Food Control 146 (2023) 109513

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Volatilomes reveal specific signatures for contamination of leafy vegetables with *Escherichia coli* O157:H7

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ARTICLE INFO

Keywords:
E. coli O157:H7
foodborne pathogens
headspace
leafy vegetables
rocket (*Eruca sativa* L.)
spinach (*Spinacia oleracea* L.)
volatilome
(E)-hex-2-enal
indole

ABSTRACT

Transmission of foodborne pathogens by plants, especially shiga toxin-producing *Escherichia coli*, has become a public health concern for ready-to-eat products. Due to the patchy distribution of foodborne pathogens on commodities, random sampling before processing and following packaging cannot reliably exclude presence of pathogenic microbes. Robust, cost-effective, and reliable methods for monitoring pathogenic *E. coli* on fresh produce are therefore urgently needed. We investigated whether volatile organic compounds are suitable for detection of *E. coli* O157:H7 contamination of leafy vegetables. Using solid-phase microextraction coupled with gas chromatography-mass spectrometry, we analyzed volatilomes of spinach (*Spinacia oleracea* L.) and rocket (*Eruca sativa* L.) leaf lysates, and of a common culture medium (lysogeny broth), in the presence and absence of *E. coli* O157:H7. Volatile profiles varied with the nutrient medium. We found higher proportions of indole, phenylmethanol, 2-methoxyphenol, ethanol, propan-1-ol, decan-1-ol, tridecan-1-ol, nonan-2-one and tridecan-2-one in headspace from inoculated compared with non-inoculated samples. This demonstrates that volatile organic compounds are suitable for detecting contamination of leafy vegetables with *E. coli* O157:H7. In future work we will focus on adapting the volatile assay for screening for *E. coli* O157:H7 contamination under different conditions, including intact and damaged baby leaves, leaf packages, or leaf batches, and on increasing its sensitivity.

1. Introduction

Shigatoxigenic *Escherichia coli* O157:H7 (STEC) is an important foodborne pathogen, causing foodborne illness at a very low infectious dose of only 10–100 cells (EFSA, 2013). Although the primary reservoir appears to be ruminants (WHO, 2018), STEC uses plants as vectors for transmission (Barak & Schroeder, 2012; Holden et al., 2009), and is hence spread in the food chain via edible plants (Holden et al., 2015; Slayton et al., 2013; Söderström et al., 2008). Between 1999 and 2019, around 44 outbreaks of microbial infections related to consumption of fresh produce were recorded in the European Union, with 64% of the cases linked to consumption of contaminated vegetables and salads (Aiyedun et al., 2020). Contamination of leafy vegetables with enteric pathogens can occur in the entire value chain, and different sources of contamination and routes of transmission have been identified (Beuchat, 2002; Castro-Ibáñez et al., 2017; Gil et al., 2015; Julien-Javaux et al., 2019; Mogren et al., 2018).

Although of enteric origin, foodborne pathogens survive epiphytically on plant surfaces and endophytically in plant tissue (Erickson, 2012; Hartmann et al., 2017; Hirano & Upper, 1983; Merget et al., 2019; Mulaosmanovic et al., 2021; Wright et al., 2017). In general, the capacity of human pathogens to colonize, grow, and internalize plant tissue depends on numerous biotic and abiotic factors, such as *(i)* plant physiology (e.g., leaf age, morphology) (Brandl & Amundson, 2008); *(ii)* interactions with resident microorganisms, which may either promote or inhibit establishment of enteric pathogens (Cooley et al., 2006); *(iii)* leaf tissue damage (Mulaosmanovic et al., 2021); (*iv*) temperature (Merget et al., 2019), and (*v*) ability of the pathogen to overcome plant defenses (Schikora et al., 2008; Spoel & Dong, 2012). As endophytes, enteric pathogens may have better access to plant nutrients and are less

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https://doi.org/10.1016/j.foodcont.2022.109513
Received 20 July 2022; Received in revised form 1 November 2022; Accepted 13 November 2022
Available online 14 November 2022

exposed to environmental fluctuations in the phylloplane (Quilliam et al., 2012). Importantly, internalized foodborne pathogens are protected against rinse water and sanitizers (Lanciotti et al., 2003; Shirron et al., 2009).

Consumption of spinach (*Spinaca oleracea* L.) and rocket (*Eruca sativa* L.) baby leaves and of other leafy vegetables is increasing (Betts, 2014). Consumer demand for leafy vegetables has resulted in an increase in global production of spinach from 4.1 million tons in 1990 to 27.3 million tons in 2018 (FAO/WHO, 2008). Since leafy greens are eaten raw and without any decontamination step between farm and fork, maintaining product safety during production, harvesting, and post-harvest handling is essential. This is challenging due to multiple steps along the leafy vegetables value chain where contamination can occur (Gil et al., 2015; López-Gálvez et al., 2009; Mogren et al., 2018), and due to cumulative damage to leaf tissue from field to bag (Mulaosmanovic et al., 2021). Classical methods for bacterial diagnostics are well established and reliable, but have certain limitations, e.g., they are destructive, costly, and require expertise (Ratiu et al., 2017). Development of robust, non-destructive, fast, efficient, timely, sensitive, and cost-effective analytical techniques that continuously monitor food safety is therefore of particular interest.

