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Topic 2.3.1 Extending shelf-life of perishable Mediterranean food products by sustainable technologies and logistics and by optimized pest and microbial control

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Bio-protective cultures and bioactive extracts as sustainable combined strategies to improve the shelf-life of perishable Mediterranean food

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P2 – Alma Mater Studiorum Università di Bologna – UNIBO

P3 – Universita' Cattolica del Sacro Cuore – UCSC

P4 – C.L.A.I. ScA – CLAI

P5 – University of Split – UNIST (vice-coordinator)

P6 – Croatian Veterinary Institute, Regional Veterinary Institute Split – CROVET

P7 – Centaurus d.o.o. – CROSME

P8 – DOMCA SAU – DOMCA

P9 – University of Ljubljana – UNILJUB

P10 – University of Maribor (Faculty of Mechanical Engineering) – UNIMB

D2.2. Report on in vitro biological activities of algae and agro-food by-products and their correlation with bioactive component composition

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1. AIM OF THE WORK

The aim of the work was to characterize and identify the biological potential of the samples collected in BioProMedFood project, the brown algae and agro-food by-products. Also, a goal was to identify the extracts that carry the bioactive properties and could be selected for potential application in the next phases of the project, to select the best source of bioactive ingredients and to determine biological activity of obtained extracts *in vitro* on common food-borne pathogens, and biogenic amines and ammonia production by bacteria.

2. REPORT ON IN VITRO BIOLOGICAL ACTIVITIES OF ALGAE

2.1 Material and methods

2.1.1. Sample collection (UNIST)

UNIST collected five species of brown algae off the coast of the island Čiovo in the Adriatic Sea from May to September 2020 (Table 2.1). During sampling the sea temperature and salinity was measured using a YSI Pro2030 probe (Yellow Springs, OH, USA). A voucher specimen of tested species is deposited in the herbarium at the University Department of Marine Studies in Split. Harvested algae were washed thoroughly with tap water to remove epiphytes, and freeze-dried by FreeZone 2.5, Labconco (Kansas City, MO, USA) (freeze drying method was selected as optimal drying method based on the preliminary work described in section 2.2.2.). All samples were then grounded (1 min in a high-speed grinder) and stored for analyses.

Table 2.1. The five species of brown algae and their markings

	Sea temperature	*Cystoseira compressa*	*Padina pavonica*	*Cystoseira amentacea*	*Dictyopteris polypodioides*	*Sargassum vulgare*
May	18.3	CCOM5	PPAV5	CAME5	DPOL5	SVUL5
June	22.4	CCOM6	PPAV6	CAME6	DPOL6	SVUL6
July	23.8	CCOM7	PPAV7	CAME7	DPOL7	SVUL7
August	26.9	CCOM8	PPAV8	CAME8	DPOL8	SVUL8
September	24.7	CCOM9	PPAV9	CAME9	DPOL9	SVUL9

1.1.2. Extraction (UNIST)

The dry algal material was mixed with 50% ethanol and extracted by microwave assisted extraction (MAE) in advanced microwave extraction system (ETHOS X, Milestone Srl, Sorisole, Italy). Extraction conditions were as follows: power and temperature were kept constant at 200 W and 60°C over 15 minutes. The extracts were than centrifuged at 5000 rpm for 8 min at room temperature and filtered, the EtOH evaporated and the extracts freeze dried.

1.1.3. Total phenolic content and antioxidant capacity (UNIST)

The TPC of algae extracts was determined by the Folin–Ciocalteu method. Briefly, 25 µL of the extract was mixed with 1.5 mL distilled water and 125 µL Folin–Ciocalteu reagent. The solution was mixed and after one minute 375 µL 20% sodium carbonate solution and 475 µL distilled water was added. The mixture was left in the dark for 2 h at room temperature. The absorbance was read at 765 nm using a spectrophotometer (SPECORD 200 Plus, Edition 2010, Analytik Jena AG, Jena, Germany). The standard calibration (0–500 mg/L) curve was plotted using gallic acid (y = 0.001x, R 2 = 0.9998). The TPC was expressed as gallic acid equivalents in mg/g of dried algae (mg GAE/g).

The antioxidant activity of was assessed with three different methods that are based on two different mechanism of action (hydrogen atom transfer - HAT (DPPH, ORAC) and electron transfer - ET (FRAP)).

The reducing activity was measured as FRAP (Ferric Reducing/Antioxidant Power). Briefly, 300 µL of FRAP reagent solution was pipetted into the microplate wells, and absorbance was measured at 592 nm. Then, a 10 µL of sample was added to the reagent and the change in absorbance after 4 minutes was measured. The change in absorbance, calculated as the difference between the final value of the absorbance of the reaction mixture after a certain reaction time (4 min) and the absorbance of FRAP reagent before sample addition, was compared with the values obtained for the standard solution (Trolox). Results were expressed as micromoles of Trolox equivalents (µM TE)

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability of extracts was measured in 96-well microplates. DPPH radical solution (290 µL) was pipetted into microplate wells, and absorbance was measured at 517 nm. Then, a 10 µL of sample was added to the wells and the decrease in the absorbance was measured after 1 h using the plate reader. The antioxidant activity was expressed as inhibition percentages of DPPH radical (% inhibition).

The oxygen radical absorbance capacity (ORAC) method was performed to determine the antioxidant capacity of extracts by monitoring the inhibition of the action of free peroxyl radicals formed by the decomposition of 2,2-azobis (2-methylpropionamide) -dihydrochloride (AAPH) to the fluorescent compound fluorescein. Briefly, 150 µL of fluorescein and 25 µL of sample (or Trolox, or blank) were pipetted into microplate wells and thermostated for 30 minutes at 37 °C. After 30 minutes, 25 µL of AAPH is added and the change in fluorescence intensity is measured every minute during 80 min at 485 and 520 nm. Extracts were diluted and the results were expressed as µM of Trolox Equivalents (µM TE).

1.1.4. Antimicrobial testing against foodborne pathogens (UCSC)

The minimum inhibitory concentration (MIC) values were also determined against *Listeria monocytogenes* (UC8160), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (DSMZ UC3674), *Staphylococcus aureus* (ATCC6538), *Bacillus cereus* (UC4044). The MIC values, which represent the lowest concentration that completely inhibits the growth of pathogenic microorganisms, were determined by a micro-well dilution method on Muller-Hinton broth, based on the optical density at 620 nm (OD620). Absorbance A, also known as optical density (OD), is the quantity of light absorbed by a solution). The most common way to assess microbial growth in solution is the measurement of the optical density. The method is based on absorbance detection mode and basically determines which portion of light passes through a sample, more specifically through a suspension of microorganisms. So lower or negative values of OD indicated no bacteria growth.

Preparation of microdilution plate

Five microplates for each pathogen were prepared, one per Algae at each different month (see scheme). Algae solution for MIC were prepared from a stock solution contained 40 mg/ml il 50% of ETOH solution. Serial two-fold dilution were performed in Muller Hilton broth in order to obtain a concentration range from 20 to 0.156 mg/ml. Twenty-five µl were dispend in a well of microdilution plate to obtain a final concentration as illustrated in the next schema.

Preparation of pathogen inoculum

Individual colonies were picked up on the agar plate, suspend in 5 ml of sterile saline until the solution turbidity reaches McFarland standard 0.5 or optical density at 625 nm of 0,1 by spectrophotometer (OD620nm), approximately 108 CFU/ml.

The bacterial suspension was them dilute 500 times in the recommended medium (Muller Hilton) and 25 µl were distribute in all microtiter wells except for the column 12 (alga blank). The MICs were performed in a final volume per well of 50 µl.

An initial OD determination was performed before incubation to obtain the time 0 inoculum values. The plates were then incubated at 37 °C for 24 h and the OD measurement was performed.

- The time 0 inoculum values are the OD measurement for the column 11 before incubation.
- The positive control (column 11) contained only the inoculum (25 µl bacterial suspension in MH+ 25 µl of MH broth).
- The blank for each alga at each month was also included in the microtiter (column 12) only contained 25 µl of the respective alga solution and 25 µl of MH broth.

The MIC value was calculated by obtaining the mean of two replicates minus the value of the algae blank. Negative OD values are considered absence of bacterial growth.

The MIC is the lowest algae concentration at which there is no growth. MIC was considered the negative OD values or OD lowest than the inoculum at time 0, because when compared with the positive control indicated no bacterial growth.

1.1.5. Antimicrobial testing against spoilage bacteria and fungi (DOMCA)

In the research of antimicrobial effect of the algae samples, additional work was done by DOMCA. The microorganisms used for the study were obtained from the CECT (Spanish Collection of Type Cultures) and wild isolates from our collection (DMC Research) (Table 2.2). Concerning wild strains, they were originally isolated from the food industry and stored with 20% glycerol at −70 °C. The culture media used were BHA (brain heart agar) and selective culture medium supplied by Biokar Diagnostics (Allone, France).

Table 2.2. References and uses of the strains used.

Microorganisms	Reference	Isolated	Culture Media
Escherichia coli	CECT 405	Minced meat isolate	MacConkey Agar
Listeria innocua	CECT 4030	Cheese isolate	Compass *Listeria*
Lactobacillus alimentarium	DSMZ 20181	Fish isolate	MRS Agar

Candida sake	DMC-01	Sausaces isolate	Sabouraud cloranfenicol
Penicillium expansum	DMC-03	Fruit isolate	Rosa de Bengala Agar

Agar diffusion test

For the agar diffusion test, cultures of different microorganisms' strains were performed in selective medium, incubating them at 37 °C for 24 hours for bacteria and 25 °C for 4 days for fungi. Previously, a sterilized cellulose disc (6 mm diameter Whatman® antibiotic test discs) impregnated with extract (20 µL of 25% extract/disc) had been placed in the center of culture medium plates. The inhibition zone of bacterial growth is proportional to the degree of inhibition produced. All assays were performed in duplicate.

Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

The MBC (Minimum Bactericidal Concentration) was used to determine the lowest concentration of an antimicrobial agent that reduces the viability of the initial bacterial inoculum by 99.9%. The method used for this study was broth microdilution according to the National Committee for Clinical Laboratory Standards. Decreasing concentrations (25, 12.5, 6.25, 3.12 and 1.5%) of extracts were used and inoculated with different bacterial strains to obtain a final concentration of 5 Log10 CFU/mL. As negative control, another well without bacteria or any antimicrobial agent was used. Finally, every well with no cell growth (measured by absorbance at 620 nm) was tested by being cultured in selective agar plates and incubated, in order to determine the MBC.

1.1.6. Growth inhibition kinetics, anti-adhesion and anti-biofilm activity (including anti-quorum sensing) on common food-borne pathogens (UNILJUB)

The growth kinetics of the bacteria strains in the presence of the selected prepared extracts were determined using Varioscan^{TM} LUX multiplate microtiter plate reader (Thermo Fisher Scientific, Sunnyvale, CA, USA). Sub-inhibitory concentrations of the extracts were prepared in Tryptic Soy Broth (TSB; Biolife, Milan, Italy). 50 µL of working concentration of the extract was pipetted into the wells in 96-well microtiter plate (NUNC, Thermo Fisher Scientific, Sunnyvale, CA, USA). Then the same volume of inoculum (10^5 cfu/mL) was added into each

well. Positive control contained 50 µL of inoculum and 50 µL of TSB. Negative control contained 100 µL of TSB. Negative controls for the extract contained 50 µL of working concentration of the extract and 50 µL of TSB instead of bacteria. The analyses were performed in 3 technical and 2 biological repetitions. The optical density (OD) at 600 nm was measured every half hour for 24 hours using microtiter plate reader. Suitable temperature (30 °C or 37 °C) and shaking on every 5 s was set. Growth curves were drawn in Microsoft Excel.

To determine biofilm formation of selected strains in the presence of sub-inhibitory concentrations ($^1/_4$ MIC) of extracts on polystyrene, CFU plate counting method was used after 24 h or after 48 h for *C. jejuni* NCTC 11168. 100 µL of working concentration of selected extract was pipetted into 12 wells of 96-well polystyrene microtiter plate (NUNC, Thermo Fisher Scientific Inc., Sunnyvale, CA, USA). Then, 100 µL of inoculum (10^5) was added. For positive control, 100 µL of bacteria and 100 µL of broth medium (TSB/MHB) were added. After inoculation, the microtiter plate was mixed on a microtiter plate shaker (600 rpm, 1 min; Eppendorf Thermomixer Comfort, Hamburg, Germany) and incubated under following conditions: *S. aureus*, *L. innocua* and *B. cereus* for 24 h at 37 °C, *P. fragi*, *S. putrefaciens* and *S. xiamenensis* for 24 h at 30 °C, and *C. jejuni* for 48 h at 42 °C (microaerophilic conditions: 82 % N_2, 10 % CO_2, 5 % O_2). After incubation, the non-adhered planktonic cells were carefully removed from the wells and each well was washed 3-times with 200 µL 0.9 % saline solution or with PBS in the case of *C. jejuni*. After the third washing, 200 µL 0.9 % saline solution or PBS was added to each well, the wells were covered with sterile Greiner BIO-one EASYseal adhesive sealer, and the microtiter plate was sonicated for 10 min at room temperature. Then 10-fold serial dilutions were prepared. 10 µL of each dilution was transferred to agar plate (TSA/MHA) and incubated for 24 h (under the conditions described above). In the case of *C. jejuni* strain, the content was carefully removed from each well after 24 h and replaced with fresh test solutions (1/4 MIC) or broth (MHB) for the positive control, and incubated for 24 h (under the conditions described above). After incubation, the non-adhered planktonic cells were carefully removed from the wells as described above.

1.1.7. Additional antimicrobial testing and biogenic amines and ammonia production by bacteria (CUNI)

Bacterial strains

The antimicrobial properties of samples were detected using three food-borne pathogens (*Salmonella* Paratyphi A NCTC13, *E. coli* ATCC25922 and *Staphylococcus aureus* ATCC29213); one strains of pathogenic yeast (*Candida albicans*) and three fish spoilage bacteria (*Pseudomonas luteola*, *Photobacterium damselae* and *Enterococcus faecalis*) All bacterial cultures were grown in Nutrient broth (Merck 1.05443.0500).

Antimicrobial activity of extracts

Agar well diffusion method

Antimicrobial activities of extracts were detected using agar well diffusion method on Mueller-Hinton Agar (MHA, Merck 1.05437, Darmstadt, Germany). For antimicrobial activity test, powdered extracts were dissolved in sterile distilled water to give a 50 mL preparation, with final 20 mg/ml stock solutions.

The test bacteria were incubated in Nutrient broth (Merck 1.05443, Darmstadt, Germany) at 37 °C for 24 h. For diffusion method, petri plates were prepared by pouring 20 ml of MHA and inoculated with 24 h broth culture of bacteria under aseptic conditions after matching the turbidity with 0.5 McFarland. After solidified, five wells were made. Fifty microliters of each samples were poured in each of the four wells of MHA plates. Fifth (control wells) was filled with distilled water. The plates were kept for 1 h at room temperature to allow the diffusion into the medium and then incubated at 37°C for 24 h. After the incubation period, the inhibition zones formed around the wells were measured in millimetre.

Minimum inhibitory and bactericidal/fungicidal concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC/MFC) of extracts were determined using Clinical and Laboratory Standards Institute's methods (2008). One mL of extract (with stock solution of 20 mg/mL) was added to the first tube in each series and serially diluted with Mueller Hinton Broth (MHB, Merck 1.10293) until obtaining final concentrations of 0.078 mg/mL. One mL of inoculum suspension (10^6 cfu/mL) from each bacterial strain was supplemented in each tube containing extract and MHB (Picture 3). A tube including MHB and bacterial suspension without extracts was used as a positive control. As a negative control, a tube did not contain the MHB. After incubation at 35°C for 18-24 h, the tubes were evaluated for MIC of each extract. MBC/MFC was detected by sub-culturing the contents of tubes of MIC displaying no growth.

Ammonia and biogenic amine analysis

The production of ammonia and biogenic amine by bacteria was monitored using histidine decarboxylase broth (HDB). HDB consisted of 1 g peptone, 0.5 g Lab-Lemco powder (Oxoid CM0017, Hampshire, England), 2.5 g NaCl (Merck 1.06404.1000, Darmstadt, Germany), 4.01g L-histidine HCl (Sigma H8125, Steinheim, Germany) and 2.5 mg pyridoxal-HCl (Sigma P9130, Steinheim, Germany) in 500 ml distilled water and the pH was adjusted according to their optimum growth pH with 1M KOH (Riedel-deHaen 06005, Seelze, Germany) or 6% TCA (Riedel-deHaen 27242, Seelze, Germany). HDB was pipetted in 10 ml bottles and then autoclaved at 121°C in 15 min prior to use. After propagation of bacteria, 0.5 mL of bacterial cultures (~10^8 cfu/mL) was removed and put into 9 mL of the HDB. Extracts CAME6 and PPAV6 (Table 2.1.) at doses of 2% (v/v) from stock solution of 20 mg/ml were added into the HDB. The control groups of HDB were the absence of any of the extracts. Samples were incubated at 28^{o}C for 72 h.

Derivatization procedures of ammonia and biogenic amines

For derivatization of standard amine solutions, 50µL was taken (4mL for extracted bacterial culture) from each free base standard solution (10 mg/mL). One millilitre of 2M sodium hydroxide was added, followed by 1mL benzoyl chloride (2%), and mixed on a vortex mixer for 1 min. The reaction mixture was left at room temperature (24°C) for 20 min. The benzoylation was stopped by adding 2mL of saturated sodium chloride solution and the solution was extracted two times with 2mL of diethyl ether. The upper organic layer was transferred into a clean tube after mixing and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 1 mL of acetonitrile and 10 µL aliquots were injected into the HPLC. For biogenic amine analysis a Shimadzu Prominence HPLC (Shimadzu, Kyoto, Japan) equipped with a SPD-M20A diode array detector, two binary gradient pumps (Shimadzu LC-10AT), auto sampler (SIL 20AC), column oven (CTO-20AC) and valve unit FCV-11AL with a communication bus module (CBM-20A) were used. The column was a reverse-phase, ODS Hypersil, 5µ, 250x 4.6 mm (Phenomenex, Macclesfield, Cheshire, UK). The confirmation of biogenic amine production was carried out using a rapid HPLC method. For ammonia analysis, same analytic method was conducted.

1.1.8. Antimicrobial activity of the essential oils from *C. compressa* (UNIBO)

The *in vitro* antimicrobial activity of *C. compressa* EO against some foodborne pathogens (*Escherichia coli* 555, *Salmonella* 155, *Listeria monocytogenes* ScottA, *Klebsiella aerogenes* EPS8 and *Staphylococcus aureus* DSM 20231t) as well as against the tyraminogenic strain *Enterococcus faecium* FC12 was studied with broth microdilution method using microtiter plates (Corning Incorporated, N.Y., U.S.A.). For the determination of cell growth/no growth, 198 µL of BHI broth inoculated with foodborne pathogen individually at a level of approximately at 5 cfu/mL were placed into 200 µL microtiter wells (Figure 2.1). Essential oils were dissolved in ethanol and two µL of these solutions were added to each well. Microtiter plates were incubated at 30°C for *E. faecium* FC12 and *Staph. aureus* DSM 20231t and at 37°C for *E. coli* 555, *Salmonella* 155, *L. monocytogenes* ScottA, *K. aerogenes* EPS8. The presence of a visible growth after 48 h of incubation was recorded for the Minimum Inhibitory concentration (MIC) detection. In the case of no growth the Minimum Bactericidal Concentration (MBC) was determined by spotting 10 µL from each well in BHI agar plates.
When the EO concentrations did not allow to define MIC or MBC values, an additional trial using disk diffusion method was performed, to evaluate EOs activity used at higher concentrations. In this case, sterile, 6-mm-diameter filter paper disks (Schleicher and Schül, Dassel, Germany) were impregnated with 10 µL of EOs dissolved in ethanol at a concentration of 100 mg/ml or 50 mg/ml. The references bacteria were pre-cultured in BHI broth for 24 h at 30°C or 37°C, then were individually inoculated in BHI soft agar with a cell concentration of approx. 6 log cfu/g (adjusted using the Standard McFarland counting method) and dispensed into sterile plates. The medium was solidified under aseptic conditions at 30°C for two hours before placing the EO impregnated paper disks on the plates. After 24 h of incubation, all plates were observed for zones of growth inhibition, and the diameters of these zones were measured in millimeters. All tests were performed under sterile conditions in duplicate.

