Anti-biofilm potential of *Lavandula* preparations against *Campylobacter jejuni*

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Abstract

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

Keywords: *Lavandula* preparations, anti-biofilm activity, *Campylobacter jejuni*

Importance

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

1. Introduction

The food industry is in a constant battle against economic losses caused by formation and prevalence of bacterial biofilms on food manufacturing surfaces. Moreover, bacterial biofilms, as a persistence form of bacterial lifestyle in food processing environments, are a significant public health problem (1). One of the most prevalent food-borne pathogens and the major cause of human intestinal infections in developed countries is *Campylobacter jejuni* (2, 3). Human infections occur mainly by consumption of undercooked meat, especially poultry, or food that has been in contact with a contaminated surface (4).

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

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

One such commonly used crop worldwide belongs to the *Lamiaceae* family, and the genus *Lavandula*, some strains of which are *Lavandula angustifolia* Mill. (true lavender,

Lamiaceae), *Lavandula latifolia* Medik. (spike lavender) and *Lavandula × intermedia* Emeric ex Loisel. (lavandin) (11). These have been used since ancient times for medicinal purposes, and more recently in industrial processes (12, 13).

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

In addition to alternative strategies using low doses of bioactive phytochemicals to control bacterial biofilms, this study has focused also on the use of by-products of essential oil distillation. More recently, the demand for *Lavandula* essential oils has grown hugely, which has resulted in greater cultivation and production of these plants. About 200 tons of lavender oil and 1200 tons of lavandin oil are produced annually, and there has been a concomitant increase in the by-products that are accumulated as waste materials and hydrolates or aromatic waters after essential oil production (22–24). These by-products and especially waste from the agro-food industry also represent a potentially important source of effective phytochemicals (10). For example, waste materials from spike lavender have antioxidant properties (13), and lavender hydrolates have antifungal and antibacterial properties that enhance the flavour and taste of foods (25). Reuse of these waste materials will partially solve one of the major problems – their disposal (26). In addition, the reuse of such a material is environmentally friendly, as the use of disinfectants, which are commonly used in the food industry, can lead to

additional unnecessary chemicals in the environment (8). However, current studies have been focused only on planktonic cell suspensions, and there have not been any reports on anti-biofilm effects of such *Lavandula* by-products. This is important, as biofilms are the most prevailing growth lifestyle in bacteria and it is well known that in biofilms the effectiveness of antimicrobials is significantly reduced (up to 1000 fold) compared with the planktonic state (27).

In this study, *Lavandula* preparations and selected pure compounds were used to determine whether they have anti-biofilm effects against *C. jejuni* NCTC 11168. Dried flowers of *Lavandula angustifolia* were used to produce an essential oil (LEO), an ethanol extract (LEF) and the ethanol extract of *Lavandula* post-distillation waste material (LEW). First, degradation of *C. jejuni* biofilms formed on an abiotic surface using these *Lavandula* preparations was investigated. Further, strategies to target motility and adhesion of *C. jejuni* were introduced, with the aim to prevent or reduce biofilm formation in its early stages. Finally, an RNAseq approach was used, to better understand the molecular mechanisms underlying the actions of LEO against *C. jejuni* biofilms, to thus indicate innovative *Campylobacter* control strategies.

2. Materials and methods

2.1. Chemicals

Muller–Hinton (MH) agar was from BioMérieux (Marcy-l'Étoile, France), MH broth was from Oxoid (Hampshire, UK), and Karmali agar was from Biolife (Milan, Italy). Glycerol solution was from Kemika (Zagreb, Croatia), *Campylobacter* growth supplement and phosphate-buffered saline (PBS) were from Oxoid, kanamycin, dimethylsulphoxide (DMSO), resazurin, menadione, propidium iodide (PI) and TRI reagent were from Sigma Aldrich (Steinheim,

Germany), SYTO9 was from ThermoFisher (Waltham, USA) and ethanol was from Merck (Darmstadt, Germany). PureLink DNase and PureLink RNA mini kits, Qubit RNA HS, Ion Total RNA-seq kits v2, RNA-seq Barcode BC primer and Ion PI Hi-Q sequencing 200 kits were from Thermo Fisher Scientific (Carlsbad, CA, USA), magnetic beads were from NEXTflex PolyA (Perkin Elmer, Waltham, MA, USA) and High Sensitivity DNA kits were from Agilent Technologies (Santa Clara, USA). The pure compounds linalool and 1,8-cineol were from Symrise (Holzminden, Germany), linalyl acetate and p-coumaric acid were from Honeywell Fluka (Charlotte, USA), camphor was from (ICN Biomedicals Inc, Aurora, OH, USA), and (-)-borneol and *trans*-ferulic acid were from Carl Roth (Karlsruhe, Germany). The total ion chromatograms from the gas chromatography–mass spectrometry analysis showed that the following purities: linalool, 94.88%; 1,8-cineol, 99.85%; linalyl acetate, 95.64%; camphor, 94.77%; and (-)-borneol, 96.65%. The PDA total scan chromatograms from the HPLC-UV showed that p-coumaric acid consisted of the *trans* (98.33%) and *cis* (1.53%) isomers, while ferulic acid also consisted of the *trans* (97.87%) and *cis* (1.85%) isomers.