Volatile organic compounds (VOCs) released from plants can signal food aroma and quality (Lytou et al., 2019; Mastrandrea et al., 2017; Raffo et al., 2021), but may also include compounds suitable for monitoring food safety (Chen et al., 2017; Fang et al., 2021). Solid phase microextraction (SPME), combined with gas chromatography-mass spectrometry (GC-MS) is a frequently used method for analysis of VOCs from food and microorganisms (Spietelun et al., 2013; Zhang & Li, 2010). The ensemble of VOCs emitted from an organism or food matrix, including VOCs emitted from microbial metabolic activity, is termed the volatilome (Casaburi et al., 2015). Volatilomics, within the field of metabolomics, has already been employed for detection, characterization, and quantification of volatile metabolites in food science (Bonah et al., 2019, 2020; Castro-Puyana & Herrero, 2013; Chen et al., 2016, 2017; Fang et al., 2021; Fitzgerald et al., 2020; Kai, 2020; Lytou et al., 2019; Mastrandrea et al., 2017; Raffo et al., 2018, 2020, 2021; Ramírez-Guízar et al., 2017; Ratiu et al., 2020; Spadafora et al., 2020; Yu et al., 2015 and references therein). It is used primarily in assessment of quality and food safety. In general, VOC profiles are affected by production site, season, storage conditions, or different nutritional regimes, and hence different batches of the same product will exhibit distinctly different profiles (Lytou et al., 2019; Santos & Oliveira, 2017).

Bacteria produce blends of mainly alkenes, alcohols, ketones, and terpenes (Farré-Armengol et al., 2016), released as primary metabolites during growth, and as secondary metabolites that serve as signaling molecules and protection against antagonists (Kai et al., 2009; Schulz & Dickschat, 2007). Further, some microbial VOCs are specific to microbial species, growth substrate, and environmental factors (Minerdi et al., 2009; Zhang et al., 2015). SPME has been widely used to monitor the chemical signature of bacterial metabolites from e.g., *Escherichia coli* (Chen et al., 2017; Fang et al., 2021; Fitzgerald et al., 2020; Hamilton-Kemp et al., 2005; Hossain et al., 2013), *Listeria monocytogenes* (Chen et al., 2017; Tait et al., 2014; Yu et al., 2015), *Salmonella typhimurium* (Fang et al., 2021), *Staphylococcus aureus* (Chen et al., 2017; Fitzgerald et al., 2020), and *Pseudomonas aeruginosa* (Fitzgerald et al., 2020). Some VOCs emitted by bacteria have been shown to be species-specific and are therefore being considered as biomarkers for bacterial detection (Chen et al., 2017; Fang et al., 2021; Kai et al., 2009; Schulz & Dickschat, 2007; Sohrabi et al., 2014). Indole has been indicated as a diagnostic marker for identification of different members of the Enterobacteriaceae, including *E. coli*, and seems to act as an extracellular signal of biofilm formation (Di Martino et al., 2003; Wang et al., 2001). Bacterial volatiles may differ in occurrence and abundance depending on whether the bacteria are incubated on culture medium or food matrices (Fang et al., 2021). To fully identify bacterial volatiles associated with food, food substrates rather than growth medium should be used for fingerprinting studies of bacterial volatiles.

The aim of this study was to examine the possibility to use headspace analysis as a simple and efficient method to detect *E. coli* O157:H7 contamination in leaf lysates. Changes in volatilomes in the presence of *E. coli* O157:H7 were studied *in vitro* under gnotobiotic conditions, using sterile-filtered leaf lysates of spinach and rocket, with microbiological nutrient broth as a control.

2. Material and methods

2.1. Leaf lysate extractions

Six batches of washed and packaged ready-to-eat baby leaves of spinach (*Spinacia oleracea* L.) and rocket (*Eruca sativa* L.), obtained from commercial suppliers, were used for the experiments. For lysate extraction, 25 g of leaves were rapidly macerated on ice using a mortar and pestle. The macerated samples were placed in filter bags (Separator 400, Blender Bags, 180 mm × 300 mm x 65 μm, Grade, England) together with 50 mL of 0.85% NaCl (VWR, Belgium). The mixture was homogenized in a smasher (AES Laboratoire, Chemunex, France) for 2 min in fast mode. The lysate obtained was centrifuged (Blackman Coulter Avanti J-20, USA, 5000×*g*, 10 min, 4 °C) and the supernatant was immediately filter-sterilized (pore size: 0.22 μm, Acrodisc Syringe Filters, Pall Corporation, USA). Finally, 10-mL aliquots of lysate were transferred to triplicate sterile centrifugation tubes (Eppendorf, 13 mL).

2.2. Preparation of inoculated and non-inoculated samples

Escherichia coli O157:H7 (strain register no. E81186, obtained from the Swedish Institute for Communicable Disease Control (SMI), Stockholm, Sweden), which is negative for verotoxin-1 and verotoxin-2, but positive for the *eae*-gene, was used for inoculation. This strain is tagged with green fluorescence protein (GFP), which is expressed when the strain is grown in the presence of 0.1% L-arabinose, and is resistant to ampicillin . For the inoculation experiments, *E. coli* O157:H7 from glycerol stocks (stored at −80 °C) was grown overnight at 37 °C in lysogenic broth (LB) (Sigma-Aldrich St. Louis, USA) supplemented with 100 μg/mL ampicillin (Sigma-Aldrich Steinheim, Belgium), on a rotary shaker (Minispin rotary shaker; VWR International AB, Stockholm, Sweden) at 180 rpm. The inoculated broth was centrifuged (3000×*g* at 4 °C for 15 min), and the cells were washed with 0.85% NaCl and resuspended with 0.085% NaCl. Cell density was adjusted by adding NaCl (0.085%) to an optical density at 620 nm (OD_{620}) of 1.0, corresponding to log 9.7 CFU mL^{-1} (Expert 96™ spectrophotometer; AsysHiTech, Eugendorf, Austria) (El-Mogy and Alsanius, 2012). The final concentration of the inoculum suspension was set to log 7.7 CFU mL^{-1} of *E. coli* O157:H7 in 0.085% NaCl. Aliquots of 100 μL of the inoculum were transferred to two of three sterile tubes containing 10 mL spinach or rocket lysate (see above) or 10 mL of LB. The third non-inoculated tube of lysate or LB served as a control. The inoculum strength in each tube was verified through serial dilution (10^{-1}-10^{-4}) and plating on LB supplemented with ampicillin (100 μg/mL agar) and L-arabinose (1.0 g L^{-1} agar, Merck KGaA, Darmstadt, Germany) and solidified with 15 g/L of Bacto agar (DIFCO, Becton, Dickinson and Company, Sparks, USA). All tubes were sealed with parafilm and incubated at 37 °C for 72 h.