2.2 Results

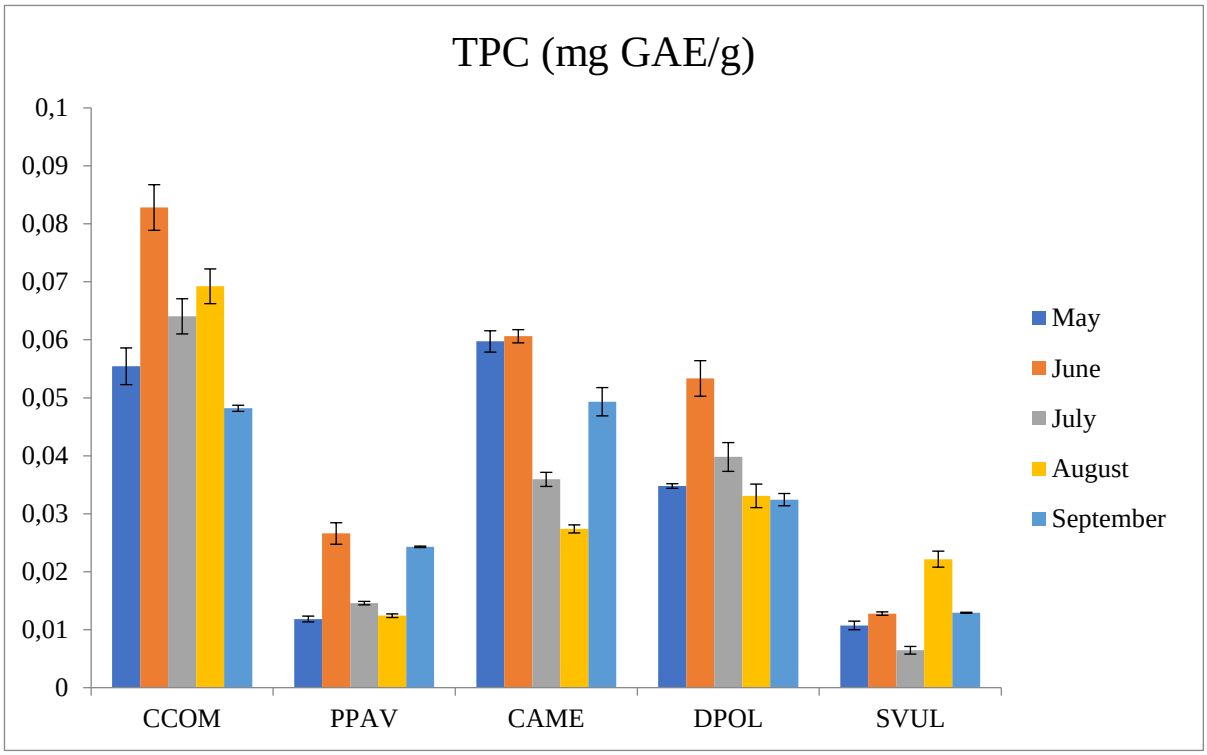
2.2.1. The total phenolic composition and antioxidant activity (UNIST)

Change in TPC through the months is evident in all collected algae (Figure 3.2. A). All algae had the highest TPC in June, except *S. vulgare*, with highest results for *C. compressa* and C. *amentacea*. *S. vulgare* had the lowest TPC compared to other algal species.

FRAP results (Figure 3.2. C) can be correlated with TPC for all algae, except *D. polypodioides*. *C. compressa* in June had the highest reducing ability. Overall, both algae belonging to the genus *Cystoseira* had significantly higher FRAP results than other analyzed algae.

Algae ability to inhibit DPPH radical is shown in Figure 2.1.B. *C. compressa* had the highest results for May, July and August. *D. polypodioides* and *C. amentacea* had the highest inhibition in June and September, respectively. Overall, *P. pavonica* and *S. vulgare* had the lowest percentage of inhibition among analyzed algae. The results of algae ability to scavenge peroxyl radicals, the predominant free radicals found in biological systems, is shown on Figure 2.1.D. Algae extracts were 200-fold diluted for ORAC assay. *P. pavonica* from June had the highest scavenging activity. High activity was also recorded for *C. compressa* in August and *D. polypodioides* in June. Results for all algae varied through collecting months.

(A)



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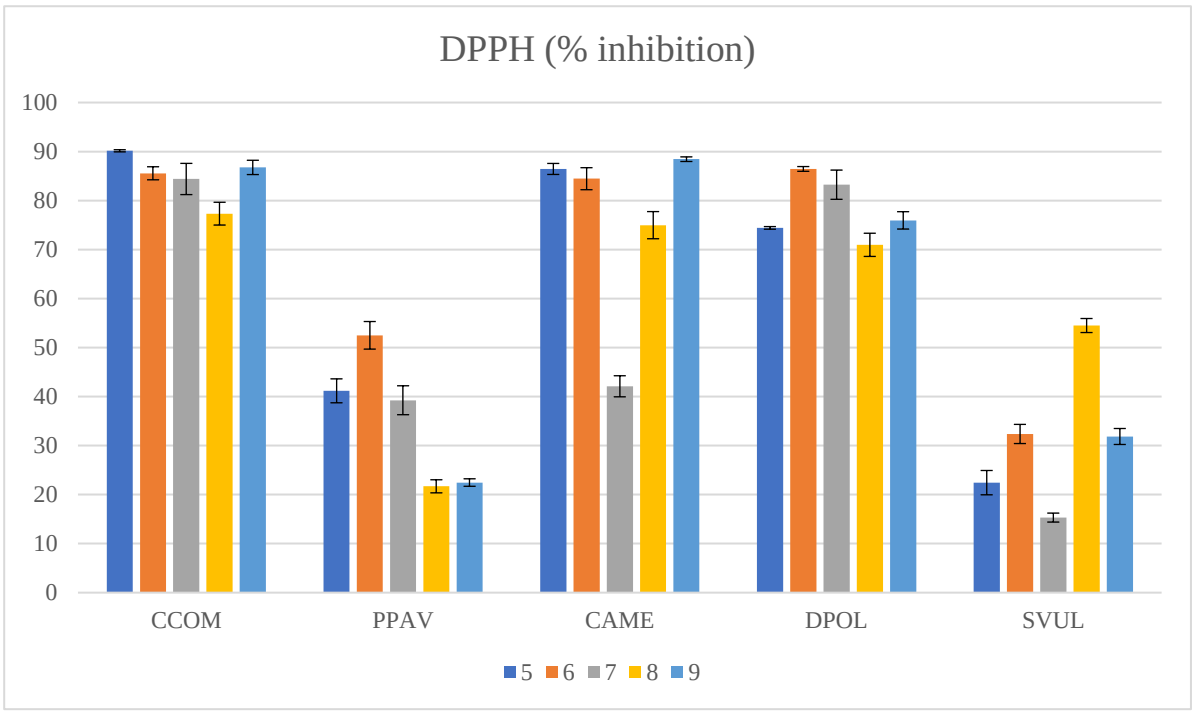
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80
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40
30
20
10
0
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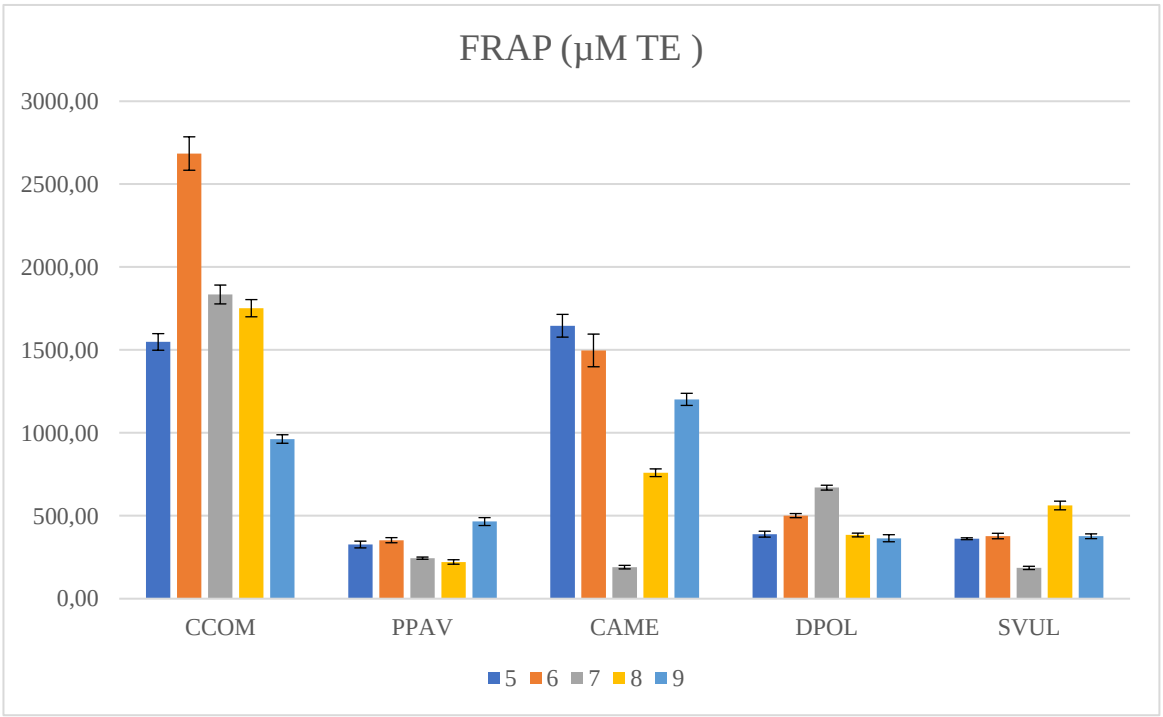
PPAV
CAME
DPOL
SVUL
5 6 7 8 9

(C)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(D)

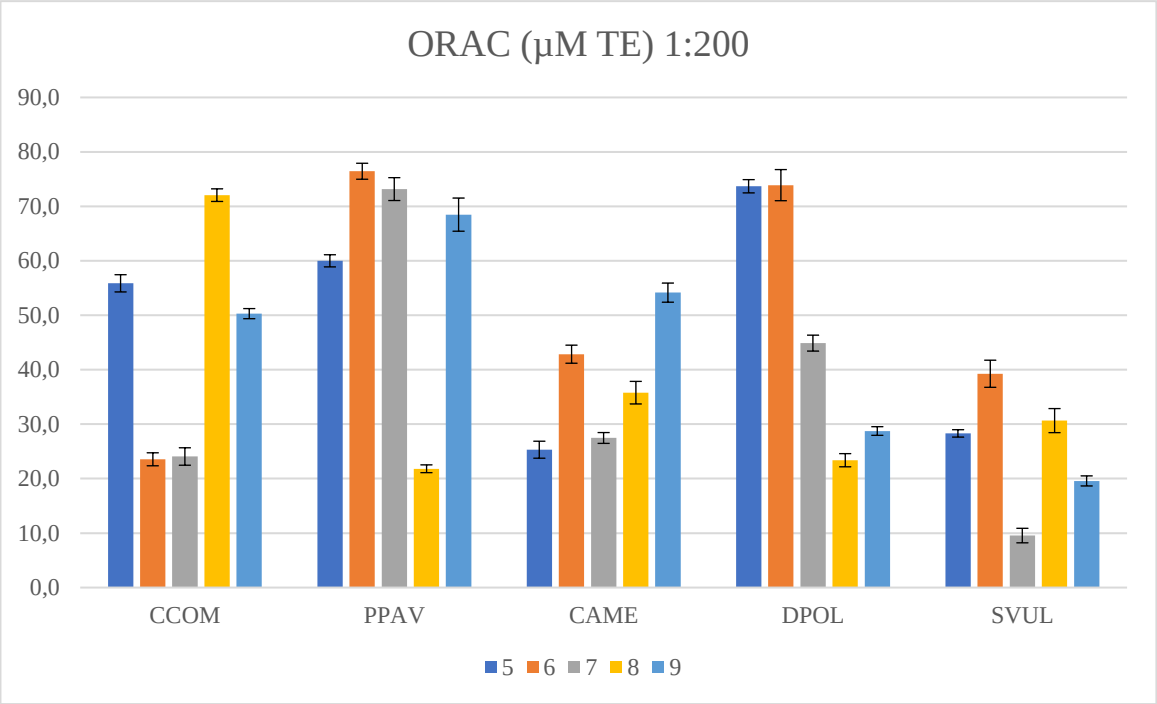


Figure 2.1. Total phenolic content (A, TPC), and antioxidation (B-D) of the algae *Padina pavonica* (PPAV), *Cystoseira compressa* (CCOM), *Cystoseira amentacea* (CAME), *Dictyopteris polypodioides* (DPOL), and *Sargassum vulgare* (SVUL) over 5 months.

2.2.2. The antimicrobial activity of the algae against foodborne pathogens (UCSC)

The capacity of algae extracts to inhibit some major food pathogen growth was assessed. Table 2.3 shows the MIC values (mg/ml) for the ethanol algae extract. All ethanol extracts tested showed inhibitory activity against pathogenic bacteria tested *Listeria monocytogenes* UC8160, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* DSMZ UC3674, Staphylococcus *aureus* ATCC6538 and *Bacillus cereus* UC4044.

The MIC values showed high variability and were dependent on both, type of alga and the month of sampling. The highest MIC values were needed to inhibit *Salmonella typhimurium* DSMZ UC3674 except for the *Cystoseira compressa* (CCOM) extract from May (0,078 mg/ml). Lower MICs are required to inhibit *Staphylococcus aureus* ATCC6538 and *Escherichia coli* ATCC 25922. While the MICs to inhibit *Bacillus cereus* and *Listeria monocytogenes* were very varied with ranging from 0,078 to 10 mg/ml). The most active for algae extract was the September sampling.

The algae extract present higher potential as natural preservatives due to their ability to inhibit pathogenic bacteria.

Table 2.3. Results of the minimal inhibitory concentration (MIC mg/mL) of the algae against foodborne pathogens.

Algae sample	***Listeria monocytogenes (UC8160)***	***Escherichia coli (ATCC 25922)***	***Salmonella typhimurium (DSMZ UC3674)***	***Staphylococcus aureus (ATCC6538)***	***Bacillus cereus* (UC4044)**
PPAV5	0,078	0,078	10	0,078	5
PPAV6	10	5	10	1,25	2,5
PPAV7	5	2,5	10	0,312	1,25
PPAV8	10	1,25	10	0,312	5
PPAV9	1,25	0,625	2,5	0,078	0,625
CCOM5	0,156	0,156	10	0,312	1,25
CCOM6	<10	0,078	0,078	0,156	2,5
CCOM7	2,5	2,5	5	1,25	0,078
CCOM8	1,25	0,625	10	0,156	2,5
CCOM9	2,5	1,25	2,5	0,078	2,5
CAME5	0,078	0,078	10	0,078	0,312
CAME6	2,5	0,156	<10	1,25	0,625
CAME7	1,25	1,25	10	0,675	1,25
CAME8	1,25	1,25	10	1,25	10
CAME9	2,5	0,625	2,5	0,078	0,625
DPOL5	0,078	0,078	10	0,078	10
DPOL6	10	0,625	<10	2,5	10
DPOL7	2,5	2,5	10	2,5	2,5
DPOL8	10	5	10	0,625	5
DPOL9	2,5	0,625	5	0,625	2,5
SVUL5	0,078	0,156	10	0,078	5
SVUL6	10	5	5	2,5	5
SVUL7	2,5	2,5	5	2,5	2,5
SVUL8	10	10	10	5	10
SVUL9	5	0,625	5	0,625	2,5

2.2.3. The antimicrobial activity of the algae against spoilage bacteria and fungi (DOMCA)

Table 2.4. Results of the antibiosis tests on solid medium, of extract against reference strains.

Extracts	*E. coli*	*L. innocua*	*Lb. alimentarium*	*C. sake*	*P. expansum*
CCOM5	-	-	-	-	-
PPAV5	-	-	-	-	-
CAME5	9 ± 1	9 ± 1	-	-	-
DPOL5	-	9 ± 1	-	-	-
SVUL5	-	-	-	-	-
CCOM6	-	-	-	-	-
PPAV6	-	-	-	-	-
CAME6	10 ± 1	-	-	-	-
DPOL6	-	-	-	-	-
SVUL6	-	-	-	-	-
CCOM7	10 ± 1	8 ± 1	-	-	-
PPAV7	-	-	-	-	-
CAME7	-	-	-	-	-
DPOL7	-	-	-	-	-
SVUL7	-	-	-	-	-
CCOM8	-	-	-	-	-
PPAV8	-	-	-	-	-
CAME8	-	9 ± 1	-	-	-
DPOL8	-	-	-	-	-
SVUL8	-	-	-	-	-
CCOM9	-	-	-	-	-
PPAV9	-	-	-	-	-
CAME9	-	-	-	-	-
DPOL9	-	-	-	-	-
SVUL9	-	-	-	-	-

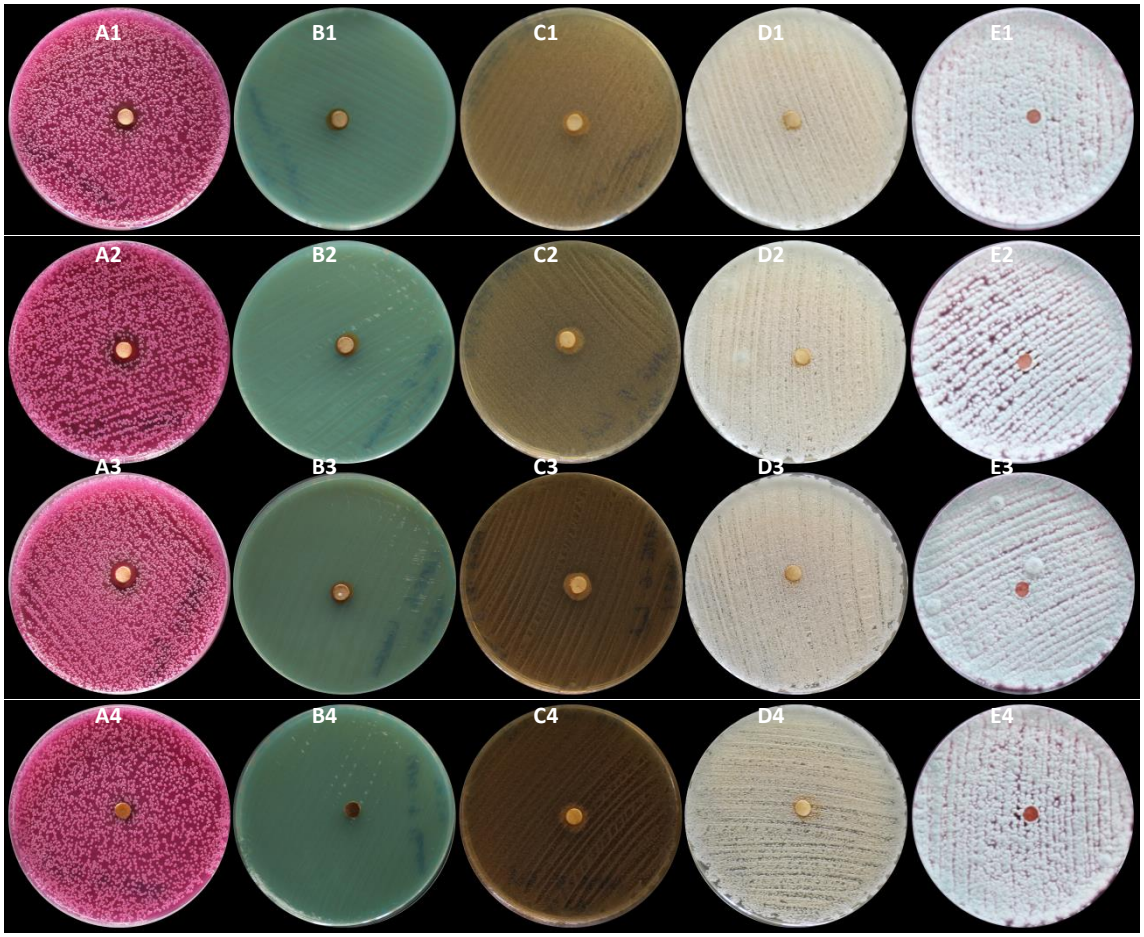


Figure 2.2. Zones of inhibition (mm) **1)** CCOM7 **2)** CAME5 **3)** CAME6 y **4)** DPOL5 against: **A)** *E. coli* CECT 405 **B)** *L. innocua* CECT 4030 **C)** *Lb. alimentarium* DSM 20181 **D)** *C. sake* DMC-01 y **E)** *P. expansum* DMC-03.

