Universidade de Aveiro Departamento de Biologia
Ano 2018

Maria Inês Ramos Pilreira Baptista

Novas abordagens para controlar e detetar estirpes enterotóxicas de *Staphylococcus aureus*

New approaches to control and detect enterotoxic *Staphylococcus aureus* strains

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Adelaide Almeida, Professora Auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro, da Doutora Sílvia Rocha, Professora auxiliar do Departamento de Química e do Doutor Jorge Saraiva, Investigador Auxiliar do Departamento de Química.

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palavras-chave

Staphylococcus aureus, Processamento por alta pressão, Enterotoxinas, Intoxicação alimentar, Resistência, Recuperação, Inativação, Eficácia de redução, Metabolismo microbiano, Compostos orgânicos voláteis do exometaboloma microbiano.

resumo

Anualmente, em todo o mundo, existem 25 milhões de casos de doenças transmitidas por alimentos fazendo com que a segurança alimentar seja um tópico de grande importância. Apesar do esforço considerável para melhorar a segurança alimentar, a incidência de intoxicações e infeções transmitidas por alimentos é ainda bastante elevada. *Staphylococcus aureus* é uma bactéria patogénica oportunista que possui a capacidade de crescer em vários tipos de alimentos, bem como a capacidade de produzir um grande número de fatores de virulência, incluindo enterotoxinas, as quais são responsáveis pela intoxicação alimentar estafilocócica. Esta é uma das doenças alimentares mais predominante a nível mundial.

Com o aumento da procura de alimentos mais seguros, têm sido desenvolvidas novas tecnologias para a sua preservação. Entre essas tecnologias encontra-se o processamento por alta pressão (PAP), que é um método não térmico que possibilita a inativação ou o controlo de microrganismos patogénicos e de microrganismos responsáveis pela deterioração dos alimentos, ao mesmo tempo que mantém as propriedades dos alimentos. A deteção rápida e específica de microrganismos patogénicos (ou de microrganismos que podem provocar deterioração de alimentos) tornou-se cada vez mais importante na indústria alimentar. Atualmente, para detetar e identificar bactérias são utilizados métodos tradicionais baseados em testes bioquímicos e/ou serológicos e também em métodos moleculares baseados em análise de DNA ou RNA. No entanto, estes métodos são dispendiosos, demorados e/ou laboriosos. Consequentemente, é necessário desenvolver novos métodos alternativos como por exemplo, com base na metabolómica microbiana, que pode ser explorada como ferramenta para deteção de microrganismos na indústria alimentar. A metabolómica microbiana utiliza os metabolitos libertados pelos microrganismos, podendo permitir não apenas a deteção e distinção de espécies microbianas mas também das suas estirpes.

Um dos objetivos deste trabalho foi avaliar a eficácia do tratamento por PAP, na inativação de estirpes não enterotóxicas e enterotóxicas de *S. aureus*. Deste modo, foi 1) avaliada a eficácia dos diferentes tratamentos (diversas pressões e tempos de pressurização); 2) avaliado o impacto dos tratamentos nos fatores de virulência, na capacidade de fermentação do manitol e na suscetibilidade à meticilina; 3) avaliado o desenvolvimento de resistência após vários ciclos sucessivos de PAP, e 4) avaliada a capacidade de recuperação da viabilidade após 14 dias de tratamento. Neste trabalho também se caracterizou o exometaboloma volátil de *S. aureus* e avaliou o seu potencial para distinguir as estirpes enterotóxicas de não enterotóxica. Assim, 1) o perfil do exometaboloma volátil de *S. aureus* foi caracterizado utilizando uma técnica avançada de cromatografia em fase gasosa; 2) o perfil de *S. aureus* foi analisado no total, avaliando a presença de compostos específicos já descritos para esta espécie bem como a sua origem metabólica; 3) aplicou-se um método de análise estatística multivariada, de forma a obter um conjunto de compostos voláteis de forma a distinguir as diferentes estirpes, nomeadamente distinguir as estirpes enterotóxicas das não enterotóxicas; e 4) este conjunto de compostos voláteis foi analisado em detalhe de forma a compreender as diferenças entre estirpes e assim justificar a sua distinção.

Os resultados da inativação por PAP mostraram maior barotolerância da estirpe não enterotóxica (ATCC 6538), não sendo esta completamente inativada a 600 MPa durante 30 minutos (mantendo uma viabilidade de aproximadamente 4.0 log $UFC.mL^{-1}$). Utilizando estas condições de tratamento, as duas estirpes enterotóxicas (2153 MA e 2065 MA) foram completamente inativadas. Tanto a estirpe ATCC 6538 como a estirpe 2153 MA (com uma enterotoxina) suportaram 10 ciclos de pressurização sucessivos, contrariamente à estirpe 2065 MA (com três enterotoxinas) que foi totalmente inativada ao fim de 4 ciclos, com um decréscimo de 9.2 log $UFC.mL^{-1}$. O tratamento por PAP não afetou os fatores de virulência testados, nem a capacidade de fermentação do manitol e a suscetibilidade à meticilina de nenhuma das estirpes. Nenhuma das estirpes foi capaz de recuperar a viabilidade após 14 dias de incubação, em nenhum dos 10 ciclos de tratamento.

O estudo do exometaboloma volátil de *S. aureus* permitiu detetar 240 compostos, pertencentes a 10 famílias químicas, tendo como principal origem metabólica a degradação de aminoácidos, o metabolismo do piruvato e o stresse oxidativo. A análise em detalhe do exometaboloma volátil permitiu selecionar 10 compostos voláteis que têm sidomais frequentemente reportados noutros estudos sobre o exometaboloma volátil de *S. aureus*. Após análise estatística multivariada, foi possível distinguir as estirpes testadas com base no número (ou na ausência) de enterotoxinas. As estirpes ATCC 6538 e 2153 MA são mais similares entre si, encontrando-se separadas da estirpe 2065 MA. Esta distinção deve-se ao facto desta última estirpe produzir maiores concentrações de compostos voláteis resultantes da degradação de aminoácidos de cadeia ramificada, enquanto a estirpe ATCC 6538 produz maiores concentrações de compostos voláteis resultantes da degradação da metionina.

Em conclusão, os resultados deste estudo mostraram que o tratamento por PAP é eficaz para controlar estirpes enterotóxicas de *S. aureus*, não permitindo o desenvolvimento de resistência nem a recuperação da viabilidade após tratamentos sucessivos. Embora os fatores de virulência não tenham sido afetados pelo tratamento por PAP, as estirpes enterotóxicas foram mais facilmente inativadas quando comparadas com a estirpe não enterotóxica. Concluiu-se ainda que o exometaboloma volátil de *S. aureus* é bastante complexo e que através do estudo do exometaboloma é possível distinguir estirpes enterotóxicas de estirpes não enterotóxicas. Foi possível selecionar um conjunto de 10 compostos que poderão potencialmente vir a ser utilizados como biomarcadores da presença de *S. aureus*.

keywords

Staphylococcus aureus, High pressure processing, Enterotoxins, Staphylococcal food poisoning, Resistance, Recovery, Inactivation, Reduction effectivness, Microbial metabolomics, Volatile exometabolome, Exometabolome microbial volatile organic compounds.

abstract

With 25 million cases of foodborne diseases occurring annually worldwide, food safety is a major concern. Despite considerable efforts to improve food safety, outbreaks of foodborne diseases due to the presence of pathogenic microorganisms, such as *Staphylococcus aureus*, are well described in the literature. *S. aureus* is a well-adapted opportunistic pathogen, which is able to grow in numerous types of food, producing an extensive number virulence factors, including enterotoxins, which are responsible for staphylococcal food poisoning. This type of food poisoning is one of the most prevalent foodborne diseases in the world.

With an increased demand for safer food, new food preservation technologies have been developed. Among these technologies is high pressure processing (HPP), which is a non-thermal food preservation method that enables the inactivation or control of pathogenic microorganisms and microorganisms responsible for food spoilage, maintaining food properties.

The quick and specific detection of pathogenic microorganisms (or of microorganisms responsible for food spoilage) has become increasingly important in the food industry. Usually the detection, and identification of bacteria is performed using traditional methods based on biochemical and/or serological tests and also on molecular methods based on DNA or RNA analysis. However, these methods are costly, time consuming and laborious. Consequently, it is necessary to develop new alternative methods, such as those based on microbial metabolomics, which can be used as a tool to detect microorganisms in the food industry. Microbial metabolomics uses the metabolites released by microorganisms, being able to allow not only the detection and distinction of microbial species, but also the distinction of their strains. One of the objectives of this work was to evaluate the efficacy of HPP treatment for the inactivation of non-enterotoxic and enterotoxic *S. aureus* strains. Thus, to accomplish this objective, 1) the effectiveness of different treatments (different pressures and holding times were evaluated); 2) the impact of the treatments on virulence factors, fermentation of mannitol and methicillin susceptibility was evaluated; 3) the development of resistance along several successive HPP cycles was evaluated, and 4) the recovery capacity after 14 days of treatment was also assessed. Other objective of this study was to characterize the volatile exometabolome of *S. aureus* and evaluate its potential to distinguish the enterotoxic strains from the non-enterotoxic strain. For this purpose, 1) the profile of the volatile exometabolome of *S. aureus* was characterized using an advanced gas chromatography technique; 2) the *S. aureus* profile was analyzed as a whole and evaluated the presence of specific compounds already described for this species as well as its metabolic origin; 3) a multivariate statistical analysis method was applied in order to obtain a set of volatile compounds responsible for the distinction of the three strains used; and 4) these set of volatile compounds were analyzed in detail in order to explain the differences between strains, thus justifying their separation.

The results of inactivation by HPP showed a higher barotolerance of the non-enterotoxic strain (ATCC 6538), not being completely inactivated at 600 MPa for 30 minutes (maintaining a viability of approximately 4 Log CFU.mL^{-1}). The two enterotoxic strains (2153 MA and 2065 MA) were completely inactivated using these treatment conditions. Both strains ATCC 6538 and 2153 MA (with an enterotoxin) were able to withstand 10 successive pressurization cycles, whereas the strain 2065 MA (with three enterotoxins) was completely inactivated after 4 cycles, with a decrease of 9.2 log CFU.mL^{-1}. The HPP treatment did not affect none of the tested virulence factors, the mannitol fermentation ability and methicillin susceptibility of any of the strains. Moreover, none of the strains were able to recover their viability after 14 days of incubation in any of the treatment cycles.

The study of the volatile exometabolome of *S. aureus* allowed the detection of 240 volatile organic compounds, belonging to 10 chemical families, having as main metabolic origins the degradation of amino acids, the metabolism of pyruvate and oxidative stress. It was also possible to find 10 of the most reported volatile compounds in studies concerning the volatile exometabolome of *S. aureus*. The detailed analysis of the volatile exometabolome allowed selecting 10 volatile compounds that have been reported more frequently in other studies concerning the volatile exometaboloma of *S. aureus*. The multivariate statistical analysis, allowed to distinguish the strains based on the number (or absence) of enterotoxins. The strains ATCC 6538 and 2153 MA are more similar to each other, being separated from the strain 2065 MA. This distinction is due to the latter strain has larger amounts of volatile compounds, resulting from the degradation of branched-chain amino acids, while the strain ATCC 6538 showed higher amounts of volatile compounds with origin in the degradation of methionine.

In conclusion, the results showed that HPP is effective in the control of *S. aureus*, not allowing the development of resistance or recovery of viability after successive treatments. Although the virulence factors were not affected by HPP treatment, the enterotoxic strains were more easily inactivated than the non-enterotoxic strain. It was also concluded that the volatile exometabolome of *S. aureus* is quite complex and that the exometabolome analysis allows to distinguish enterotoxic strains from non-enterotoxic strains. It was possible to select a set of 10 compounds that can be potentially used as biomarkers of *S. aureus*.

"The saddest aspect of life right now is that science gathers knowledge faster than society gathers wisdom."

Isaac Asimov

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PUBLICATIONS

Papers in peer reviewed journals (3)

I Baptista, RP Queirós., Â Cunha, SM Rocha, JA Saraiva, A Almeida, *Evaluation of resistance development and viability recovery by enterotoxic and non-enterotoxic Staphylococcus aureus strains after repeated cycles of high hydrostatic pressure*, Food Microbiology, 2015, 46, 515–520.

I Baptista, RP Queirós., Â Cunha, SM Rocha, JA Saraiva, A Almeida, *Inactivation of enterotoxic and non-enterotoxic Staphylococcus aureus strains by high pressure treatments and evaluation of its impact on virulence factors*. Food Control, 2015, 57, 252–257.

I Baptista, SM Rocha, Â Cunha, JA Saraiva, A Almeida, *Inactivation of Staphylococcus aureus by high pressure processing: An overview.* Innovative Food Science & Emerging Technologies, 2016, 36, 128–149.

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Poster communications (3)

I Baptista, RP Queirós., Â Cunha, SM Rocha, JA Saraiva, A Almeida, *High pressure processing in the inactivation of enterotoxic bacterial strains,* FEMS 2013 – 5th Congress of European Microbiologists; 21-25 July, 2013; Leipzig, Germany; P1221;

I Baptista, RP Queirós., Â Cunha, SM Rocha, JA Saraiva, A Almeida, *High pressure processing in the inactivation of enterotoxic Staphylococcus aureus,* MicroBiotec'13 – Portuguese Congress of Microbiology and Biotecnology; 6-8 December, Aveiro, P041.

I Baptista, RP Queirós., Â Cunha, SM Rocha, JA Saraiva, A Almeida, *Evaluation of resistance development and viability recovery by enterotoxic and nonenterotoxic Staphylococcus aureus strains after repeated cycles of high hydrostatic pressure,* TEMPH2014 – Trends in Environmental Microbiology for Public Health; 18-21 September, Lisboa, P2-4.

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ABBREVIATIONS AND SYMBOLS

AA	Amino acid
ANOVA	Analysis of variance
a_w	Water activity
BA	Blood Agar
BCAA	Branched-chain amino acid
BHI	Brain heart infusion broth
BPA	Baird-Parker Agar
CA-MRSA	Community-Associated Methicillin-Resistant Staphylococcus Aureus
CDC	Centers for Disease Control and Prevention
CDM	Chemically defined medium
CFU	Colony-forming unit
DNA	Deoxyribonucleic acid
EIA	Enzyme immunoassay
EFSA	European Food Safety Authority
ECDC	European Centre for Disease Prevention and Control
GC	One-dimensional gas chromatography
GC × GC-ToFMS	Comprehensive two-dimensional gas chromatography with time of flight mass spectrometry detection
GC-FID	Gas Chromatography – flame Ionization detector
HCH	Hierarchical Clustering Heatmaps
HPP	High pressure processing
HS	Headspace
LC	Liquid chromatography
MCC-IMS	Multi capillary column—ion mobility spectrometry
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MSA	Mannitol salt agar
MSE	Mean square error
MSI	Metabolomics standards initiative
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MVOC	Microbial Volatile Organic Compounds
NB	Nutrient Broth
NMR	Nuclear magnetic resonance spectroscopy
PBS	Phosphate-buffered saline
PC	Principal component
PCA_m	Plate count agar medium

PCA	Principal component analysis
PI	Propidium iodide
PLS-DA	Partial Least Square Discriminant Analysis
PTR-MS	Proton Transfer Reaction – mass Spectrometry
Q2	Quality-of-fit criterion
RNA	Ribonucleic acid
SE	Staphylococcal enterotoxins
SE*l*	Staphylococcal like enterotoxins
SEM	Scanning electron microscopy
SESI-MS	Secondary electrospray ionization-mass spectrometry
SFP	Staphylococcal food poisoning
SIFT-MS	Selected-ion flow-tube mass spectrometry
SPME	Solid-phase microextraction
TEM	Transmission electron microscopy
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UV	Ultra-violet
VAP	Ventilator-associated Pneumonia
VFA	Volatile fatty acids
VIP	Variable Importance in Projection
VISA	Vancomycin Intermediate Staphylococcus Aureus
VBNC	Viable but nonculturable
VRSA	Vancomycin resistant Staphylococcus aureus
WHO	World Health Organization
WRT	Weight randomization test
σ^B	Sigma B

THESIS OUTLINE AND OBJECTIVES

Thesis outline

Gastrointestinal diseases are responsible for 25 million infections each year, with diarrheal diseases as the second leading cause of death in the world (1). In fact, foodborne diseases, along with waterborne diseases, are responsible for the death of approximately 1.8 million people every year, most of whom are children, and accountable for economic losses in food industry, health systems, tourism and also for the consumer (2). Sets of norms and rules have been developed over the years, in order to guarantee safe food for the consumer. Although food contamination with foodborne pathogens might be avoided through the development and implementation of such procedures, food preservation methods are extremely important to control food contamination by pathogenic microorganisms and also to control microorganisms responsible for food deterioration. These methods have been used since antiquity and include heating, salting, freezing, drying, freeze-drying, irradiation, fermentation, canning, the addition of antimicrobials and chemicals and more recently, ionization radiation, pulsed electric fields, ultra-violet (UV) decontamination, pulsed high intensity light, high intensity laser, pulsed white light and high pressure processing (HPP) (3). Nowadays HPP progressively appears as a commercially viable alternative food preservation method in order to answer consumer demand for safes but also fresher and nutritious food (4,5).

Despite considerable efforts to improve food safety, foodborne diseases outbreaks linked to the presence of pathogenic bacteria at harmful levels in food has been well documented, highlighting the need for technological interventions in order to address food safety risk posed by these pathogens in the final food products. Among them, is *Staphylococcus aureus*, which is responsible for one of the main foodborne diseases, staphylococcal food poisoning (SFP) (6). *S. aureus* is an opportunistic pathogen, able to grow in many types of food, that produces a wide number virulence factors and is able to develop continuously resistance features, turning it into a well succeed foodborne pathogen (7). Furthermore, *S. aureus* is well known for quickly developing resistance to antibiotics, from penicillin to methicillin-resistant *S. aureus* (MRSA) strains, passing through vancomycin-intermediate *S. aureus* (VISA) strains to community acquired MRSA, until vancomcyin-resistant *S. aureus* (VRSA) strains (8), thus being melodramatically called a superbug by non-scientific (and also some scientific) press, which is mainly a multidrug resistant bacteria (9,10)

Although many efforts concerning the prevention of *S. aureus* in foodstuff, either by respecting the norms or using food preservation methods, there is still a high probability that food might be contaminated by this pathogen. The conventional methods for the detection of non-enterotoxic and enterotoxic strains of *S. aureus* both in foodstuff and in clinical samples,

are time consuming and not always sensitive, thus the need for a method which is able to distinguish the two types of strains as quickly as possible, either MRSA from MSSA, VISA from VRSA or even enterotoxic from non-enterotoxic strains, arises. Microbial metabolomics, using microbial volatile organic compounds (MVOC) or, as it will be referred over this document, volatiles, resorting to headspace (HS) solid phase microextraction (SPME) combined with comprehensive two-dimensional gas chromatography with time of flight mass spectrometry detection (HS-SPME/GC × GC-ToFMS) (11,12), has the potential to achieve such a distinction.

In order to reduce the risk of SFP, it is essential to develop alternative food processing methods, such as HPP, and an alternative *S. aureus* detection/distinction method, such as HS-SPME/GC × GC-ToFMS.

The present PhD thesis is organized in six chapters, according to the organizational sequence illustrated in Figure 1.

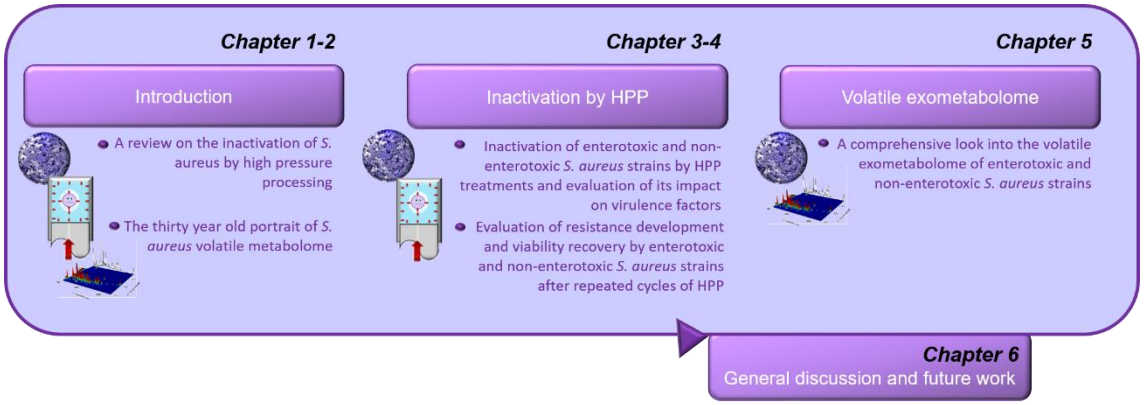


Figure i -PhD thesis workflow: from the introduction, to the work performed with HPP, to the assessment of the volatile exometabolome until the integration of the work performed with concluding remarks and future work.

Chapter 1 presents an in-depth literature review of *S. aureus*, SFP and HPP. The first part of this chapter describes the characteristics of *S. aureus*, its importance as an etiological foodborne opportunistic pathogen, the many diseases it causes, including the description of SFP and the chemical and genetic features of enterotoxins (SE). The second part describes HPP as an alternative food preservation method with the development of the factors conditioning the effectiveness of HPP in *S. aureus*. This chapter was published in the journal Innovative Food Science & Emerging Technologies with the DOI: 10.1016/j.ifset.2016.06.008

Chapter 2 offers an insight into the world of volatile microbial metabolomics of *S. aureus*. This chapter makes a small introduction to concepts related with metabolomics, focusing mostly on the concept of microbial metabolomics. Furthermore, it takes a look into the 30 year old picture of *S. aureus* volatile metabolome, including the volatiles reported over the years as well as their metabolic origin. Finally, with the data achieved from beholding such picture, a

suggestion of a possible volatile pattern of biomarkers that characterizes *S. aureus* is done. This chapter is under preparation to be submitted for publication.

Chapter 3 evaluates the efficiency of different HPP treatments in the inactivation of the three *S. aureus* strains (one non-enterotoxic and two enterotoxic) and assesses the impact of HPP on virulence factors. The results revealed that the non-enterotoxic strain was more baroresistant than the two enterotoxic strains and that none of the tested virulence factors was affected by HPP. Factors that probably explain these results are presented. This chapter was published in the journal Food Control with the following DOI: 10.1016/j.foodcont.2015.04.022

Chapter 4 reports the development of resistance and the recovery of viability of the three previously used *S. aureus* strains after several cycles of HPP. The strains were submitted to ten consecutive HPP cycles using the survivors of the previous cycle, and their viability was assessed in every cycle. Furthermore, to evaluate if the strains were able to recover from the HPP cycles, colonies were counted up to fourteen days. The results revealed that two of the strains were able to survive over the ten cycles while one was completely inactivated in the fourth cycle. The results also showed that none of the strains was able to recover from the HPP treatment even after fourteen days. This chapter was published in the journal Food Microbiology with the following DOI: 10.1016/j.fm.2014.09.016

Chapter 5 aimed to profile the volatile exometabolome of the same *S. aureus* strains and to detect if differences between the non-enterotoxic strain and the enterotoxic strains existed. The exometabolome profile comprised 240 volatiles belonging to several chemical families, which were putatively identified, making this study the first ever reporting such a high number of volatiles for *S. aureus*. The volatiles found had mainly origin in amino acid (AA) degradation pathways and were also linked to oxidative stress. Furthermore, using variable importance in projection (VIP), achieved from partial least square discriminant analysis (PLS-DA), it was possible to create a hierarchical heatmap cluster (HHC) showing the distinction between strains accordingly to the number of SE. This chapter is also under preparation for submission.

Chapter 6 integrates the key points of this work and highlights the main conclusions of both HPP in *S. aureus* and its volatile exometabolome, as well as the limitations of this work. In line with these conclusions, perspectives for future work, since *S. aureus* is such a major opportunistic pathogen with much research needed not only in the area of food microbiology but also in clinical microbiology, in order to answer the many pendent questions.

Objectives

The main objective of this PhD thesis was to apply HPP treatments into three different strains of *S. aureus* (one non-enterotoxic, one with one SE and one with three SE) and, by using microbial metabolomics, to characterize the volatile exometabolome of the same strains. Under this framework, the particular objectives can be summarized as:

• To evaluate the impact of HPP of different treatments on non-enterotoxic and enterotoxic strains of *S. aureus*.

• To assess the effect of HPP treatments on non-enterotoxic and enterotoxic strains of *S. aureus* virulence factors. The ability to ferment mannitol and susceptibility to methicillin were also evaluated.

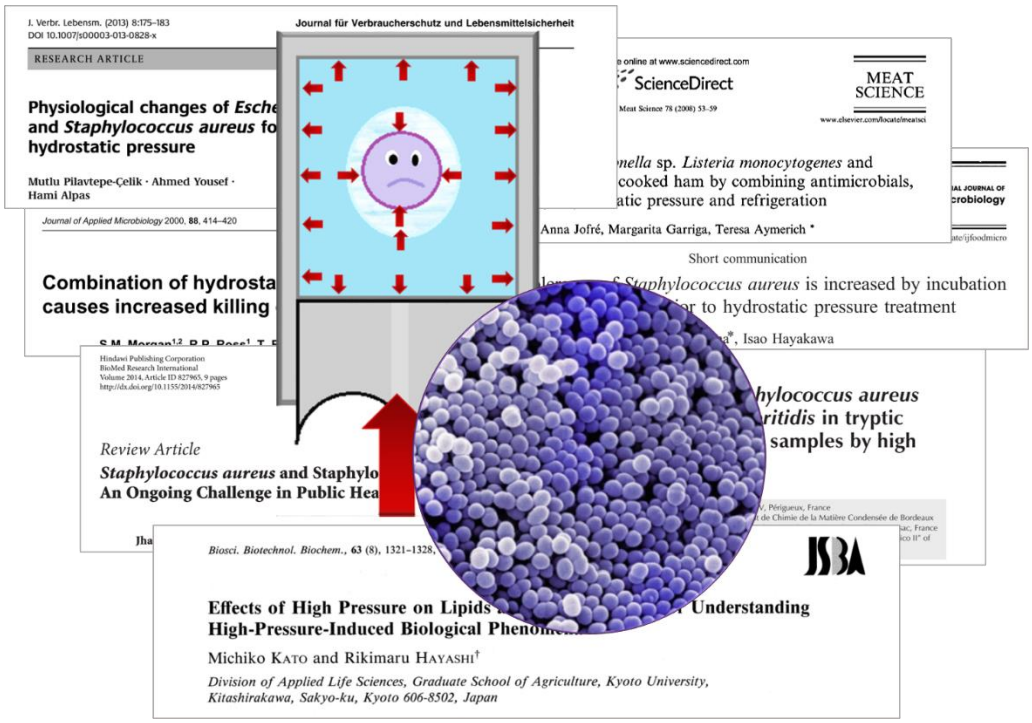
• To evaluate the endurance of non-enterotoxic and enterotoxic strains of *S. aureus* to consecutive cycles of HPP treatments and to determine if these strains recover their viability.