2.2. *Lavandula* preparations

Dried flowers of *Lavandula angustifolia* were from Kottas Heilkräuter (Control number KLA91232, Vienna, Austria), from which the LEO, LEF and LEW were produced. LEO was prepared by hydrodistillation (Meyer-Warnod, 1984), with 2 h distillation of 30 g flowers in 1 L water in a Neo-Clevenger-type distiller, and then stored at 4 °C. LEF and LEW were prepared by 4 h to 6 h ethanol extraction (Soxhlet extraction) of 20 g flowers or *Lavandula* post-distillation waste material in 500 mL 96% ethanol, to provide LEF and LEW. These were then concentrated in a rotary evaporator (Laborota 4000; Heidolph Instruments, Germany), at 40 °C and 175 mbar pressure, and stored at 4 °C.

2.3. *Phytochemical analysis of LEF and LEW*

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

Mass spectra were recorded in negative ion mode for the m/z range from 50 to 2000 amu, with data-dependent fragmentation (normalised collision energy, 35%). Mass spectrometry conditions were set to: capillary temperature, 350 $^{\circ}$ C; source temperature, 300 $^{\circ}$ C; sheath and auxiliary gas flow, 40 and 10 arbitrary units (machine settings), respectively; source voltage, 3.5 kV; and capillary voltage, -17 V. The compounds eluted were determined by their ultraviolet and mass spectra, and in comparison with the literature (24, 28, 29).

2.4. *Gas chromatography–mass spectrometry analysis of LEO*

Identification of the main compounds in LEO, and determination of the purities of the linalool, 1,8-cineol, linalyl acetate, camphor and (-)-borneol, was carried out by gas chromatography–mass spectrometry. LEO (10 μ L) was dissolved in 990 μ L hexane, and further analysed by gas chromatography (7890 A; Agilent Technologies, Santa Clara, USA) and mass spectrometry

(5975 C VL MSD; Agilent Technologies, USA), operating at 70 eV, with ion source temperature 230 °C, and interface temperature 280 °C. A split injection (injection volume, 0.2 µL; split ratio, 50:1) at 240 °C injector temperature was used for LEO. A fused silica capillary column was used (5% phenyl, 95% methyl polysiloxane; HP-5MS; 30 m × 250 µm × 0.25 µm; Agilent J & W, USA). The temperature programme was as follows: 1 min at 60 °C, then raised to 220 °C at 3 °C/min. The carrier gas was helium 5.6 at a flow rate of 0.9 mL/min. Data acquisition was performed using Agilent GC/MSD ChemStation Version E.02.02 for the mass scan range of 40 u to 400 u. The compounds were identified by their retention indices according to Adams (30), and by comparing their mass spectra with spectral data libraries (30–32) and the laboratory own database.

2.5. Bacterial strains and growth conditions

Campylobacter jejuni NCTC 11168 (National Collection of Type Culture) was used in this study. The strain was stored at -80 °C in 20% glycerol and 80% MH broth. Prior to the experiments, the strain was sub-cultivated on Karmali agar for 24 h at 42 °C under micro-aerobic conditions (85% N₂, 5% O₂, 10% CO₂). The strain was further sub-cultured micro-aerobically in MH broth for 24 h at 42 °C, and bacterial counts were determined by spectrophotometric measurements of absorbance at 600 nm. The inoculum was prepared in MH broth at 10⁵ CFU/mL for determination of the minimal inhibitory concentrations (MICs), as well as for the assays that targeted *C. jejuni* motility and adhesion. For counting, the *C. jejuni* NCTC 11168 strain was plated on MH agar under the appropriate conditions (as described above), and the colonies were counted and expressed as colony forming units (CFU)/mL.

2.6. Antimicrobial susceptibility

The MICs were determined by the broth microdilution method, as previously described (33). Stock solutions of LEO, LEF and LEW *Lavandula* preparations and the pure compounds linalool, linalyl acetate, camphor, (-)-borneol, 1,8-cineol, p-coumaric acid and *trans*-ferulic acid were prepared in DMSO at 40 mg/mL. The DMSO in the MH broth did not exceed 1%.

2.7. Targeting *Campylobacter* biofilm degradation

To determine the effects of the *Lavandula* preparations for degradation and removal of *C. jejuni* biofilms, a novel approach based on culturing *C. jejuni* on an air/liquid interface was developed, with measurement of the relative glass surface coverage after treatment with the *Lavandula* preparations. Optimal *C. jejuni* growth conditions were established on the air/liquid interface (e.g., optimal O₂ concentration). Twelve-well microtiter plates (Sarstedt, Nümbrecht, Germany) were used, with the following added to each well: 3.0 mL MH broth, supplemented with 0.4% [v/v] *Campylobacter* growth supplement (which contained 0.125 g sodium pyruvate, 0.125 g sodium metabisulphite, 0.125 g ferrous sulphate). The medium was inoculated with 1% [v/v] *C. jejuni* NCTC 11168 overnight culture that contained about 1×10^8 CFU/mL. Autoclaved microscopy coverslips (20 × 20 mm; Brand, Wertheim, Germany) were used as the model for the glass surface, which were inserted and tilted to the side of each microtiter well after inoculation of the medium. To prevent desiccation and to minimise the proportion of dead cells, the cultures were incubated without shaking in a micro-aerobic atmosphere for 72 h at 42 °C, in a damp environment. The spent medium in each well was replaced with fresh medium every 24 h.