Although both tests are simple to perform and easily reproducible, the evaluation of the antimicrobial activity of algae extracts is complex due to their rheology, which can reduce their dilution capacity, as well as cause an irregular distribution through of the agar. In order to obtain more precise results, the CMBs of these extracts were determined. The results against each of the strains tested are shown in Table 2.5.

The results obtained indicated that CCOM and CAME extracts show a discrete antimicrobial activity. CCOM6 extract proved to be the most effective against the bacterial tested at a concentration of 6.25%, followed by CAME extract. None of the extracts showed a significant antifungal activity.

Table 2.5. Results of minimum bactericidal/fungicidal concentration (MBC/MFC) of extracts against reference strains. Results are expressed as percentage.

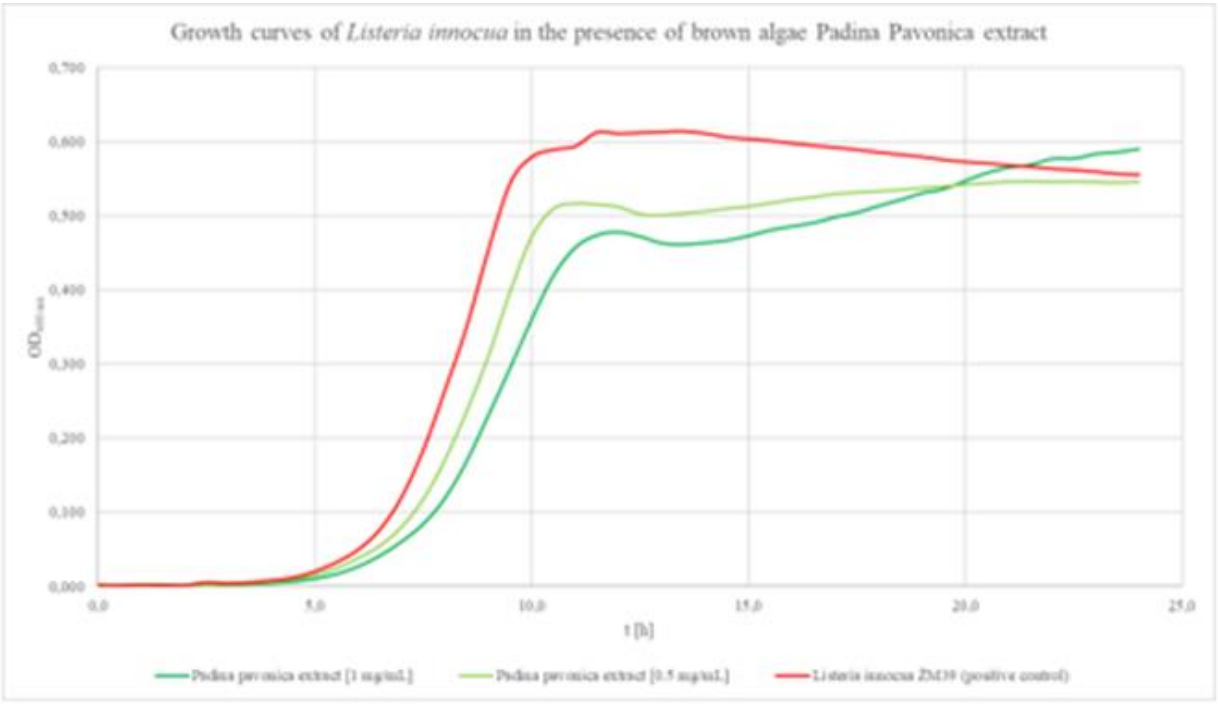
Extracts	*E. coli*	*L. innocua*	*Lb. alimentarium*	*C. sake*	*P. expansum*
CCOM5	25	12.5	25	25	> 25
PPAV5	> 25	> 25	> 25	> 25	> 25
CAME5	25	12.5	25	> 25	> 25
DPOL5	25	25	25	25	> 25
SVUL5	25	12.5	25	> 25	> 25
CCOM6	12.5	6.25	25	25	> 25
PPAV6	> 25	25	25	> 25	> 25
CAME6	25	12.5	25	25	> 25
DPOL6	25	25	25	25	> 25
SVUL6	25	25	25	25	> 25
CCOM7	12.5	6.25	25	25	> 25
PPAV7	> 25	25	25	> 25	> 25
CAME7	25	12.5	25	25	> 25
DPOL7	25	25	25	25	> 25
SVUL7	> 25	25	25	>25	> 25
CCOM8	12.5	6.25	25	25	> 25
PPAV8	25	25	25	> 25	> 25
CAME8	25	12.5	25	> 25	> 25
DPOL8	25	25	25	25	> 25
SVUL8	> 25	25	25	> 25	> 25
CCOM9	25	12.5	25	25	> 25
PPAV9	25	25	25	> 25	> 25
CAME9	25	12.5	25	> 25	> 25
DPOL9	25	25	> 25	> 25	> 25
SVUL9	> 25	25	25	> 25	> 25

2.2.4 Growth inhibition kinetics, anti-adhesion and anti-biofilm activity (including anti-quorum sensing) on common food-borne pathogens (UNILJUB)

Brown algae *P. pavonica* (PPAV6) was selected for further studies based on the results of the antimicrobial testing (section 2.2.2.). Its activity was better against Gram-positive bacteria, the lowest MIC values was determined against *L. innocua.* This was additionally confirmed with growth inhibition curves which were determined at two selected sub-inhibitory concentrations (1/2 and ¼ MIC) (Figure 2.3.).

Table 2.6. Antimicrobial activity of brown algae *P. pavonica* extract against selected common foodborne pathogens expressed as minimal inhibitory concentration (MIC [mg/mL]) and minimal bactericidal concentration (MBC [mg/mL]).

Sample	*S. aureus* ATCC 25923		*L. innocua* ŽM39		*B. cereus* ŽMJ164		*E. coli* ATCC 11229		*S.* Typhimurium ATCC 14028		*C. jejuni* NCTC 11168	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
PPAV6	10	>10	2.5	5	>4	>4	/	/	/	/	0.5	1



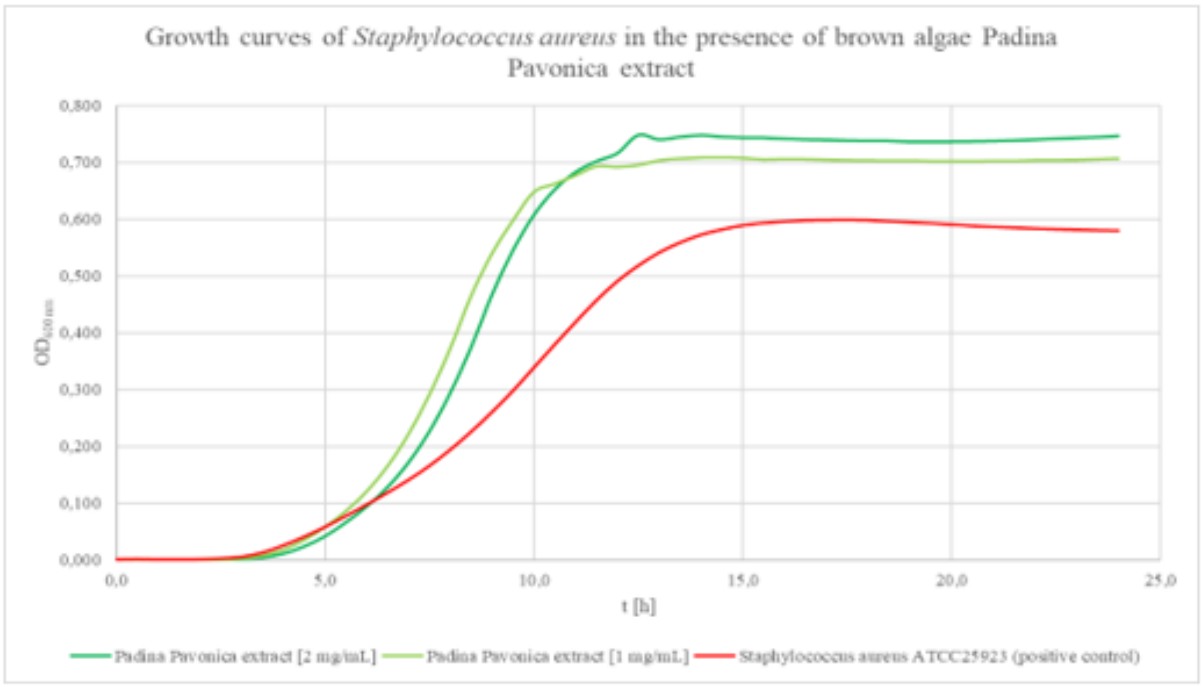


Figure 2.3. Growth inhibition curves of *Listeria innocua* and *Staphylococcus aureus* in the presence of brown algae *Padina Pavonica* (PPAV6) extract in sub-inhibitory concentration.

2.2.5. Additional antimicrobial testing and biogenic amines and ammonia production by bacteria (CUNI)

Inhibition zones of algae extracts

Table 1. shows inhibition zones of algae extracts CAME6 and PPAV6 against tested microorganisms. Came 6 extract was more effective than PPAV 6 extract on inhibition of bacterial growth ($P<0.05$). Among food-borne pathogens, *Staphylococcus aureus* was the most resistant bacteria for both extracts. Came 6 extracts showed 14.75 and 14 mm of inhibition zone against *Salmonella* Paratyphi A and *E. coli*, respectively, whilst PPAV 6 extract exerted no inhibition zone at stock concentration used (20 mg/ml). Inhibition zone of Came 6 extracts towards *Candida albicans* was 13.5 mm. Among fish spoilage bacteria, *Pseudomonas luteola* was the most sensitive strain followed by *Photobacterium damselae* towards algae extracts. *Enterococcus faecalis* was more susceptible against Came 6 than PPAV 6. Dussault et al., (2016) investigated of the 9 algal extract for their antibacterial activities against Gram-negative and Gram-positive food-borne pathogen bacteria. They reported algal extracts from *Padina* spp. and *Ulva* spp. showed an antimicrobial effect on gram-positive *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*, while it did not have an antimicrobial effect on Gram-negative *Escherichia coli* and *Salmonella enterica serovar Typhimurium*.

Table 2.7. Inhibition zones of algae extracts

Microorganisms	Algae extracts	
	CAME6*	PPAV6
Salmonella Paratyphi A	$14.75^{x} \pm 0.96^{x}$	-
E. coli	14.00 ±0.71	-
Staphylococcus aureus	-	-
Candida albicans	13.25 ±0.83	-
Pseudomonas luteola	20.25 ± 0.83^{a}	10.25 ± 0.43^{b}
Photobacterium damselae	16.75 ± 0.83^{a}	6.00 ± 0.71^{b}
Enterococcus faecalis	12.00 ±0.79	-

[x] No inhibition zone, *Table 2.1.

[x]: mean, [y]: standard deviation, Control wells (distilled water) were not included in the table because they do not show any antimicrobial effects. Means followed by different letters are significantly different ($P < 0.05$) among groups ([a–b])

MIC and MBC/MFC of algae extracts

Table 2 shows minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of algae extracts. Came 6 extract was found to be more bacteriostatic and bactericidal/fungicidal effect than PPAV6. Bacteriostatic and bactericidal effect of PPAV 6 on food-borne microorganisms were above 20 mg/ml. MIC of Came 6 was in range from 0.625 for *Pseudomonas luteola* to 20 mg/ml for *Staphylococcus aureus*. Bactericidal dose of Came 6 was low (5 mg/ml) for fish spoilage bacteria especially for *Pseudomonas luteola* and *Photobacterium damselae.* Bacteriostatic dose of PPAV 6 against *Photobacterium damselae* and *Pseudomonas luteola* were 10 and 20 mg/ml, respectively

Table 2.8. MIC and MBC/MFC determination of treatment groups on tested microorganisms

Microorganisms	Algae extracts			
	CAME6*		PPAV6	
	MIC mg/ml	MBC/MFC mg/ml	MIC mg/ml	MBC/MFC mg/ml
Salmonella Paratyphi A	10	>20	>20	>20
E. coli	10	10	>20	>20
Candida albicans	10	>20	>20	>20
Pseudomonas luteola	0.625	5	20	20
Photobacterium damselae	5	5	10	20
Enterococcus faecalis	5	10	20	>20

*Table 2.1.

Ammonia and biogenic amine production by microorganisms in the presence of algae extracts

Ammonia production by microorganisms were in range from 31.85 mg/L for *Staphylococcus aureus* to 131.50 mg/L for *Pseudomonas luteola* (Table 3). Presence of algae

extracts in the HDB resulted in significantly lower ammonia accumulation by microorganism, apart from *Staphlococcus aureus* in the presence of Came 6 algae ($P<0.05$). PPAV 6 extracts generally induced the lowest amount of ammonia accumulation in HDB ($P<0.05$). The used food borne pathogens and fish spoilage bacteria produced all amine tested particularly dopamine and agmatine. Putrescine and cadaverine production were the highest by *Enterococcus faecalis* and *Staphylococcus aureus* (>38 mg/L). Existence of both extracts led to considerably lower putrescine and cadaverine production by *Candida albicans* and fish spoilage bacteria ($P<0.05$). However, *Salmonella* Paratyphi A and *E. coli* produced higher content of putrescine and cadaverine in the presence of PPAV6 and Came 6, respectively. Among fish spoilage bacteria, *Enterococcus faecalis* had good ability to produce spermidine and spermine in HDB. Algae extracts mainly PPAV 6 were generally suppressed spermine and spermidine formation in HDB. *Staphylococcus aureus* produced tryptamine at considerably level (438.24 mg/L) and presence of PPAV 6 extract in HDB caused significantly lower level of tryptamine production by bacteria (39.26 mg/L). Histamine was formed in the range of 0.33 mg/L by *Salmonella* Paratyphi A and 41.56 mg/L by *Staphylococcus aureus*. Apart from *Salmonella* Paratyphi A and *E. coli*, presence of algae extracts mostly PPAV 6 induced notably lesser histamine accumulation in HDB. Dopamine and agmatine were main amines formed by bacteria and *Candida albicans* (200-1300 mg/L*)*. Dopamine and agmatine production were generally suppressed by algae extracts. However, presence of Came 6 and PPAV 6 extract resulted significantly higher dopamine production by *Candida albicans* and *Salmonella* Paratyphi A, respectively. Tyramine production was highest by *Staphylococcus aureus* (26.72 mg/L) and *Candida albicans* (20.42 mg/L). PPAV 6 extract led obvious decreases in tyramine accumulation by most of bacteria. However, stronger increases in tyramine production by *Salmonella* Paratyphi A were observed in the presence of PPAV 6 in HDB ($P<0.05$). Higher tyramine production by *E. coli*, *Staphylococcus aureus* and *Pseudomonas luteola* was also found in the existence of Came 6 extract.

Table 2.9. Ammonia and biogenic amine production by bacteria in the presence or absence of algae extracts (Came6: *Cystoseira amentacea* 6 (2020), PPAV6: *Padina pavonica* 6 (2020)

	AMN	PUT	CAD	SPD	TRP	SPN	HIS	2-PHEN	DOP	SER	TYR	AGM	Groups
SP	47.68±4.41^{a} 1.76±0.05^{c} 25.99±0.32^{b}	19.38±1.15^{b} 0.13±0.01^{c} 82.25±7.34^{a}	12.81±0.31^{b} 7.35±0.48^{b} 84.57±5.63^{a}	0.43±0.01^{b} 0.67±0.01^{b} 52.45±3.47^{a}	9.78±0.46^{b} 9.46±0.19^{b} 232.47±10.32^{a}	1.26±0.00^{b} 0.10±0.01^{c} 12.89±0.40^{a}	0.33±0.04^{c} 2.74±0.02^{b} 7.06±0.58^{a}	9.42±0.01^{b} 0.00±0.00^{c} 36.59±2.55^{a}	447.72±15.76^{b} 229.92±3.01^{c} 786.44±68.36^{a}	12.04±0.79^{b} 15.91±1.03^{a} 17.95±0.58^{a}	11.27±1.13^{b} 5.33±0.13^{b} 65.00±4.31^{a}	359.29±19.48^{a} 274.87±16.46^{b} 0.00±0.00^{c}	C CA PP
EC	60.82±3.19^{a} 13.67±0.76^{b} 3.87±0.19^{c}	24.74±0.36^{a} 21.20±1.70^{b} 1.74±0.13^{c}	15.44±0.93^{b} 45.42±2.63^{a} 0.75±0.06^{c}	14.38±0.46^{b} 79.11±6.77^{a} 0.04±0.00^{c}	122.95±10.09^{b} 234.17±13.41^{a} 3.56±0.13^{c}	3.08±0.03^{b} 15.34±0.98^{a} 0.45±0.02^{c}	2.89±0.14^{b} 19.29±1.79^{a} 0.19±0.02^{b}	13.31±0.57^{b} 47.20±1.08^{a} 0.00±0.00^{c}	486.51±22.03^{a} 398.28±31.40^{b} 154.92±10.46^{c}	11.14±0.16^{b} 30.03±0.97^{a} 4.25±0.21^{c}	15.72±0.32^{b} 28.55±2.51^{a} 2.30±0.22^{c}	652.27±62.26^{a} 217.89±14.13^{b} 108.39±7.35^{b}	C CA PP
SA	31.85±1.31^{b} 57.59±2.12^{a} 11.51±0.80^{c}	38.62±2.76^{b} 76.14±4.47^{a} 7.34±0.10^{c}	47.15±1.09^{b} 54.22±2.49^{a} 12.57±0.13^{c}	51.52±2.66^{a} 53.22±4.34^{a} 20.07±0.38^{b}	438.24±35.92^{a} 373.84±32.42^{a} 39.26±3.17^{b}	4.18±0.22^{b} 16.43±0.59^{a} 1.92±0.15^{c}	41.56±1.22^{a} 9.80±0.21^{b} 2.75±0.07^{c}	1.94±0.20^{c} 51.61±1.87^{a} 28.22±1.09^{b}	478.71±41.60^{ab} 526.92±41.05^{a} 402.51±23.50^{b}	39.96±0.39^{a} 22.03±0.30^{b} 23.22±1.02^{b}	26.72±0.77^{b} 31.27±1.61^{a} 17.86±1.41^{c}	451.04±12.78^{b} 758.63±23.51^{a} 316.91±26.45^{c}	C CA PP
CA	74.71±2.53^{a} 7.46±0.11^{b} 3.37±0.06^{b}	7.39±0.67^{a} 0.08±0.02^{b} 0.19±0.01^{b}	16.16±0.32^{a} 2.66±0.14^{b} 0.41±0.01^{c}	7.41±0.39^{a} 0.02±0.00^{b} 0.37±0.02^{b}	4.50±0.14^{a} 2.02±0.03^{b} 0.00±0.00^{c}	9.86±0.47^{a} 0.00±0.00^{b} 0.00±0.00^{b}	4.67±0.02^{a} 1.01±0.06^{b} 0.11±0.01^{c}	29.77±3.53^{a} 0.00±0.00^{b} 0.09±0.00^{b}	222.84±15.35^{b} 319.51±10.67^{a} 25.01±0.11^{c}	12.61±0.03^{a} 8.57±0.05^{b} 0.13±0.02^{c}	20.42±1.81^{a} 3.11±0.01^{b} 4.05±0.07^{b}	770.34±20.08^{a} 163.97±9.76^{b} 135.11±10.79^{b}	C CA PP
PL	131.50±7.05^{a} 3.23±0.07^{b} 4.27±0.33^{b}	0.82±0.05^{a} 0.33±0.02^{b} 0.09±0.01^{c}	3.78±0.17^{a} 0.78±0.00^{b} 0.94±0.09^{b}	5.82±0.37^{a} 0.10±0.01^{b} 0.23±0.02^{b}	44.64±3.73^{a} 0.50±0.00^{b} 0.96±0.02^{b}	8.53±0.73^{a} 0.00±0.00^{b} 0.00±0.00^{b}	4.89±0.18^{a} 0.04±0.00^{b} 0.00±0.00^{b}	37.93±0.75^{a} 0.10±0.00^{b} 0.00±0.00^{b}	490.38±21.61^{a} 9.38±0.20^{b} 36.99±1.68^{b}	2.95±0.10^{a} 0.00±0.00^{c} 0.94±0.05^{b}	1.21±0.07^{c} 4.96±0.08^{a} 1.76±0.01^{b}	689.28±54.57^{a} 157.37±10.85^{b} 61.98±4.29^{b}	C CA PP
PD	55.19±1.38^{a} 10.35±1.00^{b} 3.44±0.31^{c}	2.50±0.09^{a} 0.53±0.04^{b} 0.14±0.00^{c}	7.30±0.48^{a} 1.55±0.20^{b} 0.62±0.00^{b}	7.48±0.25^{a} 1.44±0.04^{b} 0.06±0.01^{c}	47.31±3.27^{a} 0.85±0.07^{b} 0.00±0.00^{b}	0.05±0.01^{a} 0.02±0.00^{b} 0.00±0.00^{c}	5.02±0.07^{a} 0.12±0.01^{b} 0.00±0.00^{b}	23.53±1.02^{a} 0.17±0.01^{b} 0.00±0.00^{b}	567.60±42.15^{a} 89.88±5.90^{b} 37.60±1.36^{b}	21.53±2.17^{a} 1.80±0.11^{b} 0.24±0.00^{b}	10.11±0.74^{a} 3.90±0.16^{b} 2.03±0.01^{c}	741.87±27.01^{a} 284.00±22.88^{b} 89.56±7.68^{c}	C CA PP
EF	32.58±2.15^{a} 8.84±0.65^{b} 3.72±0.20^{c}	57.72±3.04^{a} 8.07±0.30^{b} 0.17±0.01^{c}	46.96±2.27^{a} 12.59±0.67^{b} 0.85±0.08^{c}	114.57±1.74^{a} 14.15±0.79^{b} 0.13±0.00^{c}	0.60±0.01^{b} 156.56±5.98^{a} 0.00±0.00^{b}	16.36±1.59^{a} 1.78±0.08^{b} 0.00±0.00^{b}	11.67±0.05^{a} 2.35±0.05^{b} 0.01±0.00^{c}	76.75±5.58^{a} 14.98±0.11^{b} 0.08±0.00^{c}	130.84±112.31^{a} 152.96±7.83^{b} 19.42±0.81^{b}	43.71±2.36^{a} 6.16±0.46^{b} 0.37±0.03^{c}	12.70±0.09^{a} 3.95±0.11^{b} 1.12±0.08^{c}	531.92±27.48^{a} 215.13±8.76^{b} 57.61±3.15^{c}	C CA PP

