Detection of *Listeria monocytogenes* in cut melon fruit using analysis of volatile organic compounds.

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ABSTRACT

Ready-to-eat fresh cut fruits and vegetables are increasingly popular, however due to their minimal processing there is a risk of contamination with human pathogens. *Listeria monocytogenes* is of particular concern as it can multiply even at the low temperatures used to store fresh cut products pre-sale. Current detection methods rely on culturing, which is time consuming and does not provide results in the time frame required. Growth of bacteria on a substrate alters its chemical composition affecting the profile of volatile organic compounds (VOCs) emitted. Use of VOCs as a detection method has been hampered by lack of sensitivity and robust sample collection methods. Here we use thermal desorption gas chromatography time of flight mass spectrometry (TD-GC-TOF-MS) followed by analysis with PerMANOVA to analyse VOC profiles. We can discriminate between fresh cut melon cubes inoculated with 6 log CFU /g of *L. monocytogenes* and uninoculated controls, as well as melon cubes inoculated with < 1 log CFU /g of *L. monocytogenes* stored for 7 days at 4 ^{o}C and following equilibration for 6 h at 37 °C. This is a substantial advance in sensitivity compared to previous studies and additionally the collection method used allows remote sampling and transport of the VOCs, greatly facilitating analyses.

200 words

Key words: detection method; *Listeria monocytogenes;* GC-MS; ready-to eat fruit salad; postharvest storage; volatile organic compounds.

1. Introduction

Ready-to-eat fresh cut fruits and vegetables are increasingly popular products, and due to consumer demand for nutritious, fresh, healthy and easy to consume produce, the market for pre-cut produce has increased in last two decades (James and Ngarmsak, 2011). However, processing steps such us trimming, peeling, cutting, and packaging for distribution of ready-to-eat fruits and vegetables can be a vehicle for the transmission of human pathogens (Beuchat and Brackett, 1991). The main human pathogens of concern in the safety of fresh cut produce are *Salmonella spp*, *Escherichia coli* and *Listeria monocytogenes* (Potter et al., 2012). *L. monocytogenes* is of particular concern because it is able to multiply even at the low temperatures typically used in the supply chain for fresh cut ready-to-eat salads (Oliveira et al., 2010; Fang et al., 2013). The failure to detect contaminated food can have serious consequences, and several outbreaks of human listeriosis attributed to consumption of fresh fruits and vegetables have been reported in recent years (Beuchat and Brackett, 1991; Mukherjee et al 2006). Most recently, a multistate outbreak in the USA, which caused 32 deaths and 1 miscarriage, has been associated to consumption of melon contaminated with *L. monocytogenes* (McCollum et al 2013). In addition, recalling products after they have left the processor results in serious economic loss (Potter et al 2012). Detection of contamination with human pathogens early in the supply chain would trigger intervention to remove the sources and, therefore reduce substantially health risks and fresh produce loss.

Conventional detection methods for contamination of food products with pathogenic bacteria are largely based on culturing which can take up to 48 h, and identification of the microorganisms involved can take even longer (Deisingh and Thomson, 2002). While molecular approaches such as PCR are quick, the target DNA sequence for amplification must be known and unique for the microbe under examination (Cocolin et al. 2013; Galimberti et al. 2015). Another option is the analysis of volatile organic compounds (VOCs) produced by pathogenic contaminants that could provide a useful system for their rapid detection and identification (Tait et al., 2014).

Detection of bacteria through VOCs analysis can in principle be directly applied to the matrices of interest without culturing bacteria in different media and can be pathogen/substrate diagnostic. However, identification of bacteria growing on a food

matrix adds complexity to the problem and the low levels of contamination that need to be detected require very low detection limits. Although human pathogens such as *L. monocytogenes* are not considered spoilage organisms, they will use food substrates to provide their metabolic needs, breaking down sugars and carbohydrates first, then proteins. This breakdown process of food molecules by the microorganisms induces production of VOCs such us alcohols, ketones, hydrocarbons, esters, and amines (Doyle, 2007) that are likely specific both to the bacterium and the substrate. For example in a study on spoilt mango fruits it was found that some VOCs were associated with the microorganisms that were spoiling the fruit (Ibrahim, 2011b). Some bacteria produce specific VOCs: for example production of 2-aminoacetophenone by *Pseudomonas aeruginosa* and indole by *E. coli* (Cox and Parker, 1979; Kai et al., 2009). Bianchi et al. (2009) and Concina et al. (2009), using dynamic headspace followed by GC-MS or an electronic nose, showed that tinned tomatoes contaminated with *E. coli*, or *Saccaromyces cerevisiae* were associated with the presence of five VOCs (ethanol, β-myrcene, o-methyl-5-styrene, 6-methyl-5-hepten-2-ol and 1-octanol). However, although Yu et al. (2000) could detect specific VOCs emitted by *E. coli* O157 grown in culture media, by SPME (solid phase micro extraction) followed by GC-MS, they were not always detectable when the bacteria were grown on strawberries. The association of single compounds to specific microorganisms often falls short in specificity or robustness, and it seems likely that methods based on the analysis of whole bouquets or modules of several compounds may be more useful than the identification of single diagnostic compounds (Tait et al., 2014).

A range of methods for VOC collection and data analysis have also been applied, the most common being SPME followed by GC-MS. However SPME fibres are subject to saturation and are moreover delicate and cannot be stored, precluding remote sample collection (Tait et al., 2014). Using the whole VOC profile which may include hundreds of compounds can improve specificity when analysed using multivariate statistical analysis such as PCA (principal component analysis). Refinements of these tools could further improve limits of detection and specificity of the VOC as a detection method.

Melons are an important component of ready-to-eat fresh fruit salads, prized for their characteristic aroma. The aim of this study was to identify volatile compound markers

associated with melon fruit inoculated with *L. monocytogenes.* We used two new tools for this work: thermal desorption gas chromatography time of flight mass spectrometry (TD-GC-TOF-MS) and multivariate statistical analyses. The TD-GC-TOF-MS provides a highly robust and transportable collection method with greatly reduced saturation effects. The combined multivariate data analyses methods developed for ecological and gene analysis studies enabled comparison across the whole bouquet of VOCs and its deconstruction.

2. Materials and Methods

2.1 Bacterial strain, growth conditions and microbiological analyses.

L. monocytogenes strain LQC 15257, belonging to serotype 4b, previously isolated from a strawberry sample was used throughout this study. Long-term storage took place at -20 °C in nutrient broth supplemented with 50 % glycerol. Before experimental use, the strain was grown twice in Brain Heart Infusion broth (Biolife, Milan, Italy) at 37 °C for 24 h.