Visible biofilms were formed at the air/liquid interface on the glass coverslips after 48 h of incubation, and were then treated for 24 h with LEO at 0.5× MIC, MIC or 2× MIC, as well as with LEF and LEW ethanol extracts at 2× MIC, 4× MIC and 8× MIC, following MIC determine as bacterial suspensions (see sections 2.5. and 2.6.). Non-treated biofilms from *C.*

jejuni NCTC 11168 were used as the negative controls. The biofilms were analysed under confocal microscopy after 72 h of incubation.

After incubation the microscopy coverslips were first washed in PBS to remove weakly adhered cells. This was performed by carefully and slowly submerging the microscopy coverslips in PBS, twice in a row by holding them with forceps. The excess liquid was then removed by tapping the edge of the glass on a paper towel. The microscopy coverslips were then stained with 50 μ L of a mixture of 20 mM PI and 5 mM SYTO9, for 15 min [Live/Dead test; (34)] in an aerobic atmosphere at room temperature in the dark. The mixture of the PI solution and SYTO9 was prepared by adding 3 μ L PI and 3 μ L SYTO9 into 1 mL physiological solution. PI cannot enter intact cells, while dead cells with a compromised cell membrane allow the passage of PI. When PI binds to DNA it emits red fluorescence upon excitation. On the other hand, SYTO9 stains DNA in cells green, regardless of their membrane integrity. Thus, dead cells appear under fluorescence microscopy as red and only those that are only green are alive (34).

Confocal microscopy (LSM 800 Axio Observer Z1 inverted microscope; Zeiss, Germany) was performed using the 20 $\times$ /(NA 0.4) objective. SYTO9 fluorescence was excited with a 488 nm diode laser, and PI fluorescence with a 561 nm diode laser, operated at 0.5% power. We always considered the upper 600 μ m of the biofilm-covered glass surface, starting at the air/liquid interface in the direction towards the liquid. Experiments were performed in three or more biological replicates, and 10 images were acquired for each sample (5 $\times$ 2 mosaic images; total analysed surface, 1470 μ m $\times$ 607 μ m). In controls, this contained >400,000 viable cells. The images for each slide were analysed using the Fiji software (version 1.52c), to obtain the biofilm surface cover. Individual channel images were converted to binary format by manually setting the threshold value that fully separated the biofilm from the background. Random noise was reduced for all of the images using the ‘despeckle’ function. The biofilm

surface coverage was obtained by using our ImageJ custom macro, where surface coverage of biofilm was calculated by first summing the areas covered by green cells (SYTO9) and red cells (PI) and then subtracting the area of cells that were simultaneously green and red. The area covered by red cells was under 2% in all cases.

2.8. Targeting *Campylobacter* adhesion

The adhesion of *C. jejuni* NCTC 11168 was analysed under treatments with the *Lavandula* preparations and pure compounds at subinhibitory concentrations. Inocula were prepared as described above (section 2.5) and treated with the LEO, LEF and LEW *Lavandula* preparations and pure compounds at 0.25× MIC. They were then transferred (200 µL) to 96-well polystyrene microtitre plates (Nunc 266 120 polystyrene plates; Nunc, Denmark) and incubated micro-aerobically at 42 °C for 4, 8 and 24 h. The supernatants with non-adherent cells were removed from each well and rinsed three times with PBS. Then, 200 µL PBS was added, with sonication for 10 min (28 kHz, 300 W; IskraPlo, Šetnjernej, Slovenia). The adhesion of cells was examined as CFU/mL, as previously described (35). The untreated culture acted as the negative control.

2.9. Targeting *Campylobacter* motility

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

2.10. RNA sequencing

2.10.1. RNA isolation and quantification

The molecular mechanisms of LEO action in *C. jejuni* NCTC 11168 were analysed for treatment with subinhibitory concentrations of the LEO *Lavandula* preparation. *C. jejuni* NCTC 11168 was grown micro-aerobically in 42 mL of MH broth for 16 h at 42 °C to the middle exponential phase, and then treated with LEO at 0.25× MIC for 30 min. The negative control was the culture treated with 1% DMSO under the same conditions, and the positive control was the non-treated culture. The cells were harvested by centrifugation (5000× g, 5 min, 4 °C), and resuspended to 1 mL cell suspension, which contained approximately 10⁹ CFU/mL. Cell lysis for isolation of total RNA was performed using RNA isolation (TRI) reagent (Sigma Aldrich), DNase treatment using PureLink DNase kits (Thermo Fisher Scientific), and purification of isolated RNA using PureLink RNA mini kits (Thermo Fisher Scientific), following the manufacturer instructions. Quantification and qualification of the total RNA quality was determined using Qubit RNA HS assay kits (Thermo Fisher Scientific) and fluorimeter measurements (Qubit v4; Thermo Fisher Scientific). Messenger RNA (mRNA) was enriched from the total RNA using magnetic beads (NEXTflex PolyA; Perkin Elmer).