*Data are expressed as mean value of three samples, Mean value±Standard deviation. AMN, ammonia; PUT, putrescine; CAD, cadaverine; SPD, spermidine; TRP, tryptamine; PHEN, 2-phenylethyl amine; SPN, spermine; SER, serotonin; DOP, dopamine; AGM, agmatine. SP: *Salmonella* Paratyphi A, *EC: E. coli, SA: Staphylococcus aureus*, CA: *Candida albicans*, PL: *Pseudomonas luteola*, PD: *Pseudomonas damseale*; EF: *Enterococcus faecalis*, C: Control, CA: CAME6 extract, PP:PPAV6 extract

$^{a–d}$ Indicate significant differences ($p<0.05$) between control and treated group in a row

2.2.6. Antimicrobial activity of the essential oils from *C. compressa* (UNIBO)

C. compressa EO did not hindered the pathogens' growth at the concentrations tested (≤2 mg/ml) with broth microdilution method. For these reasons, an additional trial with higher concentrations using disk diffusion method was performed. The results showed that *C. compressa* EO was active against Salmonella at both concentrations tested (100 and 50 mg/ml), with an inhibition halo of 2 mm. Moreover, *Listeria monocytogenes* ScottA growth was affected by the four EOs when employed at the highest concentration (100 mg/ml), independently of the month in which the algae were collected. On the contrary, the inhibition of *E. coli* 555 depended on the seasonal variable. Indeed, only the EO from the algae collected in August was able to affect the growth of this strain (inhibition halo of 3 mm).

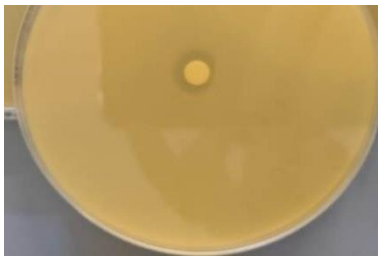


Figure 2.4. Inhibition activity *Cystoseira compressa* EOs against *Listeria monocytogenes* ScottA tested with disk diffusion method.

2. REPORT OF THE BIOACTIVE COMPONENT COMPOSITION IN AGRO-FOOD BY-PRODUCTS

3.1. Material and methods

3.1.1. Sample collection (UNIST, CROSME, UNILJUB, UNIBO, CUNI)

The agro-food by-products were collected by different partners according to Table 3.1 from traditional agro-productions (berry wine production, cherry and aronia juice production, wine and olive oil production, rosehip extract production, juniperus distillate production) and wastes

from these industries. All samples were shade-dried for approximately four to six days before being pulverized.

Table 3.1. List of the agro-food by-products collected by different partners

	Matrix	Collecting partner	Extraction method*	Mark
1.	Blackberry* whole 2020	UNIST	MAE	PRIMA_01
2.	Blackberry leaves 2020	UNIST	MAE	PRIMA_02
3.	Blackberry juice by-product 2020	UNIST	MAE	PRIMA_03
4.	Blackberry juice by-product 2020	UNILJUB	MAE	PRIMA_04
5.	Aronia juice by-product (production 2019)	UNILJUB	MAE	PRIMA_05
6.	Aronia juice by-product (production 2020)	UNILJUB	MAE	PRIMA_06
7.	Cherry juice by-product (production 2020)	UNIST	MAE	PRIMA_07
8.	Rosehip extracts production by-product 2020	UNILJUB	MAE	PRIMA_08
9.	Grape pomace 2020	CUNI	MAE	PRIMA_09
10.	Olive pomace 2020	CUNI	MAE	PRIMA_10
11.	Olive leaves Lastovka FS 2020	UNIST	UAE	PRIMA_11
12.	Olive leaves Levantinka FS 2020	UNIST	UAE	PRIMA_12
13.	Olive leaves Oblica VS 2020	UNIST	UAE	PRIMA_13
14.	Olive leaves Moraiolo Toscana 2020	UNIBO	UAE	PRIMA_14
15.	Olive leaves Frantoio Toscana 2020	UNIBO	UAE	PRIMA_15
16.	Olive leaves Brisighella (Emilie Romagne) 2020	UNIBO	UAE	PRIMA_16
17.	*Juniperus oxycedrus* berries green 2020	UNIST	MAE	PRIMA_17
18.	*Juniperus oxycedrus* berries red 2020	UNIST	MAE	PRIMA_18
19.	*Juniperus oxycedrus* needles 2020	UNIST	MAE	PRIMA_19
20.	*Juniperus communis* extract by-product 2021	UNILJUB	MAE	PRIMA_20

*MAE - microwave assisted extraction; UAE - ultrasound assisted extraction; *Rubus fructiosa*

3.1.2. The extraction procedure (UNIST)

All the dried materials were extracted in 50% EtOH using the MAE (advanced microwave extraction system ETHOS X, Milestone Srl, Sorisole, Italy, 600 W, 5 minutes) or UAE

extraction in methanol (Transsonic Tp 310H, Elma Schmidbauer GmbH, Singen, Germany, triple extraction, UVZ bath, 40kHz, RT, 30 minutes) method. The choice of the method was done based on the total phenolic content of the MAE and UAE extracts of each sample established during preliminary studies. After the extraction, the EtOH was evaporated and the extracts freeze dried and sent to partners for further analyses.

3.1.3. Preparation of the essential oil from the selected extracts (UNIST)

The essential oils (EOs) were prepared from samples PRIMA_02, PRIMA_19 and PRIMA_20 (Table 3.1.), by hydrodistillation of dried material (100 g) that was immersed in a flask with distilled water (1000 mL). The extraction process was performed in Clevenger apparatus during 3 h. Pentane and diethyl ether (1:1, v/v) in the inner tube of the apparatus were used for trapping of the volatile compounds carried through the system by vapour. Finally, after hydrodistillation, pentane was separated, and distillate was dried over anhydrous sodium sulfate. The samples of EOs were stored at 4°C in the dark vials until analysis.

EOs were diluted for analysis as it follows:

Sample	Weight	Solvent volume
PRIMA_02 EO	20 mg	1 mL 96% EtOH
PRIMA_19 EO	20 mg	1 mL 96% EtOH
PRIMA_20 EO	10 mg	1 mL 96% EtOH

3.1.4. Total phenolic content and antioxidant capacity (UNIST)

The methodology is described in section 2.1.3.

3.1.5. Antimicrobial testing against foodborne pathogens and spoilage bacteria (UNILJUB)

The antimicrobial activity of the prepared extracts and EOs (Table 3.1.) was evaluated by broth microdilution method using 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT) and resazurin as indicators. The minimal inhibitory concentration (MIC) was the lowest concentration at which no bacterial growth was detected as reduction of colourless INT to red formazan or blue resazurin to pink resorufin. The minimal bactericidal concentration (MBC) was the lowest concentration at which no bacterial growth was observed after cultivation of bacterial suspension where no change in colour occurred. Analyses were performed with

common food-borne bacteria (*Staphylococcus aureus* ATCC 25923, *Listeria innocua* ŽM39, *Escherichia coli* ATCC 11229, *Salmonella Typhimurium* ATCC 14028 and *Campylobacter jejuni* NCTC 11168) and spoilage bacteria (*Shewanella baltica* NCTC 10735, *Shewanella putrefaciens* ŽM654, *Shewanella xiamenensis* ŽM655, *Pseudomonas fragi* ATCC 4973, *P. fragi* ZIM B1064, *P. fragi* ZIM B1072, *P. fragi* ZIM B1085, *P. fragi* ZIM B1092).

3.1.6. Growth inhibition kinetics, anti-adhesion and anti-biofilm activity (including anti-quorum sensing) on common food-borne pathogens (UNILJUB)

The methodology is similar as described in section 2.1.6.

3.1.7. Quorum sensing reduction (UNILJUB)

Campylobacter jejuni NCTC 11168 and C. jejuni 11168ΔluxS from the culture collection of University of Ljubljana were used and cultured in standard conditions (at 42 ◦C under microaerobic conditions (5% O2, 10% CO2, 85% N2) for 24 h. The changes in C. jejuni AI-2 activities or QS were inferred from the changes in the measured luminescence of the Vibrio harveyi reporter strain in relative luminescent units (RLU), as described previously (Šimunović et al., 2020). Briefly, the C. jejuni cell free suspensions (CFSs) were added to the suspensions of the reporter strain to a final concentration of 10% (v/v) (i.e., 20 µL CFS added to 180 µL reporter strain suspension). Sterile medium was used as the blank [10% (v/v) MHB, 90% (v/v) AB medium] and the negative control was 10% (v/v) MHB and 90% (v/v) Vibrio harveyi suspension. Kinetic measurements were carried out for the bioluminescence signals of the V. harveyi that were produced as a result of the QS signal that originated from the presence of the CFSs. The relative luminescence signals were measured at 15 min intervals over 20 h at 30 ◦C in white microtiter plates (Nunc, Thermo Fisher Scientific, Roskilde, Denmark incubated in a microplate reader (Varioskan Lux; Thermo Scientific, Waltham, MA, USA). The relative luminescence signals were interpreted as the QS signal in the C. jejuni CFSs (i.e., higher signals indicated higher concentrations of QS signaling molecules in the CFSs produced by C. jejuni). When added to MHB, V. harveyi produces a background luminescence signal that increases with the concentration of the culture. To find the most stable point of signal production, the CFSs from C. jejuni 11168ΔluxS, a mutant that cannot produce the quorum-sensing signal AI-2, and fresh MHB were used as the negative controls. The experiments were performed as three

independent biological replicates and three technical replicates. The data presented are means ± standard deviation from three biological replicates.

3.1.8. Additional antimicrobial testing and biogenic amines and ammonia production by bacteria (CUNI)

The methodology is similar as described in section 2.1.7.

3.1.9. Antimicrobial activity of the selected essential oils (UNIBO)

The methodology is similar as described in section 2.1.8.

3.2 Results

3.1.1. Total phenolic content and antioxidant capacity of the extracts and EOs (UNIST)

TPC and antioxidant activity of by-products extracts, measured with three different methods, is shown in Table 3.2. The highest amount of phenolics was found in blackberry leaves and *Juniperus oxycedrus* needles extracts. Both extracts had high reducing ability, as it can be seen from FRAP results. Blackberry juice by-product extracts from Slovenia and Croatia also had high FRAP results. The highest ORAC results among extracts obtained using MAE and diluted 1000-fold, was found for blackberry leaves, followed by *J. oxycedrus* needles. The highest ORAC results among extracts obtained using UAE and diluted 1000-fold was found for olive leaves Brisighella (Emilia Romagna). Olive leaves extracted with UAE had higher ORAC results than by-products extracted with MAE, in almost all cases.

All by-products extracts showed high DPPH radical inhibition, except for one olive leaves sample and *J. oxycedrus* red berries. The highest percentage of inhibition was found for olive pomace.

Lowest TPC and antioxidant activity for all methods was found in *J. oxycedrus* red berries extract.

Table 3.2. Total phenolic content and antioxidant capacity of the extracts.

Sample	TPC (mg GAE/g dried extract)	FRAP (mM TE)	ORAC (µM TE)		DPPH (% inhibition)
PRIMA_01	0.04 ± 0.00	5.03 ± 0.21	1:1000	8.80 ± 0.70	87.60 ± 0.53
PRIMA_02	0.25 ± 0.01	26.75 ± 1.42	1:1000	57.40 ± 1.62	87.72 ± 1.38
PRIMA_03	0.13 ± 0.00	12.91 ± 0.43	1:1000	16.10 ± 0.54	86.05 ± 1.89
PRIMA_04	0.13 ± 0.00	13.56 ± 0.32	1:1000	15.00 ± 1.67	84.05 ± 1.41
PRIMA_05	0.16 ± 0.01	10.12 ± 0.70	1:1000	36.10 ± 1.08	80.07 ± 0.43
PRIMA_06	0.15 ± 0.01	11.50 ± 0.94	1:1000	31.10 ± 0.85	82.35 ± 0.39
PRIMA_07	0.03 ± 0.00	1.92 ± 0.10	1:1000	9.46 ± 0.72	82.76 ± 3.23
PRIMA_08	0.13 ± 0.00	11.78 ± 0.41	1:1000	32.25 ± 1.10	88.48 ± 1.11
PRIMA_09	0.07 ± 0.00	3.81 ± 0.07	1:200	81.28 ± 3.63	80.74 ± 1.63
PRIMA_10	0.04 ± 0.00	2.76 ± 0.06	1:200	114.54 ± 2.37	92.66 ± 1.82
PRIMA_11	0.09 ± 0.00	5.25 ± 0.17	1:1000	55.45 ± 5.25	83.85 ± 2.76
PRIMA_12	0.08 ± 0.00	4.56 ± 0.09	1:1000	49.71 ± 4.20	88.83 ± 0.53
PRIMA_13	0.11 ± 0.01	9.36 ± 0.09	1:1000	75.32 ± 4.22	85.20 ± 0.75
PRIMA_14	0.10 ± 0.00	7.24 ± 0.17	1:1000	53.28 ± 1.44	85.89 ± 1.31
PRIMA_15	0.10 ± 0.00	6.63 ± 0.28	1:1000	55.59 ± 0.53	78.37 ± 2.74
PRIMA_16	0.11 ± 0.00	8.25 ± 0.20	1:1000	78.19 ± 7.62	86.77 ± 0.22
PRIMA_17	0.04 ± 0.00	2.39 ± 0.25	1:1000	10.64 ± 0.35	85.72 ± 0.45
PRIMA_18	0.02 ± 0.00	0.88 ± 0.04	1:1000	3.60 ± 0.60	51.72 ± 1.51
PRIMA_19	0.19 ± 0.00	13.22 ± 0.73	1:1000	43.56 ± 0.56	89.07 ± 0.36
PRIMA_20	0.04 ± 0.00	3.44 ± 0.11	1:200	119.23 ± 3.34	91.79 ± 0.15

3.1.2. Antimicrobial testing against foodborne pathogens (UNILJUB)

Table 3.3. Antimicrobial activity of the extracts against selected common foodborne pathogens expressed as minimal inhibitory concentration (MIC [mg/mL]) and minimal bactericidal concentration (MBC [mg/mL]).

Sample	*S. aureus* ATCC 25923		*L. innocua* ŽM39		*B. cereus* ŽMJ164		*E. coli* ATCC 11229		*S.* Typhimurium ATCC 14028		*C. jejuni* NCTC 11168	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
PRIMA01	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	0.5	0.5
PRIMA02	>2	>2	>2	>2	2	>2	>2	>2	>2	>2	0.0625	0.125
PRIMA03	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	0.125	0.25
PRIMA04	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	0.125	0.25
PRIMA05	>2	>2	>2	>2	/	/	>2	>2	>2	>2	0.5	0.5
PRIMA06	>2	>2	>2	>2	/	/	>2	>2	>2	>2	0.5	0.5
PRIMA07	>2	>2	>2	>2	/	/	>2	>2	>2	>2	2	>2
PRIMA08	>2	>2	>2	>2	/	/	>2	>2	>2	>2	0.25	0.5
PRIMA09	>4	>4	/	/	/	/	/	/	/	/	/	/
PRIMA10	>4	>4	/	/	/	/	/	/	/	/	/	/
PRIMA11	2	2	>8	>8	4	4	/	/	/	/	0.5	1
PRIMA12	4	4	8	8	8	8	/	/	/	/	0.5	1
PRIMA13	2	2	>8	>8	8	8	/	/	/	/	0.5	0.5
PRIMA14	4	4	>4	>4	8	8	/	/	/	/	0.5	1
PRIMA15	4	4	>8	>8	8	8	/	/	/	/	0.5	1
PRIMA16	2	2	>8	>8	8	8	/	/	/	/	0.5	1
PRIMA17	0.5	1	0.125	0.25	0.25	1	>2	>2	>2	>2	0.5	1
PRIMA18	0.5	2	0.25	0.5	0.25	1	>2	>2	>2	>2	1	2
PRIMA19	2	>2	>2	>2	2	>2	>2	>2	>2	>2	0.125	0.25
PRIMA20	0.25	0.25	0.25	0.25	0.0625	0.25	>4	>4	/	/	1	/

The antimicrobial activity of the prepared extracts was determined against Gram-positive and Gram-negative bacteria. Against Gram-positive bacteria, S. aureus, *L. innocua* and *B. cereus*, only juniper extracts showed good antimicrobial activity – with better activity of green and red juniper extracts compared to extract of juniper needles. The extracts from olive leaves also showed antimicrobial activity against *S. aureus* while MIC values were higher for the other two Gram-positive strains. None of the extracts had any effect on Gram-negative bacteria *E. coli* and *S. Typhimurium*. In contrast, all extracts showed antimicrobial activity against Gram-negative *C. jejuni*, with the lowest values obtained in extracts of blackberry, juniper needles and rosehip.