Microbial load of the melon was assessed by classical microbiological techniques. More accurately, total aerobic mesophilic count, yeasts/molds, enterococci, lactic acid bacteria, *Enterobacteriaceae*, pseudomonads as well as qualitative and quantitative determination of *L. monocytogenes* were performed according to Paramithiotis et al. (2010).

2.2 Plant material and sample preparation.

Melons (*Cucumis melo,* inodorous group, Honeydew cultivar) were purchased at a commercial stage (3/4 slip) from a local supermarket in Greece on two separate occasions. Melon flesh (100 g, from 3-5 melons) was cut into 4-5 cubes of approx. dimensions 3x3x4 cm and placed in a sterile container (of approx. 500 mL volume). An overnight *L. monocytogenes* culture was centrifuged (12,000 *g*; 10 min; 4 ^{o}C), washed twice with Ringer's solution (LABM, Lancashire, UK), re-suspended in the same solution and used to inoculate the melon samples at less than 1, 3 and 6 log CFU /g. Inoculation took place by spraying 500 uL of an appropriately diluted bacterial suspension in Ringer's solution. Uninoculated samples were sprayed with the same volume of sterile Ringer's solution. For the first experiment, inoculated (6 log CFU /g) and uninoculated melon cubes were stored at 4 °C for up to 14 days and at 20 °C

up to 5 days. For the second experiment, melon cubes were inoculated at 6, 3 and <1 log CFU /g and stored for 7 days at 4 °C.

2.3 VOC Sampling with TD tubes.

Melon samples were removed from storage and prepared in triplicate trays, sealed and stored at 20 °C for 1 h, or at 37 °C for 6 h or 16 h to equilibrate before sampling. VOCs were sampled using an EasyVOC™ pump (Markes International Ltd) to pass a volume of 200 mL head-space through SafeLok tubes (Markes International Ltd) packed with Tenax TA and SulfiCarb sorbents. Three biological replicates were performed for each sample. VOC samples were collected in the laboratory at the Agricultural University of Athens and transported by courier to Cardiff University.

2.4 TD-GC-TOF-MS.

A TD100 (Markes International Ltd) was used to desorb the tubes in the trap with the following conditions: desorption for 10 min at 280 °C with a trap flow of 40 mL /min. Desorption of trap at a rate of 40 °C /s to 300 °C with a split ratio of 11:1 into the GC (7890A; Agilent Technologies, Inc). VOCs were separated over 60 m, 0.32 mm ID, 0.5 μm film thickness Rxi-5ms (Restek) using the following temperature program: 5 min at 35 °C initially, 5 °C /min to 100 °C followed by 15 °C to 250 °C and a final hold of 5 min (total run time 33 min). The BenchTOF-dx mass spectrometer (Almsco International) was operated at ion source temperature of 275 °C, and a mass range of 30 to 350 m/z. A retention time standard (C8-C20, Sigma Aldrich) was prepared by injection of 1 μL of the standard mixture directly onto a collection tube (Tenax TA) and analysed under the same conditions as the samples.

GC-MS data were processed using MSD ChemStation software (E.02.01.1177; Agilent Technologies, Inc) and deconvoluted and integrated with AMDIS (NIST 2011) after first constructing a retention-indexed custom MS library. MS spectra from deconvolution were searched against the NIST 2011 library (Software by Stein et al., version 2.0g, 2011) and only compounds scoring > 80 % in forward and backward fit were included. Putative identifications were based on match of mass spectra (> 80%) and retention index (RI +/- 15) (Beaulieu and Grimm, 2001).

2.5 Statistical Analysis.

VOC data were analysed using R software version 3.1.2 (R core development team 2014) after normalisation of areas and square root transformation to reduce weight of large components. Chromatographic data tend to be highly skewed and also deliver a high number of variables (peaks in chromatogram) as compared to sample units making it inappropriate to apply standard multivariate methods. Following an approach described by Mardon et al. (2010), PerMANOVA and CAP (Anderson and Willis 2003) were used to evaluate the data and to detect differences between actual VOC profiles. Analyses were carried out in R using the 'adonis' function in the package 'vegan' (Oksanen, et al. 2013) and 'CAPdiscrim' in the package 'BiodiversityR' (Kindt and Coe, 2005) in R. Ordination plots from CAP with 95% confidence intervals were used to visualise differences between treatments. Subsequent application of Weighted Gene Correlation Network Analysis (WGCNA package in R, Langfelder & Horvath (2012)) allowed identification of sub-sets of compounds that showed differentially stronger correlation with time, temperature and inoculation.

One-way analysis of variance (ANOVA) was used to assess statistically the differences between the microbial population dynamics (Table 3).

3. Results and discussion

3.1 Growth of Listeria monocytogenes *during storage of inoculated melon cubes at 6 log CFU/g*

The microbiological quality of the melons upon cutting and following enrichment at 20 °C for 1 h was very good, and only 2.01 (± 0.19) and 2.35 (± 0.10) log CFU /g of *Enterobacteriaceae* and yeasts/moulds were enumerated, respectively. *L. monocytogenes* was inoculated at 6.54 (± 0.28) log CFU /g and monitored during storage at 4 and 20 °C. *L. monocytogenes* dominated the background biota and reached 8.83 (± 0.43) and 7.81 (± 0.37) log CFU /g after 14 days at 4 °C and 5 days at 20 °C, respectively.

3.2 Analysis of VOCs from melon cubes inoculated with 6 log CFU/g Listeria monocytogenes *following storage at two temperatures.*

VOC samples were collected directly from inoculated and uninoculated melon cubes stored at 20 °C after 3 and 5 days inoculation, and after 4 and 14 days from cubes stored at 4°C. VOC profiles of inoculated and uninoculated melon samples at day 0

were also analysed. A total of 84 VOCs were obtained across all samples (Table 1). The major compound classes were esters (63), followed by alcohols (6), alicyclic compounds (3), sulphur compounds (3), nitrogen compounds (2), ketones (2), aldehydes (1), alkene (1) and and 3 unidentified compounds.

Permutational multivariate analysis of variance (PerMANOVA) and Canonical Analysis of Principal coordinates (CAP) were used to assess the discriminatory power of the VOC profiles between the inoculated and uninoculated samples at different time points during the storage at the two different temperatures (Fig. 1). As could be expected, VOC profiles showed the largest variations between storage temperature (PerMANOVA, $P < 0.000$, $R^2 = 0.34$) and days of storage (PerMANOVA, $P < 0.000$, $R^2 = 0.22$), which accounted for 56 % of the variance of the data set. However, a small effect of inoculation was detectable (PerMANOVA, $P < 0.05$, $R^2 = 0.04$) accounting 4 % of variance. CAP analysis confirmed significant differences ($P = 0.01$) but only classified correctly 66.6 % of categories (days, temperature and inoculation combined = ten categories). Accordingly, an ordination plot of linear discriminants (LDs) 1 and 2 of the total VOC profile did not show a clear discrimination between samples (Fig. 1).