• To footprint the non-enterotoxic and enterotoxic strains of *S. aureus* through their volatile exometabolome, using a metabolomics-based strategy employing HS-SPME/GC × GC-ToFMS.

• To detect, via the volatile exometabolome, if the non-enterotoxic strain of *S. aureus* can be distinguished from and enterotoxic strains of *S. aureus*.

CHAPTER 1. Introduction

A review on the inactivation of *Staphylococcus aureus* by high pressure processing



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Context

Food safety is a major concern for consumers, food industry, health systems and governments, with 25 million foodborne diseases occurring annually worldwide (1). *S. aureus*, is an extremely versatile opportunistic pathogen being responsible for SFP due to its enterotoxic strains (7). With increasing demands for safer food, new food preservation technologies are increasingly gaining interest. In the last two decades, HPP appeared as an alternative non-thermal food preservation method promoting inactivation of some spoilage and pathogenic microorganisms, while maintaining food characteristics (4,5).

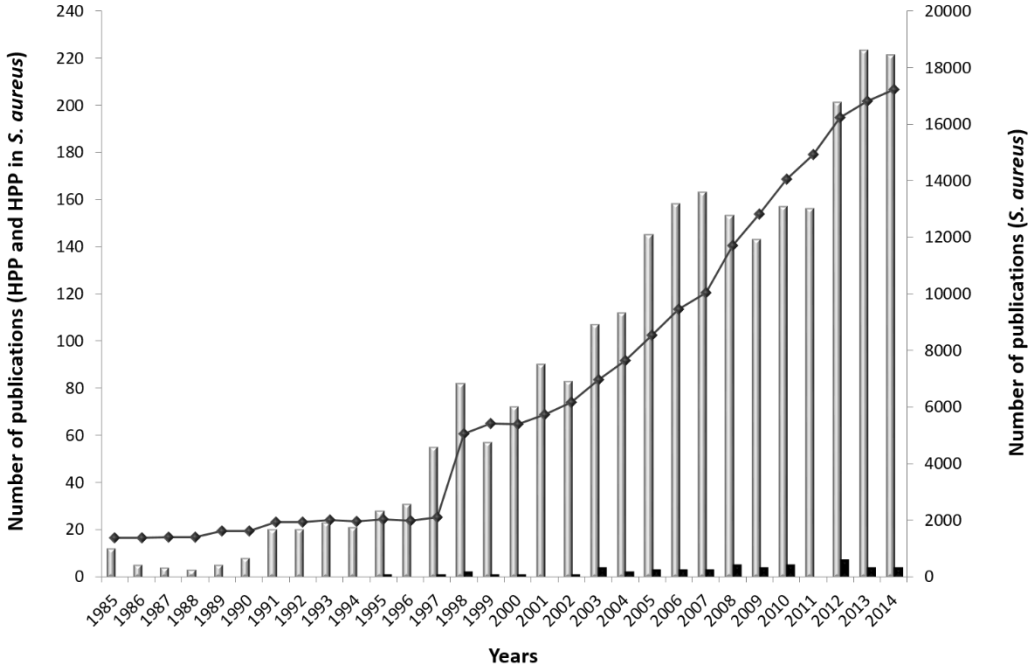


Figure 1.1 - Literature survey of published research articles using a search query in the field topic with the keywords "*Staphylococcus aureus*" for *Staphylococcus aureus* publications (dark grey line; secondary y axis), "high pressure processing" or "high hydrostatic pressure" for HPP publications (light grey bars) and "high pressure processing" or "high hydrostatic pressure" in combination with "*Staphylococcus aureus*" for *Staphylococcus aureus* inactivation by HPP publications (black bars) in topic; from 1985 to 2014 via Web of Science™.

HPP has been tested in several studies to inactivate this bacterium, and results show that *S. aureus* is one of the most barotolerant species to HPP. This bacterial species has been extensively studied from 1985 to 2014 (Figure 1.1), with the number of publications being above 7.000 per year in the last decade. Although the number of publications for HPP alone (Figure 1.1) is not as high as the number of publications for *S. aureus* alone, there is a significant amount of knowledge concerning the effects of HPP on food matrices, food components (such as proteins, enzymes and lipids) and on the inactivation of microorganisms and their ability to cope with high pressures, with the number of publications above 200 per year since 2012. However, there are specifically 57 publications concerning the effect of HPP on *S. aureus* (Figure 1.1), with

highest number of publications in 2012 (7 publications). Thus, this knowledge should not only be increased but also expanded to the food industry in order to establish effective HPP protocols for the inactivation of several strains of *S. aureus* and to different food matrices.

In this chapter, *S. aureus* biology will be approached as well as the principles of HPP. Factors that modulate HPP efficiency will be revised, firstly based on the state-of-the art described for bacteria in general and afterwards, when studies exist, for *S. aureus* specifically.

1.1. *Staphylococcus aureus*

Staphylococci are Gram-positive bacteria widely distributed in the environment, which can reside on the skin and mucosal surfaces of humans and other animals (13). The genus Staphylococcus contains at least 49 species, several of which are not only clinically significant but are also important for the food industry, agriculture and economy (14). The most pathogenic of these species is *S. aureus* (15). *S. aureus* is an exceptionally well adapted opportunistic pathogen that can survive under different conditions, with no particular nutritional or environmental requirements (16–18). This microorganism is able to withstand several months on items and surfaces and is capable to cause a large range of diseases in humans and animals (19–21).

Over the years, *S. aureus* infections have highly increased in individuals, being currently one of the main causes of human bacterial infections worldwide, with MRSA strains emerging as important etiological agents of infection not only inside (hospital acquired MRSA) but also outside of healthcare settings (community-associated MRSA) and even in a large diversity of animals (8,22–27). More recently animals have been pointed as important sources of human infections with MRSA strains (28–31) and the ability for these strains to colonize humans and for human *S. aureus* strains to colonize animals have been described (22,32–34). In fact, the presence of MRSA strains in meat products has been reported recently (35,36).

S. aureus possesses several characteristics such as its quorum sensing mechanisms and virulence factors (Table 1.1) that enable it to cause a large range of diseases (36–38). It produces a vast diversity of exoproteins (such as hemolysins, nucleases, proteases, lipases, hyaluronidase and collagenase) which grant the ability to colonize host tissues and also other exoproteins that contribute to its capacity to cause disease and inhibit the host immune responses (such as toxic shock syndrome toxin-1, SE, exfoliative toxins and leukocidin) (39,40). The genes encoding these virulence factors are regulated in a coordinated mode, revealing the strategy of this pathogen to initially establish itself in the host and, afterwards, to cause the disease (41).

1.1.1. *Staphylococcus aureus* as an etiological opportunistic pathogen

S. aureus is a ubiquitous commensal symbiont and colonizer of both humans and animals (16,25). In humans, the anterior nares of the nose are the main ecological niches of *S. aureus*

Table 1.1 - *Staphylococcus aureus* virulence factors and their function in colonizing and evading the immune responses of the host (42–47).

Virulence factor	Putative function
Cell surface factors	
Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)	Family of proteins on the basis of structural similarities and a common mechanism for ligand binding, which is mediated by two adjacent subdomains containing IgG-like folds
Staphylococcal protein A	Interference with optimization and phagocytosis, through the binding to Fc portion of the immunoglobulin
Fibronectin-binding proteins	Promotes bacterial attachment to fibronectin and to plasma clots, the adhesion to extracellular matrix and supports the invasion
Collagen-binding protein	Promotes the adherence to collagenous tissues and cartilage and prevents the classical pathway of complement activation
Clumping factor proteins	Adhesion to immobilized fibrinogen, immune evasion by binding soluble fibrinogen, degradation of C3b, adhesion to desquamated epithelial cells and nasal colonization
Serine–aspartate repeat protein	Involved in immune invasion and very likely in nasal colonization and promotes the degradation of C3b
Bone sialoprotein-binding protein	Promotes the adhesion to extracellular matrix
Capsular polysaccharides	Promotes the reduction of phagocytosis, bacterial colonization and its persistence on mucosal surfaces
Staphyloxanthin	Carotenoid pigment that confers protection against reactive oxygen species (ROS) such as O_2^-, H_2O_2 and HOCl generated by host neutrophils
Near iron transporter (NEAT) motif proteins	Promote haem capture from haemoglobin and help bacteria to survive in the host, where iron is restricted
Iron-regulated surface protein	Promotes haem uptake and iron acquisition, the adhesion to desquamated epithelial cells, the resistance to lactoferrin, the Invasion of non-phagocytic cells and accelerates the degradation of C3b
Protein A	Promotes the inhibition of opsonophagocytosis, B cell superantigen, inflammation, endovascular infection and endocarditis
S. aureus surface protein G and plasmin-sensitive surface protein	Promotes the adhesion to desquamated epithelial cells and the formation of biofilm
Adenosine synthase A	Promotes the survival inside neutrophils
S. aureus surface protein X	Promotes biofilm formation, cell aggregation and squamous cell adhesion
Serine-rich adhesin for platelets	Promotes endocarditis and endovascular infection
S. aureus surface protein C	Promotes primary attachment and accumulation phases of biofilm formation
Biofilm-associated protein (present only in bovine strains)	Promotes biofilm formation, prevents invasion of mammary gland epithelial cells and promotes aggregation on epithelial cell surfaces.
Secreted factors	
Superantigens	Promote the proliferation of large quantities of T cells causing a massive cytokine release, interfering with the host immune system by aiming the innate and adaptive responses
Staphylococcal enterotoxins	Induces emesis and gastroenteritis
Toxic shock syndrome toxin-1	Acts on the vascular system by causing inflammation, fever, and shock.
Cytolytic toxins	Promote the development of β-barrel pores in the plasma membrane and cause leakage of the cell's content and lysis of the target cell
α-hemolysin	Is inserted into in the eukaryotic membrane and oligomerizes into a β-barrel that forms a pore causing osmotic cytolysis (is mainly cytolytic to human platelets and monocytes)
β-hemolysin	Promotes hydrolyzation of sphingomyelin of the host cell plasma membrane, producing phosphocholine and ceramide
γ-hemolysin	Promotes lysis in both erythrocytes and leukocytes due to the formation of pore, by binding to the cell surface
Leucocidins E/D and M/F-PV	Promotes lysis in leukocytes due to its pore forming activity
Panton-Valentine Leucocidin	Promotes lysis in leukocytes due to its pore forming activity
Catalase	Promotes the inactivation of toxic hydrogen peroxide and free radicals produced inside phagocytic cells
Coagulase	Extracellular protein that stimulates the conversion of fibrinogen to fibrin, causing the formation of clots in mammalian plasma
Lipases	Promote lipid digestion, allowing it to grow on skin surfaces and cutaneous oil glands and biofilm formation
Proteases	Promotes the inactivation of host-derived inhibitors, modulate bacterial adhesion and cell surface proteins, acquire nutrients and spread through tissues

Nucleases	Promotes the degradation nucleic acid of the host, the evasion of neutrophil extracellular traps, the inhibition of biofilm formation through the cleavage of extracellular DNA and the prevention of biofilm initiation
Hyaluronidase	Promotes the hydrolyzes of hyaluronic acid, helping the spread through the tissues
Lecithinase	Promotes the hydrolyzes of lecithin (phospholipid), causing cell lyses
Saphylokinase	Activates plasminogen, facilitating the dissolution of the fibrin clot and interacts with the human neutrophil peptides (α—defensins) enhancing bacterial resistance to phagocytosis
Staphylococcal complement inhibitor	Binds to the C3 convertases, inhibiting their activity on the bacterial surface, thus preventing phagocytosis
Extracellular fibrinogen-binding protein	Binds to fibrinogen, interferes with platelet aggregation and also binds to C3b, preventing it to bind to activator surfaces thus inhibiting the complement-mediated lysis and opsonophagocytosis
Chemotaxis inhibitory protein of *S. aureus*	Promotes the inhibition of neutrophil and monocyte chemotaxis
S. aureus formyl peptide receptor-like 1 Inhibitor	Binds directly to the formyl peptide receptor-like 1 promoting the inhibition of specific ligands acting as an antagonist
Extracellular adherence protein	Inhibits neutrophil recruitment by interacting directly with the host adhesive proteins intercellular adhesion molecule 1, fibrinogen or vitronectin

(colonization mainly happens due to the hands when these touch contaminated surfaces or, in a much lower frequency, directly through the air) although other sites, such as the skin, perineal area, pharynx, gastrointestinal tract, vagina and the axillae might also be colonized (21,48).

There are two types of *S. aureus* nasal carriers in humans: persistent carriers (20% of individuals) and intermittent carriers (nearly 60% of individuals) (49,50); with the remaining percentage being non carriers (21). However, the reason why some individuals are non-carriers while others are persistent carriers still remains unclear (40), but studies point towards a set of factors belonging to both host and bacteria (25).Nasal carriage depends on a wide assortment of features that depend not only on availability and usage of bacterial colonization factors but also of the susceptibility, resistance and other features of the host such as genetics, sex, age, hormonal status (in women) and anatomic alterations of the nares (25,48). An interesting fact is that the population structures of nasal *S. aureus* are very similar all over the world (25). Nasal carriage is associated with most *S. aureus* infections, being the frequency of infections for carriers higher (although less severe) than for non-carriers, with *S. aureus* diseases in this last group caused mainly by the ingestion of contaminated food with enterotoxic strains of *S. aureus*. In fact*, S. aureus* has successfully colonized millions of humans (25). Furthermore, it has been described that a commensal strain can turn into a pathogenic strain through a change of state, rather than the existence of two separate strains, and this “new” strain will face an extremely severe host defense system (17,51).

When the host's body suffers some sort of disorder, *S. aureus* might become pathogenic (16,25). As a result, this bacteria is able to cause a wide range of infections in humans and other animals, including folliculitis, furunculosis, boils, impetigo, cellulitis, sepsis, deep abscesses, necrotizing pneumonia, osteomyelitis, infective endocarditis, urinary tract infections and infections of the central nervous system, and also toxic shock syndrome and SFP (21,25,41).

1.1.2. *Staphylococcus aureus* as a foodborne pathogen

SFP is caused by huge growth of *S. aureus* strains that are capable of producing SE (52). However, SFP is not directly caused by staphylococcal cells but by its SE (7), making this foodborne disease a food poisoning rather than a foodborne infection (like the ones caused by *Salmonella* spp.).

S. aureus has the ability to grow in an extensive array of temperatures, pH and NaCl concentrations, which allows its growth in many different sorts of foods, such meat (mainly poultry) and meat products, milk and dairy products, fermented food, vegetables, fish products, salted food products (e.g., ham), egg products, bakery products, sandwich fillings, among others (18,50,53). A main concern related with meat is the fact that most raw poultry meat (both fresh and frozen) are significantly contaminated with *S. aureus* (54,55) since the majority of poultry processing lines contains "endemic strains" (18). Usually, SFP occurs when the number of *S. aureus* colony forming units per gram of food (CFU·g^{-1}) is higher than 10^5, though, depending on the conditions and strain, this number can be lower (56). Food involved in SFP differ greatly from country to country (57).

Although SFP is usually a self-limiting disease, approximately 10% of the cases require hospitalization. Consequently, SFP represents a significant social burden due to hospital expenses, loss of patient working days and their productivity, and also the cost of disposing of contaminated food (18).

According to Hennekinne et al. (2010) (58), there are five conditions to induce SPF:

i. The existence of a source that contains *S. aureus* strains able of producing SE;
ii. Transfer of enterotoxic *S. aureus* from the source to the food;
iii. Food with favorable characteristics for the growth of enterotoxic *S. aureus* and generation of SE;
iv. Favorable temperature and enough time for the growth of the enterotoxic *S. aureus* and generation of its SE; and
v. Ingestion of food containing the necessary amount of SE to cause symptoms.

Since *S. aureus* does not compete with indigenous flora of raw foods, food contamination is mainly due to poor handling, allowing enterotoxic *S. aureus* to enter the food chain while processing food products and/or after improper storage (53,59). On a smaller scale, enterotoxic *S. aureus* can be transferred to food by air and dust (53). To avoid SFP, prevention must pass through strict microbiological food control, which compromises hand and environmental hygiene, identification and isolation of carriers, and proper antibiotic therapy of food handlers

(50). Swabs from the nasopharynx and/or skin lesions of food handlers should be taken when SFP is suspected (2). The isolation of enterotoxic strains of *S. aureus* from humans vary between 40 and 60% (60), making the contaminations of food by food handlers a major problem.

Even though SFP is the third most important foodborne disease present all over the world, its true incidence can be underestimated due to several factors that can go from unreported outbreaks, minor outbreaks, misdiagnosis of the illness (which is symptomatically similar to other types of food poisoning, such as the one caused by *Bacillus cereus*), improper sample collection and laboratory examination, lack of seeking assistance by the affected subjects and the lack of routine surveillance for the detection of *S. aureus* and its SE in food or the detection of SE in stools (6,18,53,61,62).

Table 1.2 - Staphylococcal food poisoning outbreaks reported in the literature.

Country, City	Period	Setting of Outbreak	Suspected source	SE	Number of diseased	Reference
Malasya, Kapar	1983	School canteen	Foodhandler	-	48	(63)
USA[a]	1986	School	Chocolate milk	SEA	850	(64)
USA, Starkville	February to April 1989	Several cafeterias	Canned mushrooms	SEA	99	(65)
USA, Texas	1992	Elementary school' canteens	Deboned chicken salad	-	1 362	(66)
Brazil, Minas Gerais	1998	Catholic priest's ordination ceremony	Chicken, roasted beef, rice and beans	SEA	4000	(67)
Japan, Osaka Prefecture	June to July 2000	Households	Powdered skim milk	SEA, SEH	13 420	(68,69)
Saudi Arabia, Al-Madinah, Al-Monawarah	March 2003	Catered buffet	Chicken and rice	-	492	(70)
Norway[a]	December 2003	Kindergarten	Mashed potato made with raw milk	SEH	8	(71)
Japan, Shiga Prefecture	2005	Restaurant	Grilled salmon	-	862	(72)
USA, Kansas	December 2005	Catered buffet at a company	Smoked sausage	SEA	138	(73)
Austria, Eisenstadt	September 2006	School canteen	Foodhandler	-	113	(74)
Republic of Paraguay, San Lorenzo, Asunción and Ciudad del Este	March 2007	Households	Milk	SEC, SED	400	(75)
Germany, Baden-Württemberg	June 2008	Catered buffet lunch at a wedding party	Pancakes filled with minced chicken	SEA	150	(76)
France[b]	October to November 2009	Households	Cheese	SEE	23	(77)
USA, Illinois	December 2010	Bakery	Cakes and desserts	SEA, SEB, SEC, SED	100	(78)
Australia, Sydney	June 2012	Catered buffet dinner at an elite sporting event	Chicken stir-fry or fried rice	-	22	(79)
USA[a]	July 2012	Military Unit Lunch Party	-	-	35	(80)
India, Mysore	December 2012	Nursing hostel	Kerala matta rice	-	171	(81)
Germany, Freiburg	April 2013	Christening party	Ice cream	SEA	13	(62)

[a] Distict or city not revealed.

[b] Several french departments (Pas de Calais, Nord, Somme, Aisne, Savoie and Gard)

SFP outbreaks (Table 1.2) vary in number and can comprise as few as 8 subjects or as many as more than 10 000. In 2012, 346 cases of SFP were reported to the European Union, corresponding to 6.4% of foodborne outbreaks, with mixed food being responsible for 31.4% of the outbreaks, followed by cheeses which were associated with 20% of the outbreaks (82). In the same year, 5 cases of SFP were reported in the United States, corresponding to 1% of foodborne outbreaks (80), with 55% of outbreaks attributed to meat and poultry dishes (6). In Australia, between 2000 and 2012, 14 cases of SFP were reported, with 29% of outbreaks associated with catered food (79).

1.1.3. Staphylococcal enterotoxins

SE are a family of serological well defined monomeric proteins (220–240 amino acids) belonging to the pyrogenic toxic superantigen family, with low molecular weight (26–30 kDa) and well conserved structures, that are water and saline solution soluble, and that are mostly produced by several coagulase producing *S. aureus* strains, which are capable of producing more than one SE (18,83,84). SE possess important properties such as the capacity to induce emesis and gastroenteritis, in addition to their superantigenecity (85). They are not only able to resist to heat and acidity, but are also stable in vast range of pH and can resist to the inactivation by gastrointestinal proteases and other proteolytic enzymes (e.g., pepsin, trypsin, rennin and papain) (57,86). In practice, these properties mean that even though *S. aureus* can be completely inactivated during food processing or through food preservation methods, the biological activity of SE remains unaffected (86). Moreover, *S. aureus* can produce SE in a wide range of temperature, pH, NaCl concentrations and water activity (87).

There are twenty one SE or SE-like (SE*l*; related SE with no emetic activity or that have not been tested yet) superantigens, SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SE*l*J, SE*l*K, SE*l*L, SE*l*M, SE*l*N, SE*l*O, SE*l*P, SE*l*Q, SER, SES, SET, SE*l*U and SE*l*V (88,89).

Currently, SEA is the serotype most frequently involved in SFP (80%), followed by SEB (10%) (50,86,87). SE can also cause toxic shock-like syndrome, being involved in allergic and autoimmune diseases (53,57,90). Moreover, SEB in particular, can be used as a potential biological warfare weapon (50,91).

There is a direct proportionality between the toxicity of a SE and the quantity ingested of that SE (92).For example, the amount of SEA necessary to cause SFP is 100 ng (93). However, the amount of SE needed to induce SFP depends also of the individual susceptibility to the SE, its weight and its health condition, the type of food, and also the SE type (50,94). Furthermore, the linearity between the number of *S. aureus* CFU.g^{-1} and the production of SE present in a sample

might be disassociated and attention must be paid to the different environments in which cells are growing, because they can affect the SE production (87).

Some strains of enterotoxic *S. aureus* isolated from food are also MRSA (95–98). However, although the number of foodstuff containing MRSA strains has become higher over the years, there is still no evidence that MRSA strains are foodborne (36).

1.2. High pressure processing

Nowadays, consumers demand not only convenient and ready-to eat food but also search natural food products with an extended shelflife and high quality, that are less processed, preservative free, with low salt, sugar or fat contents and healthier (4,87,99). Along with these requirements, the increased awareness of the risk of foodborne diseases forced the food industry to develop new non-thermal alternative food processing techniques such as ionization radiation, pulsed electric fields, UV decontamination, pulsed high intensity light, high intensity laser, pulsed white light, high pressure processing, among others (3,87,99,100).

HPP is a fairly new non-thermal food preservation technique that inactivates foodborne pathogens responsible for foodborne diseases and food spoilage by subjecting foods to pressures between 400 and 600 MPa, with or without heat (101), with minimal effect on taste, texture and nutritional characteristics (102–104). This technology also has the advantage of being applied to pre-packaged food products, highly preventing post-processing contamination (105). HPP was adopted by the food industry from the manufacturing of ceramics, artificial diamonds, superalloys and sheet metal forming (106).

Although HPP was first described to inactivate bacteria in 1895 by Royer (107) and by Hite and in 1899 (108), it was only in the 1990's that this technology began to be applied to the food industry (109). Nowadays, HPP is applied all over the world in a extensive variety of food products (99,103,110).

Over the last twenty years, considerable research has been performed and the efficacy of HPP has been proved by extending food shelf life and improving food safety. Many works have been done with vegetative cells of bacteria, mainly with several strains of *Escherichia coli*, *Listeria monocytogenes*, *Listeria innocua*, *Salmonella* spp., *Vibrio parahemolyticus* and *S. aureus*, which are important foodborne pathogens (111–113). Some works have also been done with bacterial endospores, which are pressure-resistant, with special interest to the ones performed with spores of *Clostridium botulinum, Clostridium perfrigens* and *B. cereus*, which are also important foodborne pathogens (114). Yeasts and molds (*Byssochlamys fulva*, *Byssochlamys nivea*, *Talaromyces avellaneus* and *Saccharomyces cerevisiae*) inactivation by HPP has also been

studied (105,115–117). The inactivation of viruses through HPP is not as studied as the inactivation of bacteria, however there are some published studies with Hepatitis A virus, Poliovirus, Norovirus and human Rotavirus (118–121).

The efficacy of HPP depends on many different factors such as the type of microorganism (spores are the most resistant and are in general, followed by gram-positive vegetative cells, which are followed by gram-negative vegetative bacteria, whereas molds and yeasts are more sensitive), on the bacterial shape (rods are more sensitive than cocci) and the growth of the culture (122,123).

The main advantages of this technology are (99,106):

i. The processing of food at ambient or low temperature;
ii. Instant transmittance of pressure throughout the system (independently of the size or shape);
iii. Microbial death without the need of heat and chemical preservatives/additives, improving food quality and shelf life;
iv. Creation of ingredients with new functional properties;
v. Maintenance of flavoring characteristics and nutritional components; and
vi. Being an environmental friendly process, requiring only electric energy and it does not generates waste products.

Nonetheless, as in all technologies, there are some practical challenges in the applicability of HPP to food safety, such as the high cost of HPP equipments. Furthermore, there is also a lack of information concerning the effect of HPP on toxins, allergens and nutrients (99).