3.1.3. Antimicrobial testing against foodborne spoilage bacteria (UNILJUB)

Table 3.4. Antimicrobial activity of the extracts against selected common food spoilage bacteria expressed as minimal inhibitory concentration (MIC [mg/mL]) and minimal bactericidal concentration (MBC [mg/mL]).

Sample	*S. baltica* NCTC 10735		*S. putrefaciens* ŽM654		*S. xiamenensis* ŽM655		*P. fragi* ATCC 4973		*P. fragi* ZIM B1064		*P. fragi* ZIM B1072		*P. fragi* ZIM B1085		*P. fragi* ZIM B1092	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
PRIMA_01	>2	>2	2	>2	>2	>2	2	>2	/	/	/	/	/	/	/	/
PRIMA_02	2	2	0.5	0.5	1	2	0.5	>2	0.5	>2	0.5	>2	0.5	>2	0.5	>2
PRIMA_03	2	2	1	1	1	2	1	>2	1	>2	1	>2	0.5	>2	1	>2
PRIMA_04	1	2	1	2	2	>2	1	>2	1	>2	1	>2	0.5	>2	1	>2
PRIMA_05	>2	>2	/	/	/	/	>2	>2	/	/	/	/	/	/	/	/
PRIMA_06	>2	>2	/	/	/	/	>2	>2	/	/	/	/	/	/	/	/
PRIMA_07	>2	>2	/	/	/	/	>2	>2	/	/	/	/	/	/	/	/
PRIMA_08	>2	>2	/	/	/	/	>2	>2	/	/	/	/	/	/	/	/
PRIMA_17	>2	>2	/	/	/	/	>2	>2	/	/	/	/	/	/	/	/
PRIMA_18	>2	>2	/	/	/	/	>2	>2	/	/	/	/	/	/	/	/
PRIMA_19	>2	>2	/	/	/	/	>2	>2	/	/	/	/	/	/	/	/
PRIMA_20	/	/	/	/	/	/	2	4	/	/	/	/	/	/	/	/

The antimicrobial activity of the prepared extracts was determined against Gram-negative spoilage bacteria belonging to the genera *Pseudomonas* and *Shewanella*. Among the extracts tested, blackberry extracts were found to be the most effective, with the best activity determined in extract from blackberry leaves. Juniper extract by-product from Slovenia also showed activity against *P. fragi* ATCC 4973, but the MIC was high (2 mg/mL).

3.1.4. Antioxidation and antimicrobial activity of the selected essential oils (UNIST, UNIBO, UNILJUB)

The results of antioxidant capacity of the three EOs are shown in Table 3.3. All tested oils had high ability to scavenge peroxyl radicals. Blackberry leaves EO had the highest DPPH radical inhibition. However, even that results are lower than all by-products extracts' results.

Table 3.5. The results of antioxidant capacity of the EOs.

Samples of the EOs		Conc.	ORAC (µM TE)		DPPH (% inhibition)
PRIMA_02	Blackberry leaves EO	20 mg/mL	1:1000	88,69 ± 5,77	20,26 ± 0,31
PRIMA_19	*Juniperus oxycedrus* needles EO	20 mg/mL	1:1000	63,60 ± 2,32	5,81 ± 0,61
PRIMA_20	*Juniperus sp.* extract by-product EO	10 mg/mL	1:200	110,55 ± 4,39	4,72 ± 0,71

The BlackBerry and Juniperus EOs did not show any activity against the target Gram negative bacteria (namely *Salmonella*, *E. coli*, *K. aerogenes*) at the conditions tested.

On the contrary, Blackberry EO had antimicrobial activity against Gram positive bacteria, with MIC values of 1 mg/ml for *St. aureus* DSM 20231^{t} and 1.5 mg/ml for *L. monocytogenes* ScottA and *E. faecium* FC12. Also, *Juniperus* EO was active against the strains ScottA and DSMZ 20231^{t}, being MIC 3 and 2 mg/ml, respectively. In this case, no effect on FC 12 strain

Table 3.6. Results of antimicrobial activity of blackberry (PRIMA_02) and juniperus (PRIMA_19) EOs against foodborne pathogens

Strains	PRIMA_02		PRIMA_19		PRIMA_20	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Salmonella 155	>2	NA	>4	NA	>4	NA
E. coli 555	>2	NA	>4	NA	>4	NA
Klebsiella aerogenes EPS8	>2	NA	>4	NA	>4	NA
Listeria monocytogenes ScottA	1.5	1.5	3	>4	>6	NA
Staphylococcus aureus DSM 20231^t	1	1	2	3	>4	NA
Enterococcus faecium FC12	1.5	1.5	≥4	>4	>6	NA

The antimicrobial activity of selected EOs was determined against Gram-negative spoilage bacteria, *P. fragi*, *S. putrefaciens* and *S. xiamenensis*, as well as against the common foodborne pathogen *C. jejuni*. EOs from 2021 showed good antimicrobial activity against *S. xiamenensis*, whereas the activity of juniper EO was slightly better, while MICs for *P. fragi*, *S. putrefaciens* and *C. jejuni* were higher. The antimicrobial activity of juniper EO (PRIMA_19) was determined only for *C. jejuni* strain, and showed better activity compared to the other two EOs. The *Juniperus communis* by-product EO (deriving from sample PRIMA_20) did not show any activity against the target Gram positive and Gram negative bacteria (namely *Staphylococcus aureus, Salmonella, Escherichia coli, Klebsiella aerogenes*) at the conditions tested (up to 4 mg/ml). For *Listeria monocytogenes* and *Enterococcus faecium* higher concentrations (up to 6 mg/ml) were tested, but also in this case no growth inhibition was observed.

Table 3.7. Antimicrobial activity of the essential oils (EOs) against selected common food spoilage bacteria expressed as minimal inhibitory concentration (MIC [mg/mL]) and minimal bactericidal concentration (MBC [mg/mL]).

Sample	*Pseudomonas fragi* ATCC 4973		*Shewanella putrefaciens* ŽM654		*Shewanella xiamenensis* ŽM655		*Campylobacter jejuni* NCTC 11168	
	MIC [mg/mL]	MBC [mg/mL]	MIC [mg/mL]	MBC [mg/mL]	MIC [mg/mL]	MBC [mg/mL]	MIC [mg/mL]	MBC [mg/mL]
P_19	1	1	1	1	0.125	0.125	1	1
P_02	1	2	1	>1	0.125	0.25	1	1
P_20	/	/	/	/	/	/	0.125	/

3.1.5. Growth inhibition kinetics, anti-adhesion and anti-biofilm activity (including anti-quorum sensing) on common food-borne pathogens (UNILJUB)

A) Growth inhibition kinetics

The effect of sub-inhibitory concentrations of the extracts that showed the best antimicrobial activity (based on the determined MIC values) against selected bacteria was evaluated with growth/inhibition curves. An example of growth kinetics of *L. innocua* strain in the presence of sub-inhibitory concentrations ($^1/_2$ MIC, ¼ MIC) of juniper extracts (green juniper berries – P_17, red juniper berries – P_18, juniper needles – P_19, juniper by-product – P_20) is presented in the Fig. 3.1. Further examples are attached in the Appendix. The addition of juniper extracts not only inhibited the growth of *L. innocua* strain but also prolonged the duration of lag phase and decreased the exponential growth rate as compared to the positive control. It is observed that the effect of the extract depends on its concentration – at higher concentrations, the extracts inhibited bacterial growth more effectively than at lower concentrations compared to the positive control. Nevertheless, the extracts were also found to be effective at concentrations ¼ of the MIC, with the lowest effect observed for the extract of green juniper berries, where the applied concentration was very low ($^1/_4$ MIC = 0.0313 mg/mL).

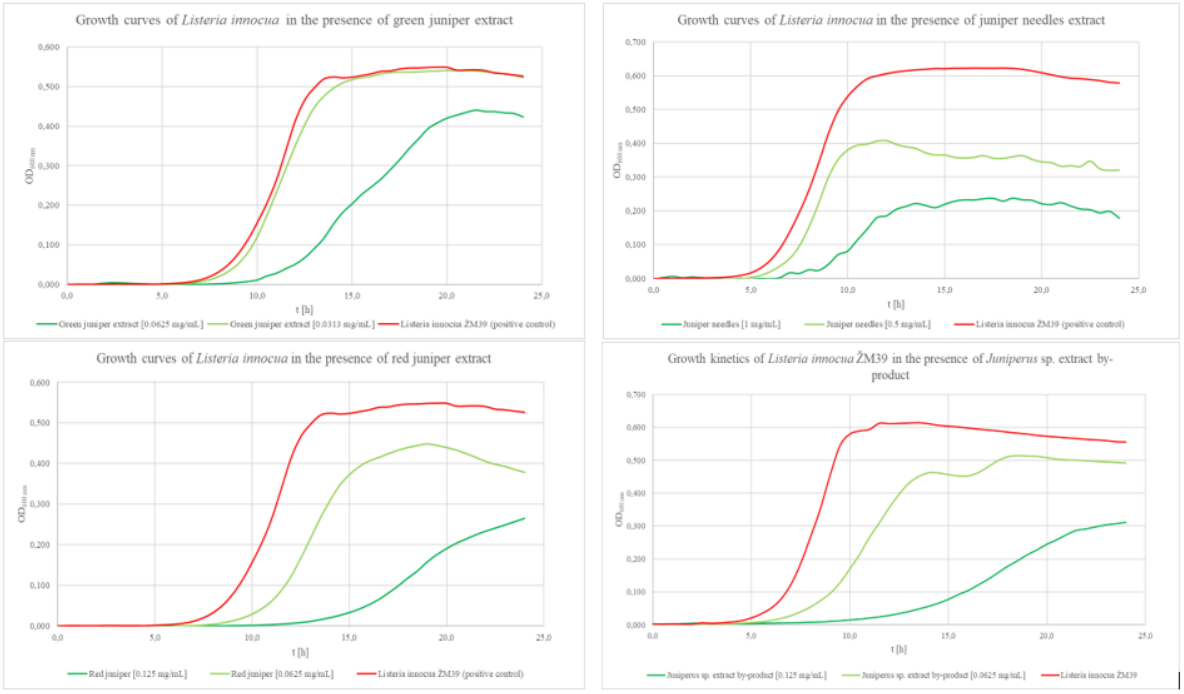


Figure 3.1. Growth curves of *Listeria innocua* ŽM39 in the presence of sub-inhibitory concentrations of different juniper extracts.

B) Anti- biofilm activity

Table 3.8. Anti-biofilm activity of selected extracts against common foodborne pathogens or food spoilage bacteria expressed as % reduction of biofilm cells.

Extracts	Reduction of biofilm cells ± SD [%]			
	Staphylococcus aureus **ATCC 25923**	***Bacillus cereus*** **ŽMJ164**	***Pseudomonas fragi*** **ATCC 4973**	***Campylobacter jejuni*** **NCTC 11168**
P_02	/	95.07 ± 1.72	75.25 ± 1.12	95.26 ± 0.41
P_03	/	/	54.99 ± 25.89	32.48 ± 7.52
P_08	/	/	/	74.49 ± 9.69
P_17	/	80.59 ± 13.36	/	61.41 ± 25.88
P_19	49.68 ± 0.32	42.77 ± 2.60	/	93.21 ± 6.52

The anti-biofilm activity of selected extracts on *S. aureus*, *B. cereus*, *P. fragi* and. *C. jejuni* strains after 24 h or 48 h of incubation was observed. The best activity was observed for blackberry leaves extract, especially against *C. jejuni* and *B. cereus* strains as it reduced the number of biofilm cells by more than 1 log or by more than 95 % compared to the control. Compared to blackberry leaves extract, the activity of blackberry juice by-product extract against Gram-negative strains was lower. Juniper needles extract showed very good anti-biofilm activity against *C. jejuni*, while the activity against Gram positive strains was weaker. Green juniper extract showed better activity against *B. cereus* strain in very low concentration compared to juniper needles extract where the concentration was 8-times higher.

C) Anti-quorum sensing activity

Reduction of the QS in *C. jejuni* NCTC 11168 after the addition of plant extracts in the concentration ¼ MIC (these concentrations were very low and ranged from 0.03125-0.250 mg/ml) was in the range from 35.8% to 87.5% for all extracts tested. **QS of *C. jejuni* was mostly inhibited by blackberry leaves extract (PRIMA_02) and extract from blackberry juice by-product (PRIMA_04). These extracts were anti-QS active in very low concentrations, 0.0156 and 0.03125 mg/ml, respectively**. The extract prepared from the by-product of rosehip extract production (PRIMA_08) was also very active, 87.5% QS inhibition was achieved at the extract concentration 0.0625 mg/ml (Table 3.9 and Figure 3.2).

Table 3.9. Reduction of the QS in *C. jejuni* NCTC 11168 after the addition of different plant extracts in the concentration ¼ MIC (subinhibitory concentration).

Sample	QS reduction average	QS reduction SD
Blackberry whole 2020 (P01)	47,0	16,2
Blackberry leaves 2020 (P02)	61,1	3,9
Blackberry juice by-product 2020 (P03)	46,7	11,3
Blackberry juice by-product 2020 (P04)	59,3	4,6
Aronia juice by-product 2019 (P05)	57,5	17,6
Aronia juice by-product 2020 (P06)	59,3	18,4
Rosehip extracts by-product 2020 (P08)	87,5	6,2
Juniperus oxycedrus berries green 2020 (P17)	35,8	4,6
Juniperus oxycedrus berries red 2020 (P18)	53,5	4,2
Juniperus oxycedrus needles 2020 (P19)	36,1	12,2

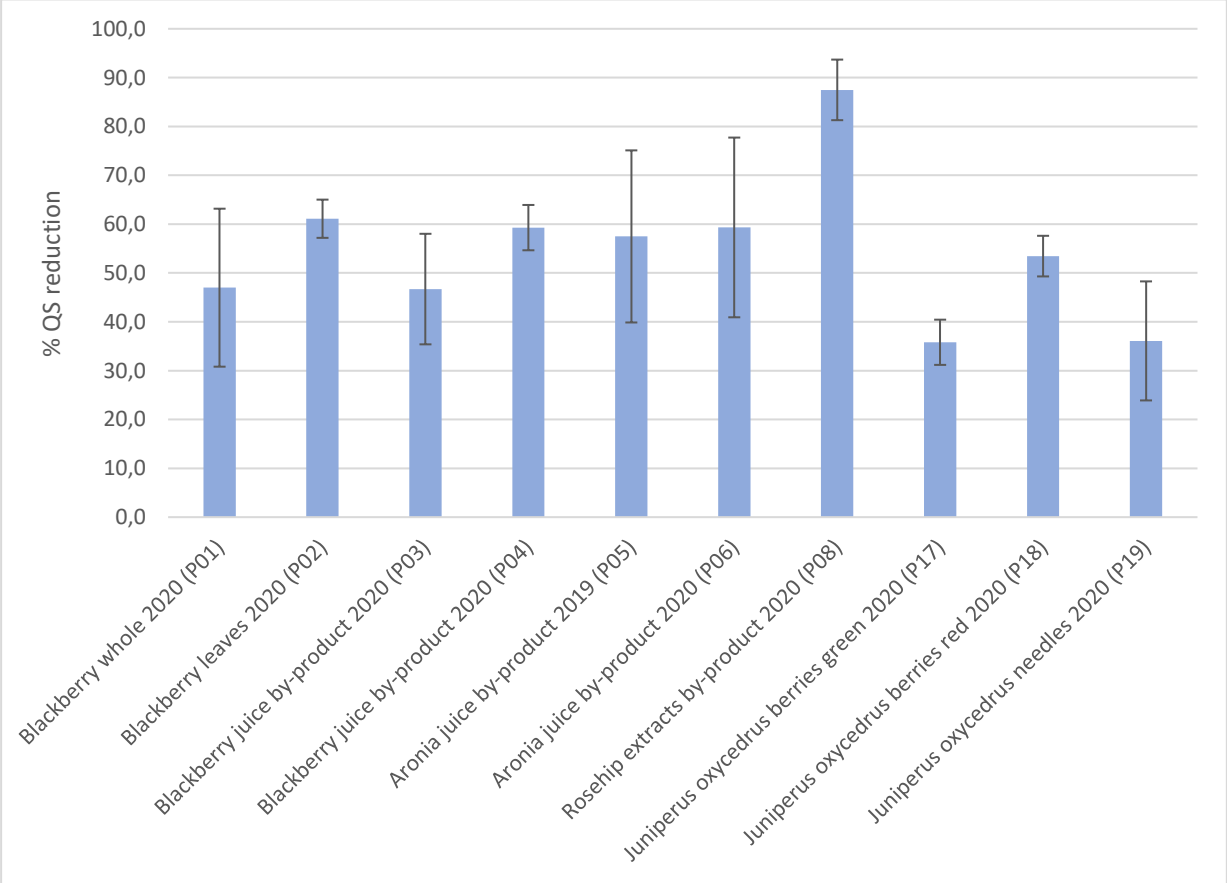


Figure 3.2. Reduction of the QS in *C. jejuni* NCTC 11168 after the addition of different plant extracts in the concentration ¼ MIC (subinhibitory concentration). Average values in % are shown ±SD

Table 3.10. Reduction of the QS in *C. jejuni* NCTC 11168 after the addition of plant extracts (PRIMA01-PRIMA11) in the concentration ¼ MIC (subinhibitory concentration).

Sample	QS reduction average	QS reduction SD	Test. konc 1/4MIC (mg/ml)	MIC (mg/ml)
PRIMA01	59,3	18,4	0.125	0.5
PRIMA02	**87,5**	**6,2**	**0.0625**	**0.25**
PRIMA03	59,3	4,6	0.03125	0.125
PRIMA04	57,5	17,6	0.125	0.5
PRIMA05	**61,1**	**3,9**	**0.0156**	**0.0625**
PRIMA06	46,7	11,3	0.03125	0.125
PRIMA08	35,8	4,6	0.125	0.5
PRIMA09	53,5	4,2	0.25	1
PRIMA10	36,1	12,2	0.03125	0.125
PRIMA11	47,0	16,2	0.125	0.5

3.1.6. Additional antimicrobial testing and biogenic amines and ammonia production by bacteria (CUNI)

For this experiment an additional sample of *Juniperus oxycedrus* needles (collected in 2021 on the same location as sample PRIMA_19) was used.

A) **Inhibition zones of plant extracts**

Inhibition zones of plant extracts against bacteria were given in Table 3.11. Among food-borne bacteria, the highest inhibition zones were observed for *E. coli* ranging value of 12.00 mm for blackberry leaf extract. In addition, *Photobacterium damselae* was more susceptible against blackberry leaf extracts than *J. oxycedrus* extracts.

B) **MIC and MBC/MBC of plant extract**

J. oxycedrus needles 2021 exerted 20 mg/ml of MIC against pathogen microorganisms (Table 5) whilst MBC was above 20 mg/ml apart from *Salmonella* Paratyphi A. However, MIC of PRIMA_19 and PRIMA_02 was similar towards *Salmonella* Paratyphi A (10 mg/ml) and E. coli (20 mg/ml). MIC and MFC of plant extracts were 20 mg/ml against Candida albicans. Among plant extracts used, *J. oxycedrus* 2021 showed the lowest MIC against Pseudomonas luteola growth, with value of 10 mg/ml. Moreover, PRIMA 19 exhibited 10 mg/ml of MIC and MBC against *Photobacterium damselae*. Plant extracts also generally showed 20 mg/ml of MIC and 20 mg/ml of MBC against *Enterococcus faecalis*.

