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A comprehensive look into the volatile exometabolome of enteroxic and non-enterotoxic *Staphylococcus aureus* strains

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Running title: Volatile exometabolome of different *Staphylococcus aureus* strains

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Highlights

- The volatile exometabolome of three strains of *S. aureus* was studied.
- The volatile exometabolome each strain comprised in average 240 metabolites.
- Hierarchical clustering analysis allowed the strains metabotyping
- Enterotoxic and non-enterotoxic strains exhibited distinct metabolomic profiles

ABSTRACT

Staphylococcal food poisoning is a disease that originates significant health and economic losses and is caused by *Staphylococcus aureus* strains able to produce enterotoxins. The aim of this work is to go further on the study of the volatile exometabolome of *S. aureus* using an advanced gas chromatographic technique. Enterotoxic and non-enterotoxic strains were assessed. The volatile exometabolome profile comprised 240 volatiles belonging to ten chemical families. This volatiles were mainly by-products of branched-chain amino acids and methionine degradation, pyruvate metabolism, diacetyl pathway, oxidative stress and carotenoid cleavage. Metabolites released by the first two pathways were produced in higher contents by the enterotoxic strains. This study add further insights to *S. aureus* volatile exometabolome, and also shows that by applying it, it is possible to distinguish strains of *S. aureus* by the number of produced enterotoxins, which is especially important from the food safety point of view.

Keywords: *Staphylococcus aureus*, Saphylococcal enterotoxin, Staphylococcal food poisoning, microbial metabolomics, volatile metabolites, volatiles, HS-SPME/GCxGC-ToFMS

1. Introduction

Staphylococcus aureus is a Gram-positive pathogen, extremely adaptable and opportunistic, with no special nutritional or environmental requirements that is able to grow in a huge array of foodstuffs and surfaces (Normanno et al., 2005). It can live as a commensal microorganism in humans and other animals, and is responsible for a wide range of diseases and producing several virulence factors, such as SE which are responsible for SFP (Grumann et al., 2014). *S. aureus* is mainly carried by humans asymptomatically, transferring this bacterium to foodstuff causing SFP outbreaks, mainly due to food workers and/or due to the lack of hygiene in places where food is prepared (Argudín et al., 2010). According to WHO, in 2014, the average SFP incidence in Australia together with Canada, France, Netherlands, New Zealand, England, Wales, and the United States of America was 77.3 per 100 000 inhabitants (WHO, 2015).

From a health point of view, symptoms of SFP are usually self-limiting and self-solved or, occasionally, can be acute enough causing hospitalization. Furthermore, SFP might also be the source of relevant economic losses not only for agro-food industries/businesses and health systems, but also for the patients (Argudín et al., 2010; WHO, 2015). Thus, even though SFP is not a high mortality foodborne disease, it is significant due to its associated morbidity and economic impact. To avoid SFP, alternative methods of identification to the conventional ones, which might take several days, are essential to detect *S. aureus* enterotoxic strains in food. Therefore, the use of a pattern of volatile biomarkers to monitor the presence of enterotoxic strains of *S. aureus*, would be an useful approach.

Microbial metabolomics is the study of the overall metabolites produced by microorganisms according to cellular state, response to an antibiotic or another chemical substance, response to different physical parameters or even differences between species and strains (Tang, 2011). Several studies of *S. aureus* volatile metabolome employing different methodologies have been reported. The first one was performed in 1986, with the objective of finding differences in volatile profiles of four bacteria, including *S. aureus*, using gas

chromatography with flame ionization (GC-FID) and one-dimensional gas chromatography-mass spectrometry (1D-GC-MS) (Zechman et al., 1986). Since then, several metabolomics studies of *S. aureus* have been performed. These were mainly designed for clinical applications such as early detection of *S. aureus*, strain discrimination, evaluation of growth conditions, among others (Allardyce, Langford, Hill, & Murdoch, 2006; Dörries & Lalk, 2013; Filipiak et al., 2012; Jia, Sohnlein, Mortelmans, Coggiola, & Oser, 2010; Liebeke et al., 2011). A wide range of analytical methods was used in these studies mainly one dimensional gas-mass spectrometry (Filipiak et al., 2012; Jia et al., 2010; Zechman et al., 1986) and nuclear magnetic resonance (Liebeke et al., 2011). Furthermore, there are already some applications using e-nose (Yusuf et al., 2014) and more advanced gas chromatographic methodology, such as comprehensive two-dimensional gas chromatography-time of flight mass spectrometry (GC×GC-ToFMS) (Gardner et al., 2011; Rees et al., 2018).

GC×GC-ToFMS is a high throughput and highly sensitive methodology, which has been proven to be a powerful tool for microbial metabolomics, specifically for the study of microbial volatile organic compounds (MVOCs) (Alves et al., 2015; Costa et al., 2016; Loots, 2014). There are two studies in which GC×GC-ToFMS was applied to compare MVOCs from different bacterial and fungal species and also different strains of bacterial species, including *S. aureus* strains ATCC 25923 and ATCC 6538, with results reporting the discrimination of species and strains (Gardner et al., 2011; Rees et al., 2018). To our knowledge, neither GC×GC-ToFMS, nor other high throughput methodologies, have been used to assess *S. aureus* volatile exometabolome and to discriminate between enterotoxic and non-enterotoxic strains. Although *S. aureus* metabolome is relatively well studied, its non-volatile fraction is better known than the volatile.

The information about MVOCs from *S. aureus* is fragmentary and, even though approximately one hundred and fifty volatile metabolites were reported, only few are in common between studies (e.g. ethanol, 3-hydroxy-2-butanone, 3-methylbutanoic acid, dimethyl disulfide, methyl 2-hydroxy-4-methylpentanoate, acetic acid, 2-propanone, 3-methyl-

1-butanol, methanethiol, ammonia, 3-methylbutanal and acetaldehyde, are reported in common by at least ten different studies). This allows to assume that the growth conditions, strains, instrumental analysis, among other factors, may have impact on the reported volatiles. Most consistently reported MVOCs of *S. aureus* include: ethanol, 3-hydroxy-2-butanone, 2-propanone, acetic acid, 3-methyl-1-butanol, 3-methylbutanoic acid, dimethyl disulfide, methanethiol, acetaldehyde and 3-methylbutanal (Allardyce et al., 2006; Dörries and Lalk, 2013; Filipiak et al., 2012; Jia et al., 2010; Liebeke et al., 2011; Zechman et al., 1986).

Due to the importance of *S. aureus* enterotoxic strains as a source of SFP the present study aims to add further insights to the volatile fraction of *S. aureus* exometabolome. To achieve this, fresh cultures of three strains (one non-enterotoxic and two enterotoxic) of *S. aureus* were studied using HS-SPME combined with GC×GC-ToFMS. Furthermore, resorting to hierarchical clustering, this study also examined if, by using the volatile exometabolome, enterotoxic strains are distinguishable from non-enterotoxic one.

2. Materials and methods

Sampling, reporting of chemical analysis, and data relative to data pre-processing, pre-treatment, processing, validation and interpretation were accomplished according to the metabolomics standards initiative (MSI) (Fiehn et al., 2007; Goodacre et al., 2007; Sumner et al., 2007). Experimental procedure was performed accordingly to Figure 1, representing the main stages for *S. aureus* exometabolome evaluation, which will be described in detail in the following sub-sections.

