; Growth potential: Log N_{max} – Log N_0; RSSL: Relative Safe Shelf-Life compared with control; sd: standard deviation; NG: conditions not supporting the growth of *L. monocytogenes*; n.a.: not apply; * not reach (mean of adjusted log cfu g^{-1} at time 10 days). Different capital letters indicate a statistically significant difference between the extracts and the control according to post-hoc multiple pairwise comparison Tukey–Kramer test.
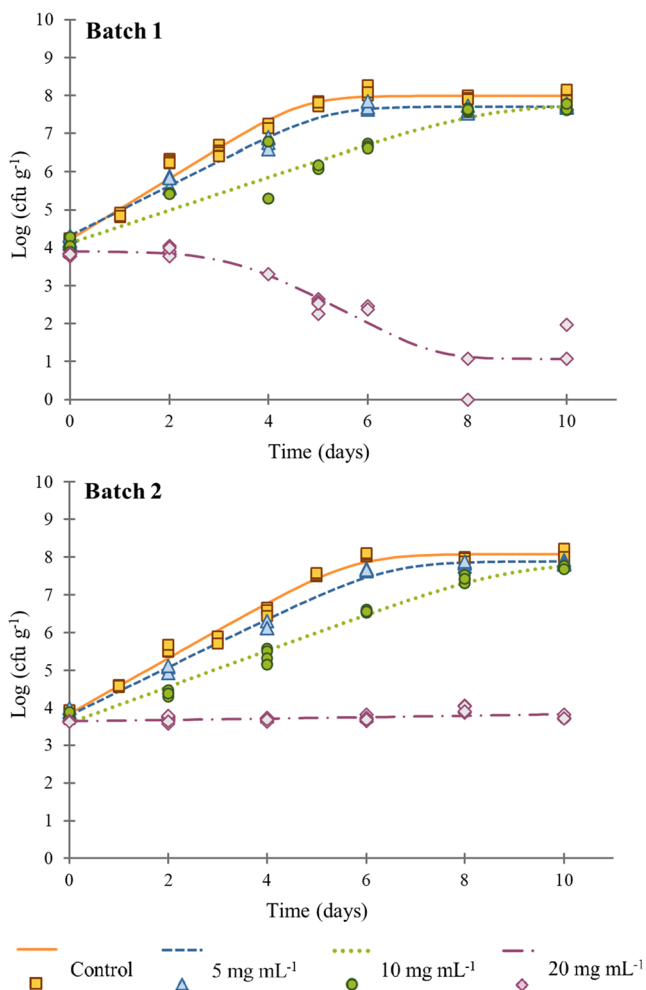

Fig. 3 Dose–response effect of crude *E. selaginoides* extract and non-polar and mid-polar subfractions on the *L. monocytogenes* growth rate during the refrigerated storage (8 ºC) of fresh cheese. Dots (triangles, circles and squares) represent the square root of the growth rate of *L. monocytogenes* obtained from the primary model fitting, while lines are the linear model fit (p < 0.05)

Effect of non-polar and mid-polar subfractions from crude extract on L. monocytogenes behaviour in fresh cheese

Results regarding the antilisterial activity of the non-polar subfraction are shown in Fig. 4, being the kinetic parameters summarised in Table 1. Slight differences in the growth of *L. monocytogenes* were observed between the control and the lowest concentration (5 mg g^{-1}). However, the effect with the intermediate concentration (10 mg g^{-1}) was more pronounced and almost halved the growth rate compared to the control. Samples with extract at the highest concentration (20 mg g^{-1}) showed the highest antimicrobial effect against *L. monocytogenes*, with variable results between the two independent batches assayed, showing a listericidal effect in batch 1 (with almost 3 log reduction of the pathogen levels during the storage) and a listeriostatic effect (no growth) in batch 2.