Table 3.11. Inhibition zones of plant extracts

Microorganisms	Plant extracts		
	Juniperus oxycedrus needles 2021	*J. oxycedrus* needles PRIMA_19	PRIMA_02
Salmonella Paratyphi A	13.00 ±0.82a	11.75 ±0.50b	11.00 ±0.82b
E. coli	15.50 ±0.50a	14.75 ±0.45a	12.00 ±0.71b
Staphylococcus aureus	6.00 ±0.71	-	-
Candida albicans	11.25 ±0.43b	6.75 ±0.56c	17.25 ±0.83a
Pseudomonas luteola	21.75 ±1.09a	16.00 ±0.71b	14.75 ±0.43b
Photobacterium damselae	9.65 ±0.41c	13.00 ±0.71b	17.50 ±0.50a
Enterococcus faecalis	14.00 ±0.35a	9.00 ±0.71c	12.25 ±0.83b

No inhibition zone ^{x}: mean, ^{y}: standard deviation, Control wells (distilled water) were not included in the table because they do not show any antimicrobial effects. Means followed by different letters are significantly different ($P < 0.05$) among groups($^{a–c}$)

Table 3.12. MIC and MBC/MFC determination of treatment groups on tested microorganisms

	Plant extracts					
	Juniperus oxycedrus needles 2021		*J. oxycedrus* needles PRIMA_19		PRIMA_02	
Microorganisms	MIC mg/ml	MBC/MFC mg/ml	MIC mg/ml	MBC/MFC mg/ml	MIC mg/ml	MBC/MFC mg/ml
Salmonella Paratyphi A	20	20	10	>20	10	20
E. coli	20	>20	20	>20	20	20
Staphylococcus aureus	20	>20	20	>20	>20	>20
Candida albicans	20	>20	20	>20	20	>20
Pseudomonas luteola	10	20	20	20	20	20
Photobacterium damselae	20	>20	10	10	20	>20
Enterococcus faecalis	20	20	20	>20	20	>20

C) **Ammonia and biogenic amine production by microorganisms in the presence of plant extracts**

For this experiment an additional sample of *Juniperus oxycedrus* needles (collected in 2021 on the same location as sample PRIMA_19) was used. Significant differences were observed among groups in terms of ammonia and biogenic amine production ($P<0.05$, Table 3.13.). Ammonia produced by all microorganisms more than 30 mg/L. The most significant amounts of ammonia were observed for *E. coli* and *Enterococcus faecalis* with a value of 910 and 685 mg/L, respectively, in histidine decarboxylase broth. Significant suppression effects of plant extracts on ammonia production were observed. Blackberry leaf extract followed by *Juniperus* spp. 2020 had higher effect on reducing ammonia accumulation by microorganism ($P<0.05$). Although the effect of plant extracts on biogenic amine production depend on microorganism and specific amine, plant extracts generally resulted lower biogenic amine accumulation. The extracts led to mostly lower ammonia and biogenic amine formation by bacteria, although the effect of ethanolic extracts of bitter melon and safflower on ammonia and biogenic amine were strain-dependent.

Putrescine and cadaverine accumulation were the lowest in blackberry leaf extract followed by *Juniperus* spp. 2020. *Juniperus* spp. 2021 extract resulted in higher putrescine production by *Salmonella* Paratyphi A, *E. coli, Staphlococcus aureus, Pseudomonas luteola* and *Photobacterium damselae*. Spermidine and spermine production by bacteria were inhibited by blackberry leaf extract. Spermidine production by *Candida albicans, Paeudomonas luteola* and *Photobacterium damselae* was increased by *Juniperus* spp. 2021 extract.

Histamine was produced below 42 mg/L. Although the presence of *Juniperus* spp. 2021 extract led to higher histamine accumulation by *Salmonella* Paratyphi A (1.36 mg/L), *Candida albicans* (12.04 mg/L) and *Photobacterium damselae* (24.44 mg/L), the blackberry leaf extract resulted significantly lower histamine production by microorganism ($P<0.05$).

Dopamine was one of the main amines produced by bacteria. All plant extracts reduced dopamine accumulation by *E. coli* and fish spoilage bacteria. Dopamine production by *Salmonella* Paratyphi A, *E. coli, Staphlococcus aureus* and *Candida albicans* was stimulated by Juniperus spp. extract. The lowest serotonin accumulation was observed for group with blackberry leaf extract. *Juniperus* spp. 2021 resulted 4-fold higher serotonin production by *Candida albicans*.

Tyramine is generally formed by Gram-positive bacteria such as lactic acid bacteria and the genus *Enterococcus*. Tyramine production by microorganisms tested was below 40 mg/L in HDB. Apart from *Salmonella* Paratyphi A and *Pseudomonas luteola*, blackberry leaf extract and *Juniperus* spp. 2020 suppressed tyramine production by bacteria and *Candida albicans*. The highest tyramine production was found by *Candida albicans* in the presence of *Juniperus* spp. 2021 extract (36.43 mg/L). Agmatine production by microorganism was limited by addition of plant extract, apart from *Salmonella* Paratyphi A and *Staphylococcus aureus* that produced higher agmatine in the presence of *Juniperus* spp. 2020.

Table 3.13. Ammonia and biogenic amine production by microorganism in the presence or absence of plant extracts

MO	AMN	PUT	CAD	SPD	TRP	SPN	HIS	2-PHEN	DOP	SER	TYR	AGM	
SP	47.68±4.41^{a}	19.38±1.15^{c}	12.81±0.31^{b}	0.43±0.01^{c}	9.78±0.46^{c}	1.26±0.00^{b}	0.33±0.04^{c}	9.42±0.01^{b}	447.72±15.76^{b}	12.04±0.79^{b}	11.27±1.13^{b}	359.29±19.48^{b}	C
	25.63±1.59^{b}	21.83±0.11^{a}	18.32±0.97^{a}	2.26±0.17^{b}	24.91±1.50^{b}	0.21±0.01^{c}	1.36±0.07^{b}	3.43±0.42^{c}	92.86±0.93^{c}	6.62±0.63^{b}	12.47±0.85^{b}	78.49±4.94^{c}	J21
	13.00±1.09^{c}	15.79±0.67^{b}	13.97±0.62^{b}	7.82±0.32^{a}	367.56±4.06^{a}	5.78±0.33^{a}	9.48±0.04^{a}	30.12±0.36^{a}	734.88±45.11^{a}	31.71±4.42^{a}	28.76±2.58^{a}	609.88±6.87^{a}	J20
	2.38±0.42^{d}	1.20±0.03^{d}	0.54±0.03^{c}	0.00±0.00^{c}	2.71±0.14^{d}	0.25±0.01^{c}	0.05±0.00^{d}	0.00±0.00^{d}	3.65±0.30^{d}	0.00±0.00^{c}	0.64±0.01^{c}	44.59±2.65^{d}	BL
EC	60.82±3.19^{a}	24.74±0.36^{b}	15.44±0.93^{b}	14.38±0.46^{b}	122.95±10.09^{a}	3.08±0.03^{a}	2.89±0.14^{a}	13.31±0.57^{a}	486.51±22.03^{a}	11.14±0.16^{a}	15.72±0.32^{a}	652.27±62.26^{a}	C
	25.00±1.81^{b}	39.82±3.14^{a}	27.23±0.37^{a}	16.19±0.73^{a}	53.10±3.06^{b}	0.99±0.10^{b}	0.94±0.00^{c}	3.83±0.14^{b}	183.85±14.08^{b}	10.76±0.66^{a}	16.40±0.60^{a}	306.11±14.67^{b}	J21
	4.32±0.25^{c}	1.42±0.08^{c}	0.75±0.02^{c}	0.18±0.01^{c}	7.92±0.35^{c}	0.28±0.03^{c}	2.37±0.10^{b}	0.05±0.07c	37.27±1.13^{c}	4.58±0.27^{b}	4.79±0.15^{b}	199.48±12.89^{c}	J20
	3.56±0.14^{c}	1.16±0.01^{c}	0.61±0.05^{c}	0.04±0.00^{c}	3.40±0.02^{c}	0.30±0.00^{c}	0.09±0.00^{d}	0.00±0.00^{c}	4.01±±0.16^{c}	0.05±0.01^{c}	0.94±0.01^{c}	52.09±1.85^{d}	BL
SA	31.85±1.31^{a}	38.62±2.76^{a}	47.15±1.09^{a}	51.52±2.66^{a}	438.24±35.92^{a}	4.18±0.22^{a}	41.56±1.22^{a}	1.94±0.20^{c}	478.71±41.60^{b}	39.96±0.39^{a}	26.72±0.77^{a}	451.04±12.78^{a}	C
	25.11±1.97^{b}	15.58±0.49^{b}	23.01±2.23^{b}	8.29±0.06^{b}	67.69±0.54^{c}	0.99±0.09^{c}	2.22±0.05^{b}	4.74±0.05^{b}	1284.49±108.52^{a}	13.07±0.39^{c}	16.19±1.39^{c}	255.50±11.62^{b}	J21
	14.97±0.80^{c}	8.99±0.92^{c}	11.54±0.52^{c}	7.06±0.21^{b}	193.75±27.02^{b}	1.41±0.01^{c}	2.39±0.07^{b}	13.34±1.16^{a}	207.54±17.45^{c}	21.89±0.66^{b}	24.33±0.01^{b}	484.52±45.67^{a}	J20
	3.59±0.22^{d}	0.72±0.03^{d}	0.60±0.05^{d}	0.13±0.02^{c}	0.96±0.08d	2.28±0.21^{b}	0.01±0.01^{c}	0.08±0.00^{d}	12.56±0.21^{d}	0.20±0.00^{d}	1.91±0.06^{d}	57.75±1.68^{c}	BL
CA	74.71±2.53^{a}	7.39±0.67^{a}	16.16±0.32^{b}	7.41±0.39^{b}	4.50±0.14^{b}	9.86±0.47^{a}	4.67±0.02^{b}	29.77±3.53^{b}	222.84±15.35^{b}	12.61±0.03^{b}	20.42±1.81^{b}	770.34±20.08^{a}	C
	22.38±0.13^{b}	4.37±0.24^{b}	20.72±2.00^{a}	14.53±1.34^{a}	0.00±0.00^{c}	9.78±0.48^{a}	12.04±0.74^{a}	36.92±3.27^{a}	1047.86±98.38^{a}	49.18±2.44^{a}	36.43±0.61^{a}	245.51±10.31^{c}	J21
	7.74±0.64^{c}	0.59±0.02^{c}	3.34±0.29^{c}	0.74±0.05^{c}	5.73±0.02^{a}	0.47±0.03^{b}	0.29±0.03^{c}	1.29±0.02^{c}	62.94±3.37^{c}	6.35±0.28^{c}	9.32±0.86^{c}	464.83±42.87^{b}	J20
	2.68±0.60^{d}	0.13±0.02^{c}	0.74±0.01^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.64±0.01^{b}	0.07±0.00^{c}	0.08±0.00^{c}	5.03±0.44^{c}	0.00±0.00^{d}	1.67±0.00^{d}	32.51±2.55^{d}	BL
PL	131.50±7.05^{a}	0.82±0.05^{b}	3.78±0.17^{c}	5.82±0.37^{b}	44.64±3.73^{b}	8.53±0.73^{a}	4.89±0.18^{a}	37.93±0.75^{a}	490.38±21.61^{a}	2.95±0.10^{d}	1.21±0.07^{d}	689.28±54.57^{a}	C
	12.43±0.23^{b}	9.03±0.59^{a}	16.92±1.23^{a}	9.55±0.70^{a}	59.83±2.59^{a}	0.96±0.02^{c}	1.18±0.05^{c}	1.45±0.07^{c}	179.65±7.92^{b}	8.52±0.75^{b}	2.65±0.13^{c}	107.78±9.86^{d}	J21
	8.33±0.50^{b}	0.86±0.20^{b}	7.17±0.59^{b}	3.36±0.16^{c}	43.88±2.95^{b}	1.12±0.04^{c}	2.15±0.22^{b}	5.70±0.25^{b}	197.01±12.20^{b}	17.15±0.22^{a}	14.81±0.67^{a}	374.17±33.91^{b}	J20
	7.24±0.36^{b}	1.12±0.09^{b}	3.65±0.17^{c}	5.15±0.34^{b}	24.68±1.80^{c}	4.32±0.10^{b}	1.05±0.05^{c}	5.58±0.37^{b}	122.43±6.58^{c}	5.15±0.39^{c}	5.21±0.31^{b}	261.75±10.16^{c}	BL
PD	55.19±1.38^{a}	2.50±0.09^{b}	7.30±0.48^{b}	7.48±0.25^{b}	47.31±3.27^{b}	0.05±0.01^{c}	5.02±0.07^{b}	23.53±1.02^{b}	567.60±42.15^{a}	21.53±2.17^{b}	10.11±0.74^{b}	741.87±27.01^{a}	C
	18.41±1.79^{b}	6.12±0.23^{a}	15.25±0.73^{a}	19.45±1.06^{a}	207.95±6.71^{a}	7.31±0.01^{a}	24.44±1.88^{a}	35.18±3.48^{a}	373.65±40.35^{b}	28.22±0.42^{a}	27.62±1.26^{a}	221.64±3.29^{b}	J21
	2.41±0.25^{c}	0.27±0.01^{c}	0.98±0.70^{c}	0.07±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.85±0.00^{c}	1.49±0.01^{c}	88.26±2.79^{c}	4.17±0.25^{c}	5.02±0.04^{c}	204.54±19.60^{b}	J20
	1.75±0.14^{c}	0.09±0.00^{c}	0.68±0.02^{c}	0.00±0.00^{c}	0.00±0.00^{c}	2.00±0.10^{b}	0.00±0.00^{c}	0.00±0.00^{c}	41.59±2.33^{c}	0.00±0.00^{d}	0.48±0.02^{d}	42.36±2.65^{c}	BL
EF	32.58±2.15^{a}	57.72±3.04^{a}	46.96±2.27^{a}	114.57±1.74^{a}	0.60±0.01^{b}	16.36±1.59^{a}	11.67±0.05^{a}	76.75±5.58^{a}	1300.84±112.31^{a}	43.71±2.36^{a}	12.70±0.09^{b}	531.92±27.48^{a}	C
	40.48±5.90^{a}	6.06±0.36^{b}	42.43±3.49^{a}	32.25±1.21^{b}	296.56±14.04^{a}	9.20±0.16^{b}	7.06±0.02^{b}	29.08±2.23^{b}	228.22±19.73^{b}	34.00±0.87^{b}	31.25±1.33^{a}	306.84±23.58^{b}	J21
	3.09±0.13^{b}	0.41±0.01^{c}	0.44±0.00^{b}	0.06±±0.00^{c}	0.00±0.00^{b}	0.07±0.00^{c}	0.00±0.00^{c}	0.11±0.01^{c}	10.97±1.15^{c}	0.39±0.02^{c}	2.00±0.06^{c}	71.94±1.79^{c}	J20
	2.83±0.27^{b}	0.97±0.08^{c}	0.70±0.06^{b}	0.10±0.01^{c}	2.40±0.09^{b}	0.25±0.00^{c}	0.01±0.00^{c}	0.20±0.02^{c}	20.25±0.82^{c}	0.09±0.00^{c}	2.48±0.19^{c}	37.57±1.31^{c}	BL

*Data are expressed as mean value of three samples, Mean value±Standard deviation. AMN, ammonia; PUT, putrescine; CAD, cadaverine; SPD, spermidine; TRP, tryptamine; PHEN, 2-phenylethyl amine; SPN, spermine; SER, serotonin; DOP, dopamine; AGM, agmatine. SP: *Salmonella* Paratyphi A, *EC: E. coli*, *SA: Staphylococcus aureus*, CA: *Candida albicans*, PL: *Pseudomonas luteola*, PD: *Pseudomonas damseale*; EF: *Enterococcus faecalis*, C: Control, J21: Juniperus spp. 2021, J20: PRIMA_19, BL: PRIMA_02

$^{a–d}$ Indicate significant differences ($p<0.05$) between control and treated group in a row

4. CONCLUSION

1) ALGAE

The algae samples showed good antioxidant and antimicrobial properties. In terms of antioxidation, a significant relationship was not found between the TPC and antioxidant potential, since it is obvious that algae samples poses other compounds that contribute to its activity. The results of antioxidant potential differed between different assays. The results of the ORAC method can related to the biological material. Further investigations will be done in WP4 Task 1 to find extracts that can repeat the antioxidant effect in a food model.

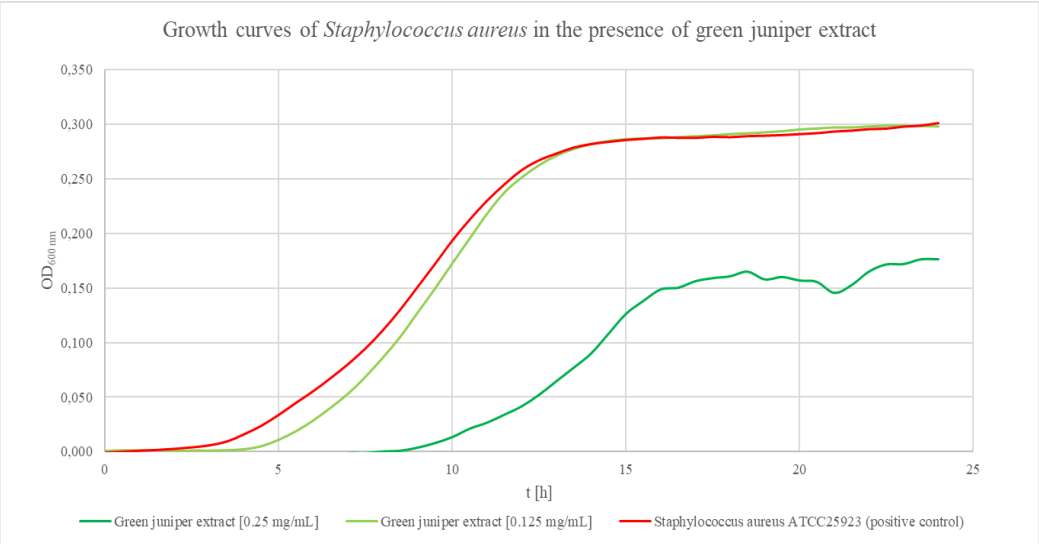
The most active antimicrobial effect was observed for algae extracts from June and September sampling. The algal extracts present high potential as natural preservatives due to their ability to inhibit pathogenic bacteria. *P. pavonica* samples showed interesting potential against *Listeria* strains and C. jejuni, and this will be further investigated. They also showed potential in inhibition of ammonia and biogenic amine production by both spoilage and pathogenic bacteria. On the other hand, *C. compressa* essential oils had a low antimicrobial activity on *L. monocytogenes* and *E. coli* but it was able to inhibit Salmonella.