In order to filter out irrelevant components and increase the discriminatory power of the data set, Weighted (Gene) Correlation Network Analysis (WCNA or WGCNA, Zang and Horvarth, 2005) was used to identify VOCs that correlated significantly with storage time, temperature and inoculation status of samples. WCNA resulted in eight groups of VOCs (modules, Fig. 2). Of these, two modules were significantly correlated with storage day (turquoise negative and green positive correlation), two with storage temperature (grey negative and turquoise positive correlation) and one with inoculation (brown positive correlation) (Fig. 2).

Overall correlations were weak and a closer inspection of VOCs relating to inoculation (brown module and taking into account the green module as well) showed a non-linear time course in concentration of the VOCs. The trend was largely similar amongst compounds in each module and showed significant differences between inoculated and uninoculated samples after storage for 3 days at 20 °C and 14 days at 4 °C (Fig. 3).

Overall WCNA allowed selection of 30 VOCs from the relevant modules taking into account individual significance of correlation with a trait (days of storage, temperature of storage and inoculation status; Supplemental Table 1, italics = total in relevant modules (53), italics bold = selected VOCS). The reduced dataset showed significant differences in the profile of the 30 compounds between storage temperature, time and inoculation status (PerMANOVA, $P < 0.000$, $R^2 = 0.18$, $P < 0.005$, $R^2 = 0.07$ and $P < 0.05$, $R^2 = 0.04$, respectively) and significant interactions occurred between days and temperature ($P < 0.000$, $R^2 = 0.23$), and between days and inoculation status ($P < 0.000$, $R^2 = 0.1$). Overall PerMANOVA analysis of the reduced profiles account for 75 % of variation of the data set but the contribution of inoculation status remained small at 4 % but discernible (Table 2).

The reduced profiles, however, separated much more clearly inoculated from uninoculated samples in CAP and an increase in correct classification from 66.6 to 76.6 % of the 10 categories across time, temperature and inoculation. LDs 1 and 2 explained most of the discrimination between samples (F = 780.5) and showed highly significant discrimination (at 95 % CI) was retained for all time points of both inoculated and uninoculated melon samples and fresh cut samples (Fig. 4). Differences in VOC profiles to the uninoculated controls occurred at very early stages of inoculation and were most significant at day 3 at 20 °C and day 14 at 4 °C. The slight overlap between inoculated samples stored for 3 days at 20 °C and for 4 days at 4 °C suggests a similarity of these VOC profiles.

3.3 Optimisation of sample collection and determination of lowest detectable inoculation level

In a separate experiment, decreasing titres of *L. monocytogenes* (6, 3 and < 1 log CFU /g) were used to identify the inoculation threshold that could be detected by the variation in VOC profiles following storage of melon cubes at 4 °C for 7 days. To improve detection, three incubation conditions prior to VOC collection were tested: 1 h at 20 °C (as was used for the first experiment), 6 h and 16 h both at 37 °C. The latter two can be considered as an enrichment, although no extra media were added to the melon cubes.

Initial microbiological enumeration showed higher counts of yeasts/moulds, compared to the first experiment, but similar levels of *Enterobacteriaceae* (Table 3).

Following storage at 4 °C for 7 days, and before enrichment, yeasts/molds and pseudomonads prevailed the surface microbiota of uninoculated melons as well as those inoculated with *L. monocytogenes* at less than 1 log CFU /g. When the pathogen was inoculated at higher populations it dominated the background microbiota. After 1 h incubation at 20 °C no significant differences in the microbial population were recorded. In contrast, after 6 h enrichment at 37 ^{o}C, pseudomonads dominated the surface microbiota of uninoculated melon cubes and cubes inoculated with < 1 log CFU /g of the pathogen. Co-domination with *L. monocytogenes* was observed after 6 h enrichment at 37 °C when the pathogen was inoculated at 3 log CFU /g while in all other cases *L. monocytogenes* dominated the background microbiota. Thus inoculation of the samples with < 1 log CFU /g of *L. monocytogenes* resulted in < 2 log CFU /g at the end of the 7 day shelf-life experiment consistent with a low level of contamination as defined by EU guidance documents (EU Working Document, 2013).

Although the overall VOC profile differed from the previous experiment, 15 individual compounds mapped onto the relevant WCNA modules identified in the first experiment using the single inoculation titre and two storage temperatures and were used for subsequent statistical evaluations (Table 1, italics).

Discrimination was non-significant in PerMANOVA for samples equilibrated for 1 h at 20 °C with a correct classification of only 50 % in CAP (Fig 5A). It was higher for samples equilibrated for 16 h at 37 °C (PerMANOVA $P < 0.001$, $R^2 = 0.46$; CAP $P < 0.05$, 75 % correct classification, Fig 5 B). Discrimination for samples equilibrated for 6 h at 37 °C was not significant in PerMANOVA but was significant in CAP ($P < 0.05$) with 66.6 % of samples correctly classified. Most importantly, all inoculum levels were clearly discriminated at 95 % CI in the ordination plot (Fig 5 C).

The VOCs derived from WCNA of the previous experiment (Section 3.2) and present in samples equilibrated for 6 h at 37 °C were: 2,3-butanediol diacetate; (E)-3-hexen-1-ol acetate; (Z)-3-hexen-1-ol acetate; 4-penten-1-yl acetate; (E)-5-decen-1-ol acetate; acetic acid; acetic acid phenylmethyl ester; hexanoic acid ethyl ester; pentanoic acid ethyl ester; 2-methyl-propanoic acid ethyl ester; 2-methyl-propanoic acid methyl ester. All of these VOCs correlated with the inoculation status. In particular 2,3-butanediol diacetate is part of the group of compounds of the brown module which showed an increase in inoculated samples. While 4-penten-1-yl acetate; hexanoic acid

ethyl ester; pentanoic acid ethyl ester; 2-methyl-propanoic acid ethyl ester are part of the green module and show the reverse trend.