2.1. Bacterial parameters

2.1.1. *Bacterial strains and growth conditions*

Three *Staphylococcus aureus* strains were used in this study: ATCC 6538, a collection strain, without SE, isolated from a human wound; 2153 MA (GenBank accession number

MG675881) (SEA) and 2065 MA (GenBank accession number MG675880) (SEA, SEG, SEI), both enterotoxic, isolated from food samples and characterized in the Centre of Biotechnology and Fine Chemistry of the Faculty of Biotechnology of the Catholic University, Portugal. These strains are all β-hemolysin, lipase, lecithinase, coagulase, thermonuclease and catalase positive, able to perform mannitol fermentation (except 2153 MA), and are methicillin sensitive. ATCC 6538 is the most baroresistant while the enterotoxic strains (2153 MA and 2065 MA) are barosensitive, and the carotenoid content follows the order: ATCC 6538>2065 MA>2153 MA (Baptista et al., 2015).

All strains were cultured at 37 °C for 18 h at 170 rpm in 25 mL of non-buffered BHI (LIOFILCHEM, Italy), conditions adapted from ISO norm 6888-3:2003 (ISO/TC 34/SC 9, 2003) in 100 mL shaking flasks, in three independent assays (a total of 9 flasks, three for each strain).

To determine viability, samples were 10-fold serial diluted (10^{-1} to 10^{-9}) in sterile 1/4-strength Ringer solution (Merck Millipore, Darmstadt, Germany). One milliliter of dilutions 10^{-5} to 10^{-9} was pour-plated in triplicate in Plate Count Agar medium (LIOFILCHEM, Italy), and plates were incubated at 37 °C for 48 h. The number of colonies were counted in the most appropriate dilution (plates containing between 30 and 300 CFU) and the number of viable cells was expressed as log CFU.mL^{-1}. Differences in cell viability between strains were statistically analyzed using ANOVA and the post-hoc Tukey test, with the SPSS 20.0. Statistical significance was considered for $\rho < 0.05$. Viability was determined in order to compare the volatile patterns for all strains under study, thus expressing their content as area/cell concentration.

2.1.2. *Extracellular pH measurements*

At pre-determined incubation times (4, 8, 12 and 18 h), 1.5 mL of each sample was removed to a 2 mL microcentrifuge tube (Labbox Labware, Barcelona, Spain) and pH was assessed. Measurements were done using 2 types of pH strips (MColorpHast™, Merck Millipore, Darmstadt, Germany): in the ranges of 4.0—7.0 and 6.5—10.0, respectively.

2.2. *S. aureus* exometabolome determination by HS-SPME/GC×GC-ToFMS

After 18 h incubation, 20 mL of each sample (BHI cultured broth) were centrifuged at 10000 rpm, at 4 °C for 15 min (centrifuge Heraeus Megafuge 16R, Thermo Scientific, United States). Next, 4 mL of supernatant were transferred via syringe with 0.22 µm filter pore (CA GyroDisc, Orange Scientific, Belgium) into 12 mL glass vials containing 0.8 g of NaCl (99.5%, Sigma-Aldrich, St. Louis, Mo., USA) and a cylindrical magnetic stirring bar of 12 x 4.5 mm (Labbox Labware, Barcelona, Spain). The vials were capped with a polytetrafluoroethylene septum and an aluminum cap (Chromacol Ltd., Herts, UK) and samples were stored at -80 °C until analysis. In addition, to exclude the medium effect on the composition of the organic volatiles released from *S. aureus*, BHI medium was analyzed using the procedure described above for the samples.

SPME and GC×GC–ToFMS experimental parameters were previously defined (Costa et al., 2016). Both SPME holder for manual sampling and coating fiber were acquired from Supelco (Sigma-Aldrich, Bellefonte, Pa., USA). The SPME device comprised a fused silica fiber coating, cross-linked with 50/30 µm divinylbenzene/carboxen™/polydimethylsiloxane StableFlex™ (1 cm), including an extensive capacity of sorbing compounds with distinct physicochemical features.

After defrost, the vials with the *S. aureus* cultures or BHI blank media were placed in a thermostated water bath at 50.0± 0.1 °C for ca. 1 min, under constant agitation at 350 rpm, and then the SPME fiber was inserted into the headspace for 30 min to allowed the extraction of the analites. Then, the SPME fiber with the sorved analytes was manually inserted into the GC×GC–ToFMS injection port and exposed for 30 seconds allowing thermal desorption into heated inlet (250 °C), with the inlet lined with a 0.75 mm I.D. splitless glass liner. Splitless injection mode was used (30 s). The GC×GC–ToFMS system, a LECO Pegasus 4D (LECO, St. Joseph, Mi., USA), contains an Agilent GC 7890A gas chromatograph (Agilent Technologies, Inc., Wilmington, De., USA), incorporating a dual stage jet cryogenic modulator (licensed from Zoex), a secondary oven, and a mass spectrometer supplied with a ToF analyzer. In the first dimension (^{1}D) an Equity-5 column

(30 m × 0.32 mm I.D., 0.25 µm film thickness, Supelco, Inc., Bellefonte, Pa., USA) was used and a DB-FFAP column (0.79 m × 0.25 mm I.D., 0.25 µ m film thickness, J&W Scientific Inc., Folsom, Ca., USA) was used in the second dimension (^{2}D). Helium was the carrier gas, at a constant flow rate of 2.50 mL.min^{-1}. The primary oven temperature was programmed from 40 °C to 140 °C (10 °C.min^{-1}; hold 1 min) and then to 200 °C (7 °C/min; hold 1 min). The secondary oven temperature program was 15°C offset above the primary oven. Both MS transfer line and MS source temperature were set at 250 °C. The modulation time was 5 s and the modulator temperature was maintained at 20 °C offset above secondary oven, with hot and cold pulses through periods of 0.80 and 1.70 seconds, respectively. ToFMS was operated at a spectrum storage rate of 100 spectra/s. The mass spectrometer was operated in the EI mode at 70 eV and detector voltage of -1480 V, using a range of *m/z* 35–350. Total ion chromatograms (TIC) were processed using the automated data processing ChromaTOF software (LECO) at signal-to-noise threshold of 100.

The data obtained was transferred into Guineu software (this software source code is published under GNU General Public License that can be downloaded from the internet (https://code.google.com/p/guineu/). This software performs score alignment based on first dimension retention time (^1t_R), on second dimension retention time (^2t_R), retention index (RI) value, spectra, and compound name.

The identification of the compounds was performed by comparing the mass spectrum of each compound detected with those in mass spectral libraries, which included an in-house library of standards and commercial databases (Wiley 275 and US National Institute of Science and Technology (NIST) V. 2.0 – Mainlib and Replib). The identification was also supported by experimentally determined linear retention index (RI) values that were compared with values reported in the bibliography for chromatographic columns similar to the one used in the present work as the ^{1}D column (Table S1 from Supplementary Information). A C_8-C_{20} n-alkanes series was used for RI determination (the solvent n-hexane was used as C_6 standard) comparing these values with reported ones in existing literature for chromatographic columns similar to ^{1}D

column above mentioned. The areas achieved were used to estimate the relative amount of each metabolite. The overall identified compounds presented similarity matches >800. To determine the relative content of each metabolite from each strain, area data from Deconvoluted Total Ion Current GC×GC were applied.

2.3. Statistical analysis

From the 315 VOCs released from the *S. aureus* headspace (Table S1 from Supplementary Information), 240 were selected for statistical purposes, thus a full data matrix was constructed with 9 observations (three strains, in three independent assays) and 240 variables (volatile metabolites or simply volatiles) (Table S2 from Supplementary Information).

The removal of the 75 VOCs (most of them belonging to the chemical families of pyrroles, pyridines, furan-like compounds, pyrazines and thiazoles) was based on the fact that they are most likely Maillard reaction products (Jousse et al., 2002; van Boekel, 2006). Furthermore, to see if these VOCs and others such as Strecker aldehydes did not exhibit significant differences between medium used as blank and *S. aureus* cultures, statistical significance was verified by analysis of variance (ANOVA) and the post-hoc Bonferroni test, using SPSS 20.0 (IBM, New York, USA). Differences corresponding to $p > 0.05$ were not considered significant and these volatiles were removed for further statistical analysis.