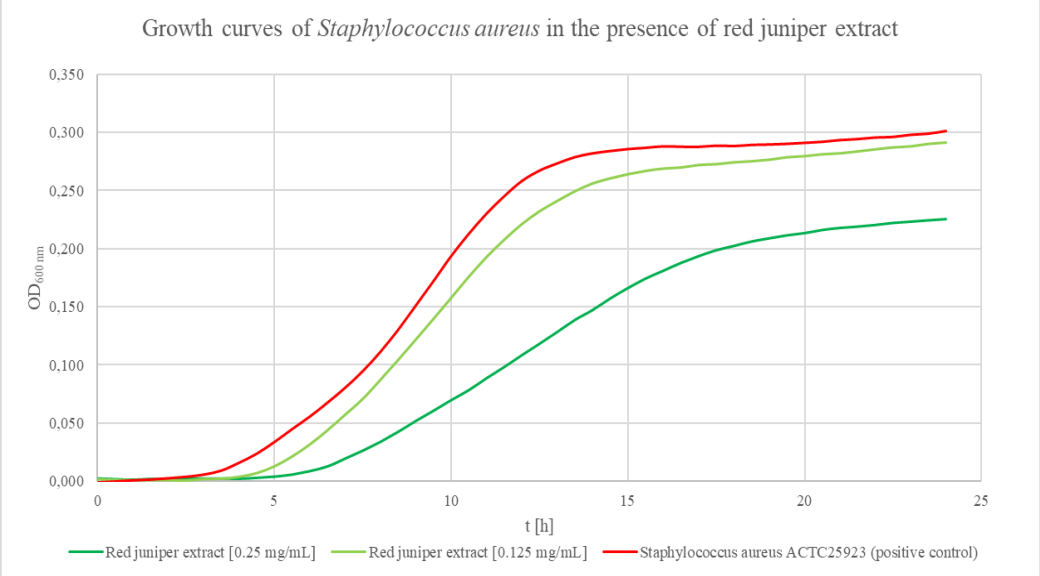
2) AGROFOOD BY_PRODUCTS

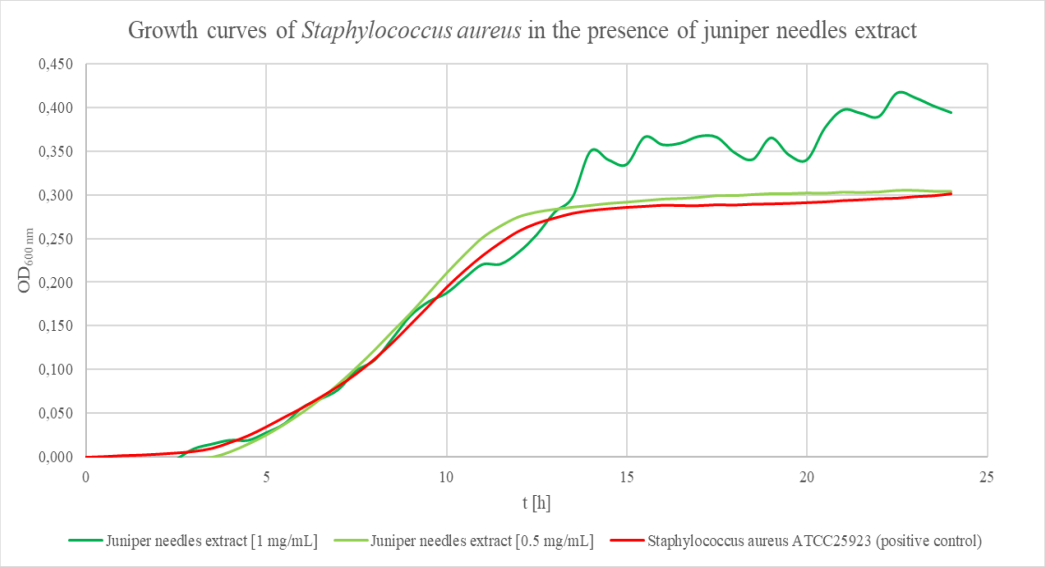
Based on the obtained results the consortium, on the 2nd GA meeting held virtually in May, concluded that the samples that will be chosen and prepared for the WPs 4 and 5 were following: blackberry by-products (primary leaves), *Juniperus oxycedrus* needles, *Juniperus communis* extract by-product. These extracts (or some of their pure components) will be used alone and in combination in different food matrices to achieve the antioxidant/antimicrobial effect needed for potential prolongation of the products shelf-life. The same natural substances will be tested for some target product combined with bioprotective strains selected in WP3 (or their supernatants).

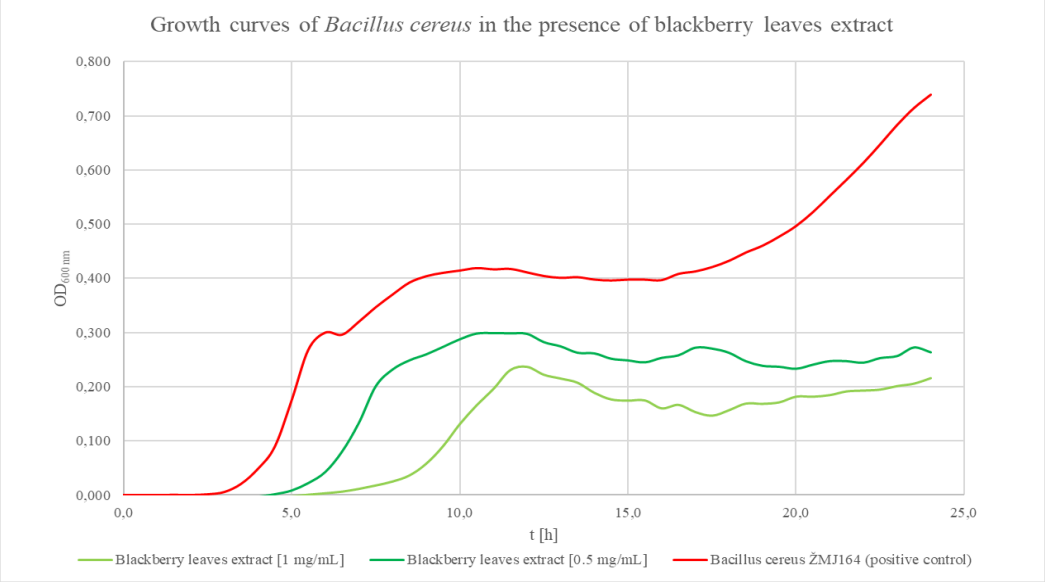
5. Appendices

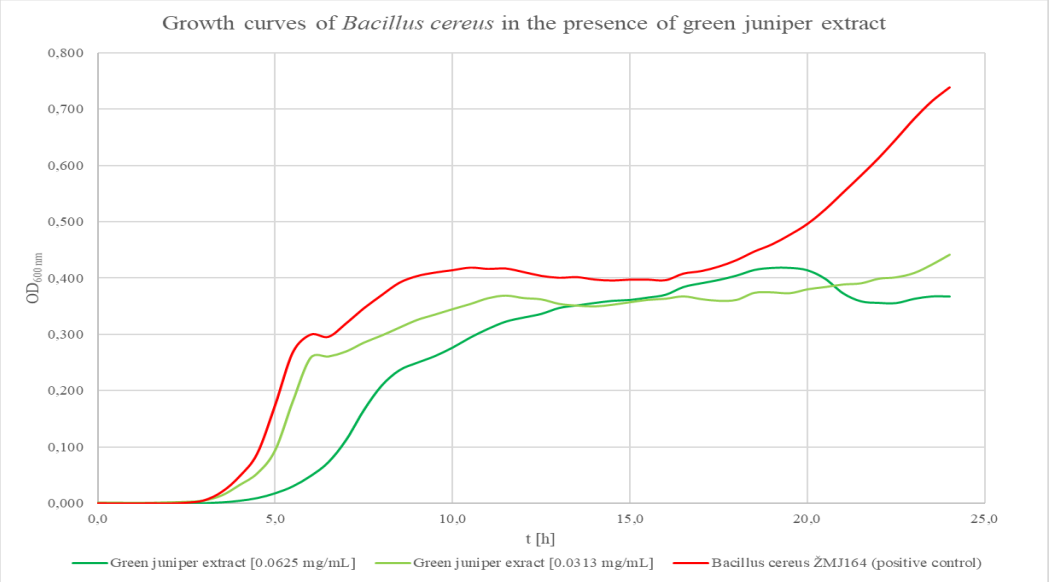
Appendix I - Growth inhibition curves:

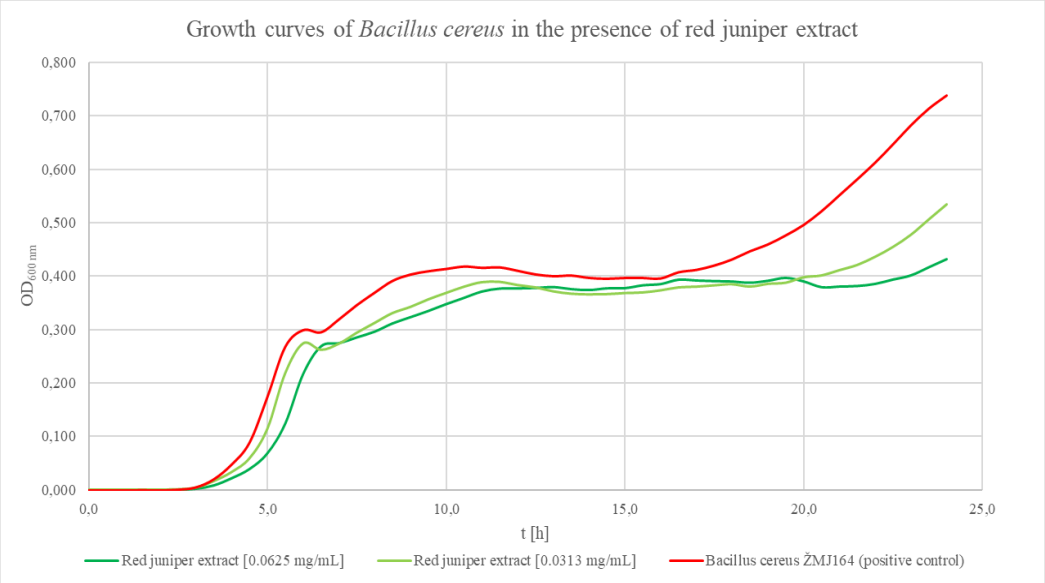


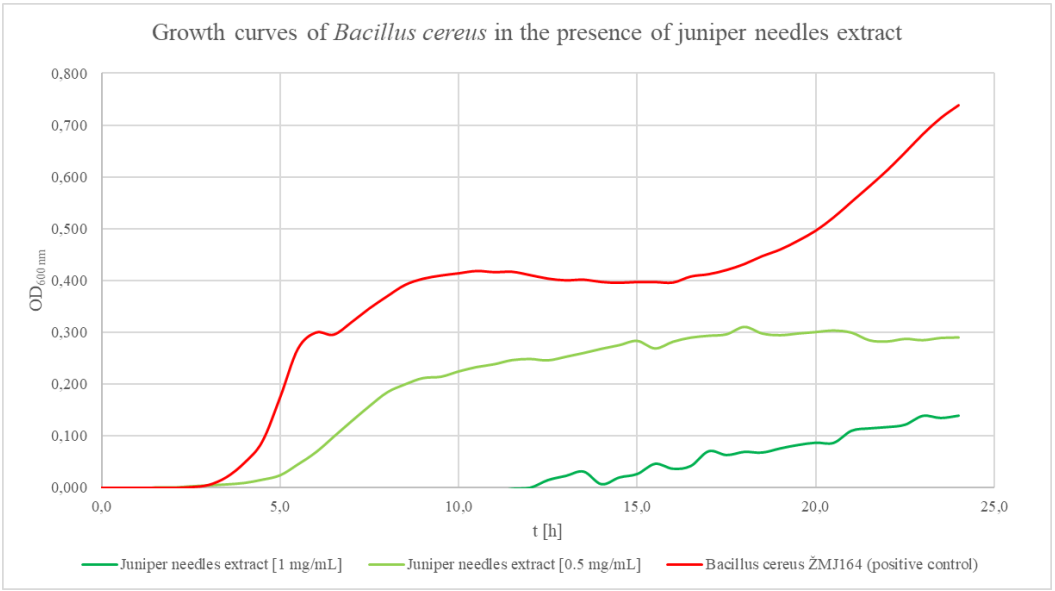


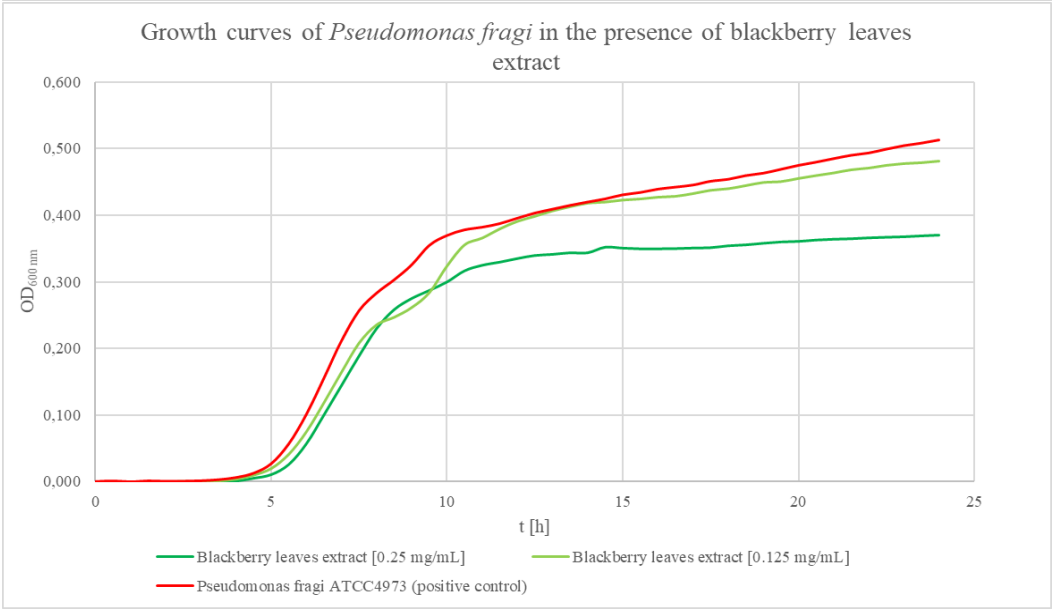


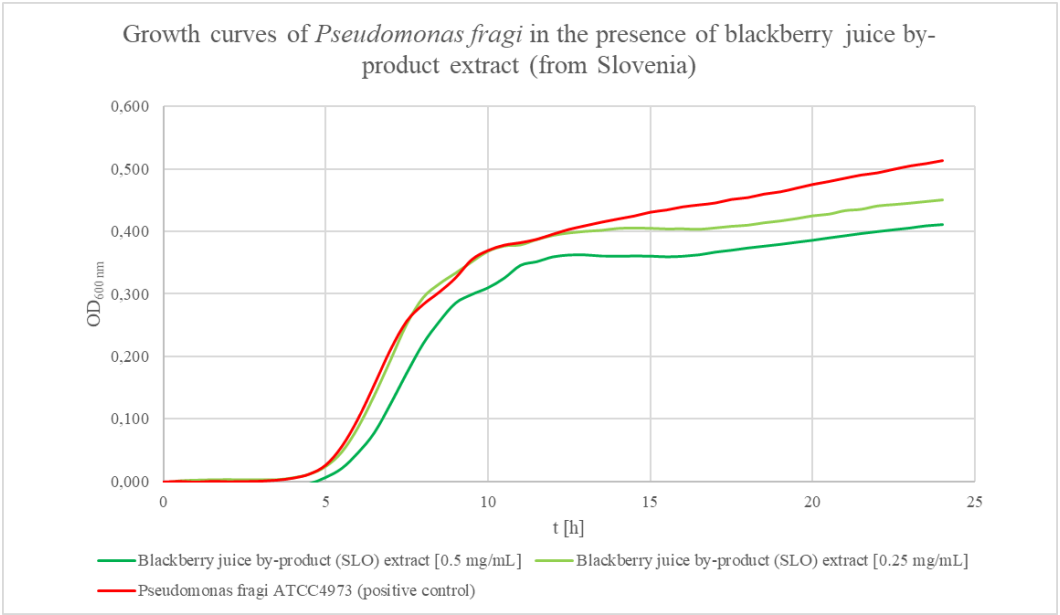

Growth curves of *Bacillus cereus* in the presence of blackberry leaves extract
0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
OD600 nm
0,0
5,0
10,0
15,0
20,0
25,0
t [h]
Blackberry leaves extract [1 mg/mL]
Blackberry leaves extract [0.5 mg/mL]
Bacillus cereus ŽMJ164 (positive control)

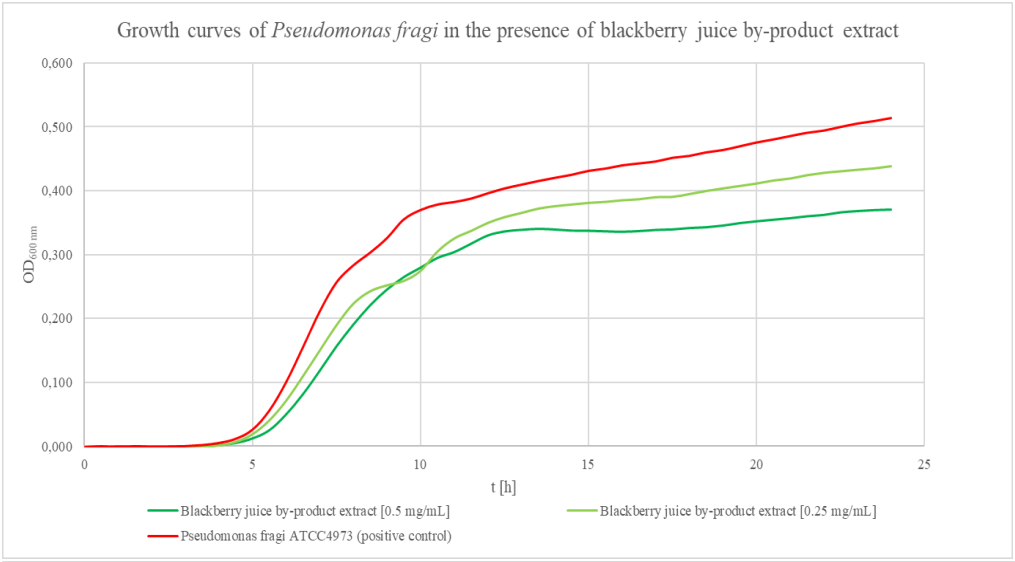

Growth curves of *Bacillus cereus* in the presence of green juniper extract
0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
OD600 nm
0,0
5,0
10,0
15,0
20,0
25,0
t [h]
Green juniper extract [0.0625 mg/mL]
Green juniper exract [0.0313 mg/mL]
Bacillus cereus ŽMJ164 (positive control)

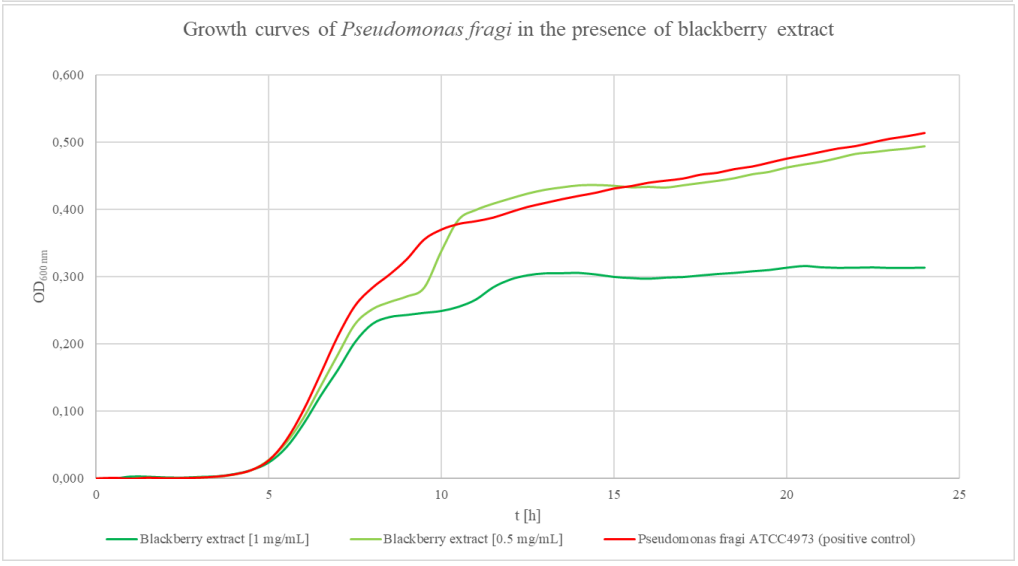

Growth curves of *Bacillus cereus* in the presence of red juniper extract
0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
OD600 nm
0,0
5,0
10,0
15,0
20,0
25,0
t [h]
Red juniper extract [0.0625 mg/mL]
Red juniper extract [0.0313 mg/mL]
Bacillus cereus ŽMJ164 (positive control)