In a recent study on tomatoes six VOCs: 4-methyloctane, 1,2,3-trimethylbenzene, 3,7-dimethylundecane, 1-hexadecanol, 2-isopropenyl-5,5-dimethyl-1,3-dioxane and n-acrylonitrylaziridine (3-aziridinoacrylonitrile), were found to be unique to tomato fruits inoculated with *L. monocytogenes* (Ibrahim et al., 2011a). None of the VOCs found in these tomatoes where found here in inoculated melon cubes, suggesting microorganism/substrate specificity.

The protocol with the short enrichment period (6 h) was successful at discriminating between uninoculated melon cubes and those inoculated with the lowest titre of *L. monocytogenes*. As a comparison Ibrahim et al (2011a) detected *L. monocytogenes* at an inoculated titre of approximately 4 log CFU/g when tomatoes were incubated at 27 ^{o}C for 7-10 days followed by chemical extraction. Thus levels of contamination detected here by collection of VOCs directly from melon cubes stored at commercially relevant low temperature with no further processing are comparable to those found previously using much higher temperature storage and chemical extraction (Ibrahim et al., 2011a). In fact our detection levels for the *L. monocytogenes* contaminated melon are comparable to detection levels reported for *E. coli* in tinned tomato using an electronic nose (Concina et al. 2009), which however were detected only after a much longer enrichment period (48 h) at 37 ^{o}C.

4. Conclusions

Changes between VOC profiles discriminated melon cubes inoculated at a titre of < 1 log CFU /g with *L. monocytogenes* from uninoculated melon after 7 days of storage at a commercially relevant temperature of 4 °C. This was achieved using a TD-GC-TOF-MS system, which provided a robust platform for remote sampling, and by equilibrating samples for just 6 h at 37 °C prior to VOC sampling. Using multivariate statistical analysis we then identified reduced profiles of VOCs, which clearly separated uninoculated from inoculated samples in CAP and could be developed into a marker panel for *L. monocytogenes* contamination of ready to eat melon fruit salads in the supply chain. Further investigations are under way to validate the VOCs in different melon cultivars, with shorter storage periods and larger sample sizes.

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Supplementary Material

Supp. Table 1: WCNA of total VOCs from melon cubes uninoculated and inoculated with *L. monocytogenes* at 6 log CFU/g

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TABLES

Table 1

VOC list from uninoculated melon cubes and cubes inoculated with *L. monocytogenes* at 6 log CFU/g (italics indicate VOCs shared by both experiments).

Compound number	Compound name	RI	CAS No.	Chemical group
C2	3-Methyl-1-butanol acetate	882	123-92-2	Ester
C6	4-Methyl-1-Hexanol acetate	1088	91367-59-8	Ester
C8	1,1-Ethanediol diacetate	902	542-10-9	Ester
C10	1,5-Diacetoxypentane	840	6963-44-6	Ester
C12	3-Methyl-2-buten-1-ol acetate	802	1191-16-8	Ester
C14	2-Butene-1,4-diol diacetate	882	18621-75-5	Ester
C15	2-Methyl-2-butenoic acid ethyl ester	959	55514-48-2	Ester
C16	2-Pentanol propanoate	989	54004-43-2	Ester
C17	2-Methyl-2-propen-1-ol acetate	796	820-71-3	Ester
C19	*2,3-Butanediol diacetate*	*1076*	*1114-92-7*	*Ester*
C20	(Z)-3-Decen-1-ol acetate	1394	81634-99-3	Ester
C21	*(E)-3-Hexen-1-ol acetate*	*1042*	*3681-82-1*	*Ester*
C22	*(Z)-3-Hexen-1-ol acetate*	*1025*	*3681-71-8*	*Ester*

C24	*3-Methylheptyl acetate*	*1154*	*72218-58-7*	*Ester*
C25	(Z)-3-Octen-1-ol acetate	1200	69668-83-3	Ester
C26	(Z)-4-Hexen-1-ol acetate	1042	42125-17-7	Ester
C27	(E)-4-Hexen-1-ol acetate	1036		Ester
C28	4-Methylcyclohexanol acetate	1108	22597-23-5	Ester
C29	(Z)-4-Octenoic acid ethyl ester	1192	34495-71-1	Ester
C30	(Z)-4-Octenoic acid methyl ester	1124	21063-71-8	Ester
C31	*4-Penten-1-yl acetate*	*891*	*1576-85-8*	*Ester*
C32	*(E)-5-Decen-1-ol acetate*	*1109*	*38421-90-8*	*Ester*
C33	9-Decen-1-yl acetate	1297	50816-18-7	Ester
C34	*Acetic acid*	*620*	*64-19-7*	*Ester*
C35	Acetic acid 1-methylethyl ester	655	108-21-4	Ester
C36	Acetic acid heptyl ester	1113	112-06-1	Ester
C37	Acetic acid octyl ester	1210	112-14-1	Ester
C38	Acetic acid pentyl ester	922	628-63-7	Ester
C39	*Acetic acid phenylmethyl ester*	*1185*	*140-11-4*	*Ester*
C41	Alkane2	1886	112-70-9	Ester
C42	Benzoic acid ethyl ester	1192	93-89-0	Ester
C45	Butanoic acid 1-methylethyl ester	846	638-11-9	Ester
C46	*2-Methyl-butanoic acid propyl ester*	*962*	*37064-20-3*	*Ester*

C47	Butanoic acid 2-methylbutyl ester	1068	51115-64-1	Ester
C48	Butanoic acid 2-methylpropyl ester	970	539-90-2	Ester
C49	3-Methyl-butanoic acid ethyl ester	856	108-64-5	Ester
C50	*Butanoic acid butyl ester*	*1013*	*109-21-7*	*Ester*
C51	Butanoic acid methyl ester	715	623-42-7	Ester
C52	Butanoic acid propyl ester	899	105-66-8	Ester
C54	Ethyl (methylthio)acetate	1012	4455-13-4	Ester
C55	Ethyl Acetate	613	141-78-6	Ester
C57	Formic acid butyl ester	771	592-84-7	Ester
C58	Heptanoic acid ethyl ester	1098	106-30-9	Ester
C59	*Hexanoic acid ethyl ester*	*1016*	*123-66-0*	*Ester*
C60	Hexanoic acid methyl ester	937	106-70-7	Ester
C61	Isobutyl acetate	830	110-19-0	Ester
C62	Methyl propionate	628	554-12-1	Ester
C63	Methyl thiolacetate	694	1534-08-3	Ester
C64	Methyl tiglate	810	6622-76-0	Ester
C65	n-Propyl acetate	706	109-60-4	Ester
C67	*Octanoic acid ethyl ester*	*1195*	*106-32-1*	*Ester*
C68	Octanoic acid methyl ester	1128	111-11-5	Ester
C69	*Pentanoic acid ethyl ester*	*904*	*539-82-2*	*Ester*