HPP stands on three basic principles (102,104,110):

i. Le Chatelier's principle, which states that the application of pressure shifts the system equilibrium toward the state occupying the smallest volume and, thus, any phenomenon accompanied by a decrease in volume will be enhanced by an increase in pressure and vice versa;
ii. Principle of microscopic ordering, which states that at constant temperature, an increase in pressure will increase the degrees of ordering of molecules of a given substance; and
iii. Isostatic principle, which states that pressure is transmitted quasi-instantaneously and uniformly throughout the sample volume independently of the size and geometry of the product, meaning that during HPP treatments all parts of the food will experience similar pressure, with pressure and temperature exerting antagonistic forces on molecular structure and chemical reactions.

The pressurization of liquid or solid foods at room temperature is usually accompanied by a moderate temperature increase, termed adiabatic heating, which is of approximately 3 °C per

100 MPa in foods with high water content or 8–9 °C in foods with high fat content (103,110). HPP treatments can be applied in a cyclic or continuous mode. The pressure, time and temperature applied are dependent of the type of product and of the expected result. Temperatures above or below ambient temperature increase the efficiency of HPP treatments for microbial inactivation (99,103,105).

1.2.1. Factors conditioning the effectiveness of HPP in *Staphylococcus aureus*

As mentioned, the efficiency of HPP depends on the type of microorganism and also on the bacterial shape (122,124). In the following sections, the parameters that modulate HPP efficiency will be discussed, firstly based on the state-of-the art for bacteria and after specifically for *S. aureus*, depending on the bibliography available. Although there is a lack of studies for *S. aureus* concerning some factors, they will be approached using other bacterial species due to its importance. Furthermore, when pertinent, comparison between *S. aureus* and other bacteria will be made. In addition, HPP inactivation kinetics will also be approached, since even though several HPP studies for *S. aureus* reported a first-order inactivation kinetics, other studies showed the need of nonlinear models to adequately fit decay curves of *S. aureus*.

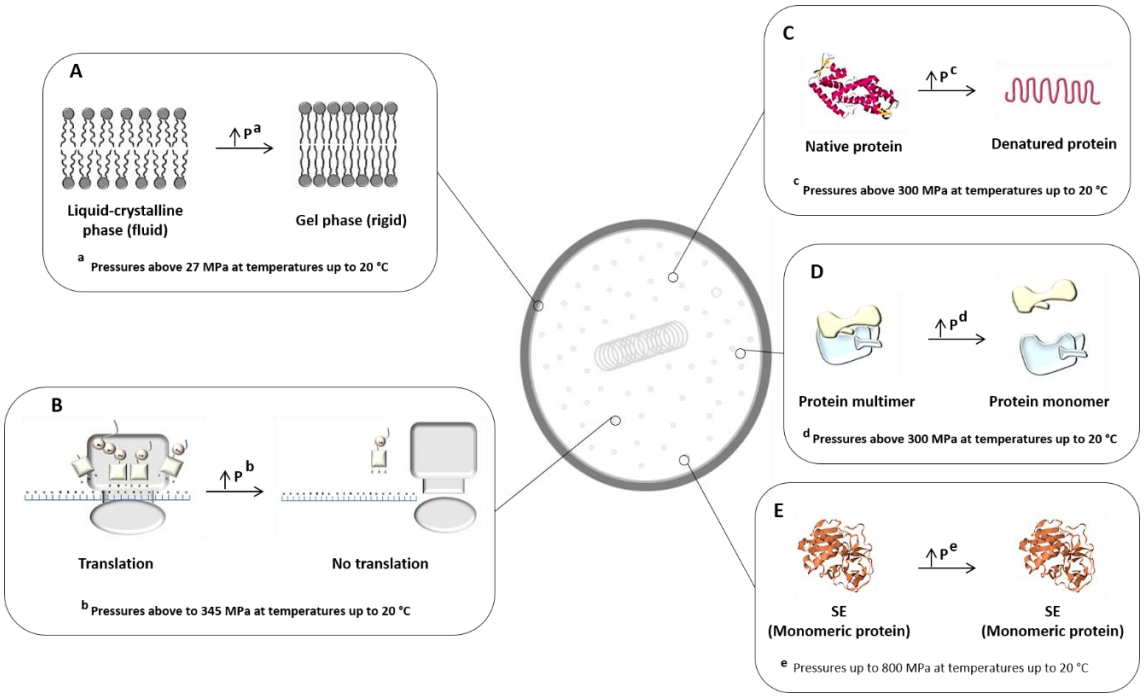


Figure 1.2 - Effect of HPP on *Staphyococcus aureus* cell structures and biomolecules; adapted from Oger & Jebbar, 2010 (125). **A**: membrane phospholipids; **B**: protein translation on ribosomes; **C**: protein folding (PDB ID: 2FNP (126)); **D**: multimeric protein; **E**: staphylococcal enterotoxin (PDB ID: 1ESF (127)). Membrane phospholipids are extremely sensitive to increasing pressures, passing from the liquid-crystalline phase into a gel state. At pressures above 345 MPa, conformational changes occur in *Staphylococcus aureus* ribosomes which will have consequences in protein translation. Although the effects of HPP on protein conformation are conditioned by the type of protein, at pressures above 300 MPa the effects are usually irreversible. Staphylococcus enterotoxins (SE) are extremely baroresistant proteins, suffering no conformational changes at pressures up to 800 MPa.

1.2.1.1. Cell structures and biomolecules

The inactivation of bacteria by HPP is a multi-target approach (Figure 1.2), causing changes in cell membranes (with special attention given to the membrane phospholipids phase transitions), cell wall, ribosomes, proteins, lipids and enzyme-mediated cellular functions (128). Thus, as HPP targets more than one cellular structure and/or function, cell death is the result of a combination of damages in different parts of the cell (129,130).

i) External cell structure (cell membrane and cell wall)

The cell membrane is the most pressure sensitive cellular component and is considered the main target of HPP inactivation in microorganisms. It is accepted that the cause of cell death is mainly due to the leakage of intracellular constituents through the permeabilized cell membrane (106,131). Nevertheless, it has been shown that bacteria can restore their membrane after pressure application if that pressure is not severe enough to affect irreversibly the membrane, as seen in *E. coli* (132). Lower fluidity of the bacterial cell membrane (Figure 1.2) causes an increase in HPP sensitivity (106). HPP effects on cell membranes will consequently alter cell permeability, transport systems, loss of osmotic responsiveness, organelle disruption and the ability to maintain intracellular pH (128). During compression, the phospholipids from bacterial membranes are compressed (130) and cell membrane proteins are degraded, which will consequently have negative effects in the membrane function (133). These effects are the main cause of sub-lethal injury created by HPP in microorganisms, which prevents them to grow on selective media and/or requires longer periods of time to recover (124).

Due to differences in the cell wall structure, Gram-negative bacteria are more sensitive to HPP than Gram-positive bacteria (133). This fact relies on the higher complexity of Gram-negative cell wall structure which consist of an inner and outer membrane with a thin layer of peptidoglycan stuck between (134). On the other hand, cell walls of Gram-positive bacteria are more elementary, consisting solely of a thick peptidoglycan outer layer (which represents 90% of the cell wall) (134). It has been shown that *S. aureus* cell membranes damage by HPP is not easy. This was shown using scanning electron microscopy (SEM) to examine cell morphological changes and it was observed that *S. aureus* cells without HPP treatment exhibited a smooth and uniform aspect opposed to loss of cell surface smoothness, irregularities and fractures when pressurized at 300 MPa or few invaginations when pressurized at 350 MPa and, only when this pressure is combined with CO_2, severe cell shrinking occurred, suggesting cell membrane damage (135,136).

This is supported by the results of propidium iodide (PI) staining that showed an increase in *S. aureus* 1.2465 cell membrane permeability from 5% to 69%, using a combination of 350 MPa and a concentration of 3.8 $NL.L^{-1}$ of dissolved CO_2, however, no cell disruption was observed (Table 1.3) (136).

Using acridine orange, it was seen that almost all *S. aureus* ATCC 25923 cells were stained orange due to little membrane permeabilization and only at pressures above 404 MPa fluorescence decreased slightly (137). Using Live/DEAD Baclight Bacterial Viability kit and fluorescent microscopy to assess membrane integrity of *S. aureus* cells pressurized between 200 to 400 MPa, the number of red cells (dead cells or cells with damaged cell membrane) increased with increasing pressures but remained very close to the number of green cells (live cells or cells with intact cell membrane), which decreased slightly even at 400 MPa (135). However, with increasing pressures from 200 to 400 MPa, changes in membrane properties were caused: disruption or increase in membrane permeability, loss of membrane integrity, denaturation of membrane-bound proteins, and pressure-induced phase transition of membrane lipid bilayer which led to a subsequent increase in *S. aureus* cell volume at pressures mainly above 400 MPa (138–140). Furthermore, due to the phase transition of membrane lipids at temperatures above 35 °C bacterial cells become more sensitive (141).

ii) Ribosomes and cytoskeleton

Another important target site are the ribosomes (Figure 1.2). Generally, HPP causes subunit dissociation in microorganism ribosomes, limiting cell viability (142,143) by inhibiting protein synthesis (124,144). In fact, cell death and ribosome injury are closely related events (143). Using differential scanning calorimetry (a technique that can be used to evaluate the effect of pressure on ribosome denaturation), a correlation between the ribosome-associated enthalpy and the loss of cell viability due to pressure treatment (50–250 MPa) was found in *E. coli* cells (143). Comparing the thermograms of unpressurized *S. aureus* cells with the thermograms of pressurized cells at 345 MPa, differences were detected through changes in specific peaks (shift to lower temperatures in pressurized cells), which suggest denaturation of the ribosome due to pressure treatment (145).

iii) Other structural and morphological cell changes

Other morphological and structural changes in microorganisms' cell include the detachment of the membrane from the cell wall, the elongation of the cell, the compression of gas vacuoles and the condensation of nuclear material (105,129).

S. aureus cells suffered an increase in their volume at pressures above 400 MPa due to changes in membrane properties (Table 1.4) (140). Under transmission electron microscope (TEM) untreated cells displayed intact cell wall, cell membrane, homogeneous cell cytoplasm and electron-transparent regions of nucleoids (Table 1.4) (92). After HPP, at pressures above 500 MPa, instead of a cell membrane with a single-thick-layer presentation, a double-track bilayer structure appeared, the cytoplasmatic material became aggregated, the nucleoids region gained a fibrous appearance and became enlarged and the interior regions became compacted (92). At lower pressures, the membrane and cell wall preserved its distinct aspect but, at higher pressures, the peptidoglycan layer collapsed and pieces of the outer layer seem to be loosened (92).

Table 1.3 - Efficiency of HPP inactivation of *Staphylococcus aureus* strains (studies performed using combined effects).

Study Matrix	HPP treatment range	HPP Complement	Strain	Inactivation Range	Reference
1% peptone solution	207 – 345 MPa/ 5 – 10 min/ 25 – 50 °C	ST	485	0.40 – 6.03[a]	(146)
			765	0.59 – 8.04[a]	
Citric acid	345 MPa/ 5 min/ 35 °C	pH 4.5	485	3.47[a]	
			765	4.00[a]	
		pH 5.5	485	1.78[a]	
			765	2.97[a]	
		pH 6.5	485	1.58[a]	
			765	2.16[a]	
Lactic acid		pH 4.5	485	4.22[a]	
			765	5.00[a]	
		pH 5.5	485	2.16[a]	
			765	3.10[a]	
		pH 6.5	485	1.70[a]	
			765	2.40[a]	
Pasteurized milk	345 MPa/ 5 min/ 50 °C	ST	485	8.34	(147)
			765	8.30	
Pasteurized orange juice		ST	485	5.50	
			765	8.30	
		Nisin plus pediocin AcH	485	8.34	
			765	8.30	
Skimmed milk	150 – 300 MPa/ 30 min/ 25 °C	ST	ATCC 6538	0.50 – 6.00[ab]	(148)

		Lacticin 3147		2.2 – 6.00[ab]	
Potassium phosphate buffer	400 MPa/ 15 min/ 20 °C	ST	LMMBM14	3.20[ab]	(149)
		Nisin		4.30[ab]	
		Bovine lactoferrin		4.10[ab]	
		Pepsin hydrolysate of lactoferrin		2.80[ab]	
		Lactoferricin		3.10[ab]	
Pasteurized milk	345 MPa/ 5 min/ 50 °C	ST	485	5.50[a]	(111)
			765	8.30[a]	
		Nisin plus pediocin AcH	485	8.34[a]	
			765	8.30[a]	
Cream of chicken soup		ST	485	5.70[a]	
			765	8.20[a]	
		Nisin plus pediocin AcH	485	8.30[a]	
			765	8.20[a]	
Sodium chloride solution (0.9% w/v)	200 MPa/ 60 min/ -20, - 10, -5, 0, 5 and 30 °C	Without pre-incubation and without chloramphenicol	IFO 13276	1.60 – 4.70[ab]	(150)
		Without pre-incubation and with chloramphenicol		1.50 – 4.90[ab]	
		With pre-incubation without chloramphenicol		1.60 – 2.70[ab]	
		With pre-incubation with chloramphenicol		1.30 – 2.95[ab]	
Potassium phosphate buffer	250 MPa/ 15 min/ 25 °C	ST	ATCC 27661	0.9[a]	(151)
		Hen egg white lysozyme		0.8[a]	
		Goose egg white lysozyme		0.2[a]	
		Cauliflower lysozyme		0.5[a]	

		Mutanolysin from Streptomyces globisporus		0.1[a]	
		Bacteriophage λ lysozyme		0.5[a]	
		Bacteriophage T4 lysozyme		0[a]	
Fuet (fermented traditional sausage)	400 MPa/ 10 min/ 17 °C	ST	CTC1008, CTC1019 and CTC1021[c]	0.5[dg]	(152)
		Enterocins A and B		0[dg]	
Phosphate-buffered saline	193 MPa/ 4x10 min/ -20 °C	ST	ATCC25923	0.70 – 1.10[a]	(153)
		Lysozyme		0.90[a]	
		Nisin		0.60 – 0.80[a]	
McIlvaine citrate-phosphate buffer		ST		0[a]	
		Nisin		0[a]	
McIlvaine citrate-phosphate buffer	193 MPa/ 35+5+10 min[h]/ -20 °C	pH 3.5	ATCC25923	1.70[ab]	(154)
		pH 4.0		1.30[ab]	
		pH 5.0		1.10[ab]	
		pH 6.0		1.00[ab]	
		pH 7.0		0.60[ab]	
Apple Juice		ST		1.30[ab]	
Fuet (fermented traditional sausage)	400 MPa/ 10 min/ 17 °C	ST	CTC1008, CTC1019 and CTC1021[c]	0.39[df]	(155)
		Enterocin AS-48		0.39[df]	
Luria Bertani	350 MPa/ 10 min/ 30 °C	ST	1.2465	0.90[a]	(136)
		CO_2		7.90[a]	
Rice pudding	500 MPa/ 5 min/ 22 °C	ST	CCUG 31966, CCUG 35601 and CCUG 41879[c]	2.90[d]	(156)
		Nisin		3.77[d]	
		Enterocin AS-48		3.30 –3.50[d]	

		Clove oil		1.80[d]	
		Cinnamon oil		1.30[d]	
Pasteurized milk	400 MPa/ 5 min/ 10 °C	ST	Sa9	0.5[ab]	(157)
		Bacteriophages (phiIPLA35 and phiIPLA88)		0.6[ab]	

ST – HPP single treatment (without complements)

[a] log CFU.mL^{-1}

[b] Exact values not given; values obtained by estimation using the data reported on graphic

[c] Cocktail of strains

[d] log CFU.g^{-1}

[f] Counts performed 7 days after pressurization

[g] Counts performed 1 day after pressurization

[h] Total time (35 min of compression plus 5 min of holding time plus 10 min of decompression

iv) Proteins

Generally, HPP can cause a wide range of effects on proteins that can go from minor conformational effects to the loss of native folding (Figure 1.2), dissociation of monomers in multimeric proteins (Figure 1.2), or even precipitation and aggregation, which depends on the protein type and processing conditions being usually reversible at pressures between 100 and 300 MPa and irreversible at pressures higher than 300MPa (99,158). Protein covalent bonds are not affected by HPP, but non-covalent bonds (ionic, hydrophobic and hydrogen bonds) are affected (103,130). For example, primary structure is minimally affected by HPP (128). However, proteins will suffer changes in their secondary, tertiary and quaternary structures, which will affect membrane proteins and some enzymes (99,128).

Due to protein denaturation, the inactivation of key enzymes is to some extent responsible for the inhibitory effects of HPP on microorganisms (124). The way to which enzymes are affected is, however, not linear and bacterial enzymes diverge greatly in their capability to resist pressure (105). Changes in the active centers of enzymes are mainly due to a change in their conformation (unfolding and, more difficultly, denaturation), which might be irreversible (134).

Denaturation of monomeric proteins, such as *S. aureus* SE may occur at extreme pressures but, generally, they do not show any changes in proteolysis (99,144). The effect of HPP on monomeric proteins is of special importance for *S. aureus* since SE appear to be highly piezotolerant monomeric proteins (83,84,159). Using enzyme immunoassays (EIA) to monitor the reactivity of suspensions of SEA to SEE it was observed that HPP treatments up to 800 MPa had no effect on the reactivity of SE at temperatures of 5 and 20 °C (159). Only at 80 °C a decrease in SE reactivity was observed (159). On the other hand, after pressurization at 600 MPa, SEA suspension was still able to induce a proliferative activity on rat thymocytes and, by increasing the temperature from 20 °C to 45 °C, the proliferative response to SEA by T-cells was significantly induced ($\rho < 0.05$) when compared to unpressurized SEA and SEA pressurized at 20 °C (160). Although the effect of HPP on proteic virulence factors is of high importance, studies are still scarce.

v) Lipids

Lipid systems are extremely sensitive biological constituents to HPP (161), due to their hydrophobic nature (162). In biological membranes, lipids are the backbone, appearing as a lamellar phospholipid bilayer matrix (163). HPP affects membrane phospholipids by reducing the curl and straightening acyl chains, causing lateral shrinkage and an increase in its thickness

(Figure 1.2) (164). During compression, above 27 MPa, the phospholipids from the membranes are compressed, favoring the passage from the liquid-crystalline phase into a gel state (Figure 1.2) and, during decompression, the dual layer conformation is lost allowing the development of pores which will cause cytoplasmic leakage (130,131). Random movements of the phospholipid acyl chains caused by HPP cause water molecules to infiltrate between the hydrophilic phospholipid head groups and into the bilayers (165).

In *S. aureus* IFO 13276, barotolerance increases when cells are able to adapt to lower temperatures (150), and also in bacteria with high unsaturated and saturated fatty acids ratio in membranes (164).

vi) Nucleic acids

Although no papers were found describing the effects of HPP in *S. aureus* nucleic acids, it is important to discuss the effect of HPP in nucleic acids. Nucleic acids are exceptionally resistant to HPP denaturation and their structure can remain undamaged at pressures up to 1000 MPa (124,166). Deoxyribonucleic acid (DNA) is extremely stable due to α-helical structures which are supported by hydrogen bonds (124,128). However, because enzymes are affected by HPP, DNA replication and transcription mechanisms, as well as protein translation, are inhibited (167). Another effect of HPP, although indirect, is the exposure of DNA to endonucleases that under normal circumstances would not be in contact with DNA (168). Nonetheless, the effects of HPP on DNA are exceptionally complex. An increase in pressure causes the release of counter ions, disrupts stacking interactions, increases the resistance of double-stranded helix (due to the stabilization of DNA hydrogen bonds) which leads to the denaturation into single strands and induces the transition of double helices from the B to Z form (165).

1.2.1.2. Bacterial strain dependence

Sensitivity to HPP is also dependent of the strains of a species (133,169). *S. aureus* follows this rule and different strains exhibit different degrees of resistance to HPP, with some strains able to present different D values (time in minutes necessary to kill 90% of the microbial population at determined temperature and pressure) under the same HPP conditions, such as the difference between the strain 485, which has a D value of 2.55 min, and the strain 765, which has a D value of 0.62 min (Table 1.4) (141), being the former strain more resistant than the latter (Table 1.3 and Table 1.4) (112,141,147).

These differences, which can be more or less higher, can be the result of inherent characteristics of each strain such as the carotenoids produced by *S. aureus* which function as antioxidants, protecting *S. aureus* against oxidative stress and stabilizing the membrane during

infection and pathogenesis (170–172). Different *S. aureus* strains produce different quantities of carotenoids which seem to be important for the resistance to HPP, as observed on a number of studies in which authors were able to relate differences in carotenoid content with HPP resistance,

Table 1.4 - Efficiency of HPP inactivation of *Staphylococcus aureus* strains (studies performed using only HPP).

Study Matrix	HPP treatment range	Strain	Inactivation Range	D[a] and z[b] values	Reference
Pork slurry	101 – 608 MPa/ 10 min/ 25 °C	ATCC 25923	0.10 – 6.30[fg]	-	(137)
Whole cow's milk	50 – 350 MPa/ 4 – 12 min/ 20 °C	ATCC 27690	0.10 – 8.61[dg]	$D_{200\ MPa/20\ °C}$ – 211.8 $D_{350\ MPa/20\ °C}$ – 15.0 $D_{300\ MPa/20\ °C}$ – 3.7 $D_{350\ MPa/20\ °C}$ – 2.56	(173)
1% Peptone Solution	345 MPa/ 5 – 15 min/ 25 – 50 °C	315	0.90[d]	-	(141)
		485	0.70 – 8.11[d]	$D_{50°C}$ -2.55	
		565	1.12[d]	-	
		582	7.80[d]	-	
		743	0.86[d]	-	
		765	1.51 – 2.70[d]	$D_{50°C}$ - 0.62	
		778	0.70[d]	-	
Ovine milk (6 % fat)	200 – 500 MPa/ 5 – 15 min / 2, 10, 25, 50 °C	CECT 534	0.10 – 7.30[dg]	$D_{450\ MPa/2\ °C}$ – 20 $D_{450\ MPa/25\ °C}$ – 16.7	(174))
Ringer Solution	400 – 500 MPa/ 15 min/ 4, 25, 50 °C	CECT 534	3.30 – 7.50[dg]	-	(175)
Ovine milk (0, 6, 50% fat)			0.50 – 7.10[dg]	-	
Cheddar cheese slurries	100 – 800 MPa/ 20 min/ 10, 20, 30°C	ATCC 6538	0 – 3.00[dg]	$D_{300\ MPa/20\ °C}$ – 38 $D_{350\ MPa/20\ °C}$ – 33 $D_{400\ MPa/20\ °C}$ – 20 Z - 359	(176)
Miniature cheeses	100 – 500 MPa/ 20 min/ 20°C		0 – 4.3[dg]	-	
Phosphate buffer			0.1 – 7.0[dg]	-	
TSBY	345 MPa/ 10 min/ 35 °C	485	2.50[d]	-	(112)
		765	3.10[d]	-	
TSB	150 – 550 MPa/ 1x15, 3x5, 5x3, 7x2 min/ 20 °C	ATCC 6538	0 – 8.9[d]	-	(177)
Sturgeon caviar	450 – 500 MPa/ 1x15, 3x5 min/ 20°C		3.50[f]	-	
Trout caviar			3.66[f]	-	
UHT whole milk	600 MPa/ 4 min/ 4 – 45 °C	ATCC 12600	4.00 – 4.30[d]	-	(178)
	600 MPa/ 8 min/ 4 – 45 °C		5.70 – 8.40[d]	-	
	600 MPa/ 10 min/ 4 – 45 °C		7.40 – 8.30[d]	-	

Apple juice	250 – 350MPa/ 5 – 30 min/ 30 –40°C	485	1.40 – 8.2[d]	-	(179)
Orange juice			0.94 – 8.2[d]	-	
Apricot juice			1.26 – 8.2[d]	-	
Sour cherry juice			0.56 – 8.2[d]	-	
Tryptone saline solution	500 MPa/ 30 – 90 min/ 20 – 25 °C	[c]	1.27 – 5.28[d]	-	(180))
	500 MPa/ 30+60, 60+90, 30+60+90 min/ 20 – 25 °C		6.86 – 8.28[d]	-	
0.1% peptone water	200 to 400 MPa/ 5 min/ 40 °C	485	0.07 - 3.24[d]	-	(140)
Human milk	500 MPa/ 6x0.5, 3x10, 6x5, 2x30, 3x30 min/ 20 – 25 °C	ATCC 6538	0 – 6.63[d]	D_{TSA} – 4.88 D_{BPA} – 5.72	(181)
0.1% peptone solution		ATCC 6538	6[d]	D_{TSA} – 5.23 D_{BPA} – 5.37	
		ATCC 25923	6[d]	D_{TSA} – 5.85 D_{BPA} – 5.08	
		ATCC 25923	6[d]	D_{TSA} – 5.85 D_{BPA} – 5.08	
Sliced cooked ham	600 MPa/ 6 min/ 31 °C	CTC1008, CTC1019 and CTC1021[h]	1.1[f]	-	(182)
Sliced dry cured ham			0.5[f]	-	
Marinated beef loin			2.7[f]	-	
Whole liquid egg	200 – 400 MPa/ 3 – 15 min/ [c]	[c]	0.92 – 2.63[d]	-	(183)
Sterile raw milk	100 – 500 MPa/ 10 – 50 min/ 25°C	ATCC 29213	5.04[dg] – 7.84[dg]	-	(92)
Sodium Phosphate	500 MPa/ 8 min/ [c]	SH 1000	0.43 – 3.8[d]	-	(184)
DMG (sodium 3,3-dimethyl glutarate)			0.48 – 4.2[d]	-	
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)			1.25[dg]	-	
MOPS (morpholinepropanesulfonic acid)			0.45[g] – 0.65[dg]	-	
Tris Buffer			0.40[g] – 1.0[dg]	-	
Non-food emulsion model	500 MPa/ 10 min/ 20 °C	ATCC 6538	6.39[d]	-	(185)
Marinated and dehydrated beef strips	550 MPa/ 2 x 1 min/ 22 °C	ATCC 25923	8[d]	D_{TSA} – 2.12 D_{BPA} – 2.45	(186)

[a] Decimal reduction time (time in minutes necessary to kill 90% of the microbial population at a certain temperature and pressure)

[b] Pressure increase required to accomplish an 1 log cycle reduction in the D value

[c] Absent information

[d] log CFU.mL^{-1}

[f] log CFU.g^{-1}

[g] Exact values not given; values obtained by estimation using the data reported on graphic

[h] Cocktail of strains

[i] log CFU per strip

i.e., strains with higher carotenoid content are more resistant than strains with lower carotenoid content (Table 1.4) (187).