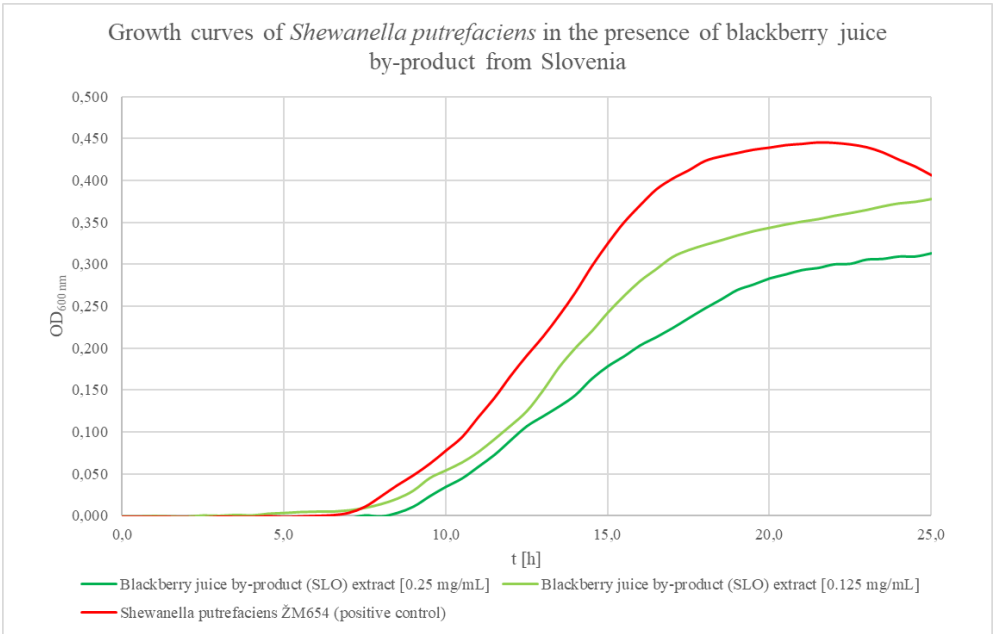

Growth curves of *Bacillus cereus* in the presence of juniper needles extract
0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
OD600 nm
0,0
5,0
10,0
15,0
20,0
25,0
t [h]
Juniper needles extract [1 mg/mL]
Juniper needles extract [0.5 mg/mL]
Bacillus cereus ŽMJ164 (positive control)

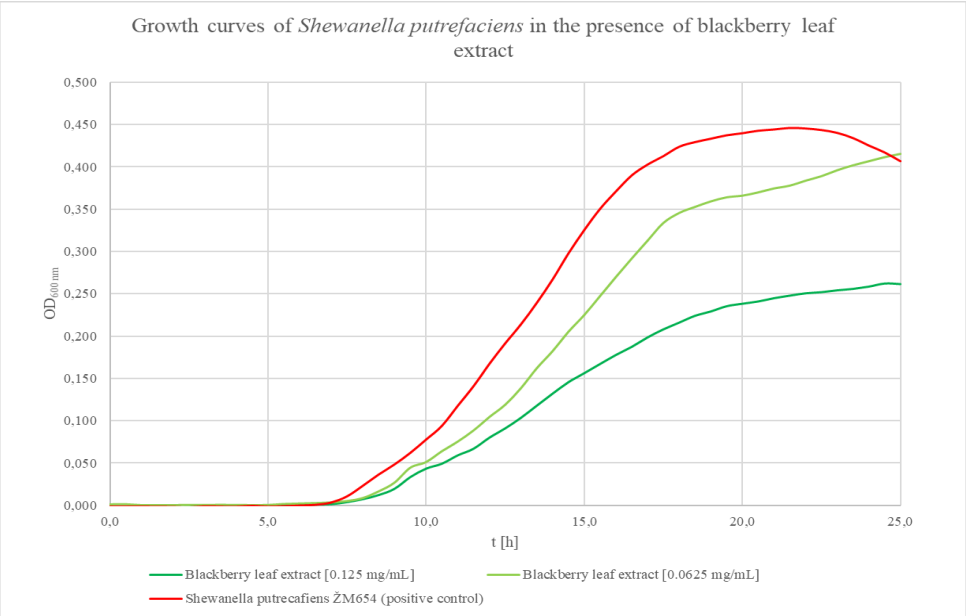

Growth curves of *Pseudomonas fragi* in the presence of blackberry leaves extract
0,600
0,500
0,400
0,300
0,200
0,100
0,000
OD600 nm
0
5
10
15
20
25
t [h]
Blackberry leaves extract [0.25 mg/mL]
Blackberry leaves extract [0.125 mg/mL]
Pseudomonas fragi ATCC4973 (positive control)

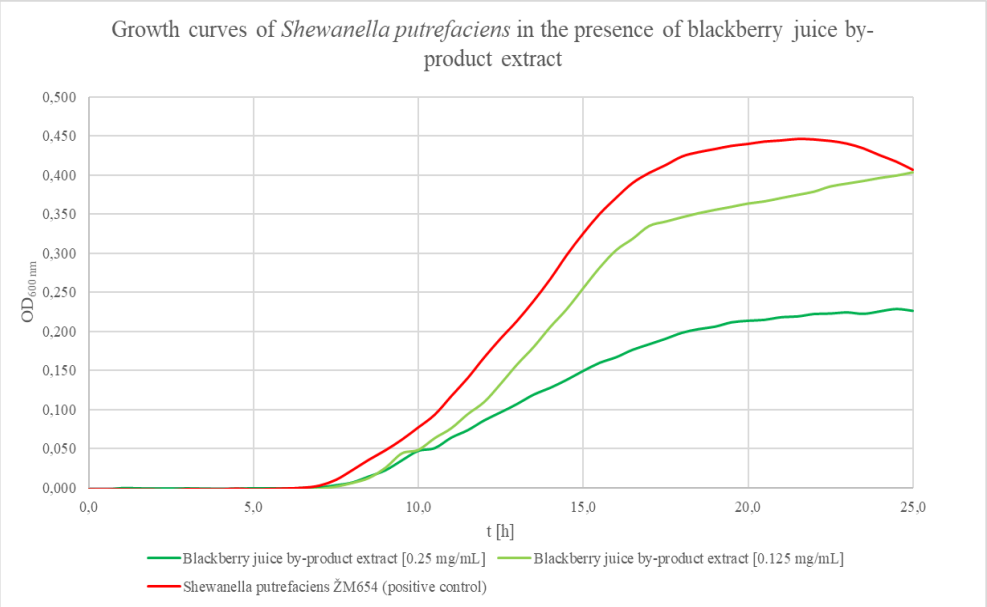

Growth curves of *Pseudomonas fragi* in the presence of blackberry juice by-product extract (from Slovenia)
0,600
0,500
0,400
0,300
0,200
0,100
0,000
OD600 nm
0
5
10
15
20
25
t [h]
Blackberry juice by-product (SLO) extract [0.5 mg/mL]
Blackberry juice by-product (SLO) extract [0.25 mg/mL]
Pseudomonas fragi ATCC4973 (positive control)

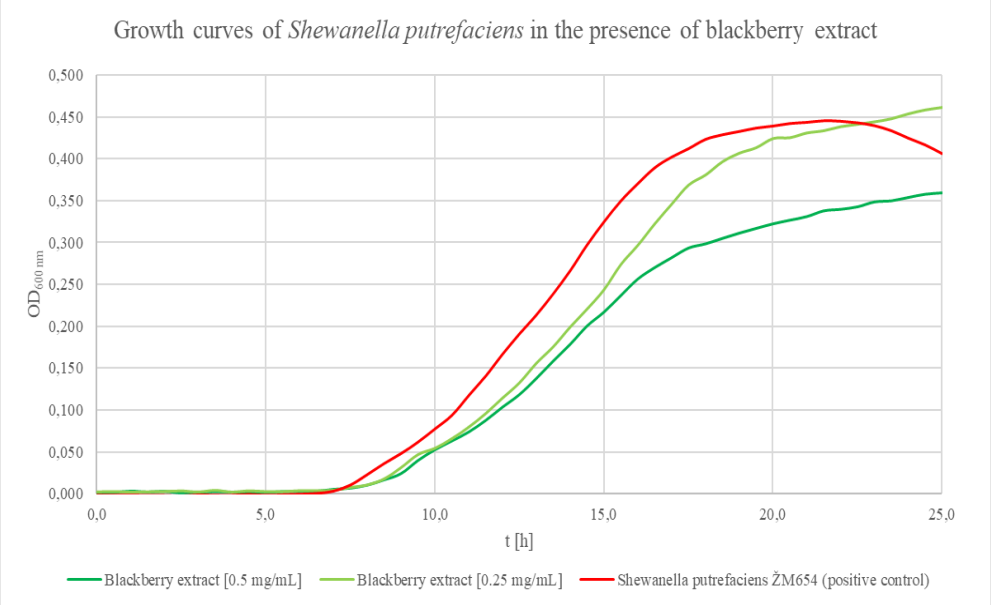

Growth curves of *Pseudomonas fragi* in the presence of blackberry juice by-product extract
0,600
0,500
0,400
0,300
0,200
0,100
0,000
OD600 nm
0
5
10
15
20
25
t [h]
Blackberry juice by-product extract [0.5 mg/mL]
Blackberry juice by-product extract [0.25 mg/mL]
Pseudomonas fragi ATCC4973 (positive control)

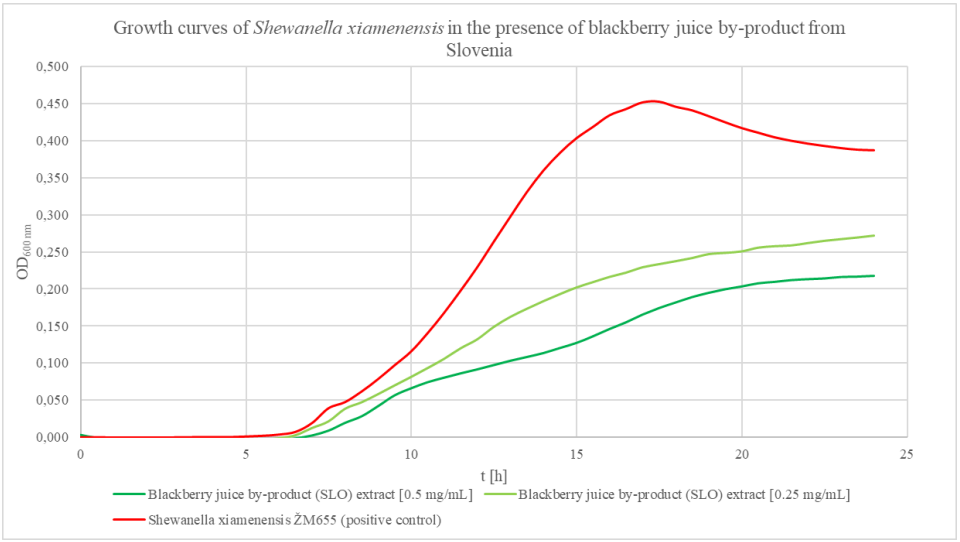

Growth curves of *Pseudomonas fragi* in the presence of blackberry extract
0,600
0,500
0,400
0,300
0,200
0,100
0,000
OD600 nm
0
5
10
15
20
25
t [h]
Blackberry extract [1 mg/mL]
Blackberry extract [0.5 mg/mL]
Pseudomonas fragi ATCC4973 (positive control)

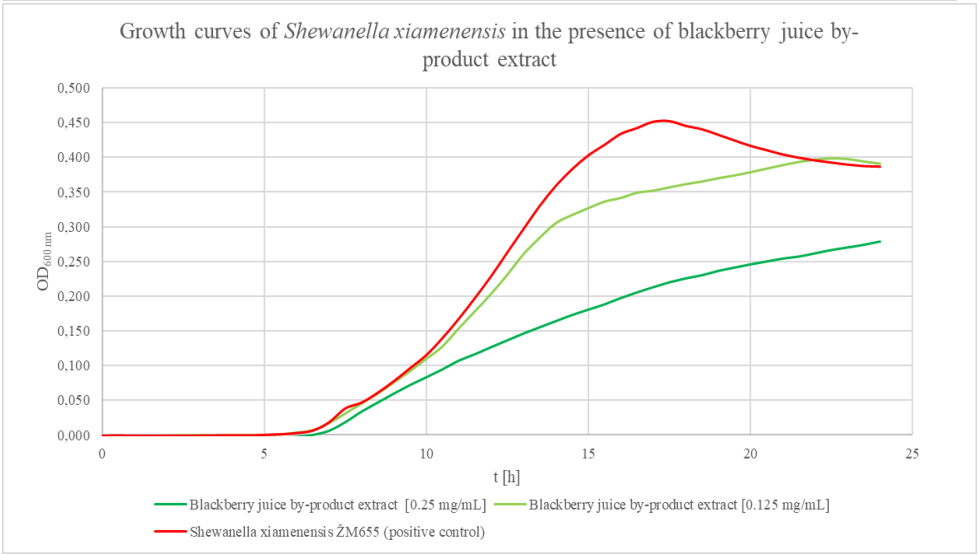

Growth curves of *Shewanella putrefaciens* in the presence of blackberry juice by-product from Slovenia
0,500
0,450
0,400
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
OD600 nm
0,0
5,0
10,0
15,0
20,0
25,0
t [h]
Blackberry juice by-product (SLO) extract [0.25 mg/mL]
Blackberry juice by-product (SLO) extract [0.125 mg/mL]
Shewanella putrefaciens ŽM654 (positive control)

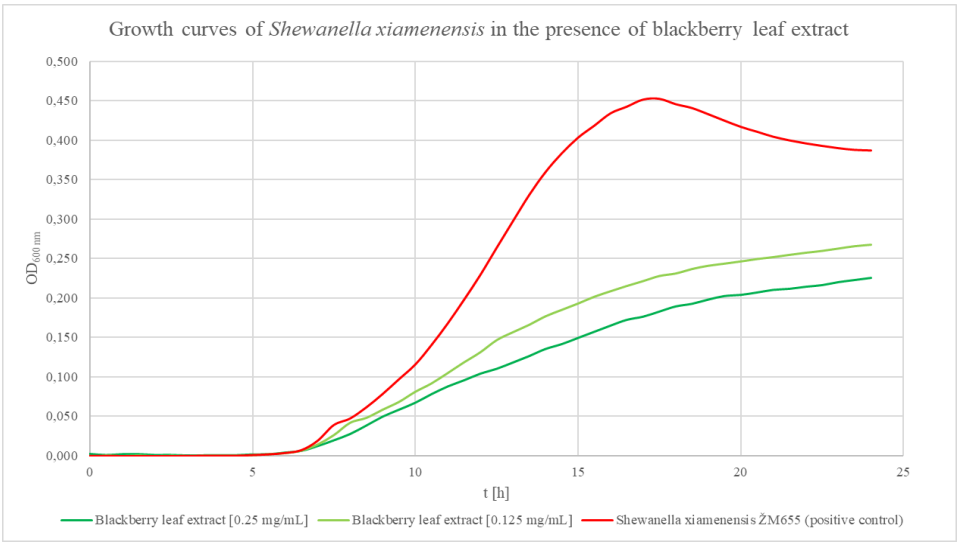

Growth curves of *Shewanella putrefaciens* in the presence of blackberry leaf extract
0,500
0,450
0,400
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
OD600 nm
0,0
5,0
10,0
15,0
20,0
25,0
t [h]
Blackberry leaf extract [0.125 mg/mL]
Blackberry leaf extract [0.0625 mg/mL]
Shewanella putrecafiens ŽM654 (positive control)

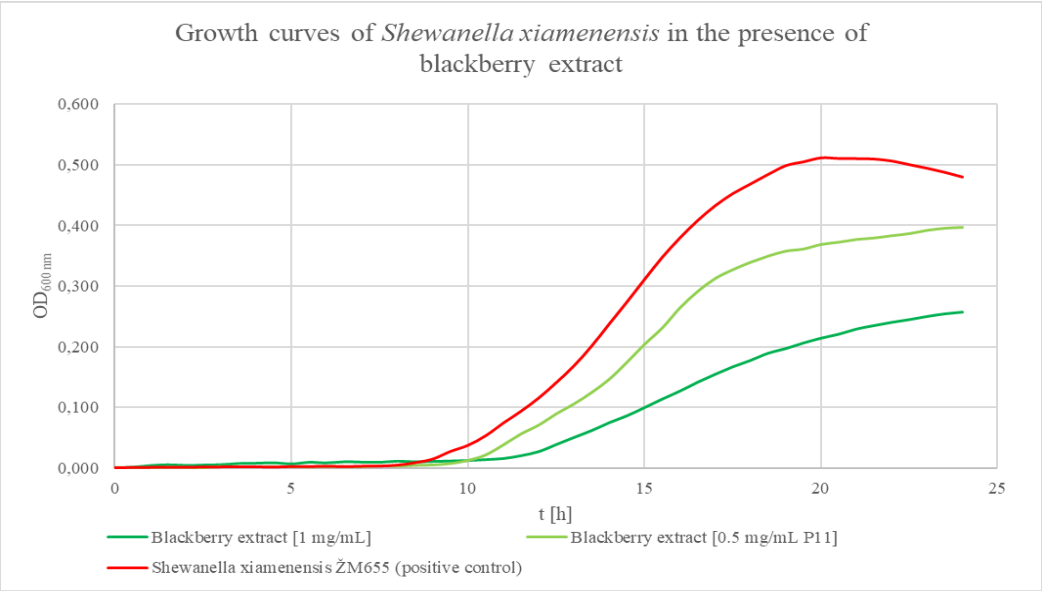

Growth curves of *Shewanella putrefaciens* in the presence of blackberry juice by-product extract
0,500
0,450
0,400
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
OD600 nm
0,0
5,0
10,0
15,0
20,0
25,0
t [h]
Blackberry juice by-product extract [0.25 mg/mL]
Blackberry juice by-product extract [0.125 mg/mL]
Shewanella putrefaciens ŽM654 (positive control)


Growth curves of *Shewanella putrefaciens* in the presence of blackberry extract
0,500
0,450
0,400
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
OD600 nm
0,0
5,0
10,0
15,0
20,0
25,0
t [h]
Blackberry extract [0.5 mg/mL]
Blackberry extract [0.25 mg/mL]
Shewanella putrefaciens ŽM654 (positive control)


Growth curves of *Shewanella xiamenensis* in the presence of blackberry juice by-product from Slovenia
OD600 nm
0,500
0,450
0,400
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
0
5
10
15
20
25
t [h]
Blackberry juice by-product (SLO) extract [0.5 mg/mL]
Blackberry juice by-product (SLO) extract [0.25 mg/mL]
Shewanella xiamenensis ŽM655 (positive control)


Growth curves of *Shewanella xiamenensis* in the presence of blackberry juice by-product extract
OD600 nm
0,500
0,450
0,400
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
0
5
10
15
20
25
t [h]
Blackberry juice by-product extract [0.25 mg/mL]
Blackberry juice by-product extract [0.125 mg/mL]
Shewanella xiamenensis ŽM655 (positive control)


Growth curves of *Shewanella xiamenensis* in the presence of blackberry leaf extract
OD600 nm
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
5
10
15
20
25
t [h]
Blackberry leaf extract [0.25 mg/mL]
Blackberry leaf extract [0.125 mg/mL]
Shewanella xiamenensis ŽM655 (positive control)


Growth curves of *Shewanella xiamenensis* in the presence of blackberry extract
0,600
0,500
0,400
0,300
0,200
0,100
0,000
OD600 nm
0
5
10
15
20
25
t [h]
Blackberry extract [1 mg/mL]
Blackberry extract [0.5 mg/mL P11]
Shewanella xiamenensis ŽM655 (positive control)