C70	Pentanoic acid methyl ester	831	624-24-8	Ester
C71	2-Methyl-propanoic acid 2-methylpropyl ester	920	97-85-8	Ester
C72	2-Methyl-propanoic acid 3-phenylpropyl ester	1397	103-58-2	Ester
C73	2-Methyl-propanoic acid anhydride	804	97-72-3	Ester
C74	*2-Methyl-propanoic acid ethyl ester*	*756*	*97-62-1*	*Ester*
C75	*2-Methyl-propanoic acid methyl ester*	*678*	*547-63-7*	*Ester*
C76	2-Methyl-propanoic acid propyl ester	859	644-49-5	Ester
C77	Propanoic acid 2-methylpropyl ester	871	540-42-1	Ester
C78	Propanoic acid ethyl ester	704	105-37-3	Ester
C79	Propanoic acid propyl ester	814	106-36-5	Ester
C1	2-Methyl-1-butanol,	737	137-32-6	Alcohol
C3	1-Decanol	1407	112-30-1	Alcohol
C5	1-Hexanol	940	111-27-3	Alcohol
C7	2-Methyl-1-propanol,	626	78-83-1	Alcohol
C9	1,4-Butanediol	773	110-63-4	Alcohol
C56	Eucalyptol	1055	470-82-6	Alcohol
C4	Propylcyclopropane,	876	2415-72-7	Alicyclic compound
C23	3-Methoxy-2,2-dimethyloxirane	705	26196-04-3	Alicyclic compound
C43	7-Methylene-bicyclo[4.1.0]heptane	1303	54211-14-2	Alicyclic compound
C53	Dimethyldisulfide,	744	624-92-0	Sulphur compound

C80	S-Methyl 2-methylpropanethioate	856	42075-42-3	Sulphur compound
C81	Thiopivalic acid	961	55561-02-9	Sulphur compound
C44	3-Methyl-butanenitrile,	727	625-28-5	Nitrogen compound
C66	N,N,O-Triacetylhydroxylamine	595	17720-63-7	Nitrogen compound
C40	Acetophenone	1091	98-86-2	Ketone
C11	3-Hydroxy-2-butanone,	704	513-86-0	Ketone
C13	2-Methyl-2-butenal	744	1115-11-3	Aldehyde
C18	8-methyl-1-decene	1085	61142-79-8	Alkene
C82	Unknown 10	1075		
C83	Unknown 5	915		
C84	Unknown 8	1030		

Table 2:

PerMANOVA analysis of VOCs resulting from WCNA.

Traits	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Temp	1	12.036	12.036	15.0213	0.18669	0.0001	***
Treat	1	3.01	3.0102	3.7569	0.04669	0.0158	*
Day	1	4.571	4.5708	5.7046	0.0709	0.0018	**
Sample	1	3.05	3.0502	3.8067	0.04731	0.0145	*
Temp:Treat	1	0.745	0.7453	0.9301	0.01156	0.4167	
Temp:Day	1	14.593	14.5931	18.2127	0.22636	0.0001	***
Treat:Day	1	6.363	6.3628	7.9409	0.09869	0.0005	***
Treat:Sample	1	3.343	3.3433	4.1725	0.05186	0.0102	*
Temp:Treat:Day	1	0.733	0.7327	0.9144	0.01136	0.4279	
Residuals	20	16.025	0.8013		0.24857		
Total	29	64.47			1		

Table 3:

Microbial populations (log CFU/g) after storage of melon for 7 days at 4°C and after different enrichment conditions prior to VOC collection. Melon was either uninoculated, or inoculated with three titres (< 1, 3 and 6 log CFU/g) of *L. monocytogenes*.

	TAMC[1]	Yeasts/molds	pseudomonads	*Enterobacteriaceae*	enterococci	LAB	*L. monocytogenes*
Initial load	4.87 (0.20)^{a}	3.76 (0.12)^{a}	< 2.00	< 2.00	< 2.00	< 2.00	absence
7d at 4°C							
Uninoculated	5.08 (0.25)^{a}	4.27 (0.56)^{ab}	4.56 (0.19)^{a}	< 2.00	< 2.00	< 2.00	Absence
10^0	5.60 (0.04)^{b}	4.56 (0.28)^{b}	4.99 (0.44)^{a}	< 2.00	< 2.00	< 2.00	< 2.00[2]
10^3	4.98 (0.14)^{a}	4.35 (0.43)^{ab}	4.85 (0.04)^{a}	< 2.00	< 2.00	< 2.00	5.12 (0.27)^{c}
10^6	7.01 (0.28)^{d}	4.79 (0.17)^{b}	4.77 (0.20)^{a}	< 2.00	< 2.00	< 2.00	7.08 (0.23)^{d}
1h at 20°C							
Uninoculated	5.22 (0.42)^{a}	4.90 (0.14)^{bc}	4.69 (0.33)^{a}	< 2.00	< 2.00	< 2.00	Absence
10^0	5.93 (0.30)^{c}	4.94 (0.35)^{bc}	4.74 (0.24)^{a}	< 2.00	< 2.00	< 2.00	< 2.00[2]
10^3	5.99 (0.18)^{c}	4.68 (0.33)^{b}	4.77 (0.49)^{a}	< 2.00	< 2.00	< 2.00	5.55 (0.16)^{c}
10^6	7.13 (0.28)^{de}	5.09 (0.54)^{bc}	4.00 (0.63)^{a}	< 2.00	< 2.00	< 2.00	7.25 (0.29)^{d}
6h at 37°C							
Uninoculated	6.94 (0.13)^{d}	5.53 (0.20)^{c}	6.41 (0.52)^{bc}	4.96 (0.32)^{b}	< 2.00	5.70 (0.20)^{a}	Absence
10^0	7.68 (0.19)^{e}	4.99 (0.32)^{bc}	6.19 (0.48)^{b}	5.10 (0.26)^{b}	< 2.00	5.78 (0.36)^{ab}	4.12 (0.19)^{b}
10^3	7.08 (0.21)^{d}	5.25 (0.37)^{bc}	6.16 (0.53)^{b}	4.10 (0.22)^{a}	< 2.00	5.73 (0.20)^{ab}	5.57 (0.20)^{c}