2.3. E. coli O157:H7 proliferation

The propagation of *E. coli* O157:H7 was followed on three nutrient media (n = 6). Duplicate tubes of LB, spinach lysate, and rocket lysate were all inoculated with log 4 CFU mL^{-1} cells mL^{-1} and incubated at 37 °C for 72 h. Samples were taken after 24, 48, and 72 h, diluted, and plated on LB solidified with 15 g/L Bacto agar and supplemented with 100 μg/mL ampicillin and 0.1% L-arabinose.

2.4. SPME headspace collection and GC-MS analysis

Headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS) was used to study volatile emissions from sterile or inoculated samples of LB, spinach lysate, or rocket lysate after 24, 48, and 72 h of incubation. For HS-SPME, a divinylbenzene (DVB)/carboxen (CAR) on polydimethylsiloxane (PDMS) coating stable fiber (Supelco, 50/30 μM), preconditioned according to the manufacturer's instructions, was used. The HS-SPME fiber was exposed to the headspace of each tube for 1 h by manually penetrating the parafilm covering the tubes. After collection, volatiles were analyzed by GC-MS (7890 GC and 5972 MS, Agilent Technologies Inc., Santa Clara, CA, USA) operated in splitless mode (30 s, injector temperature 225 °C). The GC was equipped with a fused silica capillary column (60 m by 0,25 mm; df = 0.25 μm), DB-Wax (J&W Scientific, Folsom, CA, USA). The GC oven temperature was programmed from 30 °C (3-min hold) and 8 °C/min to 225 °C (10-min hold). Helium was used as the mobile phase, at an average linear flow of 35 cm/s. The MS was operated in electron impact ionization mode at 70 eV and scan range over 29–400 m/z. The temperature of the ion source and connecting parts was set to 230 °C and 150 °C, respectively. Compounds were identified according to their retention time (Kovat's indices) and by comparing mass spectra against the mass spectral library of the National Institute of Standards and Technology (NIST, v 20) and authentic standards.

2.5. Statistical analysis

All statistical analyses were performed in R studio (version 1.3.959) (RStudio, 2020). Relative percentage of compounds was plotted using *ggplot* from the package *ggplot2* (Wickham et al., 2016).

To test for significant differences between *i)* overall volatile profiles collected from the different treatments (inoculated and non-inoculated LB, spinach lysate, and rocket lysate) and *ii)* overall volatile profiles from inoculated samples over time, we used permutational multivariate analysis of variance (PERMANOVA, based on Bray-Cutis distances calculated from amount of compounds, 999 permutations). We used pairwise PERMANOVA with Bonferroni correction for multiple testing, with the functions *adonis* and *pairwise.factorfit* from the *vegan* package (Oksanen et al., 2013). To identify groups of compounds present in higher relative percentages in one treatment or substrate compared with the others, we applied the multi-level pattern analyses *multipatt* function from the *indicspecies* package (De Cáceres et al., 2010). To visualize the overall data collected, we applied principal component analysis (PCA) from the package *ade4*, using the function *fviz_pca_ind* (Oksanen et al., 2015). We examined relationships between volatile components using partial distance-based redundancy analysis (dbRDA), with the *capscale* function of the *vegan* package (Oksanen et al., 2015). Compounds contributing to separation based on medium were omitted. Model verification was based on permutation tests for the partial dbRDA model, CAP axes and explanatory variables, using the *anova.cca* function from the *vegan* package (Oksanen et al., 2015).

3. Results and discussion

3.1. Proliferation of E. coli O157:H7 on different nutritional media

The effect of the three nutritional media (LB, spinach lysate, rocket lysate) on growth of *E. coli* O157:H7 is shown in Fig. 1. Lower growth rate and faster decline were observed when *E. coli* O157:H7 was propagated on rocket lysate, compared with LB and spinach lysate. In LB and spinach lysate, cell numbers increased significantly, from 10^4 to 10^7 CFU/mL, within the first 24 h of observation and the population then remained quite stable until 72 h. In rocket lysate, there was an increase in cell numbers from 10^4 to 10^6 CFU/mL during the first 24 h, after which viable counts decreased over the remaining observation time. This decrease was corroborated by considerable deviation between replicates, mainly at 72 h. Moreover, we observed differences in the size of *E. coli* O157:H7 colonies between the three solidified nutritional media, with rocket lysate-based medium exhibiting reduced colony size (Fig. 1). Rocket is considered a valuable source of nitrates and antioxidants, as it contains phenolic compounds and their degradation products (Villatoro-Pulido et al., 2012). Additionally, in-vitro inhibitory action has been reported for rocket leaf water extract against *E. coli* and *Staphylococcus aureus* (Qaddoumi & El-Banna, 2019) and *Clostridium perfringens* (Martínez et al., 2019). It is unclear whether the reduced number of viable counts on solidified rocket lysates seen in our study was due to a switch to viable, but not culturable, cells or persistter cells,