Other factors include the sigma B (σ^B) factor (recognized as an important factor for the resistance of *S. aureus* to heat, pulsed electric fields treatments, as well as to chemical agents). It controls the carotenoid production along with other detoxifying products such as catalases, as seen when comparing the Newton strain (which contains the σ^B factor) with its isogenic mutant (without the σ^B factor), being the shoulder duration (time during which the membrane is able to maintain its integrity and functionality) of the first one much higher than the last one (187,188).

1.1.2.4. Influence of matrices

The effectiveness of HPP is also dependent on the composition and properties of the matrix, with pH and water activity (a_w) being of high importance (105,122). Concerning the properties of the study matrix, it is reported that some food elements such as proteins, carbohydrates, and cations can have a protective effect on microbial cells, which makes the inactivation data obtained in studies performed in buffers or medium culture not directly suited to be extrapolated to real food situations (105,128).

Several studies have been done in order to compare different study matrices to determine their effect on the efficiency of the inactivation of *S. aureus* by HPP. In fact, it is possible to see that some food matrices have indeed a protective effect whilst others aid the effects of HPP. For example, the baroprotective effect of milk and other dairy products is widely recognized. In a study performed with *S. aureus* strain CECT 534 in Ringer solution and ovine milk containing several percentages of fat content (0, 6 and 50%), it was clear that inactivation rates were higher in Ringer solution, followed by 0% fat ovine milk, 6% and 50% fat ovine milk (Table 1.4) (175). In this study, it was possible to observe the baroprotective effect of milk not only due to its fat content but also due to the presence of calcium ions and protein content (175,189–191). This baroprotective effect was also seen in *S. aureus* CECT 534 (Table 1.4) which was more sensitive in phosphate buffer than in miniature Cheddar cheese and cheese slurry system (176). However, when *S. aureus* inactivation by HPP was tested in human milk, the baroprotective effect was absent and D values were slightly or considerably inferior (e.g. D values for ATCC 6538 were 4.88 min for human milk and 5.23 min for 0.1% peptone solution), depending on the strain, most likely due to the fact that human milk is known to have many antimicrobial elements that acted as a synergistic effect (181).

The baroprotective effect of matrices with low a_w (130) was observed in a study done with sliced cooked ham, sliced dry cured ham and marinated beef loin, given that the highest inactivation of a *S. aureus* cocktail (strains CTC1008, CTC1019 and CTC1021) resulting in higher inactivation in beef loin, followed by sliced cooked ham and finally, dry cured ham had the lowest inactivation (Table 1.4) (182). These results correlate with the different a_w of these products (marinated beef loin > sliced cooked ham > sliced dry cured ham). Moreover, when comparing a ham model system with phosphate buffer it was observed that D values were higher for the food model, being these values of 18.6 and 7 min, respectively, for 450 MPa (192).

Studies also show that HPP inactivation is dependent of the study matrix pH. This is clear when the inactivation of a relatively resistant strain of *S. aureus* 485 was tested in different fruit juices (Table 1.4) and results show that the lowest inactivation occurred in apricot juice (pH 3.80; 5.00 log CFU·mL^{-1}), followed by orange (pH 3.76; 5.37 log CFU·mL^{-1}) juice and, the highest inactivation was obtained for sour cherry juice (pH 3.30; 5.70 log CFU·mL^{-1}) (179). However, the baroprotective effect of food matrix is not evident when, under the same HPP conditions, the inactivation of *S. aureus* ATCC 6538 is tested in samples of sturgeon caviar, trout caviar and in tryptic soy broth (TSB). Results showed that the three different matrices did not affect the efficacy of HPP, with total inactivation for TSB and almost full inactivation for both caviar samples, making this technology suitable for the treatment of caviar (177).

The choice of the buffer used in HPP assays is also of high importance. This is seen in a study using five different buffers (two anionic, two zwitterionic and one cationic) with results (Table 1.4) showing that the inactivation of *S. aureus* SH1000 in zwitterionic buffers and cationic buffer (reduction of between 0.4 and 1.4 CFU·mL^{-1}) was much lower than that of the cationic buffers (reduction of between 3.8 and 4.2 log CFU·mL^{-1}), most likely due to the fact that the last one is more pressure sensitive (with HPP causing a pH decrease) and the first ones are pressure stable (184).

The results show that not only HPP can be quite effective inactivating *S. aureus* in low fat products, in products with acidic pH and with high water activity but also that the study matrix holds a strong influence in its effectiveness. Nonetheless, in order to apply HPP to food products not yet studied, new studies must be performed.

1.1.2.3. Microbial growth phase

The efficacy of HPP depends on the growth of the culture, with cells in the exponential phase more sensitive than cells in the stationary phase (122,124,176). In fact, bacterial cells in the exponential phase exhibit longer shoulders than cells in the stationary phase (187). For *S.*

aureus cells, the σ^B factor is likely to be involved in the differences of resistance in stationary and exponential phases since this factor, which is responsible for the control of 251 genes including stress resistance genes, is mainly expressed in the beginning of the stationary phase (187). This is supported by comparing the duration of the shoulder of *S. aureus* Newton strain and its isogenic mutant without the sigB operon (IK 184), showing that the deletion of the σ^B factor decreased significantly the shoulder length (187). Nonetheless, within the same strain, the extension of the effect of HPP is not only dependent of the growth phase but also by HPP processing parameters.

1.1.2.5. HPP processing parameters

i) Pressure value

The effectiveness of HPP in the inactivation of bacteria depends highly on the pressures used (130). Under the same temperature and pressurization times, increasing pressures usually in- crease microbial inactivation, as it is observed in most studies with *S. aureus* (Table 1.4). As consequence, D values decrease with increasing pressures, such as D values obtained for a barotolerant strain of *S. aureus* in a ham model, which decreased from 18.6 min at 450 MPa to 1.3 min at 660 MPa (192). In *S. aureus* 485, both average cell volume and average cell view area also increased with increasing pressures (Table 1.4) (140). Furthermore, for the same *S. aureus* strain, at pressures up to 250MPa cells did not exhibit significant changes in their surface and shape but, at pressures higher than 300 MPa, cell surface lost its smoothness and became irregular and fractured (135).

ii) Temperature

Processing temperature conditions the efficiency of HPP in bacteria. At temperatures between 20 and 35 °C, *S. aureus* 585 cells are not as sensitive to HPP as they are above 35 °C, as a result of the phase transition of membrane lipids (146). Bacterial cells previously subjected to other stress conditions such as sub-lethal heat or cold shock (due to the increase on the percentage of polyunsaturated fatty acids in cell membranes become more resistant to pressure, as studies with *E. coli* (129). For *S. aureus* IFO 13276 (Table 1.4), this is also valid since by adapting itself to low temperature it increased, as a consequence, its barotolerance (150). Maintaining the same pressure and pressurization time, higher inactivation is achieved by raising temperature (Table 1.3 and Table 1.4), meaning that D values become lower as it was the case of *S. aureus* CECT 534 (Table 1.4), at 450 MPa, with D values decreasing from 20 min at 2 °C to 16.7 min at 25 °C (174). A similar behavior was observed in the case of a barotolerant strain of *S. aureus*, at 400 MPa, with D values decreasing from 2.15 min at 45 °C to 1 min at 50 °C (193).

Nonetheless, at 600MPa, using low pressurization times, increasing HPP temperatures seems to have limited effect as seen on *S. aureus* ATCC 12600 (Table 1.4), for which reductions were very similar for 4 °C, 21 °C and 45 °C using 4 min and, only at pressurization times of 8 min, the temperature effect became visible (178).

iii) Holding time

HPP efficacy also depends highly on the holding time (130), *i.e.*, using higher holding times usually increases the efficiency of the process. Inactivation values for *S. aureus* CECT 534 (Table 1.4) using pressures of 500 MPa at 25 °C increased from approximately 1.7 log CFU·mL^{-1} at holding times of 5 min, to approximately 2.2 log CFU·mL^{-1} at holding times of 10 min, and to approximately 3.2 log CFU·mL^{-1} at holding times of 15 min (174). The increase in inactivation with increasing holding times is widely de- scribed in other studies (Table 1.3 and Table 1.4). However, in some studies, differences between *S. aureus* inactivation is not always significant by increasing holding time, such as the results achieved for liquid whole egg, in which at 370 MPa (Table 1.4), results showed an inactivation of 2.34 log CFU·mL^{-1} at 5 min and of 2.47 log CFU·mL^{-1} at 15 min (183).

Pressure can be applied either in a continuous way or using cycles (pulsed HPP treatment), with this last approach resulting in higher inactivation. This is the case for *S. aureus* ATCC 6538 (Table 1.4) which showed an inactivation of 1.9 log CFU·mL^{-1} when it was pressurized at a continuous holding time of 15 min, while the inactivation increased to 2.2 log CFU·mL^{-1} when 3 cycles of 5 min are applied and to 4.15 log CFU·mL^{-1} when 5 cycles of 3 min are applied (177). The same pattern was described in another study (Table 1.4), using a pressure of 500 MPa, with a reduction of 4.28 log CFU·mL^{-1} for 60 min and, using 2 cycles of 30 min, the reduction increased to 5.89 log CFU·mL^{-1}, while using 6 cycles of 5 min or 3 cycles of 10 min, inactivation was higher than 6 log CFU·mL^{-1} (180).

iv) Compression and decompression rates

Results achieved from studies relating to compression and decompression rates are scarce and conflicting (194). In microbial cells and spores, slow compressions might be able to cause stress responses turning HPP much more efficiently and fast decompressions might lead to higher inactivation due to a fast adiabatic expansion of water (114,195). In *S. aureus* and *E. coli* the combination of fast compression and slow decompression is reported to cause higher inactivation rates when compared to the opposite (194,195). However, it has also been described for *L. innocua* that inactivation rates are not affected by using either combinations (*i.e.*, fast compression with slow decompression and slow compression with fast decompression)

over the other (196). For *S. aureus* ATCC 6538, inactivation in skimmed milk and tris buffer was more effective using fast compression (33MPa/s) with medium decompression (28 MPa/s) (mainly due to mechanical injuries that occurred during pressure change in fast compression and in slower decompression due to the extension of the process time after pressurization since cells were already more sensitive because of pressure holding time) and in orange juice the inactivation was effective independently of the combinations used (194). In *E. coli*, the high effectiveness of fast compression seems to be related to the adiabatic heating during compression while the high effectiveness of slow decompression seems to be associated with the extension of processing time (194).

1.1.2.6. Antimicrobials

In order to increase HPP efficiency, antimicrobials such as bacteriocins, lysozyme, chitosan or essential oils can be used in combination with HPP (130). Depending on the antimicrobial used, the synergistic effect can be inexistent, small or high. In *S. aureus* ATCC 6538 (Table 1.3), the use of only HPP treatment at pressure 250 of MPa caused a reduction of 2.2 log $CFU \cdot mL^{-1}$ in skimmed milk but, by using the same pressure along with lacticin 3147, full inactivation (> 6 log kill) was achieved (148). In *S. aureus* LMMBM14 (Table 1.3), the synergistic effect between HPP and nisin and between HPP and bovine lactoferrin was observed, with one extra log of inactivation when compared to HPP alone, but there was no synergistic effect using pepsin hydrolysate of lactoferrin or lactoferricin with HPP (149). The use of HPP with a cocktail of two bacteriocins (nisin and pediocin AcH) caused full inactivation of *S. aureus* 485 (Table 1.3) in chicken soup cream compared to an inactivation of 5.7 log $CFU \cdot mL^{-1}$ using only HPP (111). The enterocin AS-48 shows no synergistic effect when used in a *S. aureus* multi-strain cocktail (CTC 1008, CTC 1019 and CTC 1021) inoculated in a fuet (fermented traditional sausage) (Table 1.3) (155). Using lysozyme as an antimicrobial (Table 1.3), only hen egg white lysozyme seems to have a small synergistic effect in *S. aureus* ATCC 27661, when compared to the synergistic effect of goose egg white lysozyme, or with cauliflower lysozyme, or with mutanolysin from *Streptomyces globisporus*, bacteriophage λ lysozyme and bacteriophage T4 lysozyme (151).

Nonetheless, the synergistic effect seems not only dependent of HPP conditions but also of the strains used and study matrices. This was observed when the synergistic effect of nisin was tested (193 MPa for 4×10 min at -20°C) in *S. aureus* ATCC 25923 and results show that in phosphate-buffered saline (pH 7.0) nisin in combination with HPP was unable to cause bactericidal effect on ATCC 25923 cells (Table 1.3), however there was a synergistic effect in McIlvaine citrate-phosphate buffer (pH 5.0) (154).

Oils such as clove oil and cinnamon oil (Table 1.3) are also described as possessing synergistic effect, causing an increase in HPP inactivation when compared to HPP treatment alone (156).

Bacteriophages (Table 1.3) can also be used and, although no synergistic effect was detected after HPP treatment in *S. aureus* Sa9, after 48 h the combination of HPP with the phage cocktail led to complete inactivation (approximately 9 log CFU·mL^{-1}), most likely due the fact that HPP sub-lethally injures the cells of *S. aureus* which become easier to be infected by the bacteriophages (157).

1.1.2.7. *Staphylococcus aureus* resistance to HPP

Food preservation methods apply one or more environmental stresses in foodborne bacteria in order to avoid or slow its growth (197). As response, stressed foodborne bacteria can react by adapting phenotypically or genotypically (197). Phenotypically, when wild type colonies of *S. aureus* were compared with colonies of a baroresistant sub-population (obtained by subjecting the original bacterial suspension to HPP treatments), these mutants presented a weak growth, small sized colonies, weak agglutination reactions, weak coagulation with small clumps, defective production of staphyloxanthin, identical hemolysis to wild type, higher susceptibility to tested antibiotics and diminished capability to invade the Caco-2 human colon adenocarcinoma cells, but possess, however, increased thermotolerance and barotolerance (reductions lower than 0.8 to 2.8 log CFU·mL^{-1} when compared to the wild type) (198). Genotypically, protection against the effects of HPP can be granted by mutations in regulators that are responsible for the repression of specific groups of stress-response genes (199). The *S. aureus* σ^B factor is a regulator responsible for the expression of several genes, at least 251, encoding virulence factors and stress-response proteins, and its activity is maximum in the stationary phase of growth (200,201). In *S. aureus*, the deletion of the sigB gene (IK 184 strain), which encodes σ^B, causes a reduction in the resistance to HPP when compared to the parental strain (Newton strain) (187). Hence, since *S. aureus* has the ability to adapt both phenotypically and genotypically, these fitness characteristic might be the reason why this species is highly resistant to HHP.

1.1.2.8. Recovery of damaged cells

HPP causes death to a cell when the damages inflicted surpass the capability to repair its damages (130). However, HPP may not always fully inactivate bacteria and might only cause injuries to the cells (202). Bacteria can recover from injuries under certain conditions which makes this ability extremely important from the food safety point of view since the recovery of

bacteria during storage has been described in several studies. For example, *L. monocytogenes* treated by HPP increases the expression of genes related to the repair mechanisms of DNA, protein complexes of transcription and translation, the septal ring, the system of general translocase system, flagella assemblage and chemotaxis, and lipid and peptidoglycan biosynthetic pathways (203).

The ability to repair the damages in bacterial cells after HPP is very important to avoid an overestimation of HPP inactivation (202). Usually, assessing the recovery of *S. aureus* after HPP involves the use of non-selective media [such as tryptone soya agar (TSA) or brain heart infusion (BHI)] and selective [such as media supplemented with NaCl or Baird-Parker agar (BPA)].After HPP, *S. aureus* cells, and other bacterial cells, can present 3 possible states (202):

i. Active cells - colonies are formed on both non-selective and selective agar;
ii. Primary injury - structural damages like cell wall and/or cell membrane injury; colonies are formed on non-selective agar but not on selective agar, however they can grow much more slowly in this last medium;
iii. Secondary injury - metabolic injuries; no colonies are form in either non-selective or selective agar, however, colonies might appear after a long period of storage first on non-selective agar and later on selective agar.

From pressures between 350 and 550 MPa, *S. aureus* is thought to suffer secondary injury after HPP treatments, which poses the need for studies to be done over longer periods of time in order to detect the possible repair of secondary injured cells to primary injured or to active cells (202). D values might be different when the recovery is done in selective and non-selective and, depending of the study matrix, these values might be higher in the selective media or the other way around. This is observed when the same barotolerant strain of *S. aureus* is pressurized in a food model at 450 MPa, with D values of 26.4 min for the selective media (BPA) and of 18.6 min for the non-selective media (BHI agar), while the opposite occurs in phosphate buffer (20.2 and 21.2 min for BPA and BHI agar, respectively) at 350MPa (192,193).

Given that HPP inactivation effectiveness also depends on the study matrix, the recovery of cells is also dependent of the study matrix as it can be observed that when HPP is performed in sterilized skimmed milk, orange juice and tris buffer solution, *S. aureus* ATCC 6538 cells can recover or at least maintain some viability when stored at 4 °C for 15 days in all tested matrices except in orange juice in which no viability was observed after 7 days of storage, most likely due to the acidity of the matrix (204). When using meat products as study matrix, after an initial inactivation caused by HPP, levels of a cocktail of *S. aureus* strains were able to remain constant during 120 days of storage at 4 °C in beef loin and in dry ham, however, in cooked ham, levels

decreased over storage and counts were lower than 1 log CFU.g^{-1} at the end of the 120 days (182). In a non-food emulsion model, *S. aureus* ATCC 6538 were unable to recover after being fully inactivated by HPP past 180 days of storage at room temperature (185).

The recovery of *S. aureus* is also dependent of the strain and of the conditions in which they recover, since some strains that are able to grow under non-stressful conditions are unable to grow under stressful (such as in BHI with different concentrations of NaCl, $NaNO_2$, or potassium lactate and in BHI at different levels of acidity), conditions whilst others that are able to grow under non-stressful conditions are also able to grow under stressful conditions (205). *S. aureus* 485 and 765 are able to recover in chicken soup cream with a cocktail of two bacteriocins (nisin and pediocin AcH) after a 5 day storage at 25 °C, even though full inactivation had been achieved after HPP treatment, and at the day 9 of storage levels of viability were higher than the unpressurized sample (111). In cooked ham with nisin and nisin plus potassium lactate treatment levels of *S. aureus* decreased differently ($\rho < 0.05$) over 90 days storage after HPP treatment, showing that cells were not only unable to recover but also that the effect of the antimicrobials was effective during the whole storage period (113). In fact, nisin (Table 1.3) is reported to avoid the recovery of a cocktail of *S. aureus* strains while in storage (113,156,206).

1.1.2.9. HPP inactivation kinetics

The study of HPP inactivation of microbial cells and spores has produced survival curves that were modelled by mathematical functions aiming to describe adequately and accurately the inactivation kinetics (207).Although these models have been previously mainly used in thermal inactivation they have been adapted for HPP inactivation. Therefore, this subject will be interchangeably approached giving priority to HPP inactivation kinetics of *S. aureus* when information is available. By applying accurate inactivation kinetics, both the bactericidal effect of HPP and the implementation of safe processing conditions become possible (135,206,208), while a thorough knowledge of inactivation kinetics would ideally assure a decisive application of HPP (187).

Kinetic models are mainly used to mathematically quantify microbial inactivation data achieved in HPP experiments (usually isothermal), using different processing parameters such as pressure, temperature and/or time (110).When microorganisms are exposed to HPP, or to other lethal food processing technologies, the concentration of survivors decreases with increasing processing parameters (209). Most often, the inactivation of microorganisms follows first-order kinetics (also known as the log-linear model), which means that, over time, all cells would have the same sensitivity and probability of dying over pressure treatments (135,209–

211). In a simple explanation, for cells treated with HPP, the logarithm of the surviving cells is plotted against time, producing a straight line (209). From this model one can calculate the D value, which is the time, usually in minutes, needed to decrease the number of microorganisms' cells by a factor of 10 (usually indicates as one log or decimal reduction of the number of microorganisms) and it is used as a measure of the microorganism resistance to HPP (209). From the temperature dependence of D value the "z-value", which is the temperature interval at which D value will decrease or increase by a factor of 10, can be determined (212). However, although first-order kinetics adequately describes many cases of thermal and HPP microorganisms' inactivation, the many exceptions to this model rendered it to be an exception rather than the rule. Consequently, over the last 25 years, nonlinear models for inactivation data appeared as a preferable alternative (187,207,210,211,213–215).

The use of nonlinear models is the result of important deviations from linearity due to the display of curves with a shoulder (downward concavity), tailing (upward concavity) and sigmoid shape (it begins with an upward concavity and finishes with a downward concavity or vice versa) (216). The shoulder phenomenon is the period in the beginning of the survival curve in which no measurable inactivation takes place after pressure application (217). The tailing phenomenon can be described as the stabilization at the end of a survival curve after an initial linear decrease due to the existence of a pressure resistant population in a culture, by the effects of experimental conditions, or cell-age distribution (110,218). When in rare cases both phenomena occur, survival curves with sigmoid profiles appear (187).

In fact, the complexity of effects of HPP treatments in microbial cells makes HPP inactivation difficult to follow first-order kinetics. Nowadays, it is accepted that nonlinear inactivation kinetics models explain the majority of pressure inactivation curves (207). The most commonly used nonlinear models in HPP inactivation kinetics include the Weibull (219), modified Gompertz (220), log-logistic (221), and the Baranyi (222) models. The use of these models assumes the existence of differences in the survival time due to differences in individual cells (209) and yields several independent parameters that allow the characterization of shapes as well as the response of the cells in relation to time (207). Even though first-order kinetics is not always followed, there are numerous studies where D and z values are calculated by using first-order kinetics models (211). This is true for many published papers concerning *S. aureus* inactivation by HPP (Table 1.4) and so, in this section only papers which refer and/or compare other models will be approached.

Chen (2007) (210) fitted the inactivation data of *S. aureus* (strain 210) in UHT whole milk at two different conditions (21.5 °C/600 MPa and 50 °C/500 MPa) with linear, Weibull, and log-

logistic models and computed their mean square error (MSE; smaller values of MSE indicate a better fit of the model to the data) values were computed. For the condition 21.5 °C/600 MPa the log-logistic model (MSE=0.3) allowed a more accurate description of the inactivation of *S. aureus*, followed by the Weibull model (MSE = 0.7). For the condition 50 °C/500 MPa both the log-logistic model and the Weibull model allowed an adequate description of the inactivation of *S. aureus* with a MSE value of 0.2. For both conditions, the linear model presented higher MSE values (1.7 for 21.5 °C/600 MPa and 2.4 for 50 °C/500 MPa), showing that this model should not be used to describe *S. aureus* inactivation under these conditions. Cebrián et al. (2010) (187) studied HPP inactivation kinetics of eight *S. aureus* strains under different conditions (pressures between 350 and 600 MPa and holding times up to 60min) and fitted the Baranyi model to the inactivation curves, since the study aimed to analyze different phases of inactivation (shoulders, log-linear phase of inactivation and tails). The Baranyi model allowed the authors to describe accurately these phases and consequently to separately correlate each phenomenon with the mechanisms of inactivation. In the study of Tassou et al. (2007) (192), HPP inactivation kinetics of a HPP resistant strain of *S. aureus* was examined in different matrices (phosphate buffer and ham model system). Although the log-linear model was applied to determine D and z values, the Baranyi's model was fitted to the ratio $\log10(N/N_0)$ for the estimation of the death rate constant, the standard error of fit and the correlation coefficients. The authors observed an apparent first-order kinetic behavior for both matrices, but the inactivation data was better described by the Baranyi's model. Guan et al. (2006) (178) studied HPP inactivation kinetics in *S. aureus* (ATCC 12600) at different process temperatures in UHT whole milk. By examining the survival curves they observed that the linear model was unsuitable to describe the obtained survival curves and so they fitted the data to three nonlinear models (Weibull, log-logistic and modified Gompertz) and computed their MSE. The modified Gompertz model was more appropriate to describe the inactivation of *S. aureus* at processing temperatures of 4 and 21° C, while the Weibull model was more appropriate at 45 °C. The Weibull model was also successfully applied to describe the survival curves of *S. aureus* (strain 485) in carrot juice and in peptone water (216). In the study of Viazis et al. (2008) (181), HPP inactivation kinetics was assessed in two *S. aureus* strains (ATCC 6538 and ATCC 25923) in human milk and in peptone water. For the strain ATCC 6538 first-order inactivation kinetics was observed. However, due to tailing phenomenon observed for the strain ATCC 25923, the investigators fitted the data to the Weibull model, thus demonstrating a much higher fit compared with the linear model initially applied. Yao et al. (2015) (206) also achieved a good fit of data to the Weibull model for HPP inactivation kinetics in *S. aureus* (CGMCC 1.1861, ATCC 6538) independently of the initial inoculum levels and of the matrix (saline solution or meat slurry). Furthermore, the analysis of the shape factors (n) values (a Weibull model

parameter), indicated that the survival curves of *S. aureus* fitted with Weibull model were concave upward or tailing (n < 1).

Hence, although first-order kinetics has been applied to many HPP inactivation kinetics of *S. aureus*, this model, is not always the most adequate. Data should always be fitted to adequate and accurate models, so that safer processing conditions can be estimated and assured, thus preventing risks by food sub-processing or unnecessary food over-processing, which leads to undesirable food quality losses.