10^6	8.11 (0.35)^{ef}	5.49 (0.38)^{c}	6.42 (0.38)^{bc}	5.19 (0.29)^{b}	< 2.00	5.89 (0.13)^{ab}	8.07 (0.31)^{e}
16h at 37^{o}C							
Uninoculated	8.08 (0.25)^{ef}	4.70 (0.68)^{bc}	7.16 (0.30)^{c}	6.14 (0.34)^{c}	< 2.00	6.39 (0.40)^{ab}	Absence
10^0	8.10 (0.17)^{ef}	5.19 (0.41)^{bc}	7.64 (0.27)^{c}	6.10 (0.38)^{c}	< 2.00	6.24 (0.31)^{ab}	3.57 (0.16)^{a}
10^3	8.39 (0.35)^{f}	5.49 (0.25)^{c}	7.29 (0.19)^{c}	6.16 (0.30)^{c}	< 2.00	6.42 (0.38)^{b}	8.51 (0.19)^{e}
10^6	8.29 (0.15)^{f}	5.29 (0.45)^{bc}	7.20 (0.33)^{c}	5.85 (0.34)^{c}	< 2.00	6.38 (0.14)^{b}	8.85 (0.57)^{e}

[1] Total Aerobic Mesophilic Count

[2] presence of *L. monocytogenes* was verified by selective enrichment

Within a column, different superscript letters denote significant differences (ANOVA, α<0.05).

FIGURE LEGENDS

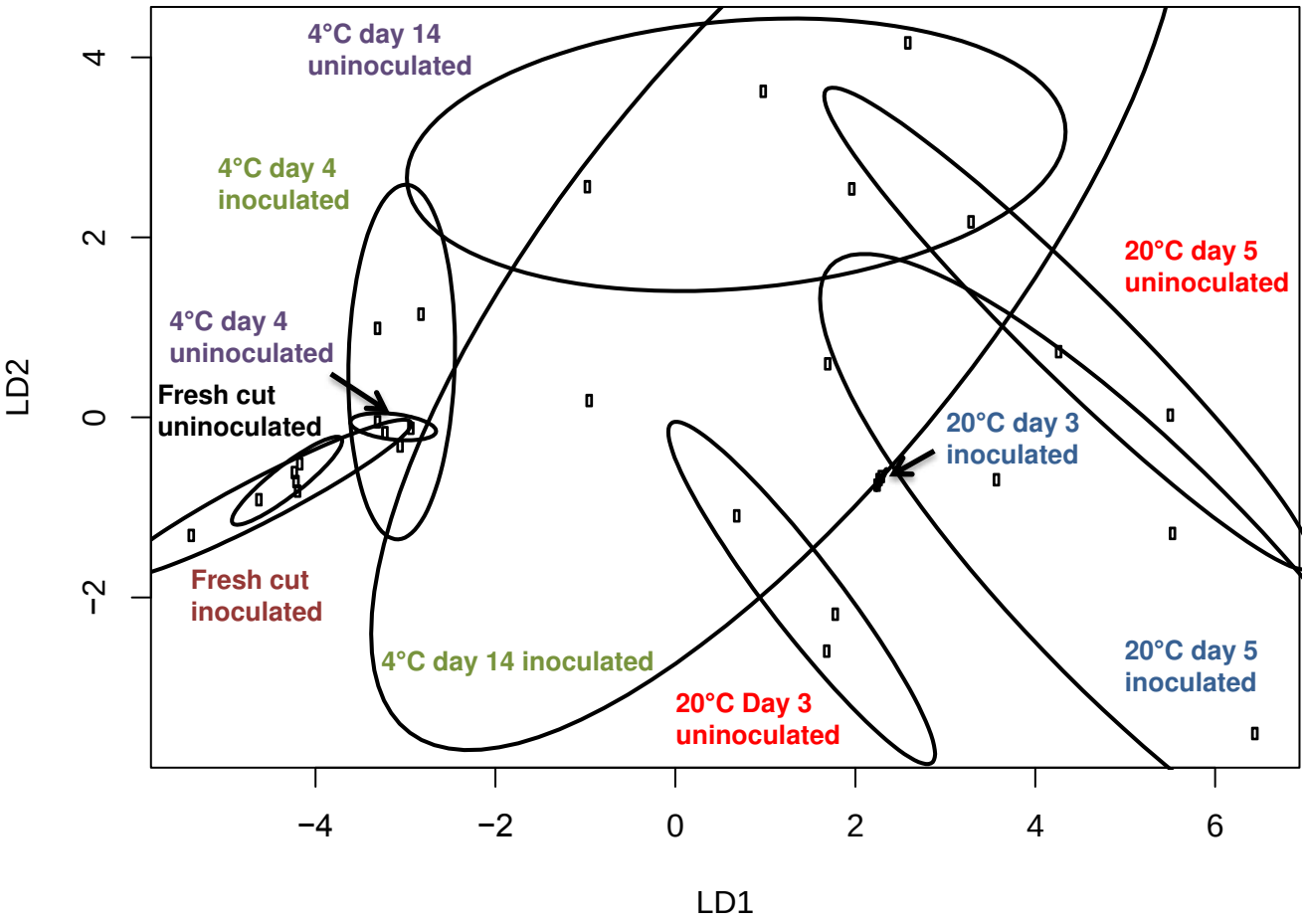
Figure 1. CAP analysis plot using the square root of the proportional abundance (% of grand total) of VOC profiles from melon cubes inoculated with 6 log CFU /g *L. monocytogenes* and stored at 4 °C for 4 or 14 days or at 20 °C for 3 or 5 days; fresh cut and uninoculated samples are also indicated. Ellipses represent the 95 % interval of confidence, n=3.

Figure 2. Module-trait relationship between VOCs and day of storage, temperature and inoculation with *L. monocytogenes* using WCNA. Numbers represent Pearson correlation, in brackets is the *P* value. Red indicates a positive correlation; green indicates a negative correlation of the module with respect to the trait.

Figure 3. Emission of VOCs belonging to the (A) brown and (B) green modules (mean ± S.E. of value summed for all VOCs) across days of storage, storage temperature (20 °C and 4 °C), and in inoculated and uninoculated melon cube samples.

Figure 4. CAP analysis plot using the 30 most significant VOCs resulting from WCNA of melon inoculated with *L. monocytogenes* using the square root of the proportional abundance (% of the grand total), Fresh cut inoculated and uninoculated samples are also indicated. Ellipses represent the 95 % confidence interval.