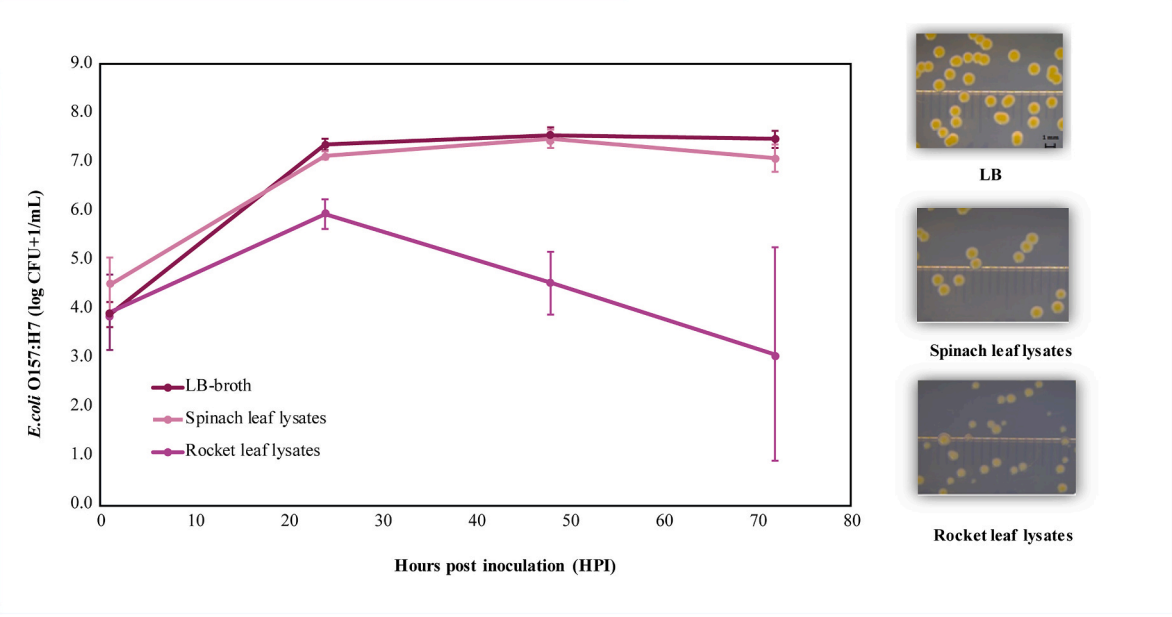

Fig. 1. Growth curves (log CFU+1/mL) and colony size of *E. coli* O157:H7 in solidified LB, spinach lysate, and rocket lysate. Each point represents the mean bacterial concentration in six replicate cultures, bars indicate the standard deviation. Colony size was determined microscopically (LEICA M165FC).

or whether it was a consequence of the compound composition in rocket lysate medium. It is intriguing to speculate about interactions between *E. coli* and the green leaf volatile (E)-hex-2-enal, a compound with known antimicrobial properties (Hatanaka et al., 1987; Patrignani et al., 2008), found in rocket lysate. According to Patrignani et al. (2008), (E)-hex-2-enal can induce noticeable modifications in bacteria VOCs production and on cell membranes. How it exerts these antimicrobial properties is not yet clear, but it has been reported that the compound can permeate bacterial membranes and, once inside the cell, react with biologically important structures (Kubo & Fujita, 2001).

3.2. Volatilomes of sterile and E. coli O157:H7 inoculated samples

We investigated the occurrence and relative abundance of VOCs emitted from the three different nutritional media, non-inoculated and inoculated, under the same experimental conditions (n = 6). By comparing volatilomes, it was possible to differentiate between nutritional media and also between samples with and without *E. coli* O157:H7. A total of 46 compounds were found in the headspace collections from sterile or inoculated samples after 24, 48, and 72 h (Fig. 2, Supplementary Table S1). The overall volatile profiles differed significantly between the three nutritional media used and between non-inoculated and inoculated samples, both overall (PERMANOVA on all percentage composition data, P = 0.001) and in pairwise comparisons (pairwise PERMANOVA with *Bonferroni* correction, P = 0.001). A significant effect of the nutritional media was confirmed by the PCA plots, where principal component 1 (PC1) discriminated according to the type of medium (14.2%), while PC2 discriminated between non-inoculated and inoculated samples (13%) (Fig. 3A and B).

The volatiles emitted from the three different non-inoculated nutritional media also differed. In the absence of *E. coli* O157:H7, LB headspace comprised of only a few compounds, dominated by benzaldehyde, undecan-2-one, phenol, butan-1-ol, 2,5-dimethyl pyrazine, and two unidentified compounds (Fig. 2, Supplementary Table S1). The volatile profiles emitted from the non-inoculated leaf lysate were rich in green leaf alcohols and aldehydes, but spinach lysate differed from rocket lysate in terms of VOCs produced. Spinach lysate had significantly higher proportions of phenol, pentan-1-ol, hexen-1-ol, and 1-methyl-4-propan-2-yl benzene, while rocket lysate contained significantly more 2-ethyl furan, (E)-hex-3-en-1-ol, and (E)-hex-2-enal (P < 0.01, Multipatt) (Fig. 2). In addition, compounds such as unknown 2, heptan-1-ol, octen-1-en-3-ol, 2-hydrobenzoaldehyde, 2-phenyacetaldehyde and (E)-4-(2,6,6-trimethylcyclohex-2-en-yl)but-3-en-2-one were only found in spinach lysate, while trisulfide dimethyl, 3-ethyl thiophene, 2-methoxy-4-propan-2-ylphenol, 1,3-ditert-butylbenzene, 1-penten-3-ol, (Z)-pent-2-en-1-ol, (Z)-hex-2-en-1-ol and (2E,4E)-hexadienal were only found in rocket lysate (Fig. 2, Supplementary Table S1).

The headspace emissions from inoculated nutritional media showed similar trends (Fig. 2). In the presence of *E. coli* O157:H7, the headspace of inoculated samples showed increased relative abundance of aromatics (e.g., of phenylmethanol), ketones (e.g., nonan-2-one, tridecan-2-one undecan-2-one) and alcohols (e.g., hexen-1-ol, (E)-hexen-3-en-1-ol, (Z)-hex-2-en-1-ol, and 3-methylbuten-1-ol in leaf lysates; ethanol, propan-1-ol, decan-1-ol, and tridecan-1-ol in LB (Figs. 2 and 4, Supplementary Table S2).