1.3. Concluding remarks

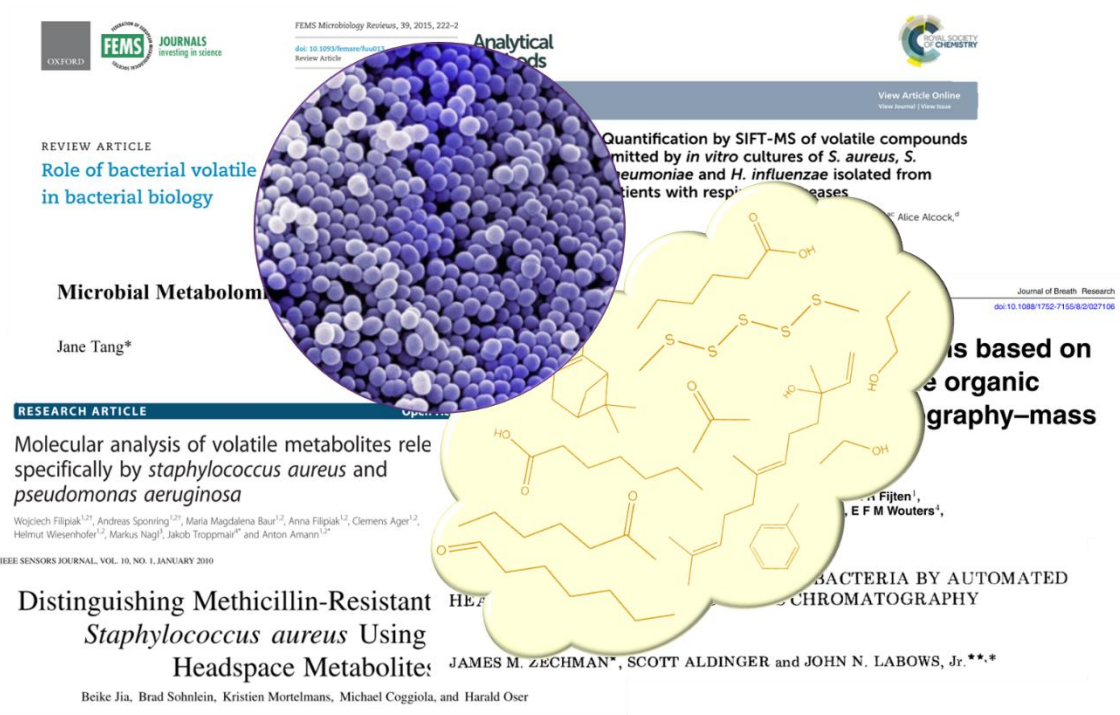
S. aureus HPP inactivation efficiency depends on several factors resembling a cascade of effects rather than being dependent of only one specific factor. Similarly, the inactivation of *S. aureus*, like that of other microorganisms, is a multi-target process. The integrity of cell structures (cell membrane, cell wall, ribosomes) and of biomolecules (*i.e.*, proteins and lipids), is affected by HPP treatment. Monomeric proteins such as SE are hardly affected by HPP, but strains of *S. aureus* with SE are more efficiently inactivated than those without SE. This resistance of SE to HPP is extremely important from the food safety point of view because even though cell death can occur SE properties remain unchanged or are enhanced if processing temperature is not high. However, if HPP is promptly applied, SE producing cells will be efficiently inactivated and will not be able to produce toxins.

When HPP only causes secondary injuries (metabolic injuries), recovery is dependent on the matrix and processing conditions used. Matrices such as Ringer solution and phosphate buffer cause higher HPP inactivation than more complex ones, which emphasizes the significance of using real food in HPP studies to avoid misestimating inactivation. Furthermore, because first-order kinetics is not always verified, and moreover pseudo first-order kinetics can occur, misleading an adequate fit, HPP inactivation data should always be carefully fitted into accurate and suitable kinetic models in order to properly estimate and assure safer processing conditions.

Barotolerance, adaptability to HPP and the ability to recover from HPP are major problems from the food safety point of view. Inactivation of *S. aureus* remains an uncharted territory with much more work to be done in order to understand its behaviour in response to HPP.

CHAPTER 2. Introduction

A thirty year old portrait of *Staphylococcus aureus* volatile exometabolome



Context

For approximately three billion years the planet Earth has been the home of the most diverse group that ever lived, microorganisms (223). Spreading over three domains (Archaea, Bacteria and Eukarya) and possessing a huge population size, microorganisms occupy very distinct ecological niches. Due to their large genetic diversity their metabolism is also extremely diverse. In a simple way, microbial metabolomics is the study of the metabolism of microorganisms under a given set of conditions (224). In this chapter, a thirty year old portrait of the volatile metabolome of *S. aureus* will be presented.

2.1. Concepts and strategies

To understand the volatile exometabolome of *S. aureus*, some concepts need to be defined as well as its assessment strategies, including the collection methods, detection methods and most commonly applied statistical methods. Hence, these themes will be approached in the next subsections.

2.1.1. Metabolome, metabolomics and microbial metabolomics

The metabolome, was defined for the first time by Oliver et al. (1998), as the comprehensive set of metabolites found in a biological cell, tissue, organ or organism, representing the end products of cellular processes, which changes accordingly to the physiology, development or pathological state of the cell, tissue, organ or organism (225). Metabolites are small molecules that offer information related to the cellular state since they are chemically dependent of the metabolism, serving thus as an imprint of the biochemical activity of the cell, tissue, organ or organism (226).

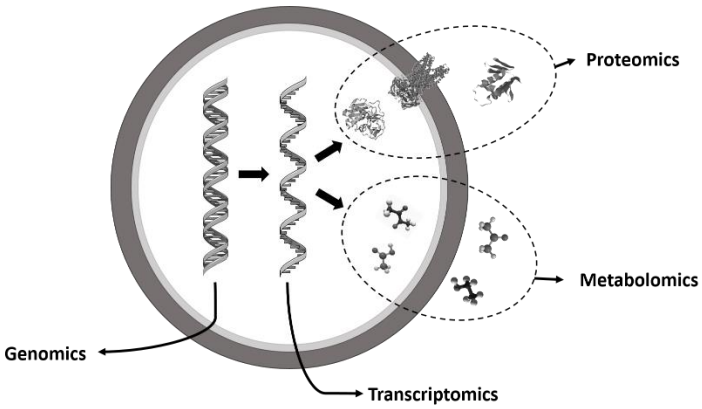


Figure 2.1 - Cell representation of the four "omics sciences" that constitute the Systems Biology discipline: genomics (DNA), transcriptomics (mRNA), proteomics (proteins) and metabolomics (metabolites).

Metabolomics, along with genomics, transcriptomics and proteomics, (Figure 2.1), is part of discipline of a larger discipline called Systems Biology (227). Metabolomics is the study of the

metabolome of a cell, tissue, organ or organism under a given set of conditions, *i.e.*, metabolomics is the comprehensive analysis in which all the metabolites (or only a part) are identified and/or quantified (228).

Microbial metabolomics studies the complete array (or just a specific part) of metabolites belonging to a microorganism and monitors the end result of interactions between its development process and the environment, thus providing a precise picture of the real physiological state of a microbial cell (224). Although microorganisms are generally simple, giving the advantage of being perfect models to develop, apply and validate metabolomic tools, making easier to integrate metabolomic data with the remaining "omics", microbial metabolomics is not an easy field due to the diversity of microbial sort of cells and the existence of three different matrices: intracellular space, extracellular space and the culture headspace, which are quite difficult to distinguish, making the dive in microbial metabolomics a difficult task (229). Furthermore, it is believed that the number of microbial metabolites discovered until now is much smaller than the reality (230), especially when only a small fraction (approximately 1%) of microorganisms is known, with the remaining 99% belong to the "microbial dark matter" (231).

Microbial metabolomics evaluates the role of microorganisms in disease (of human and other animals and plants), bioremediation, compound degradation and the interaction (positive or negative) with other organisms (232–236). Furthermore, it gives a picture of the physiological state of amicroorganism when this is subjected to specific external factor (such as the presence of an antibiotic, the growth in different culture medium, the growth phase and the presence/absence of different nutrients) (237–239). Another important application of microbial metabolomics is the discrimination of microbial species and strains, detection of silent gene mutations and in the production of biofuels (12,239–247).

Some microbial metabolites are volatile, granting specific odors to microorganisms (248). These metabolites, also known as microbial volatile organic compounds (MVOC) or simply volatiles (for easy reading), are produced by microorganisms as by-products of primary and secondary metabolisms (240,249), and are characterized by their low molecular weight (between 100 and 500 Da), high vapor pressures, low boiling points and lipophilic nature (250,251). Volatiles can be fatty acids, hydrocarbons, alcohols, aldehydes, ketones, terpenes, norisoprenoids, nitrogen and sulfur compounds (250). Volatiles have the advantage of being extracted directly from the headspace of the sample without other chemical processes or culturing (252).

Even though the potential of microbial metabolomics is enormous, there are some technical hurdles that still prevent its efficient applicability, such as the effective quenching of cell metabolism when sampling is combined with the separation of intra- and extracellular metabolites, the low ratio between microbial cell biomass and extracellular medium, the lack of standard and robust protocols that can be applied in different microorganisms and under different experimental conditions, the inexistence of a unique analytical method able to detect and identify the whole metabolome of a cell, the identification of microbial secondary metabolites, the characterization of new ones and finally their association with specific metabolic pathways is still slow process (229). Furthermore, because metabolomic analysis express the significance of changes in metabolite abundances (even the non-existence), it must be guaranteed that the intensity of each metabolite can be directly matched to the intensity of the metabolite in question in another sample, which can be, for example, a blank medium control (negative control) (228).

2.1.2. Strategies to study microbial metabolomics

Other than the primary concepts, it is highly important to define the aim of the study, since it will influence sampling, sample processing and analytical techniques (253). This means that there is the need to define a strategy before beginning the study. Thus, in light of the aim of the study, different strategies can be used (254,255):

i. Metabolite target analysis – Identification and quantitative analysis of predefined metabolites of interest concentrations, disregarding the remaining peaks existent in the sample.
ii. Metabolic profiling – Identification and approximate quantitative analysis of a set of metabolites linked by identical physical and chemical properties or connected to metabolic pathways.
iii. Metabolic fingerprinting – The analysis of samples is made without identification and quantification of metabolites. This is a quick method used to determine similarities and/or differences between samples, which can be determined visually or via chemometric techniques. It is usually referred to the analysis of endometabolome (metabolites located inside the cell). This is a fast strategy and ideally appropriated for fast evaluations.
iv. Metabolic footprinting – The analysis of samples is made outside the cell, in the metabolites expelled from the microorganism (exometabolome) into the culture medium or another specimen (food or clinical samples). With this analysis, significant information for functional genomics and strain characterization becomes available and it can also deliver a crucial understanding to cell communication mechanisms. Furthermore, it can provide information concerning the intracellular metabolic status and support the interpretation of metabolic networks and flux.

Microbial metabolomics can use several technologies, either individually or combined, depending on the aim of the study and the type of sample. In microbial metabolomic analysis, samples can be either cell pellets (endometabolome, usually mentioned as metabolic fingerprint analysis) or extracellular culture medium (exometabolome, usually referred as metabolic footprint analysis) (256). Obtaining the latter is generally quite simple, with filtration and/or rapid centrifugation being enough (256,257). However, to acquire the first, a step of quenching is necessary, mainly due to the short half-lives of intracellular metabolites, which currently poses as a problem in microbial metabolomics, with research papers dedicated on comparing several quenching and also extraction methods, with quenching procedures strongly dependent of the microorganism, meaning for example that procedures developed for bacteria might not be directly transferred for yeast (256,258–261).

Because the work concerning microbial metabolomics in this thesis is dedicated only to the volatile exometabolome of *Staphylococcus aureus* (Chapter 5), the aspects related to quenching will not be further developed.

In order to collect *S. aureus* volatiles, SPME has proven to be an extremely efficient method in capturing volatiles of different origins (262,263). SPME has the advantage of not using solvents, being inexpensive and quite simple to use, incorporating in a single step the extraction and concentration of volatiles and needing only a small sample volume (252,262). This method involves the absorption or adsorption of the volatiles onto a pre-coated fiber, like divinilbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), which is exposed in HS above the sample continuing for a determined amount of time and, afterwards, there is the release of the volatiles from the SPME fiber into an injection port (252,262,264).

Once the *S. aureus* volatiles have been collected in the SPME fiber, the next step is to analyze those metabolites. This step can be achieved using several analytical platforms that use nuclear magnetic resonance (NMR) and mass spectrometry (MS), for which the resolution, sensitivity and selectivity can be improved when coupled to gas chromatograpy (GC) or liquid chromatography (LC) (254).

In order to improve the lack of accuracy in metabolite quantification due to peak distortion, comprehensive two-dimensional gas chromatography (GC×GC) appeared as an alternative method with higher separation efficiencies reducing the problem of co-eluting metabolites and improving their quantification, having also the advantage of improving detectability (less co-elution and sharper peaks), and increasing identity assignment (265). For GC×GC time-of-flight mass analyzers coupled with MS (ToFMS), high spectral acquisition rates

while granting an elevated number of data points with a high mass resolution and high mass accuracy is provided. The GC×GC technique uses two columns, usually a nonpolar and a polar column, which are linked through a modulator, i.e., two different GC columns in stationary phase are linked via an on-column injector, the modulator, which transfers consecutively effluent from the first dimension into the second dimension (266,267). The use of GC×GC-ToFMS has been applied in microbial metabolomics with success (12,257,267).

There are many analytical platforms being used in *S. aureus* metabolomics, such as selected-ion flow-tube mass spectrometry (SIFT-MS) (243), secondary electrospray ionization mass spectrometry (SESI-MS) (268), proton-transfer-reaction mass spectrometry (PTR-MS) (269), gas chromatography with flame ionization detector (GC-FID) (270) and electronic nose (e-nose) (271).

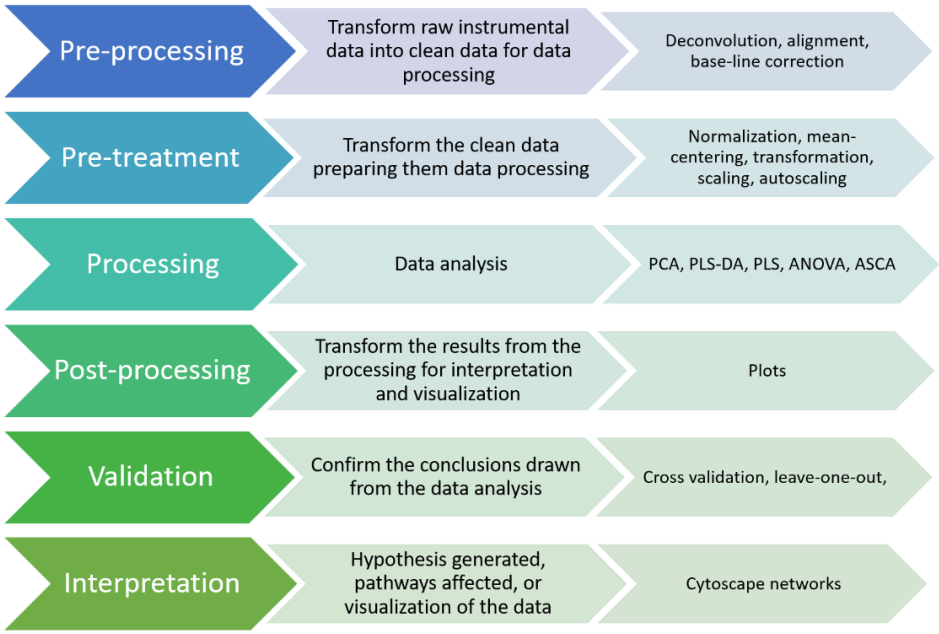


Figure 2.2 - Data processing and interpretation of a metabolomics experiment (272).

After the data is acquired from GC×GC-ToFMS equipment and in order to extract the required information, data processing and data analysis need to be applied. In Figure 2.2 are the guidelines of data processing and interpretation of a metabolomics experiment according to the metabolomics standards initiative (MSI) (272–274).

Concerning data processing, there are several statistical analysis methods used for metabolomics. This is usually performed in order to understand and give sense of data generated. Multivariate analysis is the most commonly used in metabolomics, with principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) being the two most applied statistical methods (275). PCA is an unsupervised statistical analysis, which preserves as much of the variance from the original data using the lower dimensionality (usually

two or three dimensions) output data as possible (276). PCA enables the visual assessment of the samples, which are distributed in the principal component (PC) space, using score plots (275). On the other hand, PLS-DA is supervised statistical analysis used to improve the separation between groups (275). Another common statistical analysis is hierarchical clustering, which also provides a visual judgement by dividing the observed datasets into subgroups, placing samples with similar metabolic profiles closer, i.e., in the same subgroup (275,277).

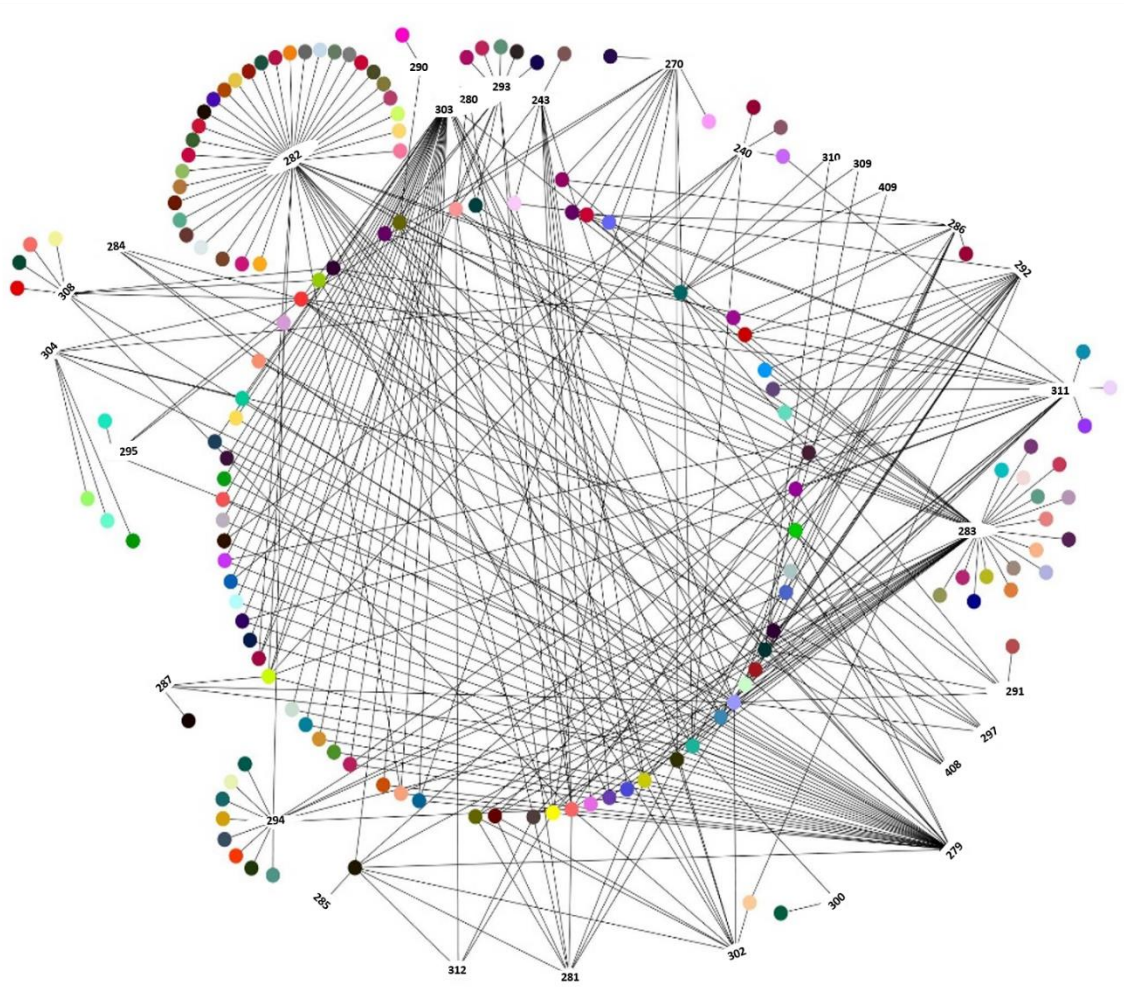


Figure 2.3 - Diagram representing how *Staphylococcus aureus* metabolomic studies are linked by shared volatiles. These same studies also have volatiles that are unique. Transparent nodes with reference numbers represent studies; black lines connect the studies between each other through the coloured nodes, which represent unique or shared volatiles (for color legend see Table A2.2, from Annexes) described for *S. aureus* in those studies.

2.2. Exploring *Staphylococcus aureus* volatile metabolome and related pathways

From 1986 to 2016, forty five studies were published either concerning the volatile metabolome of *S. aureus* or mention specific *S. aureus* volatiles as part of the study or attempted to detect *S. aureus* through its volatiles. In total, there are one hundred and fifty volatiles (Table A2.2, Annexes) described for *S. aureus*, spread over thirty three studies which reported the existence of volatiles, with the remaining twelve studies reporting only *m/z* (mass-to-charge ratio) patterns or *m/z*.

In Figure 2.3, it can be seen how these studies are not only intertwined through common reported volatiles but also possess their own unique described volatiles. While in some of the studies (Figure 2.3) the volatiles reported are almost the same as in other studies, like some sort of good Mona Lisa forgeries with some made by the same artists (243,270,278–281), in others the Mona Lisa forgeries are not as good, i.e., a considerable amount of unique volatiles is reported (282,283). Thus, all studies, in their own methodological approach, aim to find volatiles there are characteristic of *S. aureus* or its strains in order to compare them with other species or other *S. aureus* strains; or find volatiles when the aim is to see the impact of some factor (lack of glucose or oxygen during growth, use of antibiotics, among others) on *S. aureus* metabolome. In the end, most studies have a "higher purpose", which involves the application of volatiles or volatile fingerprints to detect and identify microorganisms (species and/or strains) in diseases in a faster and non-invasive way than the conventional microbiological methods.

There are two ways to address these studies. The first way divides the studies into two: those which identified *S. aureus* volatiles and those which fingerprinted *S. aureus*. The second way, also divides the studies into two different groups: the first concerning *in vitro* studies and the second concerning clinical studies, including proof of concept studies. Thus, at *in vitro* studies, are the studies that use different culture media, growth conditions or techniques to find fingerprints or footprints. The clinical studies, including proof of concept studies, are studies on which sterile clinical specimens are inoculated with cultures of microorganisms to find specific volatiles or volatile fingerprints that allow the identification of a species and/or strains and also studies with the objective of achieving an initial evaluation of a method used to detect volatiles to find the etiological agent of diseases such as pneumonia, sinusitis, and even bacteremia in clinical samples. This approach seems to be the most logical and it is the one followed in the next sub-sections. It is important to mention that whenever a study makes reference to a pathway in the origin of volatiles, this information will be given.

2.2.1. *In vitro* studies

The studies belonging to this group encompass the bulk of *S. aureus* volatile metabolomic studies. These are studies made using different culture media such as BHI, tryptic soy broth (TSB), dextrose broth (DB), nutrient broth (NB), mannitol-salt agar (MSA), blood agar (BA), chemically defined medium (CDM), among others. Within this group the volatiles of *S. aureus* come from different strains, some belonging to the American Type Culture Collection (ATCC), others to the National Collection of Type Cultures (NCTC) or even strains isolated from clinical specimens. Although this group comprises twenty eight studies, the vast majority did not address any metabolic pathways or the ones that mention mostly belong to studies done with ^{1}H-NMR. However, volatiles reported in these studies are very few and are practically the same between them, which makes the pathway described the same, i.e., pyruvate metabolism.

i. Microbial metabolomics to study *Staphylococcus aureus* antibiotic resistance

Perhaps not yet the subject of an increased attention, is the use of microbial metabolomics to understand how antibiotics affect *S. aureus* metabolism. Knowing the origin of volatiles (and the non-volatile metabolites) is indicative of the pathway (and the site in the cell) in which a determined antibiotic will find its target. This is particularly important for *S. aureus*, since this bacterium has developed resistance to many antibiotics over the years (Figure 2.4), making it of Priority 2 (high; where Priority 1 is critical and Priority 3 is medium) for the World Health Organization, in terms of research, discovery and development of new antibiotics (9).

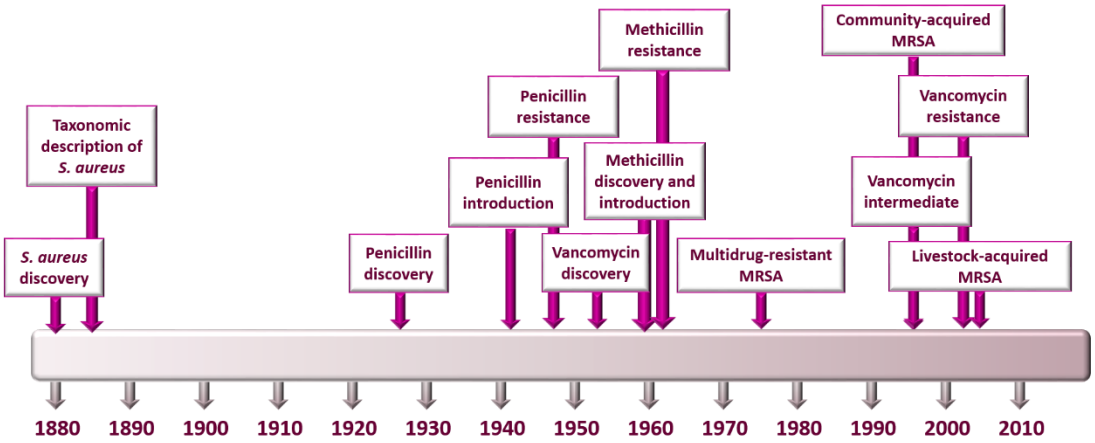


Figure 2.4 - Timeline of *Staphylococcus aureus* development of resistance to antibiotics (8,27).

In a study, performed to assess the effects of triphenylbismuthdichloride (used as an antimicrobial agent to coat catheters) on the volatile exometabolome of *S. aureus* (Sa113) acetate, ethanol and 2,3-butanediol were reported (284). The accumulation of these volatiles (and other non-volatile metabolites), which increased with increasing concentrations of

triphenylbismuthdichloride, suggested that this antimicrobial agent directed pyruvate into alternative pathways, although the mechanism of action remained unknown, indicating that triphenylbismuthdichloride affected pyruvate catabolism (284).

An interesting study also concerning antibiotics was performed with the aim of finding a volatile that could be used as a marker of bacterial growth as a fast method to test the effectiveness of an antibiotic (285). The growth of two *S. aureus* strains (ATCC 29213 and ATCC 43300, sensitive and resistant to oxacillin, respectively) with oxacillin, was tested. Methanethiol was found to be directly correlated to bacterial growth, which the authors classified as a valid maker gas for bacterial growth, which decreased with higher concentrations of the tested antibiotics (285).

ii. Microbial metabolomics to distinguish *Staphylococcus aureus* from other bacterial species

Most studies analyzed in the *in vitro* subsection have the objective of distinguishing *S. aureus* from other bacterial species.