The following strategy was used to extract relevant information from the *S. aureus* headspace volatiles: A heatmap hierarchical cluster, an unsupervised clustering analysis, was applied to evaluate the similarities among samples (i.e. strains) and, by using a chromatic scale, a graphical way of displaying the content of a metabolite is more intuitive. To achieve this, Euclidean distance measure and Ward clustering algorithm, were applied in chromatographic data previously normalized by $CFU.mL^{-1}$ and autoscaled. Then, Partial Least Squares Discriminant Analysis (PLS-DA) was applied using the same normalization criteria and VIP (Variable Importance in Projection) values were retrieved to identify the main volatiles that contribute for

the distinction between the strains under study (clusters observed from the heatmap representation). Classification model complexity (number of latent variables) of the data set (Table S2 from Supplementary Information) was computed, as well as classification rate and Q^2 (quality-of-fit criterion) were estimated by cross-validation. A set of volatiles with VIP values higher than 1.5 was defined (Table 1), and a heatmap hierarchical cluster visualization was constructed, using the chromatographic data previously normalized by $CFU.mL^{-1}$ and autoscaled.

Finally, a calibration model weight randomization test (WRT) number of enterotoxins produced by *S. aureus* was calculated using Partial Least Square regression (PLS). A set of volatiles with VIP values above 1.5 obtained as described above were used for the calculations. The model was validated using leave one out validation.

PLS-DA, VIP and heatmap hierarchical cluster visualization were achieved using MetaboAnalyst 3.0 (web interface). PLS was implemented with MATLAB, v. 7.12 (release 2011a).

3. Results and discussion

3.1. Cell growth and extracellular pH

Cell growth was similar ($p > 0.05$) for the three strains, with values of 9.15, 9.08 and 9.10 log $CFU.mL^{-1}$ for ATCC 6538, 2153 MA and 2065 MA, respectively (Figure 2 A). These values were similar to the ones reported before for the same strains and identical growth conditions (Baptista et al., 2015).

The initial extracellular pH of 7.4 decreased after 8 h of incubation to 5.5 and 5.8 for the ATCC 6538 and enterotoxic strains, respectively (Figure 2 B). The decrease of the extracellular pH during incubation time was expected, foremost because *S. aureus* is a facultative anaerobe and secondly because BHI was deliberately not buffered to mimic foodstuffs, which, in general, are not buffered. Following 8 h of incubation, pH remained relatively constant. These

observations can be explained by changes in the exometabolome, which will be discussed further ahead.

3.2. *S. aureus* volatile exometabolome profiling

S. aureus cells were grown in BHI, a high nutrient non-selective broth medium with a pH of 7.4, which supports the growth and recovery of many bacteria from a variety of clinical and non-clinical specimens, such as food products. This meaty-culture medium contains brain heart infusion and peptone as nitrogen sources (proteins), vitamin and carbon sources, glucose as carbohydrate source, sodium chloride and disodium phosphate. The sterilization of BHI, like other culture media, is performed at 121 °C for 15 min in an autoclave, promoting the formation of unwanted compounds, such as Maillard reaction products, that are formed at temperatures higher than 100 °C and consist in the condensation of sugars with amino acids (AA) (Jousse et al., 2002), both present in BHI. From the *ca*. 315 MVOCs detected (Table S1), including acids, alcohols, aldehydes, esters, hydrocarbons, ketones, terpenes, norisoprenoids, N-compounds, S-compounds, pyrroles, pyridines, furan-like compounds, pyrazines and thiazoles, of which VOCs belonging to this last five families were removed as they were most likely formed during culture medium sterilization (Jousse et al., 2002; van Boekel, 2006). Additionally, as previously explained, volatiles from the remaining families were excluded when the areas did not exhibit significant differences ($p > 0.05$) between medium used as blank and *S. aureus* cultures. This was the case of 3-methyl-1-butanal and 2-phenylacetaldehyde, which can be either Strecker aldehydes (by-products of AAs reacting with α-dicarbonyl compounds) (Monforte et al., 2018; Smit et al., 2009), or products of leucine and phenylalanine metabolism, respectively (Hazelwood et al., 2008). Other compounds such as propanoic acid, 2-methylpropanoic acid, 4-methyl-2-oxopentanoic acid, 2-aminoacetophenone and methyl dihydrojasmonate, although associated with specific metabolic pathways, were also removed due to similar areas in samples and controls. Moreover, the volatiles indole and 3-methylindole, known to be products of tryptophan catabolism (due to the action of the enzyme tryptophanase) were excluded because

S. aureus does not possess tryptophanase (Holt et al., 1993). Thus, a set of 240 volatiles (Table S2) was used to build the dataset, which was composed by 10 chemical families as shown in the contour plot in Figure 3: acids (0.8%), alcohols (13.3%), aldehydes (9.6%), esters (13.8%), hydrocarbons (21.3%), ketones (22.9%), terpenes (5.8%), norisoprenoids (1.3%), N-compounds (5.4%), and S-compounds (5.8%). This section describes the overall exometabolome of the three strains of *S. aureus*.

In the present study it was possible to identify the main volatiles previously reported in the literature for *S. aureus*. These were: ethanol, 3-hydroxy-2-butanone, 2-propanone, acetic acid, 3-methyl-1-butanol, 3-methylbutanoic acid, dimethyl disulfide, methanethiol, acetaldehyde and 3-methylbutanal (Allardyce et al., 2006; Dörries and Lalk, 2013; Filipiak et al., 2012; Jia et al., 2010; Liebeke et al., 2011; Zechman et al., 1986). This last volatile (Table S1), although identified, was removed as previously explained. It is possible that, after proper validation, these volatiles might be used as biomarkers for *S. aureus*. Finding a specific pattern of volatiles is paramount for a quick and specific detection of *S. aureus* in food and clinical samples.

Most volatiles in the dataset were neither described as part of *S. aureus* exometabolome nor described to play an important role in its metabolism before. Some of these volatiles have their origin in: branched-chain amino acids (BCAAs) degradation (such as 3-methylbutanoic acid, 3-methyl-1-butanol) (Filipiak et al., 2013, 2012; Hazelwood et al., 2008); phenylalanine metabolism, either from degradation or from cyanoamino acid metabolism (benzonitrile, phenylacetonitrile, benzyl alcohol, 2-phenylethanol, benzaldehyde, 2-hydroxybenzaldehyde, methyl benzoate, benzophenone, 1-phenylethanone) (Fenske and Imaizumi, 2016; Hazelwood et al., 2008; Lapadatescu et al., 2000; Miki and Asano, 2014; Møller et al., 1998; Paczkowski and Schütz, 2011; Schulz and Dickschat, 2007; Tavaria et al., 2002; Uddin et al., 2015); degradation of methionine(methanethiol, 3-(methylthio)-propanal, dimethyl disulfide and dimethyl trisulfide) (Bonnarme et al., 2001; Hazelwood et al., 2008; Schulz and Dickschat, 2007);

carotenoid cleavage (6-methyl-5-hepten-2-one and geranylacetone) (Barbieri et al., 2005; Wei et al., 2016); mevalonate pathway,either via geranyl diphosphate or via farnesyl diphosphate, (monoterpens and sesquiterpens, respectively) (Carrau et al., 2005; Croteau et al., 1978; Demissie et al., 2013; Dewick, 2002; Pardo et al., 2015; Rabe et al., 2013); pyruvate metabolism (acetic acid, ethanol, 1-butanol, 2,3-butanediol, 3-hydroxy-2-butanone, 2,3-butanediol) (Birkenstock et al., 2012; Carvalho et al., 2017; Chingin et al., 2015; Dörries and Lalk, 2013; Filipiak et al., 2015, 2013, 2012; Hara et al., 2014; Mader et al., 2013); and in the degradation of toluene (4-methylphenol) (Evans et al., 1991). However some of the above-mentioned volatiles are not yet associated with any pathway and no definite prove exists, as to their origin in *S. aureus*. Some of the volatiles detected in this study also play key roles in pH homeostasis (3-hydroxy-2-butanone and 2,3-butanediol) (Bore et al., 2007; Rode et al., 2010) and others (hexanal, 2-heptenal, heptanal, octanal, nonanal, decanal, undecanal, dodecanal and tridecanal) are the result of oxidative stress, including lipid peroxidation (Calenic et al., 2015; Kang et al., 2013; Montanari et al., 2013; Paczkowski and Schütz, 2011; Repetto et al., 2012), and fatty acid degradation (1-octanol, 1-dodecanol, ethyl propanoate, butyl acetate, hexadecane, heptadecane) (Audrain et al., 2015; Ladygina et al., 2006; Qin et al., 2014; Schirmer et al., 2010).