Figure 5. CAP analysis using the most significant VOCs deriving from WCNA; plots of melon cubes inoculated with increasing titres of *L. monocytogenes* and stored for 7 days at 4 °C. Uninoculated samples are also indicated. Ellipses represent the 95 % confidence interval. (A) Enrichment for 1 hour at 20 °C before VOC collection. (B) Enrichment for 16 h at 37 °C before VOC collection. (C) Enrichment for 6 h at 37 °C before VOC collection



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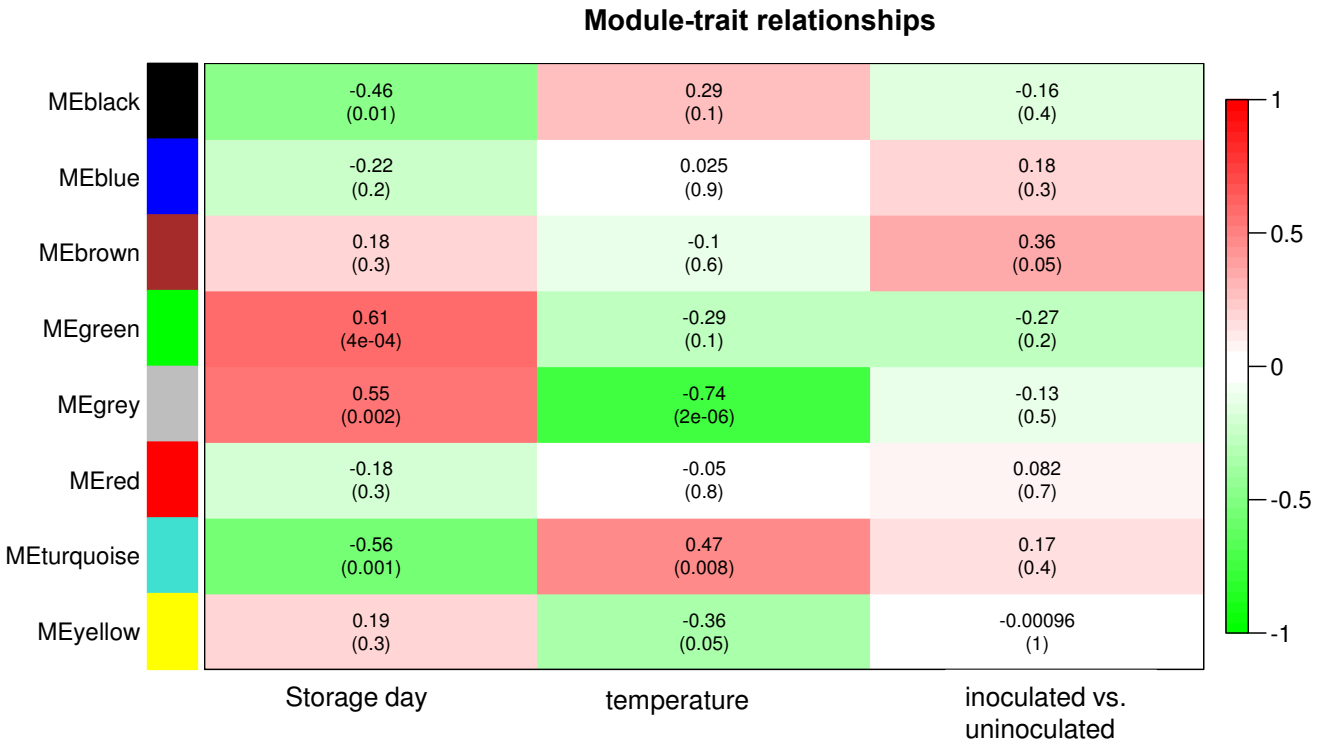

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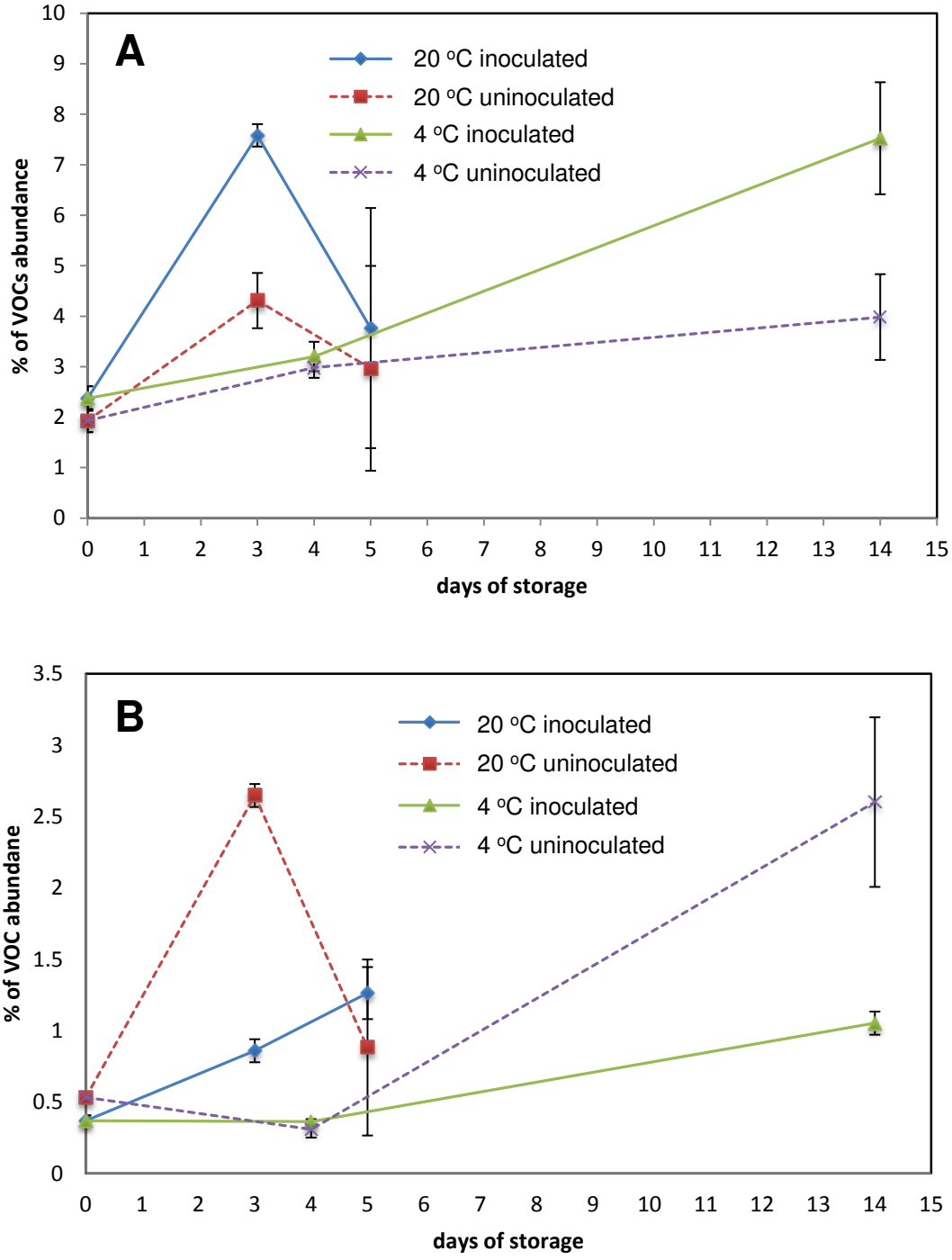