2.10.2. Ion torrent library preparation and sequencing

For next-generation RNA-sequencing (RNA-seq), transcriptome libraries were constructed using Ion Total RNA-seq kit v2 (Thermo Fisher Scientific). Briefly, mRNA samples were enzymatically fragmented and purified using magnetic beads. Afterwards, ion adaptors were hybridised onto the fragmented mRNA and ligated, and reverse transcription was performed. The prepared cDNA samples were purified using magnetic beads, and each cDNA sample was barcoded with an Ion Xpress RNA-Seq Barcode BC primer (Thermo Fisher Scientific). The cDNA libraries were purified, and the concentration and size distribution of cDNA libraries

were determined using a 2100 Bioanalyser and High Sensitivity DNA kits (Agilent Technologies). The barcoded cDNA libraries prepared were diluted to the same molar concentration, pooled in equal volumes, and amplified using an Ion OneTouch 2 System with the accompanying Ion PI Hi-Q OT2 200 kits. Sequencing was performed on an Ion Proton System, using Ion PI Hi-Q Sequencing 200 kits (Thermo Fisher Scientific). The datasets generated and analysed during the current study are available in the NCBI Sequence Read Archive (SRA) repository (<https://www.ncbi.nlm.nih.gov/sra/>) under the BioProject accession PRJNA747749 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA747749>) and SRA accession numbers; SRR15183216, SRR15183214, SRR15183213, SRR15183212.

2.10.3. Data analysis

The bioinformatics analysis was performed using a CLC Genomics Workbench (version 12.0.3) and a CLC Genomics Server (version 11.0.2). Prior to the differential expression analysis, quality control of the sequencing reads and trimming of the adapter sequences were performed using the ‘Trim Reads’ tool. Sequencing reads from each library were subjected to differential expression analysis using the RNA-Seq Analysis 2.21 tool (CLC Genomics Server 20.0.2). *Campylobacter jejuni* subsp. *jejuni* NCTC 11168 (ATCC 700819) complete genome sequence and genome annotations from the NCBI Nucleotide database (Accession number: NC_002163.1) were used as the reference genome sequence (36). To compare gene expression between samples treated with LEO and the controls, the ‘Differential Expression in Two Groups 1.1’ tool was used. Genes with absolute $\log_2$ fold-change ≥ 1 and false discovery rate (FDR) p -value ≤ 0.05 were considered as differentially expressed. Differentially expressed genes were further analysed via the STRING Consortium 2020, which provides functional enrichment analysis of protein-protein interaction networks in the STRING mapper tool (<https://string->

db.org/cgi/input.pl?sessionId=oO8HWWKY15Fd&input_page_show_search=on). To construct a network of differentially expressed genes using Cytoscape, the NCBI protein identifiers of differentially expressed genes were searched in String database for the organism *Campylobacter jejuni* subsp. *jejuni* NCTC 11168 = ATCC 700819, with a cut-off of 0.8 confidence (score) and a maximum of 10 additional interactors. Afterwards, the network was clustered using the MCL Cluster algorithm implemented in the Cytoscape plugin clusterMaker (37), to determine clusters and functional interactions. For each cluster, the built-in functional enrichment available from the stringApp was used to obtain enriched terms.

Gene enrichment analysis was performed using GO_MWU (GO-MWU, https://github.com/z0on/GO_MWU, <https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12864-015-1540-2>), which uses Mann-Whitney U tests and the Benjamini-Hochberg (B-H) FDR corrections of *p*-values to define which enriched GO categories are significantly represented by either up-regulated or down-regulated genes. GO categories with B-H FDR *p*-values of <0.1 were considered significantly enriched by either up-regulated or down-regulated genes.

2.11. Statistical analysis

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

3. Results

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

3.1. Phytochemical analysis and identification of flavonoids

The ethanolic extract of the *Lavandula* flowers, LEF, and the ethanolic extract of *Lavandula* post-distillation waste material, LEW, were analysed for their phenolic compound contents using liquid chromatography–photo diode array–electrospray ionisation mass spectrometry. LEF and LEW had phenolic acid glycosides as their major constituents. In addition, rosmarinic acid and the flavone apigenin-7-O-glucoside were detected. Flavonoids only had a minor role in the composition of LEF and LEW. Table 1 summarises the peaks that were identified for both LEF and LEW. Supplementary Figure 1 illustrates a representative UV chromatogram. Compound 4 in Table 1 is most probably identical to the o-coumaric acid-2-O-glucoside previously described for *L. angustifolia* (38), as indicated by the UV spectral data; however, m-coumaric acid as an aglycon cannot be excluded at this point.

Please insert Table 1.

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

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

Please insert Table 2.

3.2. Anti-Campylobacter activity of Lavandula preparations and pure compounds

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

Please insert Table 3.