The observed stabilization of growth medium pH after 8 h of incubation was probably the outcome of the intrinsic acidification of the cells associated with ammonium production by arginine deaminase (Stavropoulou et al., 2015). Although there is a high probability that all strains produced this volatile, it was absent from the chromatograms possibly due to its high volatility. Acetoin (3-hydroxy-2-butanone) and 2,3-butanediol detected in the exometabolome of *S. aureus* are known to raise the internal pH of the cells. These volatiles, generated in the diacetyl pathway, contribute to pH homeostasis by decreasing the levels of pyruvate, thus avoiding the formation of acids (Rode et al., 2010). The content of these two volatiles is higher in ATCC 6538 (Table S2). Conversion of pyruvate to acetoin as a mechanism of the pH

homeostasis has been described for other bacterial species such as *Lactobacillus plantarum* and *E. coli* (Bore et al., 2007).

A low extracellular pH can be responsible for oxidative stress in *S. aureus* (Bore et al., 2007). Several volatiles related to oxidative stress, namely with lipid peroxidation were detected, including a set of aliphatic aldehydes and alcanes, i.e., hexanal, heptanal, octanal, nonanal, octane and nonane (Calenic et al., 2015; Kang et al., 2013; Montanari et al., 2013; Paczkowski and Schütz, 2011; Repetto et al., 2012).

The strain 2153 MA presented highest content of volatiles related to lipid peroxidation, namely hexanal, 2-heptenal, heptanal, octanal, nonanal, decanal, undecanal, dodecanal and tridecanal (Calenic et al., 2015; Kang et al., 2013; Montanari et al., 2013; Paczkowski and Schütz, 2011; Repetto et al., 2012). The strains 2153 MA and ATCC 6538 also presented a lipid peroxidation product, octane, with similar content (Calenic et al., 2015; Kang et al., 2013; Montanari et al., 2013; Paczkowski and Schütz, 2011; Repetto et al., 2012). Other lipid peroxidation products were detected with highest content in ATCC 6538 and/or 2065 MA: nonane (in 2065 MA), tetradecanal (in ATCC 6538) and pentadecanal (similar content in both strains) (Calenic et al., 2015; Kang et al., 2013; Montanari et al., 2013; Repetto et al., 2012). These results suggest that 2153 MA strain present higher level of lipid peroxidation metabolites (Table S2).

S. aureus contains seventeen types of carotenoids, including staphyloxanthin and γ-carotene, which have an antioxidant role and are also responsible for the golden color of its cells (Marshall and Wilmoth, 1981). Oxidative stress causes carotenoid degradation via oxidation by non-specific enzymes, including lipoxygenases and peroxidases, which cleave non-specific double bonds, resulting in norisoprenoids (Hammond and White, 1970; Schulz and Dickschat, 2007). The 6-methyl-5-hepten-2-one (Table S2), already described for *Staphylococcus* spp., is the product of such sort of cleavage in γ-carotene (Barbieri et al., 2005). Contents of 6-methyl-5-hepten-2-one and geranylacetone (two out of three norisoprenoids detected) support the

antioxidant role of carotenoids (which are found in the cellular membrane) as a protection against oxidative stress.

A set of methyl ketones (2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, 2-nonanone, 2-decanone, 2-undecanone and 2-tridecanone) were detected in *S. aureus* exometabolome, in accordance to data previously reported (Bos et al., 2013; Jia et al., 2010; Watts and Butzke, 2003; Zechman et al., 1986). These compounds are considered as by-products of fatty acids β-oxidation but, until now, there is no explanation for the presence of these compounds since *S. aureus* lacks fatty acids β-oxidation pathway (Cronan, 2014). Furthermore, there is no proof that *S. aureus* and other species of Firmicutes own a different pathway with different enzymes and processes to use fatty acids (Cronan, 2014). Thus, as this subject has not yet been studied intensively it is impossible to assert the origin of the methyl ketones detected in the strains of *S. aureus* presented in this study.

3.3. Using the exometabolome to distinguish strains

Figure 4 shows the hierarchical cluster heatmap containing the dataset of 240 volatiles. The chromatic scale of the heatmap allows access the relative amount of each metabolite (from dark blue, minimum, to dark red, maximum). The dendrogram (Figure 4) built from the HCA, is an exploratory tool that reveals clustering between the strains under study, grouping the strains according to the similarities their metabolite profiles. Thus, it is possible to detect the presence of two main clusters: the first containing the 2065 MA strain and the second containing the strains ATCC 6538 and 2153 MA. The strain 2065 MA achieved the highest content for the families of acids, alcohols, esters, ketones, N-compounds and terpenes and the lowest for the families of norisoprenoids and S-compounds. The strain ATCC 6538 had the highest content for the family of hydrocarbons and the lowest for the families of alcohols, aldehydes, ketones, and N-compounds and the strain 2153 MA showed highest content for the families of aldehydes,

norisoprenoids and S-compounds and the lowest for the families of acids, esters, hydrocarbons and terpenes.

Specific volatiles from each family with highest content for each of the three strains were identified (Table S2). Among the acids, 3-methylbutanoic acid was the most abundant for all strains. The most prominent alcohol released from both ATCC 6532 and 2153 MA was 1-dodecanol, while for 2065 MA it was 3-methyl-1-butanol. Benzaldehyde, 2-methyl-4-phenylbutyric acid methyl ester, 3-ethyl-3-phenyl-1-pentene and phenylacetonitrile were the most abundant compounds for the families of aldehydes, esters, hydrocarbons and N-compounds in all strains, respectively. For ATCC 6538 and 2153 MA the monoterpene with the highest content was tetrahydrogeraniol and for 2065 MA was dihydromyrcenol. The sesquiterpene with highest content for both ATCC 6538 and 2065 MA was β-caryophyllene while for 2153 MA was nerolidol. For ATCC 6538 and 2153 MA, the norisoprenoid with highest content was 6-methyl-5-hepten-2-one while for 2065 MA was geranylacetone. Finally, among S-compounds, dimethyl disulfide had highest content in ATCC 6538 and 2065 MA whereas for 2153 MA it was 3-(methylthio)-propanal.

The Variable Importance in Projection (VIP values) were computed and a sub-dataset was constructed comprising 25 metabolites with VIP values higher than 1.5 (Table 1) resulting in the creation of another hierarchical clustered heatmap (Figure 5 A). VIP analysis allowed the decrease in the number of variables, and thus, the pre-processing time (to go from raw instrumental data to clean data for data processing) was substantially reduced, since only 25 metabolites were extracted from the raw instrumental data. This sub-dataset was comprised by alcohols, aldehydes, esters, ketones, N-compounds and S-compounds, and allowed to perceive the presence of two main clusters: the first containing the 2065 MA strain (with three SE) and the second containing the strains 2153 MA (one SE) and ATCC 6538 (without SE), as observed in Figure 4, when the full data set. Because the vertical axis of the dendrograms shows the similarity between samples, lower height corresponds to higher similarity between the samples,

thus in spite of higher differences between the strains exhibited in Figure 4, the reduced data set also reveals the strains resemblance.