In 1986, one study stood out for being pioneering, and is perhaps one of the most cited works concerning the study of bacterial volatiles (270). This study compared several clinical isolated strains of *S. aureus*, *Pseudomonas aeruginosa*, *Proteus miriabilis* and *Klebsiella pneumoniae*. Sixteen volatiles were described for *S. aureus* strains using both GC-FID and GC-MS. In fact GC-MS added four volatiles to the ones found by CG-FID. In the reported volatiles, *S. aureus* strains produced a distinctive profile that consisted of 2-methyl-1-propanol, 3-methyl-1-butanol and 3-hydroxy-2-butanone, with this last volatile being unique for *S. aureus* (270).

Another study aimed to distinguishing *S. aureus* ATCC 25923 from other bacterial species (*P. aeruginosa*, *E. coli* and *Salmonella typhimurium*) (286). The spectra of each bacterial species were qualitatively unique, possessing distinctive features that could be used to discriminate the tested bacterial species, based only on their volatile profiles (286). Thirteen volatiles were reported in total, of which eleven volatiles (Table A2.2, Annexes) were detected for *S. aureus,* from which nine volatiles were shared with other studies, such as 3-methyl-1-butanol, ethanol and acetic acid, while others, such as acetonitrile and pyrimidine, were detected only in this study (286).

Other studies were also able to positively discriminate *S. aureus* from other bacterial species. This is the case in which *S. aureus* DSM 13661 was distinguished from other fourteen bacterial species using the volatiles acquired via multi capillary column-ion mobility spectrometry (MCC-IMS) (with GC-MS confirmation) (287), or by using fingerprints obtained for

S. aureus (ATCC 25923) and three other Gram-positive bacteria, differences between spectra were found (288), and even differences between *S. aureus* ATCC 25923 from *S. typhimurium* and eleven strains of *E. coli* O157:H7, independently of the media culture used (meat extract medium; vegetable extract medium and apple extract medium) were also found (289).

An interesting study comparing mainly long-chain alcohols and other volatiles emitted from twenty four foodborne Gram positive (including *S. aureus* ATCC 12600) and Gram negative bacteria concluded that the first group of bacteria produced a lower number of long-chain alcohols, as well as other volatiles, than the latter (290). Furthermore, it was also reported that for Gram positive bacteria a more frequent detection of polysulfides (especially dimethyl disulfide) was found (290). Concerning specifically the *S. aureus* strain studied, there was a lack of production of long-chain alcohols and only two volatiles were reported: 2-tridecenone (volatile uniquely reported in this study) and dimethyl disulfide (volatile commonly reported not only for *S. aureus* but also for other bacteria) (290).

iii. Microbial metabolomics to distinguish *Staphylococcus aureus* strains

Some studies aimed to distinguish between *S. aureus* strains. One of those studies aimed to detect differences between one MSSA (ATCC 29213) and one MRSA (NRS 382) strains, using two different types of SPME fibers (282). Differences between the two strains were able to be detect, especially if the SPME exposure time was of 8 h (versus 10 min every hour for a total of ten hours) (282). This study reported 46 volatiles (Table A2.2, Annexes) in total, including commonly found *S. aureus* volatiles, like 3-methyl-1-butanol, 3-methylbutanal, ethanol, acetic acid, 3-methylbutanoic acid and acetaldehyde or unique volatiles including the halogenated compounds N,N-dimethylsulfamoyl chloride and 2,4,6-triphenyl-1,3,5-tripropylborazine, decanal, 2-methyl-3-hexanol among many others (282). Actually, this study stands out in Figure 2.3, on the top left, not only for being linked to many shared colored nodes but also for having many unique colored nodes.

A singular study is the one of Chippendale and co-workers, which used clinical isolates of *S. aureus* (four isolates), *Streptococcus pneumoniae* and *Haemophilus influenza*, to follow the development of major volatiles over an increasing period of time (24, 48 and 72 hours) and, by using the concentrations of those volatiles, were able to distinguish two strains of *S. aureus* unknown to the authors until the analysis of the PCA done to *S. aureus* and also by analyzing the concentration of the volatiles detected (243). First, using PCA as multivariate analysis of samples, it was possible to see that *S. aureus* cultures were clearly different from the medium culture (BHI supplemented with lysed horse blood) but, concerning incubation time, no individual

clusters were observed based on the concentration of the volatiles detected (243). The surprise was seen in the same PCA, which showed two different clusters of *S. aureus* (one at the positive PC1 and the second in the positive PC1 and negative PC2) revealing the presence of two groups that comprised distinct metabolic activities, which the authors attributed to probable genotypical or phenotypical differences (243). In this study, eight major volatiles were reported for *S. aureus*. Another remarkable fact, was the intake of ammonia from the medium culture in the first 24 h and, afterwards, its elevated emission, surpassing the concentration of this volatile in the medium culture, most likely produced to raise the external pH (243,291). Furthermore, 2-propanone, which is usually reported as part of the volatiles emitted by this species (Table A2.2, Annexes) was detected in the HS of *S. aureus* cultures, but none of the two groups produced this volatile (243). A distinctive feature of these groups is that one produced higher amounts of methanol, acetaldehyde, ethanol and propanol, and while this same group produced increasing concentrations of pentanal from 48 to 72 h, the other group was only able to produce higher concentrations than the medium culture of this volatile at 72 h. An important aspect of this study is that through the concentration of the volatiles detected and the PCA, it was possible to see the presence of two distinct groups of *S. aureus*.

iv. Microbial metabolomics combining both the distinction of bacterial species and strains

Here we can find at least two important studies. The first compared eleven strains belonging to eight bacterial species, including the strains NCTC 6571 and ATCC 8325 of *S. aureus* (292). The authors used only volatiles released which were significantly higher than the negative controls (blank media culture). Analyzing the resultant twenty three volatiles, significant differences, both qualitatively and quantitatively, were found between species and strains (292). The second study compared eight strains of four species of bacteria, including three *S. aureus* strains, of which the first was only described as a clinical isolate, the second strain was a MSSA and the third strain was a MRSA (293). Twenty five volatiles comprised the dataset analyzed, which was used to compare different strains, and their abundance was compared with the medium control (blank media culture). Using the first three components of PCA it was possible to explain 26.5% of the total variance, showing a separation between the medium control and the four studied microorganisms, with *S. aureus* being characterized by the release of high levels of 2-propanone, dimethylsulfide and dimethyltrisulfide (293). Concerning the MSSA and the MRSA strains, it was possible to observe that they were quite similar between each other and, only after the use of a PLS-DA with cross model validation, differences between the two strains were found using the eight volatiles most dissimilar and descriptive to compare both strains (293). Thus, the MRSA strain was characterized by high abundances of seven of the volatiles

while the MSSA was characterized by the remaining one, which was a halogenated compound (1,1,2,2-tetrachloroethane) (293). Along with this volatile, two other volatiles containing chlorine (trichloroacetic acid and 1,4-dichloroacetic acid) were found in the headspace of the strains of *S. aureus* which, according to the authors, was described for the first time for *S. aureus* (293).

v. Microbial metabolomics studies comparing different *Staphylococcus aureus* growth conditions

Volatiles reported in these studies mainly compare *S. aureus* growth conditions, such as the presence or absence of oxygen, the lack of a substrate or of an enzyme, the type of culture medium, the type of SPME fiber used to collect volatiles from the HS, the type of GC columns, among others.

Concerning the growth of *S. aureus* (ATCC 6538) under aerobic and anaerobic conditions, using ^{1}H-NMR, fourteen volatiles were reported showing that the presence/absence of oxygen had influence on volatiles coming from pyruvate metabolism, namely through ethanol and 3-hydroxy-2-butanone (294). Also the lack of an enzyme such, as formyl transferase, was able to influence pyruvate metabolism (295).

Another sort of study, done with the strain NCTC 6571, aimed to test three different SPME fibers, two different GC columns and three different growth media (240). In total eighteen conditions were tested and eight volatiles were detected in one or more conditions. The volatile 3-methyl-1-butanol was detected in seventeen of the conditions being more an exception than a rule, since the remaining seven volatiles appeared in much less conditions (240).This means that variations in methodologies (different SPME fibers, different growth media and different GC columns) influenced the volatiles detected (240).

In other study in order to evaluate how different conditions influence the volatiles produced by six bacterial species and by a total of twenty one strains, including four strains of *S. aureus*, obtained from routine samples were used (296). Using three different media culture, accordingly to the species tested: MacConkey agar for *E. coli*, *Klebsiella* spp., *Citrobacter* spp., and *P. aeruginosa*; MSA for *S. aureus* and pylori agar for *Helicobacter pylori*, it was found that the volatiles detected for all species belonged to different chemical families: acids, alcohols, hydrocarbons and ketones (296). The total concentration of those volatiles per species was assessed and the results showed that *S. aureus* produced lesser concentration of volatiles when compared to the other species, with the exception of *H. pylori* (296). Based on the spectra of the

several species, the authors concluded that the species were unequivocally different from each other, with some masses being unique of each species (296). However, one can wonder if the differences found on the volatile concentrations and the spectra of the different species were not the result of using different media culture, one for the Gram-negative bacteria (except *H. pylori*), another for *S. aureus* and another for *H. pylori*. The different media culture used have different composition, which will influence the pathways used by the different bacteria, which in turn will influence the volatiles released. Thus the "explicit" differences between bacterial species can be questioned.

In another study, the volatiles released by *S. aureus* (a clinical isolated strain) grown in NB, BHI and DB were assessed (269). By subtracting the background mass spectra of the negative culture media controls, it was possible to stay only with the volatiles released from the growth of the cultures in the different media culture (269). The authors concluded that the types, but not the concentrations, of volatiles released by *S. aureus* were not dependent of the growth broth (269). Nonetheless, the authors also reported that the time evolution and intensity of the volatiles detected as well as the growth of the culture were highly dependent of the growth medium (269), which is expected due to the different composition of the media which will influence the time in which the maximum concentration of volatiles will occur since their concentration is dependent of the growth phase of the bacterium. In fact, even the lack of glucose has influence in the central pathways of *S. aureus*, with a decrease in the release of the larger part of metabolites associated with those pathways (297).

Consequently, based on the results of Lechner et al., 2005 (296) and of O'Hara and Mayhew, 2009 (269), a gap can be found in the studies of the volatile fraction of microbial metabolomics. It seems to lack a study which compares the volatiles released from a bacterium, such as *S. aureus*, when it grows in different media, and that also compares it with other bacteria grown at the same time and in the same media, using growth curves in addition to the detection of volatiles. Preferably, this study should be done using a combination of analytical techniques to cover as many metabolites as possible (volatiles and non-volatiles) and as many pathways as possible.

Finally it is important to mention the studies performed with an electric nose (e-nose) which aim to identify different bacterial species through volatile patterns as a potential method of non-invasive detection of infections (271,298–301).

2.2.2. Clinical and proof-of-concept studies

In this subsection are presented some examples of studies performed for *S. aureus*, that use inoculated sterile clinical specimens to find volatiles or volatile fingerprints. In general, most proof-of concept studies dwelling with microbial metabolomics, namely the volatile fraction, usually have a prior phase in which the method is tested with pure cultures in samples of healthy specimen donors and, only afterwards, the method is tested in specimens of unhealthy donors.

i. Microbial metabolomics to study the volatiles released by *Staphylococcus aureus* inoculated in blood

Two studies aimed to detect volatiles released by *S. aureus* inoculated in blood, using BacT/ALERT® media bottles. The first described and quantified the volatiles released at two collection times (6 and 24 h) by five bacterial species, including the strain ATCC 25923 of *S. aureus*, in BacT/ALERT®FA media bottles, using SIFT-MS (302). Nine volatiles which were most likely to be bacterial growth markers, were quantified in both collection times. *S. aureus* was characterized by the production of acetaldehyde, ethanol, ammonia, methanethiol and dimethylsulfide (302). In this study the pattern of ammonia concentration variation was similar to that obtained in the study of Chippendale et al., 2014 (243). The concentration of ammonia was lower than that in the culture medium at 6 h and increased at 24 h, attaining a higher concentration than that in the culture medium (302). The authors found that the profile of *S. aureus* (and the other bacteria) was similar in both collection times, meaning that by using SIFT-MS, the identification could be done at 6 h (302).

The second study aimed to compare the effectiveness of SIFT-MS with the BacT/ALERT automated blood culture system, using two inoculation concentrations (5 and 100 CFU.mL^{-1}) in two types of BacT/ALERT® media bottles (FA and SN) and two collection times (8 and 24 h) for the quantification of growth indicative volatiles. For *S. aureus*, it was possible to detect bacterial growth much earlier using by SIFT-MS than the BacT/ALERT® system, between 5 h and 7 h earlier, using both inoculation concentrations and both types of media bottles (280).

ii. Microbial metabolomics to study *Staphylococcus aureus* antibiotic resistance

Also using the same bacteria as the two previous studies and using two different types of BacT/ALERT® media bottles (FA and SA), this study aimed to assess not only bacterial growth but also to evaluate antibiotic susceptibility through changes in the concentration of the volatiles detected (281). Using the concentration of the nine volatiles previously identified (302), *S. aureus* released high concentrations of ethanol, 2-propanone, acetaldehyde, acetic acid, ammonia and dimethyl sulfide (281). When the effect of flucloxacillin was tested below and

above minimum inhibitory concentration (MIC), the production of ammonia and dimethyl sulfide was inhibited at 6 h. At 22 h, aminoacetophenone, ethanol and formaldehyde were inhibited by flucloxacillin above its MIC, while below it MIC no inhibition of these volatiles was observed (281). The authors showed that like in the previous study (302), SIFT-MS can detect the volatiles characteristic of bacterial growth at 6 h in both types of media bottles and, that by using volatiles, it is possible to determine antibiotic susceptibility at 22 h (281).

iii. Microbial metabolomics in the detection *Staphylococcus aureus* in respiratory proof-of-concept studies

In proof-of-concept studies there are many that aim to find in microbial metabolomics a quicker and non-invasive method to detect the pathogen responsible for a determined respiratory disease. Thus, by using volatiles released by respiratory pathogenic bacteria, of which *S. aureus* is part, it is possible to make a diagnosis fitting the criteria above mentioned (236,268,279,283,303–308).

One of these studies uses the volatiles released by six pathogenic bacteria responsible for sinusitis, in order to detect their growth in both culture media and in infected sinus-mucus (304). In the first part of the study, the volatiles released by the six bacteria were assessed after growth in blood or chocolate agar. Furthermore, these authors also studied the blanks of both mediums and of the petri dish, which had different volatiles not related to the bacteria-released volatiles (304). *S. aureus* produced 8 volatiles from which 3-methylbutanoic acid was the most abundant and characteristic (304). Volatile analysis from sinus mucus collected from twelve patients showed that for one of the patients the flora was dominated by *S. aureus*, and at least for another, *S. aureus* would be present due to the presence of the volatile 3-hydroxy-2-butanone, although the authors described this volatile as not being that abundant in the pure cultures (304). The authors conclude that these results are positive for the creation of e-nose specific sensors for non-invasive examination of suspected sinusitis (304). Furthermore, due to differences in the abundances of some volatiles or even the appearance/disappearance of others when comparing bacteria grown in media culture and in sinus mucus, the important role of growth substrate and environment is shown (304).

Also, one of these studies compares the volatiles released from two bacterial species (*S. aureus* and *P. aeruginosa*) responsible for ventilator associated pneumonia (VAP) (303). This study stands out not only for the number of volatiles described, of particular interest for *S. aureus*, but also for attributing the pathways in the origin of some of the volatiles detected. *S. aureus* released 32 volatiles (Table A2.2, Annexes), belonging to different chemical families, including 3-methylbutanoic acid, 3-methyl-1-butanol and 3-methylbutanal, which have their

origin in the amino acid leucine (Figure 2.6) or volatiles such as acetic acid ethanol, acetaldehyde, and 3-hydroxy-2-butanone, which have their origin in pyruvate metabolism (Figure 2.6). Because some volatiles appear 1.5 to 3 hours after inoculation of bacteria and most do not appear in the exhaled breath of healthy volunteers (smokers and non-smokers), the monitoring of volatile breath markers of the tested bacteria, as well as other VAP causing microorganisms, can allow the non-invasive detection, and possibly the identification, earlier than conventional microbiological methods. Using this study as well as other studies performed using different microorganisms, these authors used the volatiles detected for a non-invasive detection of the used pathogens, including *S. aureus*, in the lower respiratory tract for the diagnosis of VAP. *S. aureus* was detected in 22.7% of the patients, with twelve of the previously detected volatiles being detected in the end-tidal air of VAP patients (279).

A noteworthy study was done with the aim of using volatiles for the detection of common etiological pathogens as a non-invasive and quick method for the analysis of breath in cystic fibrosis patients (307). In a first phase, human cells were used for the *in vitro* study, which were inoculated with the tested pathogens, including *S. aureus* DSM 20231, and volatiles were assessed. In a second phase, *in vivo* detection of volatiles were assessed in the breath of cystic fibrosis patients. In both phases, volatiles were achieved through SPME and analyzed by GC-MS. Although no identification of the volatiles was done for none of the tested pathogens, the authors were able to see differences between the microorganisms tested but they concluded that the use of volatiles for a non-invasive method, although having a great potential, still need to be improved (307).

iv. Microbial metabolomics in the detection *Staphylococcus aureus* in different specimens in proof-of-concept studies

Proof-of-concept studies were also done using other specimens. A major study was performed with the objective of testing a quick chromatographic method (GC-FID) to detect volatile fatty acids (VFA) in anaerobic blood cultures (309). To achieve this objective, BacT/ALERT®FAN media bottles (for anaerobic bacteria) were inoculated with cultures containing suspensions of twenty six bacterial species (including *S. aureus*) in sterile human blood. The two VFA identified for *S. aureus* were acetic acid and 3-methylbutanoic acid (309). A total of 288 (out of 375) clinical blood cultures were microbiologically positive (309). After being submitted to routine microbiological examination, GC-FID was used to detect VFA present. Results showed that 202 blood cultures had a single bacterial species, and of those only 9 had *S. aureus*, which released the two previously found VFA, although 3-methylbutanoic acid was only occasionally found (309). The authors concluded that using the combination of microbiologically

positive clinical blood cultures grown in anaerobic media bottles, the aerobic bacteria could be distinguished by genus and that the presence of 2-methylpropanoic acid, butanoic acid, 4-methylpentanoic acid and hexanoic acid was typical for anaerobic bacteria (309). However, the main conclusion of this study is not so directed for *S. aureus* but for the ability of GC-FID to detect 11.2% of anaerobes from negative blood cultures undetected by routine microbiological examination (309). The same kind of study was also performed for clinical exudates of different origin and results for *S. aureus* were the same as in the previous studies, with the same VFA being detected (310). Nonetheless, a higher percentage of clinical exudates (20.5%) was detected with GC-FID based on distinct VFA profiles which were reported as negative by cultivation (310).

Using another specimen, is a study that uses routine microbiological methods and volatiles to test samples of milk from cows with clinical mastitis (311). Milk was first tested using routine microbiological methods, and afterwards, if one of the main etiological agents (including *S. aureus*) of mastitis were found, samples were submitted to GC-MS, in order to analyze their volatiles. From the mastitis etiological agents found in the samples, *S. aureus* was the pathogen which produced more volatiles (nineteen in total), being distinguished from the remaining species mainly due to 3-methyl-1-butanol, 2-methylbutanal, 3-methylbutanal, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 2-butanone and 3-hydroxy-2-butanone (311). Using the volatiles produced by the analyzed bacteria, the authors found that they were different depending on the species. However, they were only able to distinguish *S. aureus*, coagulase-negative staphylococci and *E. coli* from the two remaining species of *Enterococcus* spp. (311).

Finally, in order to detect common urinary tract infection pathogens, including *S. aureus* NCTC 7447, volatiles from inoculated sterile urine were studied using SIFT-MS (312). In urine*, S. aureus* was only able to produce four volatiles, including large quantities of ammonia (312). As mentioned before, this production was most likely raise the low extracellular pH (291). In this study it was not possible to completely discriminate between the inoculated microorganisms (312).

2.3. Potential *Staphylococcus aureus* volatile biomarkers

From the studies presented in the previous section, most of them aimed 1) to detect the volatiles of *S. aureus* or its spectra and second 2) to be identify *S. aureus* in different types of samples. Besides, in other studies it has been searched for a pattern of biomarkers that enables a quick identification of *S. aureus* and other species. These studies reported that by using the

volatile metabolites the detection time of *S. aureus* in a sample could be significantly lower than that of the conventional methods. Furthermore, if a biomarker could be found for MSSA or MRSA strains or even for enterotoxic and non-enterotoxic strains, it would be a great detection innovation.

An ideal biomarker must meet several criteria: should be readily available, should be highly sensitive and specific, should vary promptly in response to a specific condition; should provide a deeper understanding about the microorganism; and it should be useful in different areas (313,314). Besides identifying *S. aureus* as an etiological agent, biomarkers can potentially be used to answer an array of different objectives: to detect *S. aureus* in food samples or in the environment, especially in hospitals, can be used to see how *S. aureus* interacts with other microorganisms and with their hosts, allowing to detect differences between colonization and disease, among many others (232–247,315).

Ideally, volatiles with the potential to serve as biomarkers must be reported independently of the growth conditions. Thus, it is important to summarize which are the most reported volatiles of *S. aureus* (Figure 2.5), being more likely linked to the metabolism of this bacterium and not the result of a specific condition or belong to the growth media artefacts or even to the container in which the research is done. In Figure 2.5, it is possible to see that the volatiles that are most frequently detected for *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 (Table A2.2, Annexes). On the other hand, also in Figure 2.5 can be seen that most of the volatiles which are closer to the center, were only reported in one (light blue) or two studies (light lilac), such as 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-propanoic acid, 2,4,7,9-tetramethyldec-5-yne-4,7-diol, 3-phenylfuran, styrene, 2-octanone, 2,6,10,14-tetramethylhexadecane, among many others (Table A2.2, Annexes).

It is, however, important to give up the concept of a biomarker and embrace the concept of a pattern of biomarkers. For example, based on Figure 2.5, it could be suggested the use of ethanol as a biomarker for *S. aureus*. However, this would be inapropriate, because ethanol is a volatile produced by other microorganisms (248). There are, however, some volatiles derived from the catabolism of leucine 3-methyl-1-butanol, 3-methylbutanal, 3-methylbutanoic acid that are commonly reported as associated with *S. aureus* specifically (Table A2.2, Annexes). Another commonly reported volatile of *S. aureus* is 3-hydroxy-2-butanone and also methanethiol and dimethyl disulfide (Table A2.2, Annexes). In fact, the combination of these

volatiles can be a pattern of biomarkers for *S. aureus*. However, more work needs to be performed in order to validate this or any biomarker pattern.

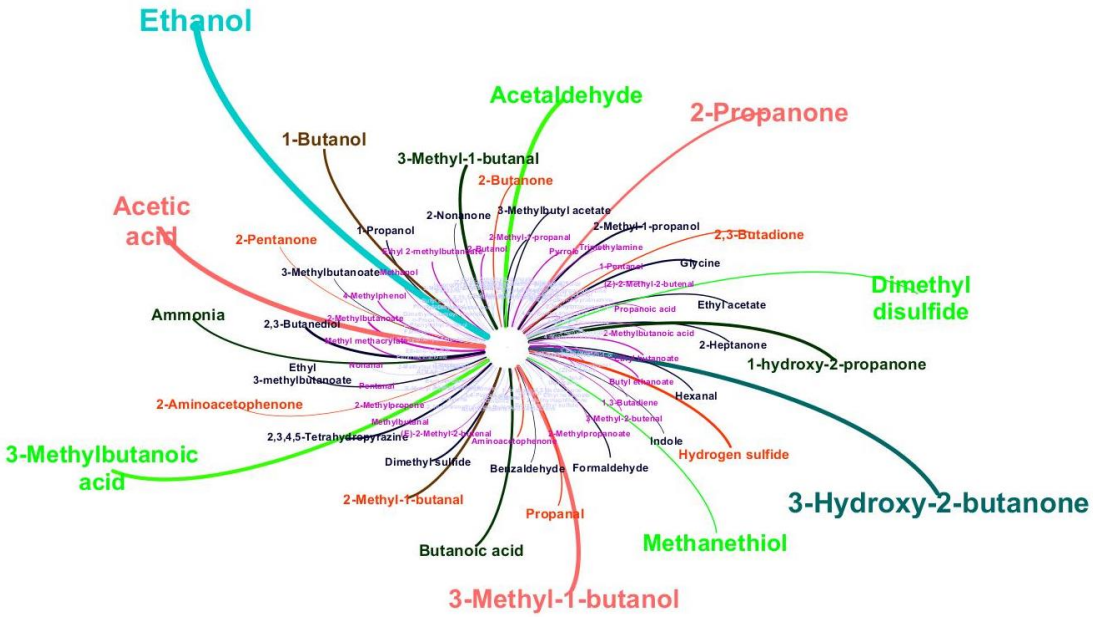


Figure 2.5 - Diagram illustrating the frequency of appearance of volatiles of *Staphylococcus aureus* by published papers (Table A2.2, Annexes). The volatiles nearer the centre with smaller letters and thinner lines are volatiles that appear with less frequency in papers. As volatiles move away from the centre and letters get bigger with thicker lines, the higher is the frequency in which they appear in papers. Volatiles with the same colours appear in the same frequency as each other.

In order to validate a possible biomarker, it is important to know its metabolic origin. All the above mentioned as the most reported volatiles are known to come from specific pathways such as pyruvate metabolism, diacetyl pathway, leucine catabolism, methionine catabolism, or derive indirectly from these pathways (Figure 2.6). However, not all pathways have been described for *S. aureus* and, in most research papers, the pathways are transposed from other microorganism for which the pathway was studied. Concerning *S. aureus*, the pathways described for the commonly reported volatiles have not yet been studied. One example of a commonly described pathway in microorganisms in general is the β-oxidation of fatty acids which is in the origin of methyl ketones. Although 2-pentanone or 2-heptanone, are reported in 4 and 3 studies, respectively, they do not originated in the β-oxidation of fatty acids pathway, because the phylum Firmicutes does not possess this pathway and an alternative pathway remains yet to be found (96).