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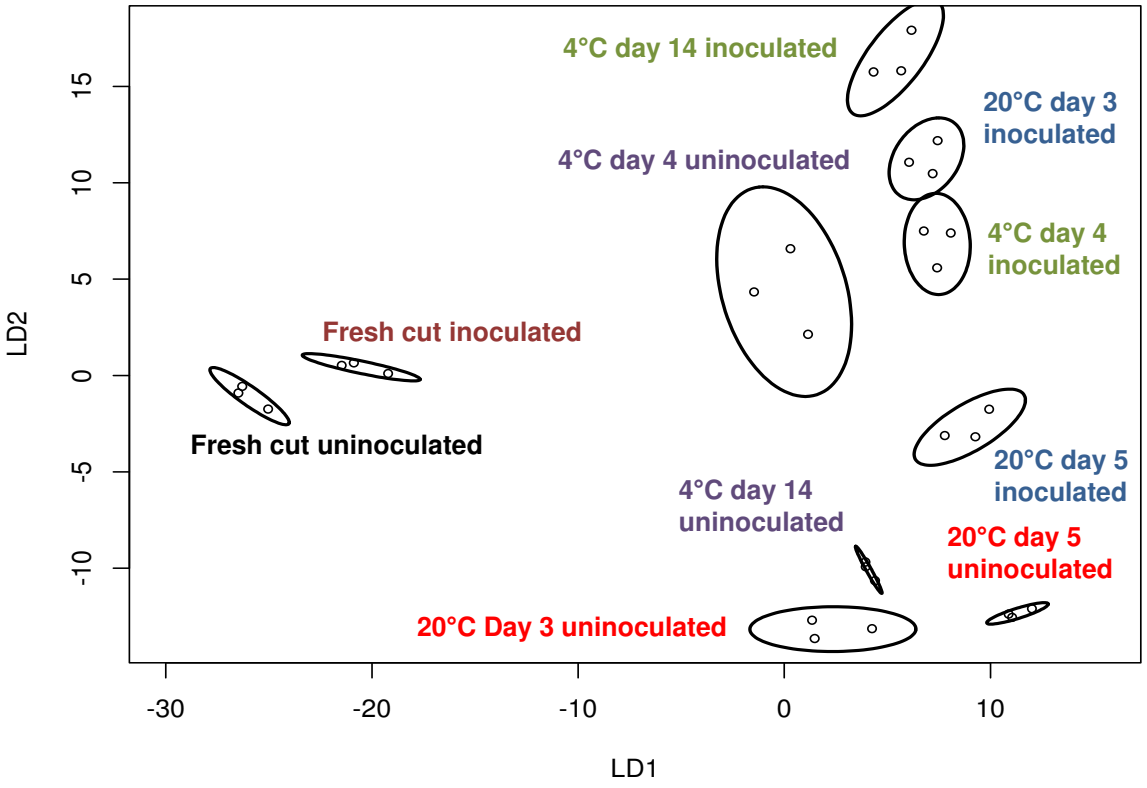

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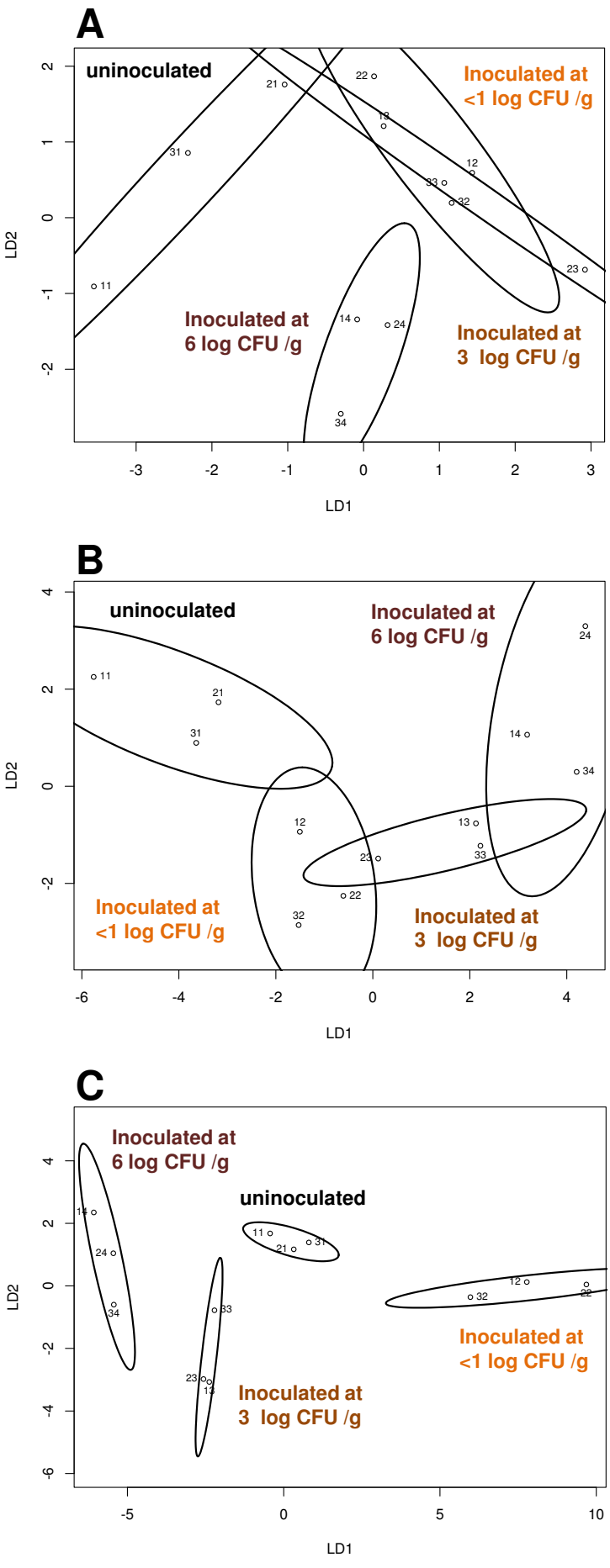

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Supplementary Table 1

Click here to download e-component: Supplemental data 1.xlsx