Most VIP volatiles were detected in all strains, with differences between them mainly due to the contents of the chemical families released by each strain. Alcohols, aldehydes, esters, and N-compounds were higher in the first cluster (2065 MA). In the second cluster, it was possible to observe that the strain ATCC 6538 had higher contents of S-compounds, while the strain 2153 MA had intermediate contents of the VIP volatiles.

In spite of the origin of almost half of the VIP volatiles is unidentified for *S. aureus*, the VIP volatiles with known origin belong to the metabolic pathways related to the degradation of BCAAs, phenylalanine and methionine and to the cyanoamino acid metabolism.

Metabolic pathways most active at the end of growth involving selected volatiles are shown in Figure 5 B with the purpose to illustrate difference between enterotoxic and non-enterotoxic strains. Volatiles originating from the degradation of BCAAs and the cyanoamino acid pathway (with origin at leucine which is a BCAA) have the highest content in the strain 2065 MA and lowest the in the ATCC 6538. Volatiles from methionine degradation have the highest content in ATCC 6538. Most of volatiles with unknown origin have higher content in 2065 MA. Thus, taking into account that volatiles originating from BCAAs metabolism (either degradation or via cyanoamino acid pathway) such as 3-methylbutanol, 2-methylpropanol and 2-methylpropanal, have highest content in the strain with more enterotoxins (2065 MA) and lowest in the strain without enterotoxins (ATCC 6538), it is probable that those volatiles might be part of biomarker pattern for strains with at least more than one SE. On the other hand, as volatiles originating from methionine degradation such as methanethiol, dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide and dimethyl pentasulfide, have the highest content in ATCC 6538 and the lowest in 2065 MA, it is possible that those volatiles are part of biomarker pattern for strains without or with only one SE.

To see if the number of SEs could be related and predicted using the sub-dataset of the 25 metabolites an exploratory study was performed using that sub-dataset to build a calibration model ($y = 0.9866x + 0.0178$, $R^2 = 0.9866$). Results suggest that this sub-dataset of 25 metabolites might be useful to detect *S. aureus* enterotoxic strains since there is are satisfactory correlation coefficients (above 0.9 for both calibration and validation). This is also supported by low prediction root-mean-square errors (0.1293 and 0.2250 number of toxins, for calibration and validation, respectively). In fact, as mentioned previously, it is possible to notice that the strain with three SEs (2065 MA) produced higher contents of BCAAs degradation by-products and lower contents of methionine degradation by-products while the opposite occurred in the strain with no SEs (ATCC 6538), *i.e.*, higher contents of methionine degradation by-products and lower contents of BCAAs degradation by-products. The strain with only one SE (2153 MA) produced intermediate contents of both BCAAs and methionine degradation by-products. To support this findings, PLS showed a strong correlation ($R^2 = 0.9866$) between the number of SEs and the content of BCAAs and methionine degradation by-products.

The explanation as to why higher contents of BCAAs degradation by-products were detected in the exometabolome of the strain with three SEs and also in the strain with one SE can be that these metabolites were the result of SE degradation (monomeric proteins). The degradation of proteins has already been described for *S. aureus* when this bacteria needs to raise the extracellular pH to consequently raise the internal pH (Bore et al., 2007; Rode et al., 2010). This mechanism was attributed to the upregulation of genes involved in the degradation of proteins, peptides and glycopeptides (Bore et al., 2007; Rode et al., 2010). Since the enterotoxic strains have in their SEs structures 20% of BCAAs, it is reasonable to hypothesize that SEs are degraded as a way to raise the extracellular pH, which will result in higher BCAAs degradation by-products when compared to the non-enterotoxic strain. In fact, SEs of 2065 MA strain (SEA, SEG and SEI) have in their sequence a total of 137 BCAAs residues (SEA: 10 residues of isoleucine, 23 residues of leucine and 13 residues of valine; SEG: 14 residues of isoleucine, 17

residues of leucine and 15 residues of valine; SEI: 18 residues of isoleucine, 18 residues of leucine and 9 residues of valine) and the SE of 2153 MA (SEA) has in its sequence a total of 46 BCAAs (SEA: 10 residues of isoleucine, 23 residues of leucine and 13 residues of valine) (Fernández et al., 2011, 2006; Schad et al., 1995). Because ATCC 6538 lacks SEs it lacks those extra BCAAs, possibly explaining why this strain has lower contents in BCAAs degradation metabolites in its exometabolome. Moreover, this strain can use the degradation of other proteins to raise its pH. More studies are needed concerning this hypothesis.

Most volatiles were detected in all three strains with exception of methyl farnesoate, which was absent in 2065 MA, and dimethyl pentasulfide, which was absent in both enterotoxic strains. Furthermore, it was possible to examine the predominance of several chemical families in each strain (Table S2, seen in the Subtotal cells; and Figure 4). The predominant families were: hydrocarbons for ATCC 6538 (non-enterotoxic); aldehydes, norisoprenoids and S-compounds for 2153 MA; acids, alcohols, esters, ketones, N-compounds and terpenes for 2065 MA.

Aromatic volatiles such as phenylacetonitrile, 2-phenylethanol or 5-methyl-2-phenyl-2-hexenal and other volatiles derived from phenylalanine had, in general, higher content in the enterotoxic strains. However, some of these volatiles, such as methylbenzene, 1,3-dimethylbenzene, naphthalene and biphenyl, might also be products of degradation of aromatic compounds present in the controls (culture medium).

Almost all volatile sulfur volatiles either originate from the AA methionine degradation, such as methanethiol and 3-(methylthio)-propanal (methional), or are derived from methanethiol, such as dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulphide, or are formed in the reaction of methanethiol with another metabolite, as for example methylthiolacetate, which is a product of methanethiol reaction with acetyl-CoA (Landaud et al., 2008). ATCC 6538 has more S-compounds originating from the degradation of methionine, an AA extremely vulnerable to oxidative stress, than the enterotoxic strains, and this might mean

that methionine sulfoxide reductases, responsible for the repair of oxidized staphylococcal proteins (Pang et al., 2014), can be less active in the ATCC 6538 strain. Some of the other volatile sulfur volatiles shown in Table S2, have been associated with bacteria while others have been described only for fungi.

4. Concluding remarks

In summary, the methodology based on HS-SPME/GC×GC-ToFMS tandem with clustering analysis provides novel data on *S. aureus* volatile exometabolome, showing higher complexity (240 volatiles) than previously reported. It consists in 10 chemical families: acids, alcohols, aldehydes, esters, hydrocarbons, ketones, terpenes, norisoprenoids, N-compounds, and S-compounds, with volatiles deriving in different metabolic pathways. Until now, no other study was able to detect and putatively identify as many volatiles as the current study for *S. aureus*. These volatiles may have origin from branched-chain amino acids and methionine degradation, pyruvate metabolism, diacetyl pathway, oxidative stress and carotenoid cleavage.

Hierarchical clustering analysis allowed the strains metabotyping, and two main clusters were observed: the first containing the 2065 MA strain and the second containing the strains ATCC 6538 and 2153 MA. Similar clustering was observed using a sub-dataset of 25 variables with high importance in projection, i.e., the VIP values. The results also allow to infer that the pathway of branched-chain amino acids is more active in the strain 2065 MA and that the pathway of methionine degradation is more active in the strains 2153 MA and ATCC 6538 Nonetheless, further studies need to be performed to confirm these findings.