The selection and identification of patterns of biomarkers will be the first step toward developing and optimizing diagnostic tools that specifically detect the presence of *S. aureus* that are cheaper than the analytical platforms available and that will be able to detect in minutes and without a specialized technician to interpret the results (293,316). Specific patterns can be

recognizable by e-noses as *in situ* diagnostic tool which can be "custom made", through the development of specific and sensitive sensors, to detect a pattern of biomarkers, improving the detection of *S. aureus* in different sort of matrices, from clinical specimens to foodstuff (316,317).

2.4. Concluding remarks

From the studies concerning the volatile fraction of *S. aureus* metabolome, it is possible to realize that the knowledge obtained from 1986 to 2016 is still small, especially when compared to other organisms such as *S. cerevisiae*, *E. coli*, among others. However, it is possible to see that using patterns of some volatiles along with their concentration allow the distinction of *S. aureus* from other bacterial species.

Most of the studies concerning the volatile exometabolome of *S. aureus* mentioned only few volatiles (one or two) while others reported as many as forty six volatiles. In these studies it is stated that the volatiles of *S. aureus* have low carbon numbers. However, analyzing Table A2.2 (Annexes) it is possible to see that *S. aureus* volatiles can have from one to fifty nine carbons.

Furthermore it was patent in some studies the importance of including a control of the used medium (blank culture medium), which can be removed/subtracted to the areas/concentrations of the volatiles in order to have a dataset which is composed only of volatiles that are significantly different from those of the control medium and, thus, have a huge probability of belonging to the metabolome of *S. aureus* instead of the used culture medium. Moreover, the results of these studies also showed that the volatiles produced are strongly conditioned by the used conditions: type of media culture, type of SPME fibers, type of GC columns, time of volatile extraction, the method of dataset selection and the design of the research. It will be important to have more studies using the same experimental conditions in order to define a selective metabolomic profile for *S. aureus* detection/identification.

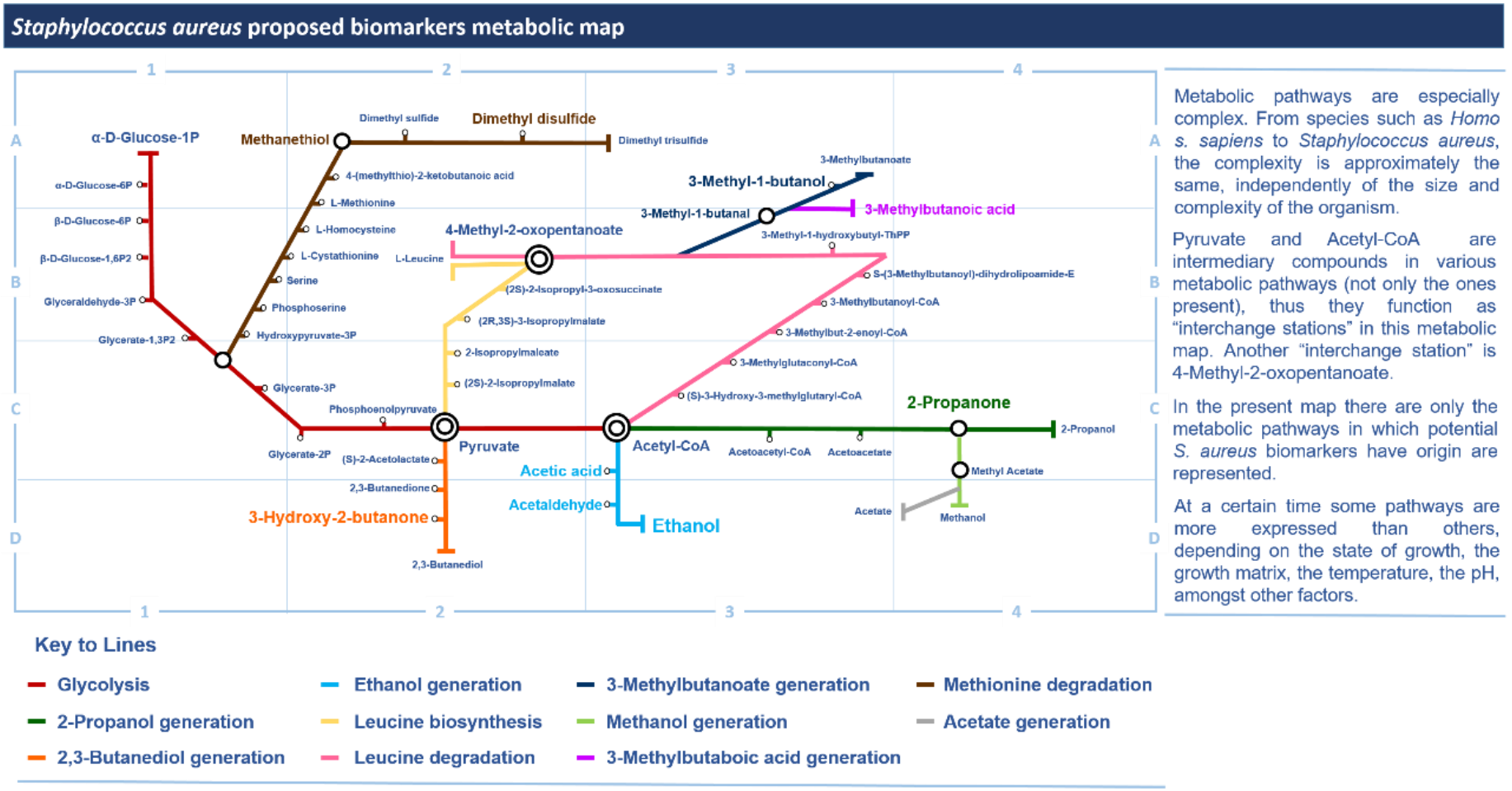
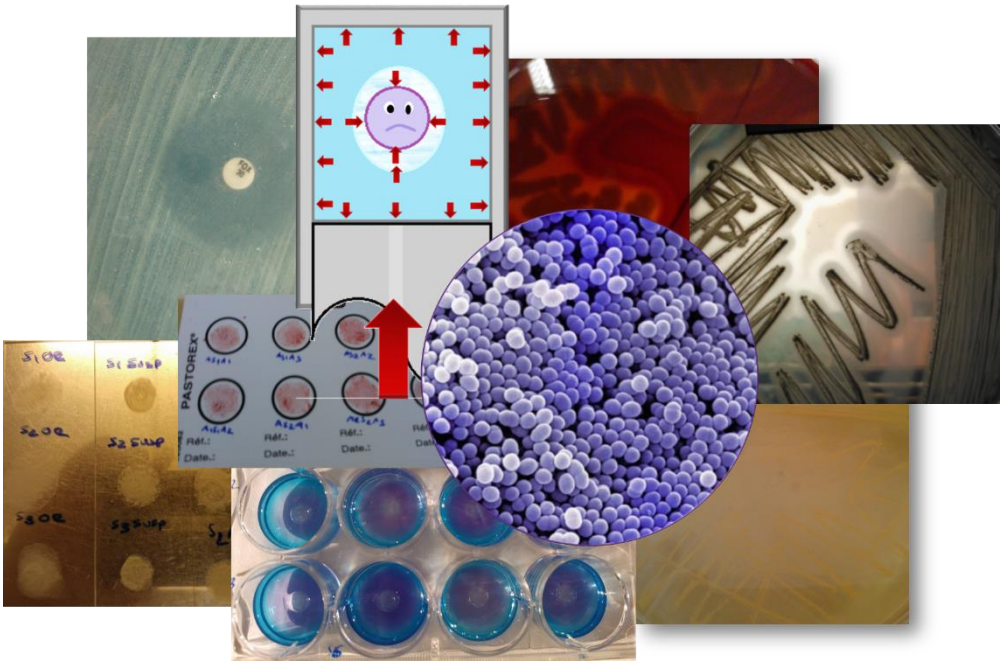


Figure 2.6 - Metabolic map of potential biomarkers in bold for *Staphylococcus aureus* represented as a subway map: different color lines correspond to different pathways, small circles (stations) correspond to metabolites (volatile or non-volatile), and big circles (stations with connection with another line) correspond to metabolites that are the starting point to another pathway or metabolites. Colored metabolite's name and size appear in more than seven papers (303,318–322).

It is also essential to know the origin of the most reported volatiles. Thus, a complete substantiated metabolomic map specific for this such problematic bacterium is yet needed. Transposing pathways from other bacteria is not enough. It is most likely that the central pathways, like glycolysis, pyruvate metabolism and degradation of AA can be equal or similar between bacterial species, or, as seen, can be more or less active in some species rather than others. However, as it can be inferred from Table A2.2 (Annexes), indole, which is a typical volatile of *E. coli*, *H. influenzae*, *K. oxytoca*, is not produced by *S. aureus* because this bacterium does not possess the enzyme tryptophanase. In fact, when biochemical identification tests are performed for *S. aureus* it is always stated that "*S. aureus* is indole negative" (323). Although this is not necessarily linear, especially when the discovery of many *S. aureus* strains even allowed the discovery of non-mannitol fermentative strains and even catalase negative strains, there are perhaps some strains which can be indole positive. This is also similar to the case of the production of methyl ketones via β-oxidation of fatty acids, which *S. aureus* does not possess (324).

Finally, the important message, is that through volatile metabolite studies it is possible to distinguish *S. aureus* from different bacterial species and it is even possible to differentiate within *S. aureus* strains,, such as MRSA and MSSA.

CHAPTER 3. Inactivation of enterotoxic and non-enterotoxic *Staphylococcus aureus* strains by high pressure processing treatments and evaluation of its impact on virulence factors



Adapted from:

I Baptista, SM Rocha, Â Cunha, JA Saraiva, A Almeida, Inactivation of enterotoxic and non-enterotoxic *Staphylococcus aureus* strains by high pressure treatments and evaluation of its impact on virulence factors.

Food Control 57 (2015): 252-257

Abstract

Staphylococcus aureus is responsible for a large spectrum of diseases, including SFP, due to its ability to produce SE. To prevent the development of SFP, effective food preservation methods are needed. HPP uses pressures, between 100 and 600 MPa, to inactivate pathogenic and spoilage microorganisms. In this study, HPP treatments (450 MPa and 600 MPa, both for 15 and 30 min, at 20 °C) were applied in three different strains of *S. aureus* in the stationary growth phase and reduction effectiveness was assessed. A non-enterotoxic strain ATCC 6538 and two enterotoxic strains 2153 MA (with enterotoxin A) and 2065 MA (with enterotoxin A, G, I) were used. It was verified that the non-enterotoxic strain was the most resistant to HPP, not being completely inactivated within 30 min at 600 MPa. Additionally, it was demonstrated that HPP had no effect on virulence factors (enterotoxins, β-hemolysin, lipase, lecithinase, coagulase, thermonuclease, catalase), and also in mannitol fermentation capacity and methicillin susceptibility. HPP treatments also proved to be less effective in the strain with higher carotenoids content (non-enterotoxic strain). The results of this study show that *S. aureus* HPP barotolerance might be related not only to the presence of enterotoxins but also to carotenoids level, although these two factors may not be the only mechanisms responsible for the distinct sensitivity/resistance to HPP shown by different strains of *S. aureus*.

3.1. Introduction

Staphylococcus aureus is a very versatile opportunistic human pathogen that causes a broad spectrum of diseases, ranging from superficial wound infections to septicemia, toxic-shock syndrome and SFP, due to a wide variety of virulence factors, invasiveness capacity and antibiotic resistance (90). SFP appears due to the capability that *S. aureus* has to asymptomatically colonize healthy individuals who inadvertently contaminate foodstuff while manipulating it (325,326). In 2008, SFP represented 5.5% of foodborne poisoning outbreaks in the European Union, being *S. aureus* the fourth most common causal agent in bacterial food poisoning outbreaks (327,328). Most strains are able to produce one or more SE (18). Twenty one SE and SE*l* types have been described (58,329), although two more have been more recently proposed (330). The most common SE isolated from food are SEA, SEB, SEC and SED, with SEA being more frequently recovered from food poisoning outbreaks (329,331). SE are a family of serologically defined, low molecular-weight monomeric proteins (26—30 kDa) (59,84), soluble in water and saline solutions (90). SE are pH resistant, thermally stable and resistant to the activity of the digestive tract proteolytic enzymes (329), allowing them to retain their activity in the digestive tract (90). Hence, heat treatments used to eliminate *S. aureus* may not eliminate SE already formed (332). Ingesting low quantities (100—200 ng) of SE can cause intoxication (327).

Almost all strains produce other virulence factors that grant them the ability to colonize and cause diseases in mammalian hosts. These proteins include hemolysins (alpha, beta, gamma and delta), thermonuclease, proteases, lipases, hyaluronidase, and collagenase (90,333). They also produce carotenoids (mainly staphyloxanthin) that are able to absorb excess energy from reactive oxygen species (ROS), protecting them against hydrogen peroxide, superoxide radical, hydroxyl radical, hypochloride and singulet oxygen and are also responsible to regulate membrane fluidity (170,171,334). MRSA strains are also a major concern since they are present in food products which can be transmitted to humans (335,336).

HPP is an alternative food processing method that preserves the products features such as color, flavor, organoleptic properties and nutritional values (337,338). Food industry uses pressures between 100 and 600 MPa and short pressurization times (3—5 min) for the inactivation of pathogenic and spoilage microorganisms (169,326). The response of microorganisms to HPP depends on microbial species/strain, cell shape, growth phase, matrix, pressure magnitude, pressurization time and temperature (103,339). HPP damaging effects are mainly verified in lipid membranes, protein structure and function and ribosomes (92,338,339). Nonetheless, monomeric proteins can be quite stable to HPP, being dependent on each

individual protein (99,340) and enzymatic reactions are differently affected (being either enhanced or inhibited) (106).

S. aureus poses a major problem for HPP implementation because it is one of the most barotolerant nonsporulated foodborne pathogen (326). There are several studies which tested different strains of *S. aureus* in different combinations of pressure, time, temperature, matrix, different growth phases and with different carotenoid content (92,205,326,338,341).

The main objective of this study was to evaluate the impact of HPP treatments on *S. aureus* virulence factors, and also in ability to ferment mannitol and susceptibility to methicillin. Furthermore, the effectiveness of HPP treatments (different pressures and different holding times) in the inactivation of enterotoxic and non-enterotoxic *S. aureus* was also evaluated. The relevance of studying HPP treatments in enterotoxic *S. aureus* strains and their virulence is due to the high importance of this species from the food safety point of view, even more because it is highly barotolerant to HPP.

3.2. Materials and methods

3.2.1. Cultivation and characterization of bacterial strains

Three *S. aureus* strains were used: a non-enterotoxic strain ATCC 6538 and two enterotoxic strains, 2153 MA (with SEA) and 2065 MA (with SEA, SEG, SEI), isolated from food products and characterized in the Centre of Biotechnology and Fine Chemistry of the Faculty of Biotechnology of the Catholic University, Portugal. All strains are β-hemolysin, lipase, lecithinase, coagulase, thermonuclease and catalase positive. The strain 2153 MA was unable to ferment mannitol. All strains are methicillin sensitive and are able to produce pigmented colonies. The three strains were grown in BHI (Liofilchem, Italy) at 37 °C for 18 h at 170 rpm until stationary phase, corresponding to a concentration of approximately 10^8—10^9 colony forming units per mL (CFU mL^{-1}). Cells were harvested by centrifugation (15,000 × g, 10 min), washed twice and ressuspended in the same volume of sterile phosphate buffered saline (PBS) (pH 7.0). Cell suspensions in PBS were used in HPP treatments.

3.2.2. High pressure processing assays

Cell suspensions were transferred with sterile glass Pasteur pipettes to 0.4 mL polyethylene tubes. For each strain, three independent samples were used and for each one, three sub-samples were prepared (n = 9) and three replicates per sub-sample were analyzed (n = 18). The tubes containing each sub-sample were placed in low permeability polyamide-polyethylene bags (PA/PE-90, Albipack—Packaging Solutions, Portugal) with 70% ethanol,

thermosealed and pressurized. HPP conditions tested were 450 MPa/15 min, 450 MPa/30 min, 600 MPa/15 min and 600 MPa/ 30 min at 20 °C in a hydrostatic press (High pressure system U33, Institute of High Pressure Physics, Poland) and the pressurization fluid was a mixture (60:40) of water and propylene glycol (DOWCAL™, Dow). Non-pressurized controls were also included for each strain in the different conditions.

3.2.3. Enumeration of viable cells

Pressurized and unpressurized samples were 10-fold serial diluted (10^{-1}—10^{-8}) in sterile Ringer Solution (Merck KGaA, Germany). One millilitre of each dilution was pour-plated in triplicate in PCA_m (Liofilchem, Italy). After incubation (37 °C for 48 h) the number of colonies was counted in the most appropriate dilution (plates containing between 30 and 300 CFU). The survivor number was reported as log CFU ml^{-1} and values were used to determine high pressure reduction effectiveness (RE), using the equation

$$RE = \log N_0 - \log N,$$

where N_0 and N represent, respectively, the number of viable cells in the unpressurized suspensions and in the pressurized suspensions.

3.2.4. Virulence factors, mannitol fermentation, methicillin susceptibility and carotenoids determination

To assess if HPP treatments affected the virulence factors of the three strains, non-pressurized controls and pressurized samples were tested for the presence/absence of virulence factors, choosing one typical colony of *S. aureus* from each sub-sample. Based on RE results, the pressure values were set at 500 MPa/15 min for 2153 MA (with SEA) and 2065 MA (with SEA, SEG, SEI) and 500 MPa/30 min for ATCC 6538, since these pressure treatments showed considerable RE, but viable cells were still possible to obtain in order to study the effect on virulence factors. The presence of SEA was determined using SET-RPLA Kit Toxin Detection Kit (Thermo Scientific, United Kingdom), according to the manufacturer's instructions, with positive results based in agglutination patterns (the presence of enterotoxins G and I were not tested since SET-RPLA Kit Toxin Detection Kit only detects the most common enterotoxins: SEA, SEB, SEC and SED). β-hemolysin was determined by streaking BA plates (Sheep Blood 7%; Liofilchem, Italy) and observing a clear/yellow zone surrounding *S. aureus* colonies. Lipase and lecithinase activities were determined by streaking BPA (Liofilchem, Italy). *S. aureus* colonies appear in black with clear zones surrounding them (lecithinase activity), and an opaque precipitation zone (lipase activity). Coagulase was tested using Pastorex Staph Plus (Bio-Rad, United States of America), with positive results shown by agglutination. Thermonuclease (TNAse) was

determined using D.N.A. Toluidine Blue Agar (Bio-Rad, United States of America), with positive results corresponding to a change in the color of halos, from blue to pink (342). Catalase production was evaluated using Catalase/Oxy Test (Liofilchem, Italy), with positive results shown by gas bubbles production. To determine the carotenoids content before pressurization, cell suspensions were collected in stationary phase of growth and optical density was adjusted to a value of 1. Carotenoids were extracted using the methodology described by Morikawa et al. (2001) (343) and quantified by measuring the absorbance at 465 nm. Mannitol fermentation was tested using MSA (Liofilchem, Italy), with positive results detected as color change (from pink to yellow). The susceptibility to methicillin was tested using the cefoxitin disk screen test, performed accordingly to the Clinical and Laboratory Standards Institute (CLSI), in which cultures with halos ≥22 mm are methicillin susceptible and cultures with halos ≤21 mm are methicillin resistant (344).

3.2.5. Statistical analysis

HPP inactivation and carotenoids content results 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 $\rho < 0.05$ were considered significant. Statistical analysis was performed taking into account the three independent assays, each one with three sub-samples and each one with three replicates.

3.3. Results

3.3.1. Bacterial inactivation by HPP

HPP treatment RE on *S. aureus* strains for all tested conditions is presented in Table 3.1. With increasing pressure and holding time an increase in RE was observed (decreasing CFU concentration), for all strains. For ATCC 6538 (Table 3.1), the RE was 2.3 log CFU ml^{-1} for suspensions treated at 450 MPa/15 min and the highest RE (5.1 log CFU.ml^{-1})was obtained at 600 MPa/30 min. Bacterial inactivation was significantly different ($\rho < 0.05$) between the non-pressurized suspension and the different conditions and between all conditions. The exception was between the treatments at 450 MPa/30 min and 600 MPa/15 min.

Both, enterotoxic strains had a similar response to HPP (Table 3.1). RE values in the samples pressurized at 450 MPa/15 min were 4.1 log CFU.mL^{-1} for the strain 2153 MA and 4.8 log CFU.mL^{-1} for the strain 2065 MA. The highest RE was observed at 600 MPa/30 min with values of 8.6 and 9.3 log CFU mL^{-1} for 2153 MA and 2065 MA, respectively. Bacterial inactivation was significantly different ($p < 0.05$) in both strains, in all tested conditions, not only between

the controls and tests but also between conditions. The exception was between 450 MPa/30 min and 600 MPa/15 min which produced a similar RE ($\rho > 0.05$).

Comparing all strains, the inactivation of ATCC 6538 was significantly lower ($\rho < 0.05$) in all treatments from the enterotoxic strains. For each HPP condition, the inactivation between the enterotoxic strains was similar ($\rho > 0.05$).

Table 3.1 - Reduction Effectiveness along different HPP conditions in three *Staphylococcus aureus* strains: ATCC 6538 (without enterotoxins), 2153 MA (SEA) and 2065 MA (SEA, SEG and SEI).

Strain	Sample condition	Log CFU.mL^{-1}	Reduction Effectiveness (log CFU.mL^{-1})
ATCC 6538	Suspension	9.17 ± 0.05	-
	450 MPa/15 min	6.85 ± 0.10	2.32 [b,c,d]
	450 MPa/30 min	5.65 ± 0.31	3.52 [a,d]
	600 MPa/15 min	5.81 ± 0.18	3.36 [a,d]
	600 MPa/30 min	4.06 ± 0.18	5.11 [a,b,c]
2153 MA	Suspension	8.59 ± 0.12	-
	450 MPa/15 min	4.46 ± 0.11	4.13 [f,g,h]
	450 MPa/30 min	2.40 ± 0.11	6.19 [e,h]
	600 MPa/15 min	2.38 ± 0.04	6.21 [e,h]
	600 MPa/30 min	N.D.	8.59 [e,f,g]
2065 MA	Suspension	9.30 ± 0.04	-
	450 MPa/15 min	4.47 ± 0.08	4.83 [j,k,l]
	450 MPa/30 min	3.89 ± 0.04	5.41 [i,l]
	600 MPa/15 min	2.99 ± 0.33	6.31 [i,l]
	600 MPa/30 min	N.D.	9.30 [i,j,k]

N.D. – Not Detected (Below the limit of detection, 1 CFU mL^{-1})

[a] Significantly different ($\rho < 0.05$) from ATCC 6538/450 MPa/15 min
[b] Significantly different ($\rho < 0.05$) from ATCC 6538/450 MPa/30 min
[c] Significantly different ($\rho < 0.05$) from ATCC 6538/600 MPa/15 min
[d] Significantly different ($\rho < 0.05$) from ATCC 6538/600 MPa/30 min
[e] Significantly different ($\rho < 0.05$) from 2153 MA/450 MPa/15 min
[f] Significantly different ($\rho < 0.05$) from 2153 MA/450 MPa/30 min
[g] Significantly different ($\rho < 0.05$) from 2153 MA/600 MPa/15 min
[h] Significantly different ($\rho < 0.05$) from 2153 MA/600 MPa/30 min
[i] Significantly different ($\rho < 0.05$) from 2065 MA/450 MPa/15 min
[j] Significantly different ($\rho < 0.05$) from 2065 MA/450 MPa/30 min
[k] Significantly different ($\rho < 0.05$) from 2065 MA/600 MPa/15 min
[l] Significantly different ($\rho < 0.05$) from 2065 MA/600 MPa/30 min

3.3.2. HPP effect on the virulence factors, mannitol fermentation and methicillin sensitivity

Virulence factors were tested in unpressurized (control cell suspensions) and pressurized samples (Table 3.2). The presence of SEA was detected in the two enterotoxic strains before and after HPP treatments. β-hemolysin, lipase, lecithinase, coagulase, thermonuclease, catalase, mannitol fermentation and methicillin susceptibility in the surviving cells were also not affected by pressurization.

Table 3.2 - Effect of HPP on *Staphylococcus aureus* virulence factors, mannitol fermentation and methicillin resistance for the strains ATCC 6538 (without enterotoxins), 2153 MA (SEA) and 2065 MA and (with SEA, SEG, and SEI). The signal "+" means "presence" and the signal "-" means absence.

Strain	Samples	Virulence Factors							Mannitol fermentation	Methicillin sensitivity (mm)
		Enterotoxin A	β-hemolysin	Lipase	Lecithinase	Coagulase	Thermonuclease	Catalase		
ATCC 6538	UPS 1[a]	-	+	+	+	+	+	+	+	34
	PS 1[b]	-	+	+	+	+	+	+	+	34
	UPS 2[a]	-	+	+	+	+	+	+	+	33
	PS 2[b]	-	+	+	+	+	+	+	+	33
	UPS 3[a]	-	+	+	+	+	+	+	+	32
	PS 3[b]	-	+	+	+	+	+	+	+	32
2153 MA	UPS 1[a]	+	+	+	+	+	+	+	-	27
	PS 1[b]	+	+	+	+	+	+	+	-	27
	UPS 2[a]	+	+	+	+	+	+	+	-	28
	PS 2[b]	+	+	+	+	+	+	+	-	28
	UPS 3[a]	+	+	+	+	+	+	+	-	28
	PS 3[b]	+	+	+	+	+	+	+	-	28
2065 MA	UPS 1[a]	+	+	+	+	+	+	+	+	29
	PS 1[b]	+	+	+	+	+	+	+	+	29
	UPS 2[a]	+	+	+	+	+	+	+	+	29
	PS 2[b]	+	+	+	+	+	+	+	+	29
	UPS 3[a]	+	+	+	+	+	+	+	+	30

PS 3[b]	+	+	+	+	+	+	+	+	30

[a] UPS 1, UPS 2, UPS 3 – Unpressurized samples 1, 2 and 3.

[b] PS 1, PS 2, PS 3 – Pressurized samples 1, 2 and 3.