From all three inoculated nutritional media (leaf lysates and LB), one similar set of distinct VOCs, namely indole, nonan-2-one, tridecan-2-one, and undecan-2-one, was produced in the presence of *E. coli* O157:H7. The nitrogen-containing compound indole was the most representative VOC in *E. coli* O157:H7-inoculated samples (Stat = 0.69, P < 0.001, Multipatt) (Supplementary Table S2). The chemical fingerprint of bacterial metabolites emitted from different substrates inoculated with *E. coli* has been investigated previously (Chen et al., 2017; Fang et al., 2021; Fitzgerald et al., 2020; Hamilton-Kemp et al., 2005; Hossain et al., 2013). Similar compounds have been reported as VOCs produced by *E. coli* O157:H7 grown on other substrates (Chen et al., 2016; Yu et al., 2000). Chen et al. (2017) reported indole, 2-nonanone, 2-heptanone, 2-undecanone, 1-decanol, and 1-dodecanol as VOCs produced by *E. coli* O157:H7 on culture media. Maddula et al. (2009) detected 2-heptanone and 2-nonanone, while Fang et al. (2021) detected indole, 2-ethylhexanol, 1-decanol, and 2,5 dimethylpyrazine.

Several studies have already identified indole as a major compound in the presence of *E. coli* (Bunge et al., 2008; Chen et al., 2017; Fang et al., 2021; Kunze et al., 2013; Maddula et al., 2009; Thorn et al., 2011). However, in our contaminated leaf lysate the amount of indole detected varied. One aspect to be considered when exploring the characteristics of bacterial VOC profiles is that the occurrence and abundance of VOCs changes throughout the growth process of bacteria, with emission of volatiles affected by cell density and growth stage (Chen et al., 2017). During bacterial proliferation, readily available carbon sources are degraded and transformed, which in turn may alter the volatiles emitted by the bacteria and the matrix (Farré-Armengol et al., 2016). Correlations between the concentrations of major VOCs and bacterial cell counts have been reported previously (Chen et al., 2017; Mayr et al., 2003). However, the relationship between total bacteria and VOCs is not always linear (Silcock et al., 2014). The concentration of VOCs is reported to be largely unchanged until the number of bacteria reaches 10^6–10^8 CFU/mL (Chen et al., 2017), 10^7 CFU/mL (Mayr et al., 2003), or 10^7–10^8 CFU/mL (Silcock et al., 2014), which is far above the infectious dose of *E. coli* O157:H7.

Some leaf compounds (e.g., aldehydes) may have a negative influence on VOC emissions by bacteria (Patrignani et al., 2008). In the presence of *E. coli* O157:H7, headspace samples from all three nutritional media we analyzed displayed a decrease in the relative percentage of aldehydes (e.g., benzaldehyde). It has been shown that aldehydes can be used as substrate for bacterial metabolism (Kunjapur & Prather, 2015; Yu et al., 2000), and that some microorganisms can naturally convert aldehydes into their respective alcohols, and thus detoxify them (Kunjapur & Prather, 2015). It has also been shown that *E. coli* O157:H7 is able to oxidize different carbon sources, which increases its survival in different microenvironments (Franz et al., 2011).

So far, only a few studies have examined production of VOCs over time in bacterial cultures (Bunge et al., 2008; Chen et al., 2017; Kunze et al., 2013; Maddula et al., 2009). In the present study, we attempted to study production of VOCs as a function of time. However, we found that the abundance of VOCs did not differ between samplings at 24, 48, and 72 h. This may be due to the majority of volatiles being formed during secondary metabolism, when bacteria are entering the stationary phase, which was already reached by *E. coli* O157:H7 in all substrates at 24–28 h (Fig. 1).

3.3. Key compounds emitted in the presence of E.coli O157:H7 as potential biomarkers for EHEC contamination

Detection of foodborne pathogens, including *E. coli* O157:H7, is a critical step in elimination of pathogens in the food supply chain. Available detection methods include conventional culture-dependent methods, immunological assays, DNA-based methods, biosensor-based methods and, more recently, spectroscopic methods and spectral imaging techniques (Bonah et al., 2020). A major advantage of VOC assay over conventional methods for detection of foodborne pathogens is its non-destructive nature. Furthermore, analysis of key volatiles present in the headspace of leafy vegetables could allow screening for presence of pathogens in larger volumes of salad, irrespective of site (package, cooling compartment/storage, or transport). To identify volatiles that are the most reliable indicators of *E. coli* O157:H7 contamination, regardless of the medium, we performed partial dbRDA by eliminating the influence of the unknown 1, unknown 3, and 2,5-dimethyl pyrazine compounds associated with LB, the 3-ethyl thiophene associated with rocket lysate, and the 2-phenyl acetaldehyde associated with spinach lysate (Figs. 5 and 6). After minimizing the influence of medium, we found that the volatiles emitted from non-inoculated samples were