Finally, it is important to point out that this is the first study of the volatile exometabolome of *S. aureus* using HS-SPME/GCxGC-ToFMS showing the difference between enterotoxic and non-enterotoxic strains. The volatiles allowing the distinction must be validated, and further work must be done using more *S. aureus* as a species and stains, and *S. aureus* in a mixture of foodborne microorganisms and also by using different culture media culture and food products.

Nonetheless, this work improves the knowledge on the *S. aureus* metabolomics which can be the basis for the development of useful methodology for staphylococcal food poisoning control.

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Figure Captions

Figure 1 - Main stages for *Staphylococcus aureus* determination of exometabolome volatile profile: growth conditions, sample preparation, metabolites extraction, GC×GC analysis and data processing. Three independent assays were performed for each strain.

Figure 2 - A) Cell viability of the three strains of *Staphylococcus aureus* under study, at 18 h of incubation (log CFU.mL-1 ± SD): ATCC 6538 (white), 2153 MA (light grey) and 2065 MA (dark grey). No significant differences were observed between strains. B) Extracellular pH values over 18 h incubation for the three strains: ATCC 6538 (black solid line with triangles), 2153 MA (light grey dashes with squares) and 2065 MA (dark dots with circles).

Figure 3 - GC×GC-ToFMS total ion chromatogram contour plot of the *Staphylococcus aureus* ATCC 6538 culture headspace volatile components. Volatiles chemical families used for statistical analysis are represented by lines and clusters. The increase in volatility (low $^{1}t_{R}$) is mainly related to the decrease in the number of carbons through the first dimension. On the other hand, an increase in the $^{2}t_{R}$ correlates to an increase in polarity through the second dimension.

Figure 4 - Hierarchical clustered heatmap visualization of the three strains of *Staphylococcus aureus* cultures headspace volatiles, organized by chemical families: ATCC 6538, 2153 MA and 2065 MA. The chromatographic area of each metabolite was normalized by $CFU.mL^{-1}$ followed by autoscaling. Each line corresponds to one metabolite and each column corresponds to each independent assay.

Figure 5 - A) Hierarchical clustered heatmap visualization of the volatiles with VIP values (Variable importance in Projection from PLS-DA) higher than 1.5 from the three strains of *Staphylococcus aureus* cultures headspace volatiles, organized by chemical families: ATCC 6538, 2153 MA and 2065 MA. The chromatographic area of each metabolite was normalized by CFU.mL-1 followed by autoscaling. Each line corresponds to one metabolite and each column corresponds to each independent assay. B) Metabolic pathways related with the VIP volatiles and their relative content in three strains of *Stapylococcus aureus* under study: S1 - ATCC 6538; S2 - 2153 MA; and S3 - 2065 MA. Relative content of metabolite is illustrated on a red (high) to blue (low) scale.