3.3. Degradation of pre-formed biofilms by the Lavandula preparations

To determine the effects of these *Lavandula* preparations on *C. jejuni* biofilms, a novel approach was developed based on culturing *C. jejuni* at the air/liquid interface for 48 h. After treatment with the *Lavandula* preparations, this allowed measurement of the relative area of the glass surface covered by the biofilm using confocal laser scanning microscopy. The images of the untreated cultures showed the biofilm structure of the viable bacteria, which were mainly dispersed into small microcolonies (Figure 1). The influence of different concentrations of LEO, LEF and LEW on the biofilm structure and the cell viabilities were determined after 24 h of treatments (Figure 2). Interestingly, when the biofilms were exposed to LEO, LEF and LEW, there was significant degradation of the biofilms across all of these treatments. LEO was the most effective, as it effectively removed the bacterial biofilms. LEF and LEW had similar effects for *C. jejuni* biofilm removal (Figure 2). After the treatments of the *C. jejuni* biofilms with LEF and LEW at 8× MIC, the relative surface cover with live cells was 0.016 ± 0.005 to 0.031 ± 0.002 , respectively. Surprisingly, for LEO, the same was seen for concentrations as low as 1× MIC and 2× MIC, with relative surface cover of 0.043 ± 0.005 and almost zero, respectively. Interestingly, even for LEO at 0.5× MIC, the relative cover of the glass surface was affected, with a relative surface cover of 0.38 ± 0.15 . Additionally, the lowest concentration of LEW was more effective compared with LEF, with relative surface cover for LEW at 2× MIC of 0.51 ± 0.09 compared with LEF at 2× MIC of 0.88 ± 0.01 .

Please insert Figure 1. (colour)

Please insert Figure 2.

3.4. Modulation of Campylobacter adhesion by Lavandula preparations and pure compounds

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

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

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

Please insert Figure 3.

3.5. Modulation of *Campylobacter* motility by *Lavandula* preparations and pure compounds

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

Please insert Figure 4.

3.6. Effects of LEO on the transcriptome of *C. jejuni* NCTC 11168

It is important to evaluate the mechanisms and the potential targets behind these reductions in biofilm-related contamination, and for eradication of biofilm-related infections. According to the effects on the *C. jejuni* biofilm degradation and on motility and adhesion in the early phase of biofilm formation, we selected LEO to extend this study to the molecular mechanisms behind these actions against *C. jejuni* NCTC 11168. For the biofilms, LEO at its MIC was already sufficient, while to effectively reduce *C. jejuni* motility and adhesion to polystyrene, LEO was effective at subinhibitory concentrations (i.e., $0.25 \times \text{MIC}$). Thus, the mechanism of these LEO actions against *C. jejuni* were evaluated with the major focus on the genes modulated and the cell processes they are involved in.

Campylobacter jejuni NCTC 11168 in exponential growth phase was treated with LEO for 30 min, and with a DMSO control to eliminate any influence of the solvent used for LEO.

The transcriptomic analysis was for the differentially expressed genes in comparison to control cultures ($P \leq 0.05$), with a view to predicting their generic functions, and of greater interest, also their known functions. Strain-specific RNA-seq was analysed using Ion Torrent technology. The reads were mapped to the annotated *Campylobacter jejuni* subsp. *jejuni* reference genome, with the data available at NCBI, under the BioProject accession number PRJNA57587.

3.6.1. Differential gene expression for *C. jejuni* NCTC 11168 treated with LEO

Detailed analysis showed that 326 genes were differentially expressed in *C. jejuni* NCTC 11168 when treated with LEO, with normalisation to the DMSO control ($\log_2FC \geq 1$; $P \leq 0.05$). Among these, 138 genes were up-regulated and 188 genes were down-regulated (Supplementary Table 1).

The LEO treatment up-regulated 42 genes that are involved in synthesis of ribosomal and export proteins in *C. jejuni* (Supplementary Table 1, Supplementary Figure 2). This led to global changes in gene expression of various pathways, including the TCA cycle and those of pyruvate metabolism, nicotinate and nicotinamide metabolism, folate biosynthesis, aromatic amino acid biosynthesis, and terpenoid and porphyrin biosynthesis (Supplementary Figure 2). The genes in the main efflux system of *C. jejuni* involved in β -lactam resistance were also up-regulated (i.e. *cmeA*, *cmeB*, *cmeC*; Supplementary Figure 2).

Of the 188 down-regulated genes, 73 are involved in synthesis of transmembrane proteins and are important for transport of different proteins and ions. Many genes involved in the iron uptake system were also down-regulated, including the ABC transporters and TonB box (i.e., *chuA*, *chuB*, *tonB3*, *tonB2*, *exbB1*, *exbB2*, *Cj0178*, *ceuC*; Supplementary Table 1, Supplementary Figure 2). Moreover, four genes involved in stress defence were down-regulated (i.e., *cstA*, *hrcA*, *dcuA*, *ppk*; Supplementary Table 1).

It is worth noting that 24 of the differentially expressed genes are involved in *C. jejuni* motility and biofilm formation (Table 4). Most of the genes that are involved in flagellar assembly of *C. jejuni* were down-regulated, and 18 of them had fold-change lower than -1 ($\log_2FC$) (Figure 5).

Please insert Table 4.

3.6.2. Gene ontology enrichment analysis

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

Please insert Table 5.

4. Discussion

In the present study *Lavandula* preparations (i.e., LEO, LEF, LEW) were used, with aim to modulate the *Campylobacter* properties that are responsible for the initial phase of the multifactorial event of biofilm formation on abiotic polystyrene and glass surfaces. In doing so, we used a new approach of culturing and study of biofilms formed at the air/liquid interface.