Results of the mid-polar subfraction are shown in Fig. 5, being the kinetic parameters summarised in Table 1. The lowest concentration of the mid-polar subfraction (10 mg g^{-1}) showed minor effect on the growth of *Listeria* as well as for the crude extract and non-polar subfraction. Moreover, the time to 2 log increase (5.44 ± 0.13 days) and the RSSL doubled since the growth rate decreased to 0.37 ± 0.01 days^{-1} at the intermediate concentration (25 mg g^{-1}). Additionally, best results were also observed at the highest concentration (50 mg g^{-1}) as the growth rate
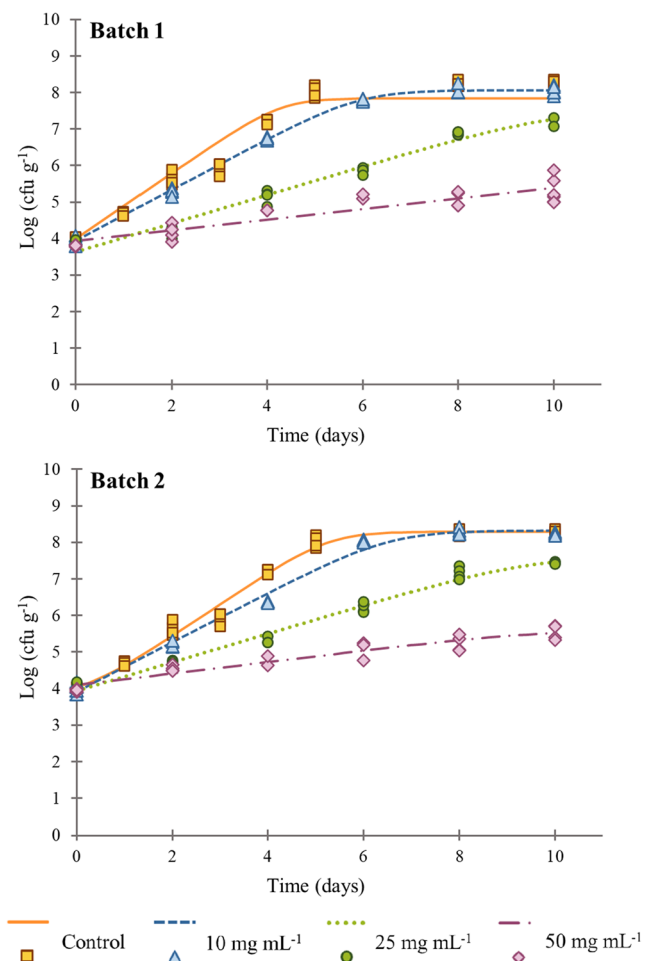
Fig. 4 Behaviour *of Listeria monocytogenes* inoculated in fresh cheeses stored at 8 °C in presence of the non-polar subfraction at three concentrations for the two independent batches performed. Symbols represent the experimental observed data and lines the fit of the primary growth model

Fig. 5 Behaviour of *Listeria monocytogenes* inoculated in fresh cheeses stored at 8 °C in presence of the mid-polar subfraction at three concentrations for the two independent batches performed. Symbols represent the experimental observed data and lines the fit of the primary growth model

decreased 4 times comparing with the control. This meant more than fourfold increase of the safe shelf-life with respect to the control without extract.

Discussion

A traditional Catalan fresh cheese called "mató" was selected as a food matrix, since its pH and a_w values together with low NaCl content and the absence of other antimicrobial preservatives, determine the inclusion of this product into 1.2 category of RTE products that supports the growth of *L. monocytogenes* according to the European Regulation (EC) 2073/2005 (EC 2005). The composition

and the volume of the solvent mixture used for the addition of crude extract into the food product was carefully selected to have a minimal effect on the a_w and pH values of the "mató" cheese matrix under experimental conditions. Moreover, storage temperature at 8 ºC was chosen to assess a worst-case scenario with a slight abuse of the refrigeration temperature corresponding to a reasonably foreseeable condition of the consumer stage (EURL Lm 2021; Jofré et al. 2019).

Total aerobic microorganisms and Enterobacteriaceae counts of the commercial product under the limit of detection ensured the microbiological hygienic quality of the commercial cheese, avoiding possible interferences between *L. monocytogenes* and endogenous microbiota. Furthermore, no colonies counted in control samples confirmed the hygienic handling conditions.