Fig 1

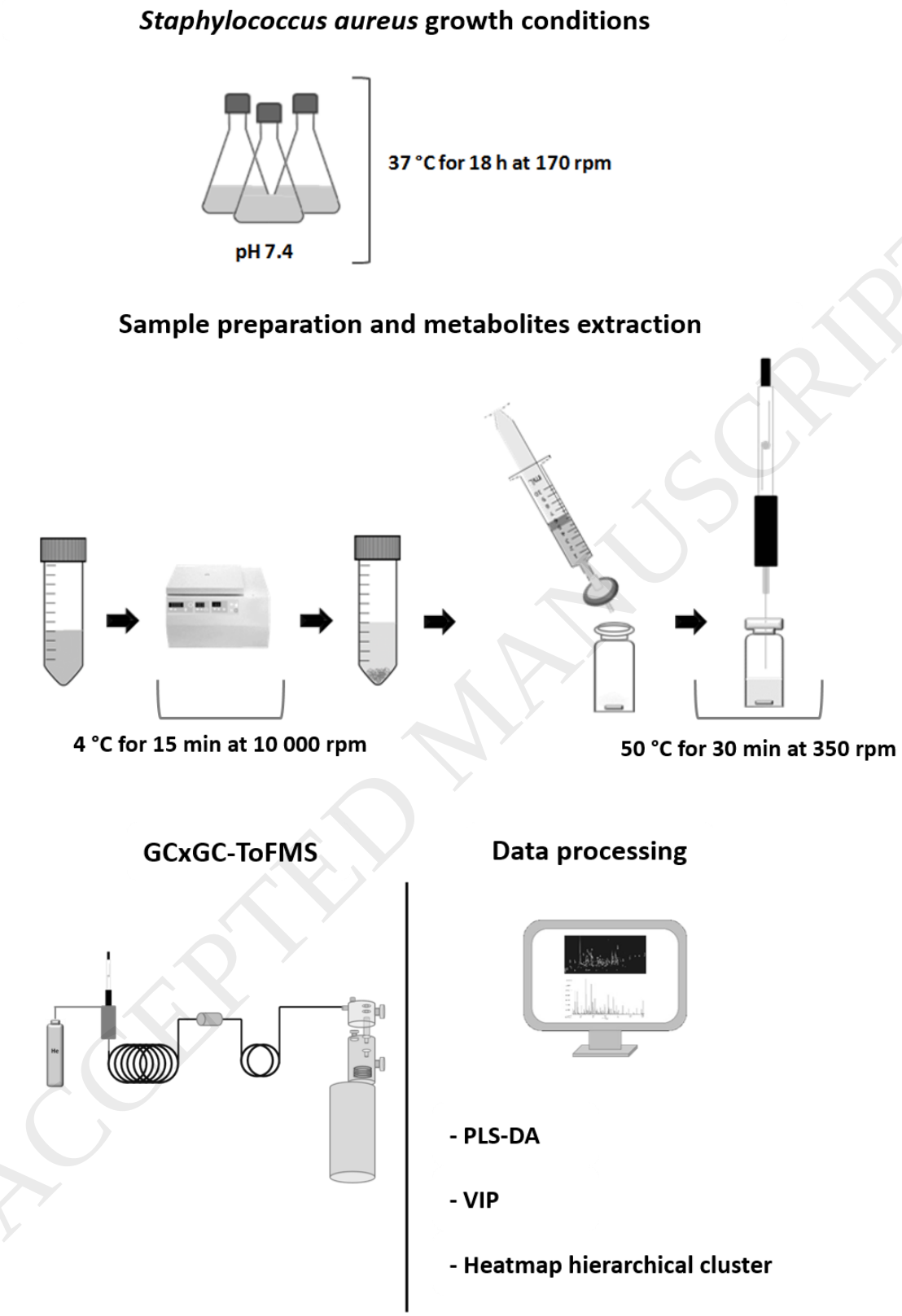


Fig 2

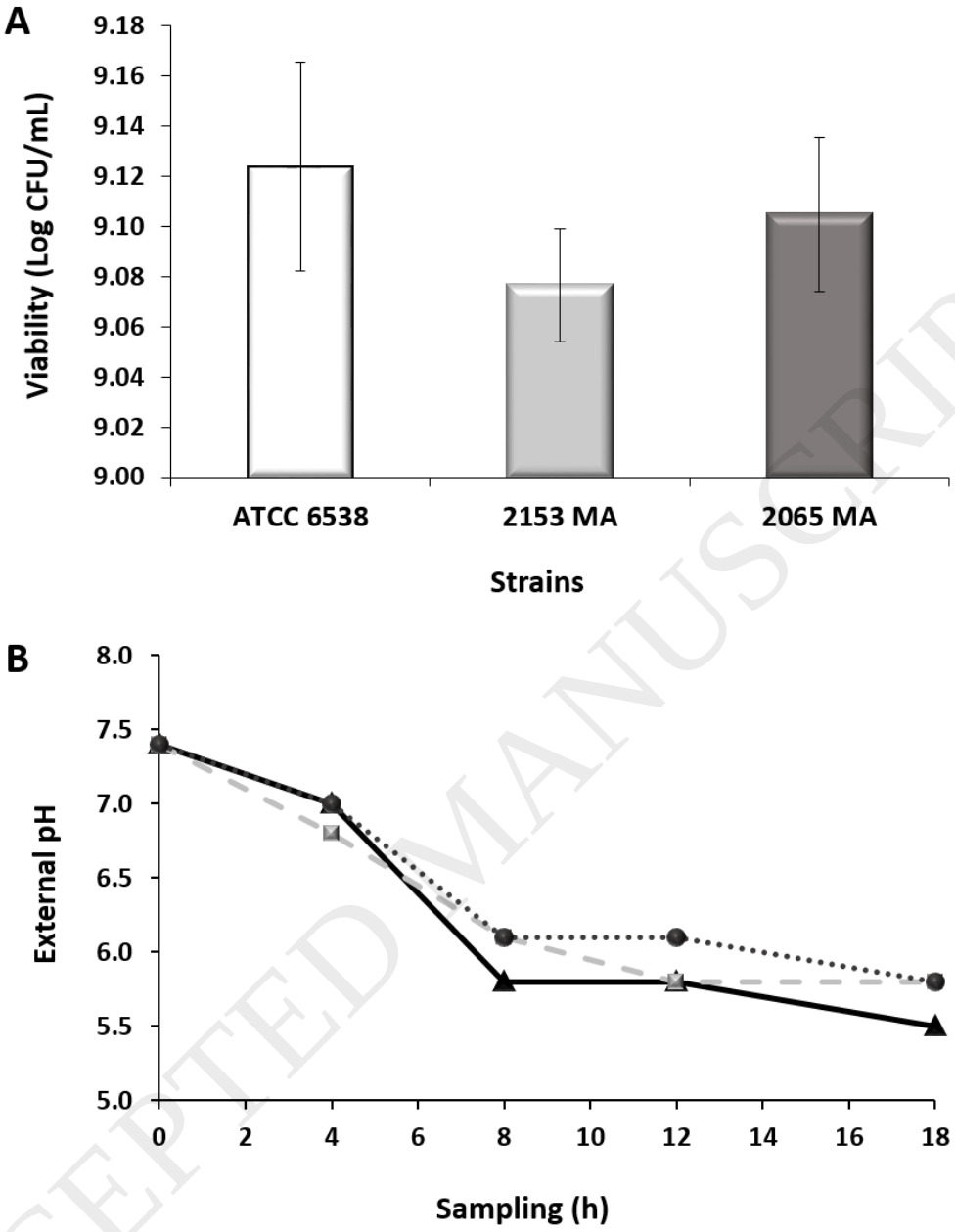


Fig 3

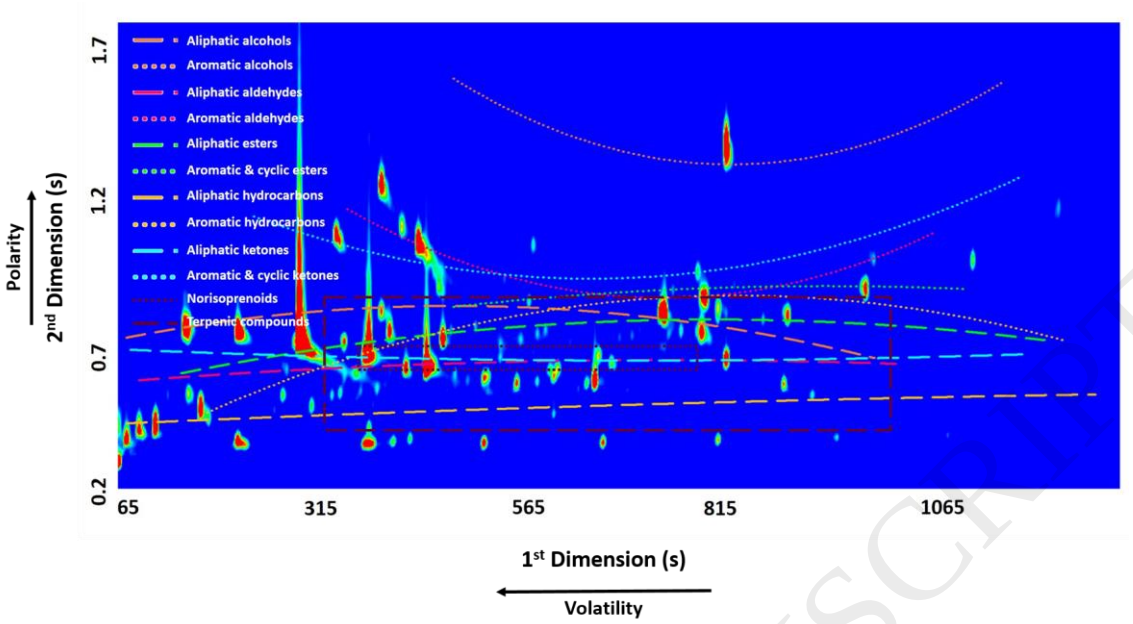
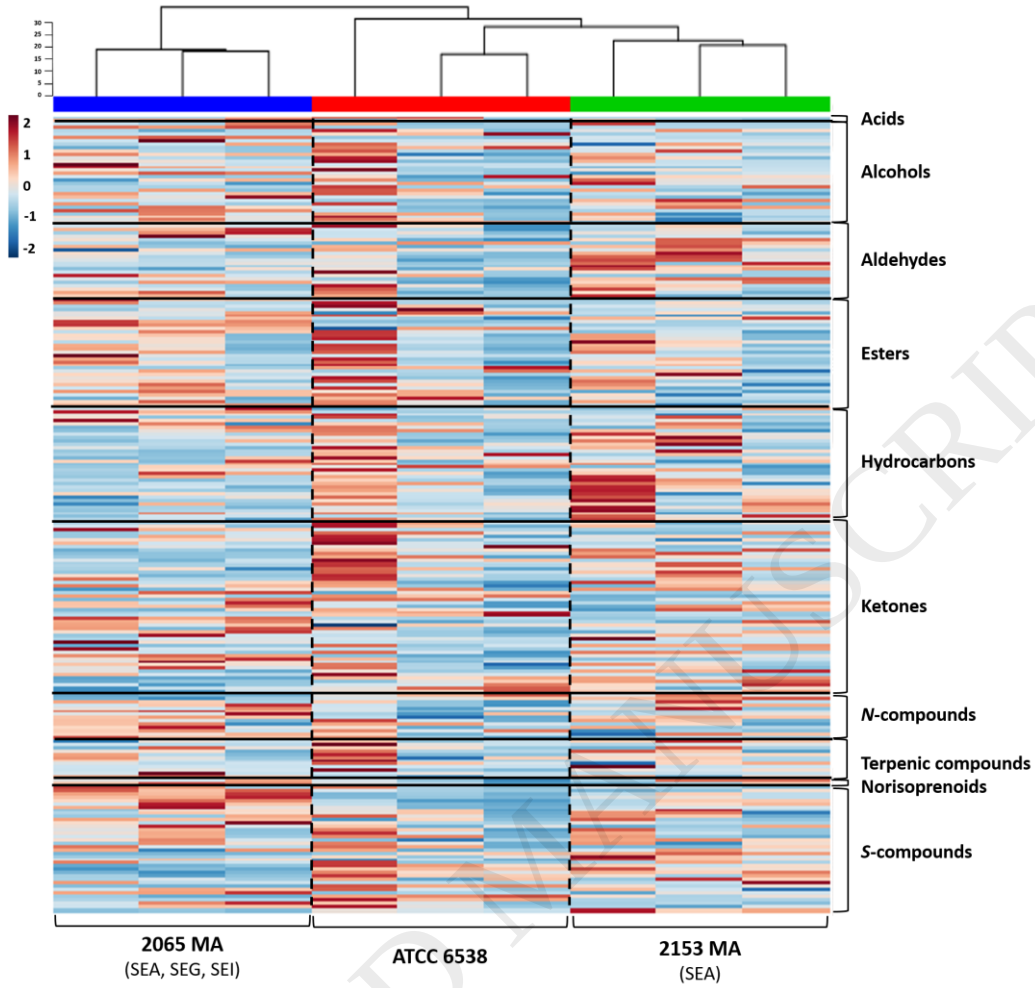
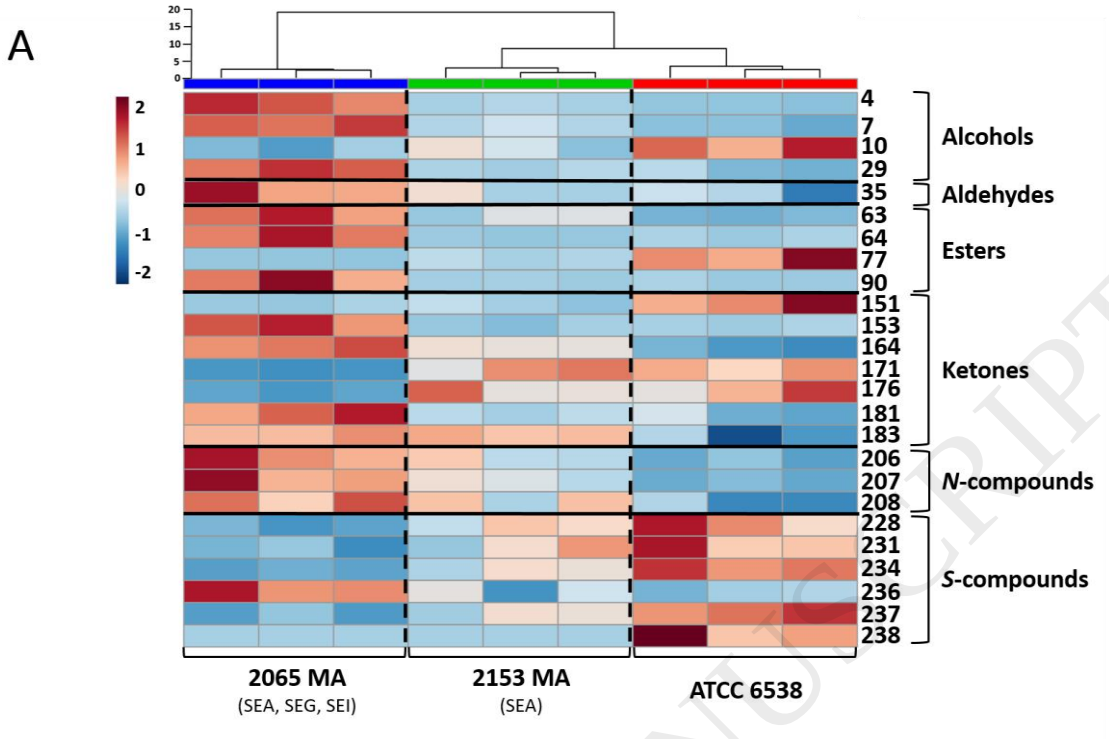