Also, we investigated LEO, LEF and LEW as effective control strategies for biofilm degradation and removal under conditions that do not provide selection pressure for further development of antibiotic resistance of *Campylobacter* spp.

The occurrence of persistent and resistant *C. jejuni* cells in their biofilm form is associated worldwide with severe infections and gastrointestinal diseases in humans (4). The attachment of biofilms to different abiotic surfaces is responsible for major problems in the food industry, as the reversible binding turns into irreversible bonding and biofilm formation, with possible further dispersion and contamination of the food matrix (9, 39). Therefore, alternative strategies to either remove pre-formed biofilms or inhibit biofilm formation based on alternative antimicrobial agents of natural origin represents an innovative control approach, as we have described here.

For this purpose, dried flowers of *Lavandula angustifolia* were used to produce LEO, the essential oil, LEF, the ethanol extract of flowers prior to distillation, and LEW, the ethanol extract of *Lavandula* post-distillation waste material. Interestingly, LEF and LEW had similar chemical compositions. As expected, their main constituents were phenols, with the detection of rosmarinic acid, the flavone apigenin-7-O-glucoside, and a number of phenolic acid glycosides. Torras-Claveria et al. (24) reported a comparable composition for *Lavandula* × *intermedia* waste material, which in their case contained glucosides of coumaric, ferulic and caffeic acids, in addition to rosmarinic acid, chlorogenic acid and a number of flavone glycosides. As the identification in the present study was derived from mass spectrometry and UV data, the sugar moieties were designated only as hexosides, although glucosidation is most likely to occur, according to other reports for *Lavandula* spp. (24, 38). The complete chemical profile was determined here for the essential oil, LEO, with 1,8-cineol, linalool and linalyl acetate as the major compounds. This confirms the origin of LEO as the genus *Lavandula* (17), although the high levels of 1,8-cineol and linalool oxides indicate a high degree of oxidation

for LEO, most likely due to extended storage after purchase of the plant material. On this basis, the chemical composition of LEO was only partially comparable with extensive previous studies in which linalool and linalyl acetate have been seen as the main components (12, 40–42). According to the chemical compositions of LEO, LEF and LEW, the following seven main compounds were selected for further testing: 1,8-cineol, linalool, linalyl acetate, camphor, (-)-borneol, p-coumaric acid and *trans*-ferulic acid. The antibacterial effects of these pure compounds were then compared with the antibacterial effects of the *Lavandula* preparations (LEO, LEF and LEW) against *C. jejuni*.

Linalyl acetate had the strongest effect, while (-)-borneol, camphor, 1,8-cineol had comparable antibacterial effects to LEO. Similarly, Blažeković et al. (12) showed that essential oils of *L. × intermedia* Budrovka and *L. angustifolia*, as well as linalool and linalyl acetate, have antibacterial effects against numerous Gram-positive and Gram-negative bacteria. For comparison, the *Lavandula* preparations and pure compounds used in the present study had stronger antibacterial effects against the Gram-negative *C. jejuni*. However, according to the higher levels of oxidised compounds in LEO compared with the essential oils from *L. × intermedia* Budrovka and *L. angustifolia* tested by Blažeković et al. (12), a stronger antimicrobial effect might have been expected. At the same time, antibacterial effects of plant preparations can depend strongly on the type and storage of the plant material, the method of preparation of the plant material, the type of microorganisms used, the inoculum volumes, and the culture medium used, along with the pH, temperature and incubation time (43, 44).

The moderate but comparable antibacterial effects of the ethanol extract LEF and the waste material LEW, as well as for p-coumaric acid and *trans*-ferulic acid, showed that LEW contains a diverse pool of bioactive compounds with antimicrobial properties. This is consistent with previous studies on post-distillation thyme waste, pinot noir grape skins and seeds, and post-distillation juniper fruit waste, where the advantages of agricultural waste material as

promising antimicrobial agents with potential industrial applications were demonstrated (35, 45).

The *Lavandula* preparations in this study were effective for degradation and removal of the *C. jejuni* biofilms. LEO had the strongest effects on these mature *C. jejuni* biofilms. Similarly, Dănilă et al. (46) reported that lavender essential oil is an effective anti-biofilm agent against *Staphylococcus epidermidis* clinical strains. It has also already been shown that various essential oils are good anti-biofilm agents on various surfaces (47, 48). As showed here, LEO is rich in secondary metabolites, which are probably responsible for the degradation of the extracellular polymer matrix of the biofilms (49). Part of the *C. jejuni* polymers in the extracellular matrix can also form a capsule (50). Indeed, this study has shown that the two major proteins that are involved in the formation of the capsule in *C. jejuni* (i.e., KpsM, KpsS) were down-regulated. High levels of numerous secondary metabolites might also modulate the synthesis and activity of enzymes, to cause capsule degradation, while disturbing the uptake of nutrients. These can also alter the pH gradient of the cell cytoplasm, and thus cause cell death (51).