A broad mixture of valuable bioactive compounds, including lipophilic pigments (chlorophyll *a* and fucoxanthin), polyphenols, polysaccharides and other uncharacterised compounds have been extracted and assessed previously from *E. selaginoides* using a mid-polarity extraction medium including 0.73% fucoxanthin, 7.97% polyphenolic compounds and 3.57% carbohydrates (Rubiño et al. 2022b). Antimicrobial activity has been previously reported for these compounds (Pérez et al. 2016), as well as antilisterial activity of crude extract from *E. selaginoides* in in vitro assays (Rubiño et al. 2022b).

Antilisterial activity was reported testing chemical standard and purified fucoxanthin (1 mg mL^{-1}) from *Himanthalia elongata* (Rajauria and Abu-Ghannam 2013). No more references testing antimicrobial activity of fucoxanthin against *L. monocytogenes* have been found in literature, however, other works have also reported activity of both commercial and purified fucoxanthin against other Gram-positive and Gram-negative target strains by disk diffusion and microdilution methods, with the effect stronger against Gram-positive strains (Karpinski and Adamczak 2019; Liu et al. 2019).

Antimicrobial activity of phenolic compounds from brown macroalgal species *Laminaria digitata*, *Saccharina latissima* and *H. elongata* against spoilage and foodborne pathogenic bacteria, including *L. monocytogenes*, was reported by Cox et al. (2010), as well as antilisterial activity of ethyl acetate fractions rich in phenolic compounds obtained from *Ecklonia cava* and *Eisenia bicyclis* with MIC values of 128–256 µg mL^{-1} (Cox et al. 2010; Nshimiyumukiza et al. 2014; Kim et al. 2018). Among polyphenolic compounds in brown algae, phlorotannins are reported to be the predominant class and have been described as molecules with antimicrobial potential found in algal extracts since their -OH groups can bind with the -NH groups of bacterial proteins and cause cell lysis (Ford et al. 2020). Although they are only composed of monomers of phloroglucinol (1,3,5-trihydroxybenzene), there is limited knowledge about the macroalgae-derived phlorotannin profile because of their polymeric nature with diverse molecular weights together with the complexity of their molecular structure (Heffernan et al. 2015). Thus, quantification of extracted phlorotannins is usually recorded for total phenolic content. Currently, only low-molecular-weight phlorotannins (2–8 phloroglucinol units) have been described in the literature. Nevertheless, the occurrence of six phlorotannins has been reported in purified ethyl acetate fraction of *E. bicyclis*, such as eckol (EK), fucofuroeckol-A (FFA), 7-phloroeckol (7-P), dioxinodehydroeckol (DD), phlorofucofuroeckol (PFF) and dieckol (DE). Noticeable antilisterial activity was reported for FFA and PFF with MIC values that ranged 16–32, 32–128 µg mL^{-1}, respectively, whereas the activity against *L. monocytogenes* of 7-P, DD and DE was moderate (64–128 µg mL^{-1}) and minor in the case of EK (128–256 µg mL^{-1}) (Kim et al. 2018).

Crude extract used in this study also co-extracted a proportion of polysaccharides (Rubiño et al. 2022b), which was assumed to be probably linked to polyphenols (Ayrapetyan et al. 2021). Brown macroalgae are a rich source of polysaccharides with diverse molecular structures such as alginates, laminarin and fucoidan (sulphated polysaccharide) (Stiger-Pouvreau et al. 2016). The antimicrobial effect against *L. monocytogenes* of laminarin-rich extracts from *Laminaria hyperborea* with MIC values of 2.6–7.0 mg mL^{-1} and in acidic extracts of *Ascophyllum nodosum* at MIC values of 59.7–66.8 mg mL^{-1} has been reported by Kadam et al. (2015). Antimicrobial activity of commercial fucoidan (74.2 g mol^{-1} weight-average molar mass) from *Fucus vesiculosus* against *L. monocytogenes* KCTC 13,064 achieving MIC values of 250 µg mL^{-1} has been also described (Jun et al. 2018). Antimicrobial activity was also reported in sulphated polysaccharides, mainly fucoidan, extracted from brown macroalgae including *Sargassum* sp., *Saccharina japonica*, *Spatoglossum asperum* and *F. vesiculosus* (Cabral et al. 2021). Poveda-Castillo et al. (2018) also evaluated the effect of commercial purified fucoidan (purity ≥ 95%) from *F. vesiculosus* against *L. monocytogenes* in apple beverage. Fucoidan added at concentration levels of 100 and 1000 µg mL^{-1} reduced bacterial population in 0.40 ± 0.02 and 0.70 ± 0.01 log respectively after 12 days of incubation at 8 ºC.