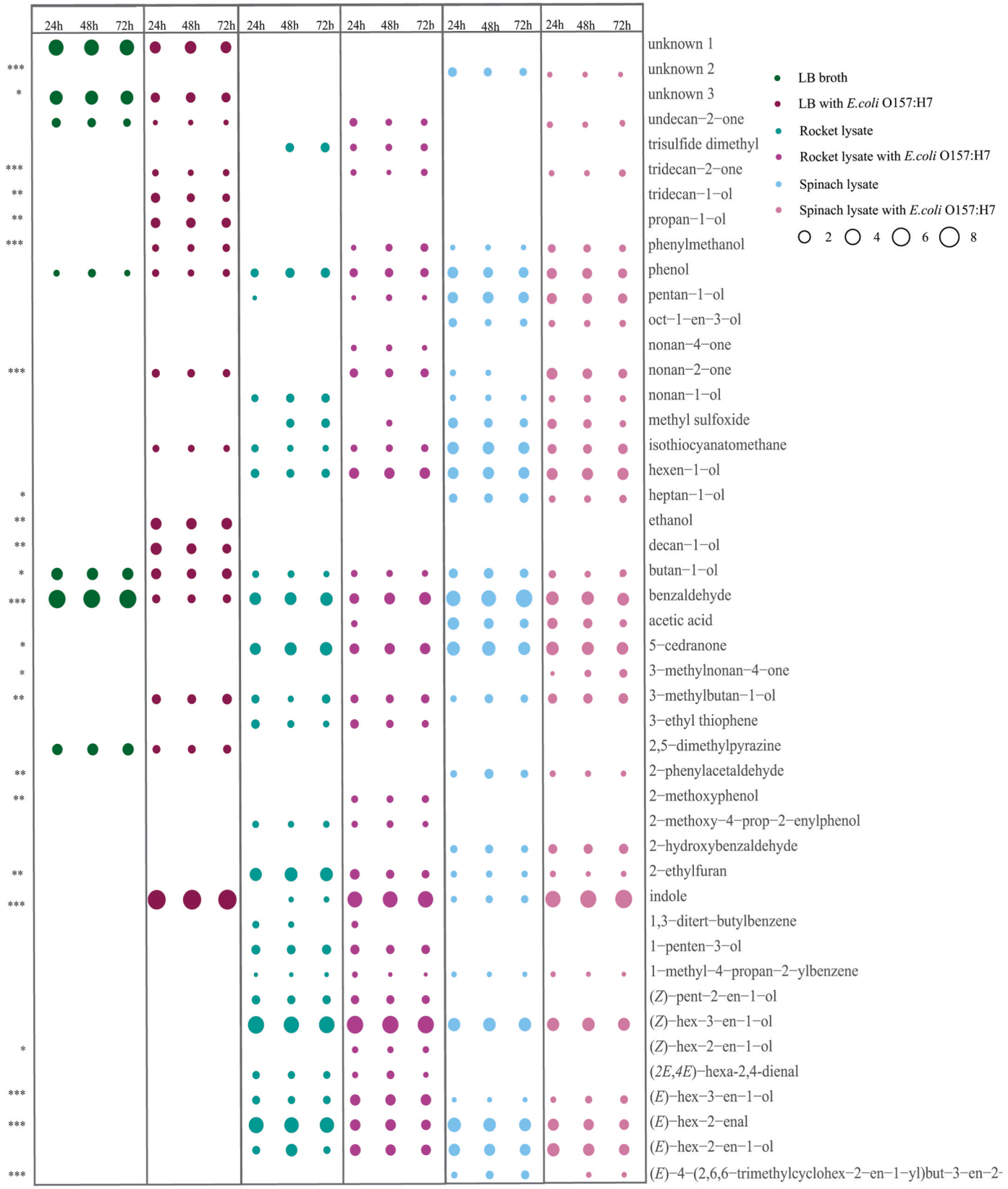

Fig. 2. Volatilome composition from LB-broth and leaf lysates (rocket and spinach) with and without inoculation of *E. coli* O157:H7. Samples were taken after 24 h, 48 h and 72 h post inoculation. (see Supplementary Table S1). Size of the circles represent the results from the square root of relative abundance of compounds (rows) detected in each treatment (columns). Asterisks (*) indicates significant differences in the relative abundance of compounds detected between the treatments (***P $<$ 0.001, **P $<$ 0.01, *P $\leq$ 0.05, Multipatt).