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

The *Lavandula* preparations were successful for the degradation of these mature *C. jejuni* biofilms, and were further used to determine whether they can be used to prevent biofilm formation. The first phase of biofilm formation is the attachment of the cells to the surface (39). Here, these *Lavandula* preparations reduced the adhesion of *C. jejuni* to polystyrene after 4, 8

and 24 h of incubation. These reductions in the adhesion were by $\geq 1 \log_{10}$ CFU/mL, which satisfies the recommendations proposed by the European Food Safety Authority (3). These results are also comparable with the effects of lavandin essential oil and ethanol extracts from *Urtica dioica* on the adhesion of *C. jejuni* (21).

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

Further, the effects of the *Lavandula* preparations and pure compounds were monitored for *C. jejuni* motility, which is important for both colonization of surfaces and survival in the environment through biofilm formation (10, 52). All three of the *Lavandula* preparations and all pure compounds indeed reduced the motility of *C. jejuni*. The *Lavandula* preparations all had similar effects on *C. jejuni* motility. Linalyl acetate and (-)-borneol had similar effects to LEO, while LEO had stronger anti-motility effects than linalool and camphor. This also suggests that the combination of the pure compounds in LEO is important for these anti-motility effects. For LEF and LEW, these both had stronger anti-motility effects than p-

coumaric and *trans*-ferulic acid, which suggests that these two compounds only contribute to the higher anti-motility effects of the combined components of LEF and LEW.

The RNAseq data provided new insights into the influence of LEO on gene expression in *C. jejuni*. It can be noted that many ribosomal genes were up-regulated, which could be the result of a global change in the transcriptome that caused transcriptional reprogramming. Transcriptional reprogramming might indicate that the bacteria are under intense stress (53). When bacteria are under stress, the stringent response is activated, which affects the expression of ribosomal genes that are necessary for stress resistance (54). Up-regulation of the CmeABC efflux system confirms that *C. jejuni* was under severe stress while exposed to LEO. Nevertheless, it can be seen that this transcriptional reprogramming affected the gene expression of many pathways, including the TCA cycle, and pyruvate metabolism, nicotinate and nicotinamide metabolism, folate biosynthesis, aromatic amino acid biosynthesis, and terpenoid and porphyrin biosynthesis (Supplementary Figure 2). Many transmembrane proteins were down-regulated, including proteins involved in the iron-uptake system. Askoura et al. (55) have shown that iron acquisition genes are down-regulated during acid stress, so it can be hypothesised that exposure of *C. jejuni* to LEO also led to stress responses in these bacteria. It is important to note that the iron-uptake system is involved in the colonisation, biofilm formation and pathogenicity of this pathogen (56, 57), so down-regulation of this pathway might lead to reduction of these processes. Gene *cstA*, which encodes a carbon starvation protein, was also down-regulated, and this gene is known to be involved in *C. jejuni* motility and autoagglutination (58). Three other down-regulated genes are also important for the stress response of *C. jejuni*: *ppk*, *dcuA* and *hrcA* (59, 60). Down-regulation of these genes might also contribute to biofilm reduction.

The analysis here of the transcriptome of *C. jejuni* NCTC 11168 following treatment with LEO showed that most of the genes involved in flagellar assembly and modification of

flagella were down-regulated (Figure 5), which helps to explain these observations at the physiological level. Some of the down-regulated genes (e.g., *folK*, *Cj0719c*, *maf4*, *Cj1467*) are also part of a network of genes that are important for flagellar synthesis. It is known that *C. jejuni* requires functional flagella for initial attachment and further biofilm formation (61), so it is necessary to modulate genes involved in flagellar assembly to reduce biofilm formation. Motility in *Campylobacter* is regulated by a chemotactic signalling system that allows the organisms to follow favourable chemical gradients in environments (62). It is interesting to note that *Cj0262c* was up-regulated, which is the gene that encodes the transducer-like protein and is involved in chemotaxis of *C. jejuni* (63). Furthermore, its closely related Tlp1-3 transducer-like proteins that are also involved in the *C. jejuni* chemotaxis sensory system, motility, and colonisation (64) were not differentially expressed. From the results obtained at the molecular and physiological level, it can be concluded that LEO did not influence chemotactic behaviour and consequently chemotactic motility of *C. jejuni*.

Furthermore, LEO also reduced the expression of genes that are important for formation of proteins and lipopolysaccharides of the outer bacterial membrane (i.e., *kpsM*, *kpsS*, *bamD*, *waaC*, *lgt*), and are involved in the initial attachment of these cells to abiotic and biotic surfaces (65). In contrast, up-regulation was seen for four of the main genes that are involved in the synthesis of outer membrane proteins and are important for cell adhesion and biofilm formation (i.e., *peb2*, *peb3*, *Omp50*, *porA*) (34). Overall these data indicate that down-regulation of the flagellar genes, together with lower expression of genes involved in the iron-uptake system and in stress defence, and thus reduced cell adhesion and motility, are the most interesting targets to combat biofilm formation. These data confirm that bacteria are under severe stress while exposed to such plant materials. These insights into complete transcriptome analysis opens up new aspects and possibilities for research of new targets that should help in the control of this pathogen.

The results of the present study show the potential use of this *Lavandula* ethanol extract of post-distillation waste material, LEW, against pre-formed mature biofilms, as well as against the adhesion and motility mechanisms involved in biofilm formation. These results offer new solutions for the use of such plant waste materials that still contain high amounts of bioactive molecules. The re-use of LEW might thus provide economic benefits in different industrial fields (e.g., pharmaceuticals, food industry) and might also help solve one of the global problems here: waste disposal (66, 67).