The identified presence of potential antimicrobial compounds of diverse nature in crude extracts of *E. selaginoides* studied in this work allows for the consideration that the observed antimicrobial effect could not be attributed to a single compound. The assumption is reinforced by the differences in dose-dependent effect that were also observed for the assayed subfractions. Thus, the concentration effect of the crude extract and the mid-polar subfraction on the growth rate of *L. monocytogenes* was significantly different to the observed for the non-polar subfraction (Fig. 4). It should be expected that an additive, synergic and/or antagonist action of the individual active compounds are affected by the interaction among the wide structural diversity of compounds occurring in the crude extract, such as polyphenols and polysaccharides. Indeed, the minimization of these interactions in the non-polar subfraction could be responsible of the observed differences in the antimicrobial effect between extracts.

Currently, few studies evaluating the potential of including whole macroalgae and/or their compounds/extracts against foodborne pathogens and spoilage bacteria are found in literature, aiming to assess the antimicrobial functionality of macroalgae in food matrices. Furthermore, although studies in this sense are still at an early stage, one of the main factors that limits the use of these innovative ingredients as

food preservatives is the impact that can cause over the sensory attributes of food, influencing the acceptability by consumers (Cabral et al. 2021). Further purification steps could allow to get compounds with higher purity minimizing the impact in organoleptic features; i.e., Poveda-Castillo et al. (2018) reported that added commercial purified fucoidan with antilisterial activity in apple beverage remained organoleptic properties intact at all concentrations tested.

Legislative context according to L. monocytogenes control policies in RTE products

Considering the present results in the framework of current EU food safety regulations (EC 2005, 2007), the results of our study provide the scientific justification to include cheeses formulated with the highest concentration of crude extract of *E. selaginoides* and the non-polar subfraction into the category 1.3 of RTE food unable to support the growth of *L. monocytogenes* according to the Regulation (CE) 2073/2005 (EC 2005), as no more than 0.5 log units of pathogen growth are observed during the refrigerated storage of the product (EURL Lm 2021). Similarly, according to Health Canada policies (Health Canada 2011), also the formulation of cheese with high concentrations of extracts allowed to consider the product in the health risk 2. More specifically cheese containing crude extract and non-polar subfraction would be included in the category 2B as growth was inhibited during the studied shelf-life. In this last category, products receive a lower priority with regards to industry verification and control, as well as regulatory oversight and compliance activities. In other countries such as USA, applying zero tolerance against the occurrence of *L. monocytogenes* in RTE food (FSIS 2003a, b), use of high concentrations of the three extracts would allow the classification of the operating procedure into the Alternative 2b by the application of an AMA able to reduce or inhibit the growth of *L. monocytogenes* (less than 2 log increase during the stablished shelf-life).

Conclusions

This study showed that crude extract together with the non-polar subfraction of *E. selaginoides* at the highest concentration tested could totally inhibit the growth of *L. monocytogenes* artificially inoculated in fresh cheese ("mató") during the refrigerated storage. Moreover, a listericidal effect was observed with the non-polar subfraction, which could even reduce *L. monocytogenes* level by ca. 3 log after 10 days of refrigerated storage in one batch. The observed

dose-dependent effect provides useful information to justify proper concentrations in their application as a *L. monocytogenes* control strategy. Results of the present study extend knowledge for the application of brown macroalgae extracts as biopreservatives to minimize the risk of foodborne pathogens as *L. monocytogenes* in food products, emphasizing that a tailor-made approach based on specific product formulations, is important for validation and implementation of natural antimicrobials. Further research based on the purification of these extracts is needed to describe the active principle/s, which could allow to minimize the observed variability in the antimicrobial effect and address the drawbacks associated with the impact of the extracts on the sensory characteristics of the food matrix.

Declarations

Competing interests The authors declare no competing interests.

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