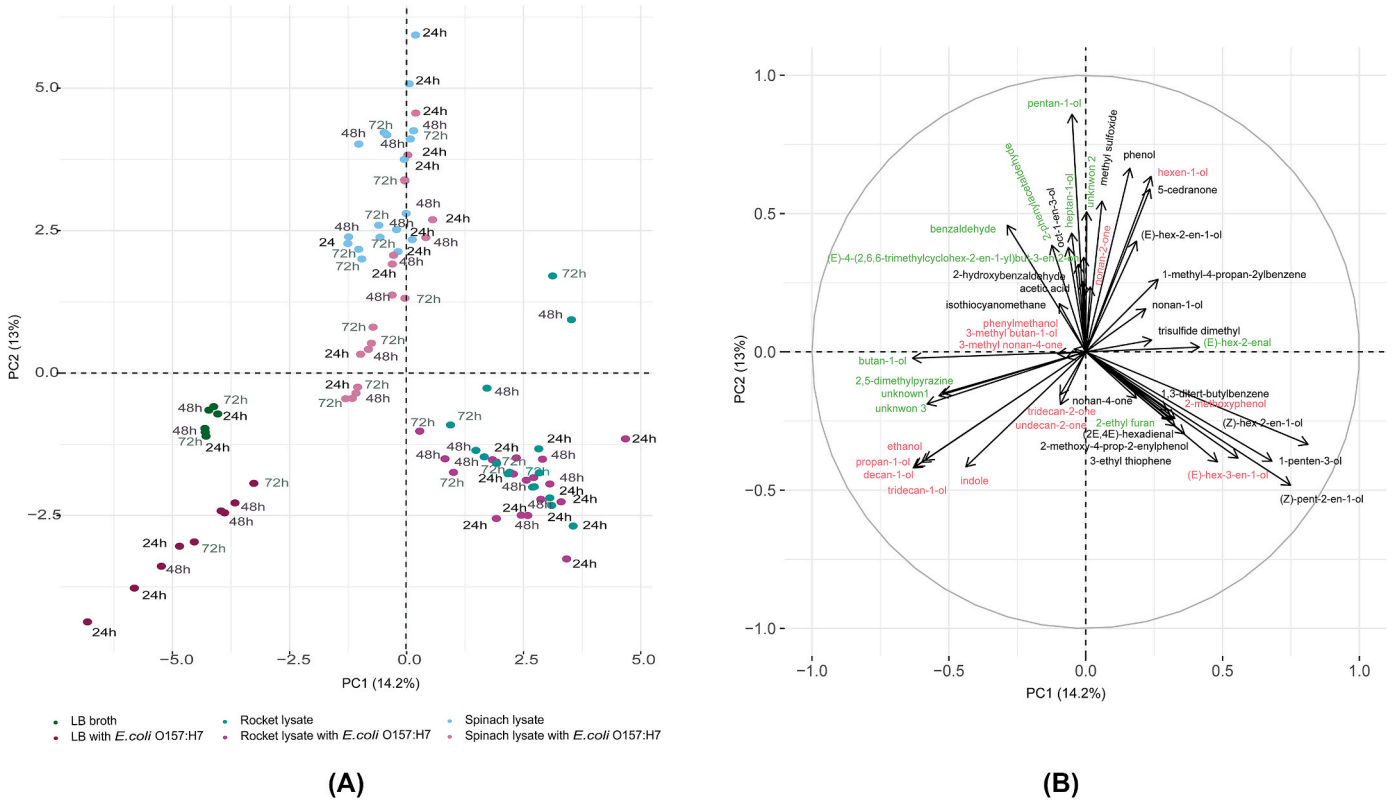

Fig. 3. Principal component analysis (PCA) on the volatilome of LB-broth and leaf lysates (rocket and spinach) with and without inoculation of *E. coli* O157:H7. PC1 and PC2 summarized 27,2% of the overall variance of the dataset. **A.** Variable plot: represents the contribution of each compound to the main variance in the dataset. Positively correlated compounds are grouped together; negative correlated compounds are grouped on opposite quadrants. Compounds with high distance from origin (long arrows) are well-represented on the factor map. In green: compounds significantly contributing to the overall odour profiles of samples without inoculation; in red: compounds significantly contributing to the overall odour profiles of samples with inoculation (Multipatt, $P < 0.05$). **B.** Score plot: each point represents one volatile sample.

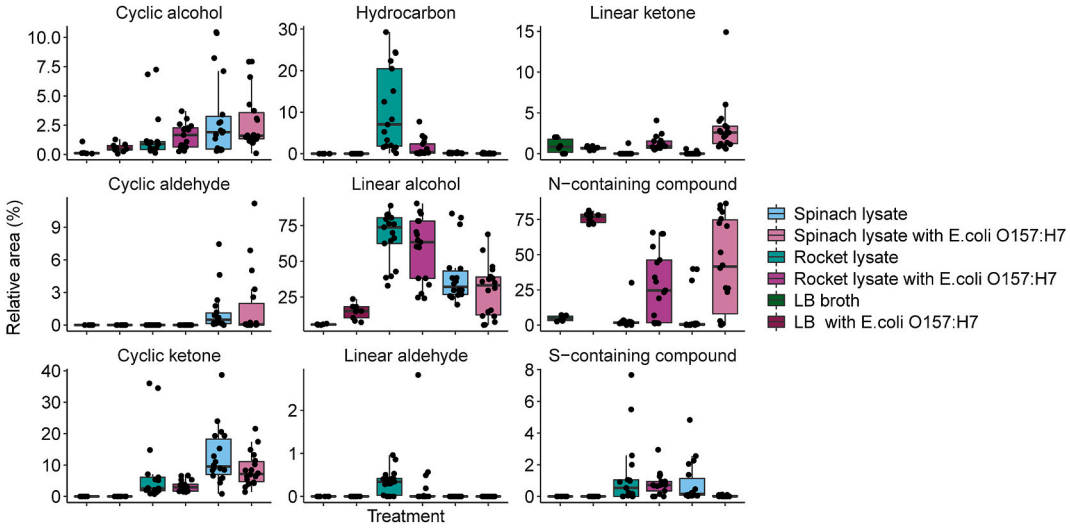

Fig. 4. Relative abundance of groups of volatile organic compounds across treatments.

grouped on the positive side of the CAP2 axis from those emitted from inoculated samples (Jaccard distance: $F = 9.761781$, $P = 0.001$). Indole, phenylmethanol, 2-methoxyphenol, ethanol, propan-1-ol, decan-1-ol, and tridecan-1-ol contributed most to separation along CAP2 axis (Figs. 5 and 6, Table 1). Correspondingly, higher proportions of these compounds were found in inoculated samples compared with uninoculated samples, irrespective of growth medium (Fig. 2 and Supplementary Table S1). Thus, these seven compounds and the ketones such as nonan-2-one and tridecan-2-one (Table 1) should be investigated further as potential biomarkers for *E. coli* O157:H7 contamination of leafy vegetables, using whole spinach and rocket leaves as a matrix.

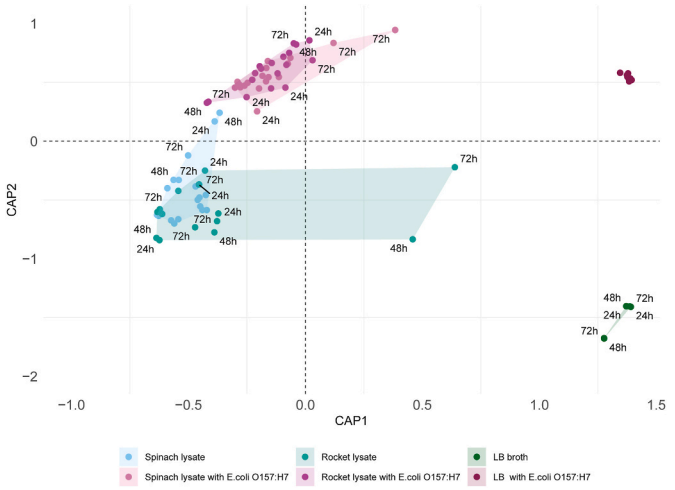

Fig. 5. Partial distance-based redundancy analysis plots showing separation between inoculated and non-inoculated samples. Partial dbRDA based on Jaccard distance in volatile composition, each colour represents a different treatment. $F_{model} = 14.605$ $P_{model} = 0.001$, $\lambda_{CAP1} = 6.062$, Proportion explained$_{CAP1}$ = 0.3435, $F_{CAP1} = 136.3368$, $p_{CAP1} = 0.001$, $\lambda_{CAP2} = 2.9605$, Proportion explained$_{CAP2} = 0.0.1677$, $F_{CAP2} = 66.5826$, $p_{CAP2} = 0.001$.