5. Conclusion

Campylobacter jejuni is shown to be sensitive to *Lavandula* preparations and selected pure compounds. *Lavandula* preparations are shown to be particularly effective in the fight against one of the world's most common food-borne pathogens, *C. jejuni*. The *Lavandula* preparations have relatively potent anti-biofilm properties. Moreover, both physiological and molecular approaches confirmed modulation of the first steps in biofilm formation (i.e., adhesion, motility). Also, it has been shown that the post-distillation waste material of *Lavandula* flowers has particular anti-biofilm effects against *C. jejuni*, which suggests that such waste material can be re-used for industrial purposes. Therefore, further efforts can now be directed towards such innovative approaches for alternative strategies and novel targets against bacterial biofilms, to find and develop new and effective agents with anti-biofilm activities.

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Author Contributions

DR conceived and conducted experiments. FB and UK analysed data. AK, ID and SSM designed and coordinated research. DR wrote manuscript. All authors have read and approved the manuscript.

Competing Interests Statement

The authors declare that they have no conflict of interest. The funders had no role in the design of the study; in the collection, analysis or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Table 1. Identification of the main common flavonoids in the *Lavandula* ethanolic extract, LEF, and in the ethanolic extract of waste material from hydrodistillation, LEW.

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

Table 2. Identification of the main components of the *Lavandula* essential oil, LEO.

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

^a Linear Retention Index relative to n-alkanes on HP-5-MS column; ^b Compounds identified by mass spectral libraries [(30–32); laboratory database] and RI comparison (30); ^c Quantification by normalisation (area percent method) without considering calibration factors.

Table 3. Minimal inhibitory concentrations against *C. jejuni* NCTC 11168 for the *Lavandula* preparations (LEO, LEF, LEW) and pure compounds.

Treatment	Minimal inhibitory concentration (mg/L)
<i>Lavandula</i> essential oil (LEO)	0.25 ±0.06
<i>Lavandula</i> ethanol extract (LEF)	1.00 ±0.25
<i>Lavandula</i> waste material (LEW)	1.00 ±0.25
Linalool	0.25 ±0.06
Linalyl acetate	0.20 ±0.05
Camphor	0.50 ±0.12
(-)-Borneol	0.25 ±0.06
1,8-Cineol	0.50 ±0.12
p-Coumaric acid	1.00 ±0.25
<i>trans</i> -Ferulic acid	1.00 ±0.25

Table 4. Differentially expressed genes (false discovery rate, $P \leq 0.05$) that are important for biofilm formation of *C. jejuni* NCTC 11168.

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

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

Category	Code	Term	Differentially expressed ^a	FDR p-value	Regulation
Biological process	GO:0040011	Locomotion	5/32	0.054026	Down
	GO:0071973; GO:0097588; GO:0001539; GO:0048870; GO:0006928	Cilium or flagellum-dependent cell motility	3/23	0.012148	Down
	GO:0065007	Biological regulation	19/83	0.062751	Down
	GO:0044271	Cellular nitrogen compound biosynthetic process	65/183	1.62E-10	Up
	GO:0006430	Transfer RNA aminoacylation	2/22	0.006264	Up
	GO:0006807	Nitrogen compound metabolic process	87/433	2.16E-08	Up
Molecular function	GO:0046873	Metal ion transmembrane transporter activity	2/16	0.061105	Down
	GO:0015103	Organic anion transmembrane transporter activity	4/10	0.085316	Down
	GO:0019843	rRNA binding	27/39	1.51E-15	Up
	GO:0003735	Structural constituent of ribosome	36/52	1.00E-15	Up
	GO:0005198	Structural molecule activity	37/59	1.14E-14	Up
	GO:0097159; GO:1901363	Organic cyclic compound binding	93/436	0.006437	Up
	GO:0003676	Nucleic acid binding	54/175	4.16E-05	Up
	GO:0000049	tRNA binding	6/18	0.009744	Up
	GO:0005488	Binding	113/588	0.048065	Up
	GO:0140101	Catalytic activity, acting on a tRNA	2/43	0.048065	Up
	GO:0004812; GO:0016875	Aminoacyl-tRNA ligase activity	2/22	0.005088	Up
	GO:0140098	Catalytic activity, acting on RNA	9/64	0.069198	Up

^a, number of genes differentially expressed (absolute $\log_2$ fold-change ≥ 1 ; false discovery rate [FDR] $P \leq 0.05$) relative to total number of genes in enriched category

Figure legends

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

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

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

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

Figure 5. Pathway map of the *C. jejuni* NCTC 11168 flagellar assembly (cje02040). This assembly showed up (red) and down (green) regulated genes in *C. jejuni* after exposure to the essential oil LEO at 0.25× MIC for 30 min (see Table 3). Those genes are essential for locomotion and flagellum-dependent motility, which represent two features necessary for biofilm formation of *C. jejuni*. KEGG map of differentially expressed genes involved in flagellar assembly was constructed using ‘pathview’ (68) and the KEGG genome database (<https://www.genome.jp/kegg/pathway.html>).

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.