Fig 4





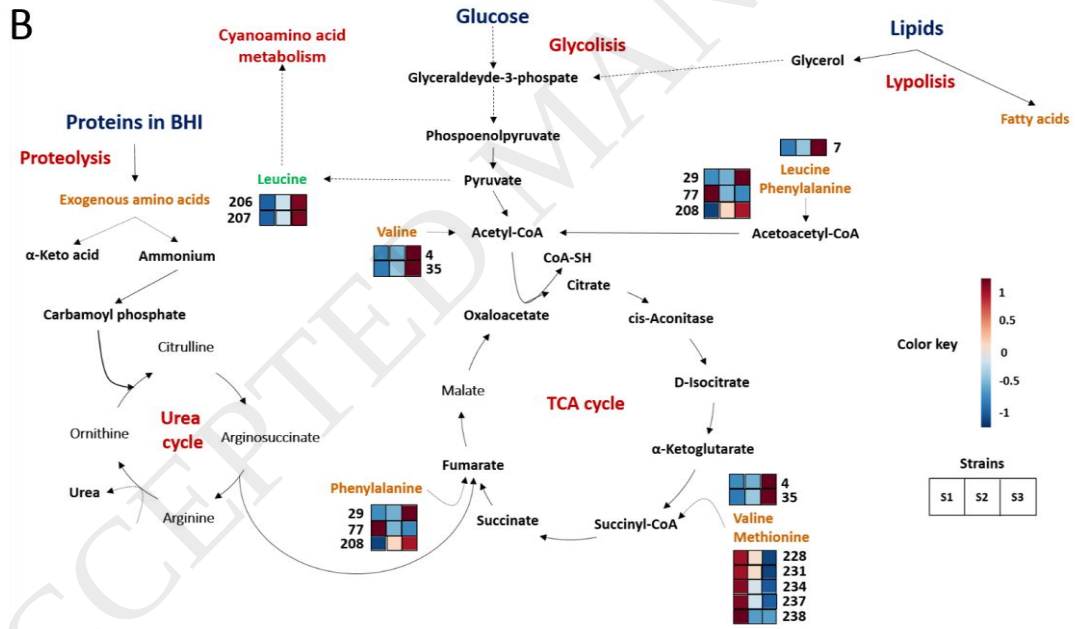


Table 1 - Sub-data set of volatiles, respective VIP values (Variable Importance in Projection) and metabolic pathways related with their origin.

Peak number	Metabolites	VIP value	Pathways	Reference
164	2-Nonanone	2.11	-	-
234	Dimethyl trisulfide	2.06	Methionine degradation	Schulz & Dickschat, 2007
237	Dimethyl tetrasulphide	2.03	Methionine degradation	-
7	3-Methylbutanol	2.00	Leucine degradation	Filipiak et al., 2012
63	3-Methyl-3-butenyl acetate	1.98	-	-
207	3-Methylbutanal oxime (isomer)	1.94	Leucine degradation	Ganjewala et al., 2010
206	3-Methylbutanal oxime (isomer)	1.94	Leucine degradation	Ganjewala et al., 2010
10	2-Methyl-3-hexanol	1.92	-	-
4	2-Methylpropanol	1.92	Valine degradation	Hazelwood et al., 2008
29	2-Phenylethanol	1.90	Phenylalanine degradation	Hazelwood et al., 2008
228	Methanethiol	1.90	Methionine degradation	Hazelwood et al., 2008
208	Benzonitrile	1.87	Phenylalanine degradtion	Hazelwood et al., 2008
181	1-Phenyl-1,2-propanedione	1.84	-	-
77	Methyl benzoate	1.82	Phenylalanine or biphenyl or toluene degradation	Møller et al., 1998
183	4-Phenyl-2-butanone	1.75	-	-
236	6-(Methylthio)hexa-1,5-dien-3-ol	1.74	-	-
35	2−Methylpropanal	1.74	Valine degradation	Hazelwood et al., 2008]
64	Methyl-2-hydroxy-4-methylpentanoate	1.74	-	-
90	Butyl cyclopropanecarboxylate	1.74	-	-
153	2,3-Heptanedione	1.74	-	-
151	3-Hydroxy-2-pentanone	1.74	-	-
171	2-Tridecanone	1.73	-	-
231	Dimethyl disulfide	1.73	Methionine degradation	Schulz & Dickschat, 2007
238	Dimethyl pentasulfide	1.69	Methionine degradation	Schulz & Dickschat, 2007
176	2-Hexadecanone	1.69	-	-