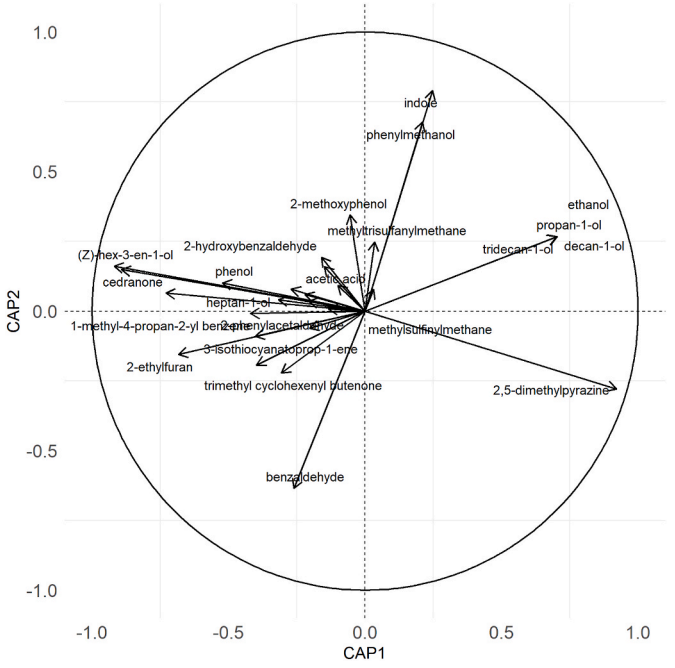

Fig. 6. Partial distance-based redundancy analysis plots showing contribution of volatile components in separation. Vectors show the strength of relationship between predictor variables and dbRDA axis. $F_{model} = 14.605$ $P_{model} = 0.001$, $\lambda_{CAP1} = 6.062$, Proportion explained$_{CAP1} = 0.3435$, $F_{CAP1} = 136.3368$, $p_{CAP1} = 0.001$, $\lambda_{CAP2} = 2.9605$, Proportion explained$_{CAP2} = 0.0.1677$, $F_{CAP2} = 66.5826$, $p_{CAP2} = 0.001$.

4. Conclusions

Isolation and identification of microorganisms is currently used for monitoring contamination of leafy vegetables by *E. coli* O157:H7, which is time-consuming and costly. The preliminary results obtained in this study indicate that headspace analysis may instead become a more cost-effective decision-making and monitoring tool in the future. Using spinach and rocket leaf lysates as a proxy for baby leaves, we obtained empirical evidence showing that it is possible to use volatilome profiles to discriminate between sterile and *E. coli* O157:H7-contaminated samples. The strain *E. coli* O157:H7 produced a volatile signature (consisting of a particular set of compounds) irrespective of the substrate used for growth and an increase in aromatics, ketones, and alcohols. Emission of the volatile compounds indole, phenylmethanol, 2-methoxyphenol, ethanol, propan-1-ol, decan-1-ol, tridecan-1-ol, nonan-2-one and tridecan-2-one contributed most to separation between *E. coli* O157:H7-contaminated and non-contaminated samples in this study. Future investigations are planned to assess the sensitivity of the assay and to adjust it to different sample sizes and types of leafy vegetables, including precut, mixed, and ready-to-eat products. For future routine use, chemical analysis could possibly be replaced with electronic noses, which could be calibrated and fine-tuned using synthetic copies of the key compounds identified here.

Table 1
Compounds significantly associated with *E. coli* O157:H7 irrespective of the growth medium. The db-RDA shows the correlation coefficients of vectors fitted to the first two constrained axes (CAP1 and CAP2) and the significance of permutation tests. n.f. indicates that no significant correlations were found for the compound using this method. The multilevel pattern analysis (Multipatt) shows *stat* that represents the strength of the association of each compound with the inoculated samples and variates from 0 (low) to 1 (high), and the p-value that provides a measure for significant difference on the relative abundance of the compound found between inoculated and non-inoculated samples.

Compounds	**CAS**	db-RDA vector fitting			Multipatt analysis	
		CAP1	**CAP2**	***p-value***	***Stat***	***p-value***
indole	120-72-9	0.298	0.954	<0.001	0.69	<0.001
phenylmethanol	100-51-6	0.296	0.955	<0.001	0.38	<0.001
2-methoxyphenol	90-05-1	−0.157	0.987	<0.005	0.33	<0.001
ethanol	64-17-5	0.935	0.354	<0.001	0.32	<0.01
propan-1-ol	71-23-8	0.934	0.354	<0.001	0.31	<0.01
decan-1-ol	112-30-1	0.934	0.354	<0.001	0.29	<0.01
tridecan-1-ol	112-70-9	0.935	0.354	<0.001	0.28	<0.01
tridecan-2-one	112-70-9	n.f.	n.f.	n.f.	0.36	<0.001
nonan-2-one	821-55-6	n.f.	n.f.	n.f.	0.42	<0.001

CRediT authorship contribution statement

Maria Sousa: Conceptualization, Data curation, Visualization, Formal analysis, Investigation, Methodology, Writing – original draft. **Emina Mulaosmanovic:** Visualization, Formal analysis, Writing – review & editing. **Anna Laura Erdei:** Visualization, Formal analysis, Writing – review & editing. **Marie Bengtsson:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Peter Witzgall:** Supervision, Writing – review & editing. **Beatrix W. Alsanius:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We thank Professor Göran Birgersson, Department of Plant Protection Biology, Unit of Chemical Ecology, Swedish University of Agricultural Sciences, for technical advice. The project was supported by the Swedish Research Council Formas, Stockholm (grant ID: 2017-00511), which is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.109513.

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