Carotenoid content was only determined for the unpressurized samples. Absorbance was 0.053 for ATCC 6538, 0.017 for 2153 MA and 0.036 for 2065 MA. Thus, carotenoid content was significantly different ($\rho < 0.05$) between strains and it was highest for the nonenterotoxic strain.

3.4. Discussion

One of the objectives of this study was to observe the behavior of the three *S. aureus* strains to different HPP treatments. Similarly to other studies, an increase in pressure and holding time of treatments caused higher *S. aureus* inactivation (177,205,326,345). Furthermore, O'Reilly et al. (2000) (345) and Fioretto et al. (2005) (177) reported identical inactivation for the non-enterotoxic strain (ATCC 6538). Differences in inactivation of different *S. aureus* strains by HPP, have also been described the studies of Alpas et al. (1999) (346) and Cebrián et al. (2010) (326).

ATCC 6538 was not completely inactivated in the most severe HPP condition tested (600 MPa/30 min) and was more barotolerant to the different treatments than the enterotoxic strains. Under the condition 600 MPa/30 min, the strains 2153 MA and 2065 MA were inactivated to the limit of detection (LOD) of the method (1 $CFU.mL^{-1}$). In view of these results, differences between strains were not only related with the presence/absence of SE but also with the carotenoids content, which was higher for the most barotolerant strain and lower for the most barosensitive strains. Cebrián et al. (2010) (326) also explained the different barotolerant behavior of *S. aureus* strains to HPP based on carotenoids content, with strains with lower carotenoids content more susceptible to inactivation. Our results are in accordance with that study. Actually, the fact that carotenoids content is related with the resistance of *S. aureus* to several stress factors was reported in several studies which show that, mutant strains that are unable to produce carotenoids are more susceptible to those stress factors (171,172,347,348). So, it seems that in this study, for the tested strains, barosensitivity, lower carotenoids content and SE presence are related. Nonetheless, for the enterotoxic strains, the number of SE did not affect significantly ($\rho > 0.05$) their barosensitivity. The fact that barotolerance of several strains of *S. aureus* is dependent of more than one feature is not new. Although the exact reasons behind these differences are not yet understood, the σ^B factor is known to grant resistance to stress factors such as heat and pressure to *S. aureus* since this factor controls 251 genes and/or operons (188,201).

The present research study focused mainly on the ability that surviving bacteria maintained the capability to produce virulence factors, their sensitivity to methicillin and to ferment mannitol. In light of the results obtained at the tested conditions, HPP had no effect in

the capability of surviving cells to produce SEA or any other virulence factors and maintained its biochemical characteristics. Since this detection, alongside the methicillin resistance/susceptibility were performed in the surviving cells, it would be expected that the bacteria, even if injured during HPP treatments, would be able to continue producing those virulence factors and other enzymes. Furthermore, along with the spherical shape, the high impermeability of *S. aureus* membrane seems to provide protection against 350 MPa pressure, as shown by the study of Wang et al. (2010) (349), in which cells maintained their general morphology.

The effect of HPP on proteins is dependent of the type of protein and of the protein itself. For example, monomeric proteins are more stable to HPP than oligomeric proteins, with the first ones being able to withstand higher pressures (depending on each individual protein tolerance) whilst pressures of less than 300 MPa can cause dissociation into individual subunits in the last ones, leading to denaturation (340). Furthermore, at pressures between 400 and 800 MPa, monomeric proteins can unfold and aggregate although, reversible unfolding occurs for many proteins (350,351). If the effect of HPP was tested directly on SE, it would not been expected that SEA suffered many changes, since it is a small monomeric protein (99). This was observed in the study of Margosh et al. (2005) (159), where in HPP treatments between 0.1 and 800MPa for 30 min at 20 °C, staphylococcal SE suffered no change in their immuno-reactivity. It was also observed in the study of René-Trouillefou et al. (2010) (160), in which SEA solutions were submitted to 600 MPa/15 min at 20 °C, that there was no significant immunoreactivity difference between non-pressurized and pressurized SEA. Furthermore, it was observed that under 600 MPa/15 min at 20 °C spectral changes (fully reversible under decompression) occurred in SEA (160).

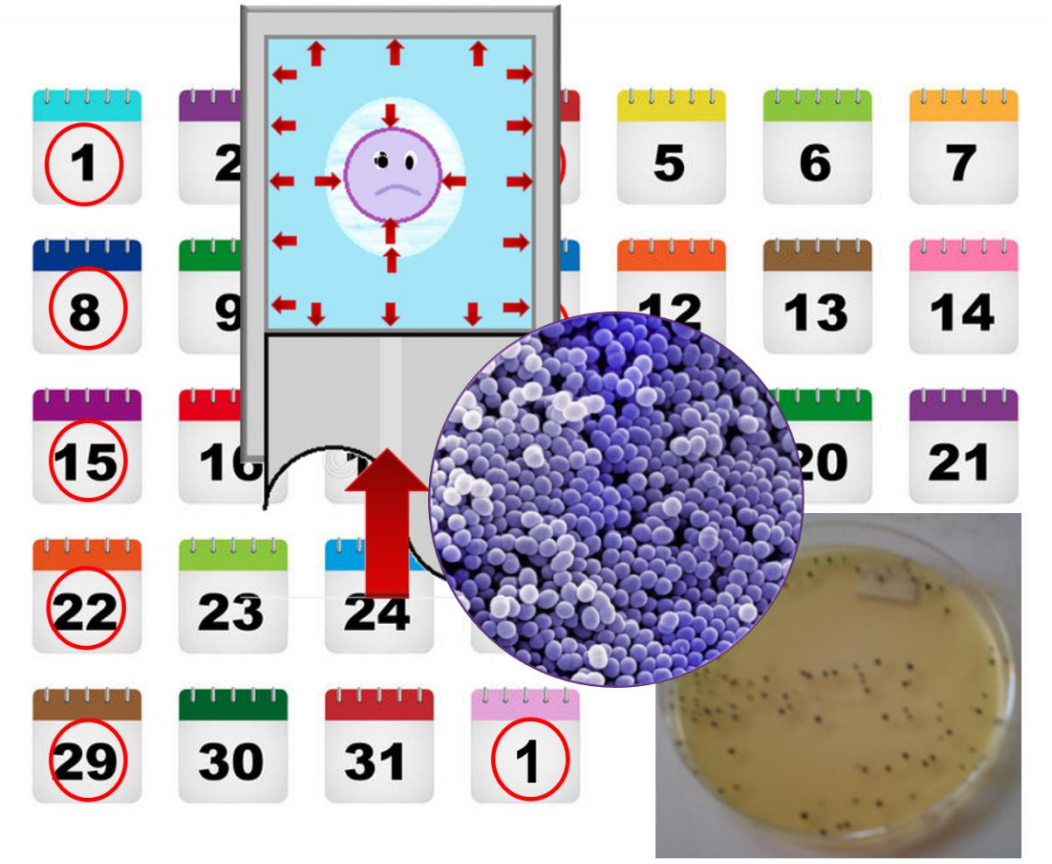
Although the longer pressurization times and the matrix (PBS) used in this study are for research purposes and are not comparable to the ones used in food industry, they are selected as a the first step to study the effect of HPP technology in inactivation of enterotoxic and non-enterotoxic *S. aureus* and evaluation of its impact on virulence factors. Further steps should include studies in foods prompt to be contaminated with *S. aureus* to check its HPP resistance behavior and, when HPP conditions are established, apply those conditions in food industry.

3.5. Conclusions

In conclusion, *S. aureus* enterotoxic strains with lower carotenoids content and SE were more easily inactivated by HPP treatments. Furthermore, the surviving cells have not lost their capability to produce SE and other factors/characteristics of virulence. The results of this study

allow to state that SE presence and carotenoids level are some of the distinctive features of each strain that might influence the barotolerance. In fact, these two features are dependent of the σ^B factor, which was already recognized to be responsible for differences in barotolerance between several strains of the same species. As these differences seem to be the result of more than one feature distinctive of each strain, more assays using several distinct strains are necessary in order to confirm the relationship between carotenoid production level and SE gene content.

CHAPTER 4. Evaluation of resistance development and viability recovery by enterotoxic and non-enterotoxic *Staphylococcus aureus* strains after repeated cycles of high pressure processing treatments



Adapted from:

I Baptista, SM Rocha, Â Cunha, JA Saraiva, A Almeida, Evaluation of resistance development and viability recovery by enterotoxic and non-enterotoxic *Staphylococcus aureus* strains after repeated cycles of high hydrostatic pressure.

Food Microbiology 46 (2015): 515-520

Abstract

In this work, the development of resistance and the recovery of growth after several consecutive cycles of high hydrostatic pressure (HPP) were for the first time evaluated in different strains of Staphylococcus aureus. Three strains of this important and highly resilient to HPP foodborne pathogen were used: a non-enterotoxic ATCC 6538 strain, treated with 600 MPa for 30 min at 20 °C, and two enterotoxic strains, 2153 MA (with enterotoxin A) and 2065 MA (with the enterotoxins A, G and I), treated with 600 MPa for 15 min at 20 °C. After the first treatment, surviving colonies were used to produce new bacterial cultures. This procedure was repeated nine times more for each bacterium or until total inactivation occurred. The inactivation profile of non-enterotoxic strain and the two enterotoxic strains did not change after consecutive cycles, but the toxic strain with three enterotoxins was completely inactivated after the fourth cycle. The three strains did not recover their viability after 14 days. The results indicate that HPP effectively inactivates non-enterotoxic and enterotoxic strains of *S. aureus* after a single treatment. The surviving bacteria did not develop resistance after 10 cycles of pressurization and did not recover their viability after 14 days of incubation.

4.1. Introduction

HPP treatment is an emerging food preservation method able to meet not only the increasing consumer demand for microbiologically safe foods, but also to produce foods with fresh-like appearance and with minimal modification of nutritional and organoleptic properties (110,337,338,352,353). HPP treatments of food are carried out with intense pressure (commercially ranging from 100 to 600 MPa) with or without heat, inactivating efficiently microorganisms and, consequently, extending food products shelf-life (128,169).

The susceptibility of microorganisms to high hydrostatic pressure varies considerably depending on the pressure range applied, temperature and duration of the treatment (103,354–356), but also depends on each microorganism characteristics, growth phase and suspending medium (205,338,346,356). It is generally assumed that Gram-positive bacteria and cells in the stationary growth phase are more resistant than Gram-negative and cells in the exponential growth phase (128). It has also been stated that bacterial protective effect against HPP may be given by carbohydrates, proteins, lipids and other constituents of the suspending medium or food (which, when caught up by the bacteria decrease their sensitivity to HPP) (338,357,358).

HPP affects bacterial cellular processes such as protein and DNA synthesis, membrane-associated processes, macromolecular quaternary structures (e.g., protein denaturation) and cellular membrane structure. Cell external structures are the primary sites that are damaged by pressure, altering cell permeability, transport systems, osmotic pressure and ability to preserve pH, resulting in leakage of cell contents (92,203,338,339,359,360). As the main targets of HPP are the external structures, microorganisms seem to have low chance to develop resistance. However, it has been shown that some cells can repair the sub-lethal damage induced by HPP, allowing them to proliferate once they have restored the injury. Injury recovery of Gram-positive (*L. monocytogenes* and *S. aureus*) and two Gram-negative (*E. coli* O157:H7 933 and *S. enteritidis* FDA) foodborne pathogens in HPP treated milk during storage was observed (202). Recovery of *E. coli* B in phosphate saline buffer after treatment with HPP was also reported (361).

S. aureus was selected for being an opportunistic human pathogen that causes a broad spectrum of infections (362), being the fourth most common causal agent in bacterial food poisoning outbreaks (327,328). *S. aureus* has no particular nutritional and environmental requirement for growth and has also the ability to grow in a vast range of temperature, pH and NaCl concentration (18,363,364). Besides, some strains produce heatstable enterotoxins that are powerful gastrointestinal toxins, causing SFP (52,90,325,329,362).

S. aureus bacterial development of resistance and the growth recover after several cycles of HPP have not yet been evaluated. Hence, the aim of this study was to evaluate if enterotoxic and non-enterotoxic strains of *S. aureus* that survived a previous HPP treatment can develop resistance to repeated cycles and are able to recover their viability.

4.2. Materials and methods

4.2.1. Preparation of bacterial cultures

Three *S. aureus* strains were used in this study: one non-enterotoxic strain (ATCC 6538) and two enterotoxic strains isolated from food (2153 MA, with SEA; and 2065 MA, with SEA, SEG and SEI). *S. aureus* cultures were grown in BHI (LIOFILCHEM, Italy) at 37 °C for 18 h at 170 rpm to achieve a concentration of approximately 10^8—10^9 colony forming units mL^{-1} (CFU mL^{-1}) reaching the stationary phase. Cells were harvested by centrifugation (15,000 × g 10 min), washed twice and resuspended in the same volume of sterile phosphate buffer solution (PBS) (pH 7.0) in order to achieve concentrations of 10^8—10^9 CFU mL^{-1}.

4.2.2. High pressure processing assays

Prior to HPP treatments, cell suspensions were transferred with sterile glass Pasteur pipettes to 0.4 mL polyethylene tubes. For each strain, three independent samples were used and for each one, three sub-samples were prepared (n = 9). The tubes containing each of the 3 sub-replicates set were placed in low permeability polyamide-polyethylene bags (PA/PE-90, Albipack—Packaging Solutions, Portugal) sterilized distilled water which were then thermo-sealed and pressurized. Based on the results achieved in CHAPTER 3, the pressurization conditions required to achieve a bacterial inactivation corresponding to ca. 5 to 6 log reductions (from ≈ 9 initial logs), ATCC 6538 strain was pressurized at 600 MPa for 30 min at 20 °C and the strains 2065 MA and 2153 MA were pressurized at 600 MPa for 15 min at 20 °C in a hydrostatic press (High pressure system U33, Institute of High Pressure Physics, Warsaw, Poland). The pressurization liquid was a mixture of 60% water and 40% propylene glycol (DOWCAL™, Dow). With such a procedure, it was intended to obtain a sizeable number of colonies, which were used to study the possible development of resistance and bacterial recovery. Non-pressurized controls were also included in the experiments.

4.2.3. Enumeration of viable cells

For each sub-sample treated and for untreated samples 10-fold serial dilutions (10^{-1}—10^{-8}) were made in sterile PBS. One milliliter of each dilution was plated on PCA_m (LIOFILCHEM, Italy) in duplicate. The plates were incubated at 37 °C for 48 h and the number of colonies was

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}. The number of survivors was reported as log CFU mL^{-1}. The RE of high pressure inactivation of the pathogen strains was calculated using the equation

$$RE = \log N_0 - \log N,$$

where N_0 represents the average number of viable cells in the untreated suspensions and N the number of viable cells in the pressurized suspensions.

4.2.4. HPP resistance assays

To verify the development of resistance to HPP, a new set of enterotoxic and non-enterotoxic bacterial cultures was produced from an isolated colony obtained after each cycle of exposure to HPP treatment. In order to obtain a bacterial inactivation corresponding to ca. 5 log of reductions, the bacterial suspension was exposed to HPP in the same conditions of the aforementioned HPP assay. This allowed to test if the bacteria affected by HPP were able to develop resistance to HPP. After each cycle, survivor colonies were removed from PCA_m and incubated in BHI at 37 °C for 22 h at 170 rpm. Unpressurized controls were also included in the experiments. This procedure was repeated for ten consecutive cycles. For each strain, and for each of the 10 cycles, three independent samples were used and for each sample three sub-samples were done (n = 9).

4.2.5. HPP viability recovery assay

In order to evaluate if pressurized cells could recover viability after HPP treatments, enterotoxic and non-enterotoxic bacterial suspensions were subjected to HPP in the conditions described above. After the initial enumeration (48 h), the plates used to count viable cells were incubated for 14 days at 37 °C and the colonies were recounted after 5, 8, 11 and 14 days of incubation. This counting strategy was used after each pressurization cycle and the concentration of viable bacteria was determined. For each strain, three independent samples were counted, each one with three subsamples (n = 9).

4.2.6. Statistical analysis

HPP inactivation data from the resistance and recovery assays were statistically analyzed using analysis of variance (ANOVA) and the post-hoc Tukey test, with the SPSS 20.0 (IBM, New York, USA). Statistical significance was considered for $\rho < 0.05$.

4.3. Results

4.3.1. HPP resistance assay

The application of HPP treatment produced reductions in the counts of the three strains, being the magnitude dependent on each strain under study ($\rho < 0.05$). In each cycle, no significant differences among the 3 independent samples (ANOVA, $\rho > 0.05$) was observed for the three strains. Though, the efficiency of bacterial inactivation after the first treatment was almost the same ($\rho > 0.05$), the HPP treatment time was different for the non-enterotoxic strain and for the strain with one toxin (Fig. 1). After 30 min of treatment at 600 MPa, the RE of the non-enterotoxic strain was 4.5 log (out of 8.7 log) and, after 15 min at 600 MPa, the RE of the two enterotoxic strains was 4.7 log and 5.5 log (out of 8.3 and 9.2 log, respectively), for the strain with one toxin and the strain with three toxins, respectively (Figure 4.1). For this first cycle, for all tested strains, the results are in accordance to the ones obtained in past studies of the same group of authors (CHAPTER 3), in which RE values were approximately the same when only one cycle HPP treatment was applied. The number of surviving bacteria, after the second cycle for the non-enterotoxic strain (ATCC 6538) and after the first cycle for the enterotoxic bacterium with 1 toxin (2153 MA), was approximately the same. The efficiency of inactivation was not significantly different in the subsequent HPP cycles (ANOVA, $\rho > 0.05$) for these two strains (with exception for the seventh cycle for non-enterotoxic and sixth cycle for enterotoxic strains). The efficiency of inactivation for the strain with three toxins (2065 MA) was not constant during the 4 consecutive treatments (Figure 4.1), increasing with the number of cycles and, after 4 cycles

of treatment, no surviving bacteria were detected. For this strain, the efficiency of inactivation was significantly different among the four consecutive HPP cycles (ANOVA, $\rho < 0.05$).

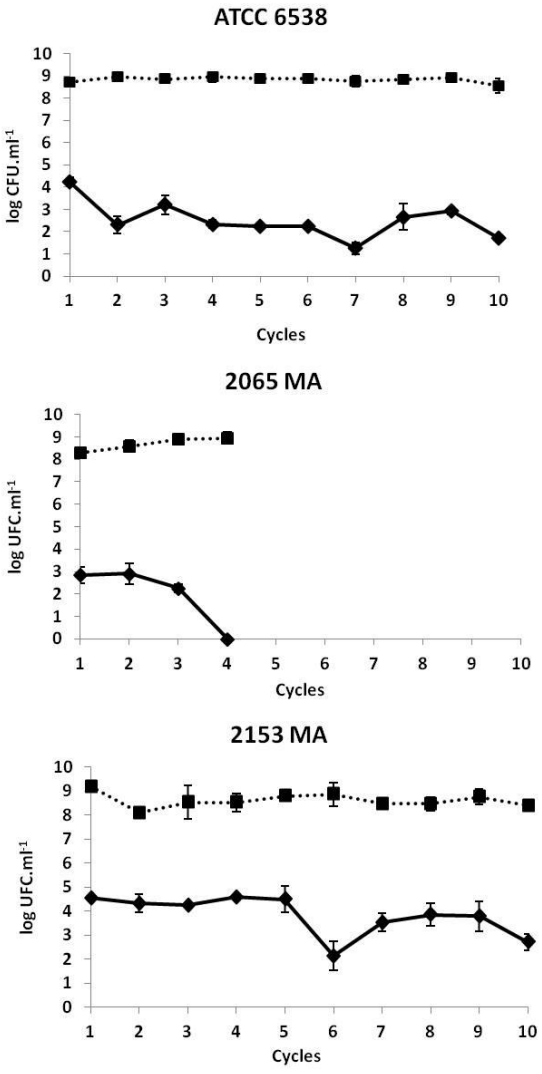


Figure 4.1 - Counts of the three strains of *Staphylococcus aureus* under study (log CFU mL^{-1} ± SD) before and after pressurization over cycles. Treatment at 600 MPa for 30 min (strain ATCC 6538) and 600 MPa for 15 min (strains 2065 MA and 2153 MA). The dotted lines and squares are the counts before pressurization (N_0) and the continuous line and diamonds are the counts after pressurization (N). Values represent the mean of three independent samples with three sub-replicates each and two replicates for each sub-replicate; error bars indicate the standard deviation of the three samples. The value zero means that no colonies were detected, which means that the counts were below the limit of detection of the method (<1 CFU. mL^{-1}).

4.3.2. HPP viability recovery assay

The plates used to enumerate viable cells in the HPP resistance assays were counted past 2 (initial enumeration), 5, 8, 11 and 14 days of incubation at 37 °C for each of the 10 cycles. Values of viable cells counts over different recovery times are shown in Table 1. Although, after the first cycle of partial HPP inactivation, the bacterial concentration of the non-enterotoxic strain and of the enterotoxic with one toxin strain were significantly reduced to 4.2 and 4.5 log, respectively (ANOVA, $\rho < 0.05$), and the survivor bacteria did not recover their viability during the following 14 days of incubation at 37 °C. The number of colonies counted after the first 2 days of incubation was similar (ANOVA, $\rho > 0.05$) to that obtained after the 14 days. The same profile of variation was observed for the nine consecutive cycles of HPP treatment for these two strains (Table 4.1). For the strain with three toxins, although the number of colonies decreased between the consecutive cycles (ANOVA, $\rho < 0.05$), during the following 14 days of incubation at 37 °C the number of colonies did not vary significantly (ANOVA, $\rho > 0.05$) in each cycle.

4.4. Discussion

In general, the development of resistance to HPP by microorganisms should be considered as an unlikely event, because this is typically a multi-target process and cell external structures are the primary sites that are affected by pressure (92,203,339,365,366). In this study, the nonenterotoxic and the two enterotoxic strains, which present different susceptibility to HPP (CHAPTER 3), did not develop resistance to HPP treatments. Although in this study longer pressurization times were applied in comparison to the currently applied by the food industry (3—5 min), the levels of bacteria used in the experiments were also much higher (between 10^8 and 10^9 CFU.mL^{-1}) than those found in food [the range of *S. aureus* counts that can produce the amount of toxin able to induce staphylococcal food poisoning is between 10^3 and 10^5 CFU mL^{-1} (367)]. Using lower bacterial concentrations, corresponding to those observed in food, will allow the use of lower pressure values and pressurization time to efficiently treat contaminated food (368).

The results of the resistance assays showed that, after consecutive treatments none of the strains exhibit any evident signs of resistance. When the non-enterotoxic strain and the strain with 1 toxin, were subjected to ten consecutive HPP cycles, the fraction of survivor bacteria did not show considerable variation between cycles. After the first and the second treatments, respectively, for the strain with 1 toxin and for the non-enterotoxic strain no significant changes in the efficiency of inactivation were observed. For the strain with 3 toxins, substantial variations between the consecutive cycles were observed. The number of colonies decreased and,

consequently, the efficiency of inactivation increased, and bacteria were inactivated below the limit of detection (<1 CFU.mL^{-1}) after the fourth treatment cycle (reduction of ≈ 9 log). Pressure can potentially lead to simultaneous damages on molecules of the external structures and also to the nucleic acid (DNA), avoiding the development of resistance to HPP. The bacteria would require multi-site mutations to become resistant, an event with significantly lower probability than single-target mutation which is often sufficient for conferring resistance. Up to now, the development of bacterial resistance to HPP after consecutive cycles of HPP treatment has not been reported for *S. aureus*.

There are, however, some studies on bacterial growth after HPP treatment (202,360,366), which are, in fact, targeted for bacterial growth recovery and not for the development of resistance. Nonetheless, there is one study of Hauben et al. (1997) (359), in which barotolerant resistant mutants of *E. coli* MG1655 were selected and the survival of these mutants after HPP treatments was assessed. In this study, while the mutants were able to survive at increasing pressures as a result of the development of barotolerance, the viability of the wild type decreased, since it did not develop any resistance to pressure. Consequently, our finding is of particular interest, indicating that HPP treatment is a safe food method to control *S. aureus* growth, under the conditions tested.

Although none of the three bacterial strains developed resistance to HPP after consecutive cycles of treatment, the efficiency of inactivation was significantly different for the three strains. The non-enterotoxic strain and the strain with one enterotoxin were not completely inactivated after the ten consecutive cycles, indicating that these two strains are more tolerant to HPP than the strain with three toxins. The results obtained for the first cycle are in agreement with those obtained in other studies using *S. aureus* collection strains and strains isolated from food, carried out in the range of 100 — 600 MPa with or without heat, in which this bacterium was not completely inactivated by HPP treatments (205,326,338,369). In the pressurization conditions of this study, an average of 2.5—3.8 log of the non-enterotoxic strain and the strain with one enterotoxin survived for ten HPP cycles (corresponding to a RE of ≈ 6.3 and 4.8 log cycles, respectively).

The efficiency of inactivation slightly increased along repeated HPP cycles, with an average RE of 2.4 and 1.0 log, respectively, for non-enterotoxic strain and strain with one enterotoxin. The highest RE was observed after the first cycle for both strains (approximately 4.5 log) but, for the non-enterotoxic strain, which was subjected to a longer time under pressure (30 min against 15 min for the other two strains) a higher RE between the first and the second cycles was also observed (6.7 log). However, after the second treatment the reduction for the

non-enterotoxic strain and strain with one enterotoxin was not significant, which indicates that these

Table 4.1 - Counts of the three strains of *Staphylococcus aureus* under study (log CFU.ml^{-1} ± SD) after 2, 5, 8, 11 and 14 days of incubation at 37 °C for each cycle (n = 9). Treatment at 600 MPa for 30 minutes (strain ATCC 6538) and 600 MPa for 15 minutes (strains 2065 MA and 2153 MA). Values represent the mean of three independent samples with three sub-samples each and two replicates for each sub-sample; error bars indicate the standard deviation of the three samples.

Strains	Cycle	N_0[a]	2	5	8	11	14
ATCC 6538	1	8.73 ± 0.09	4.24 ± 0.21	4.26 ± 0.21	4.27 ± 0.21	4.27 ± 0.21	4.27 ± 0.21
	2	8.97 ± 0.09	2.30 ± 0.40	2.35 ± 0.36	2.37 ± 0.36	2.37 ± 0.36	2.37 ± 0.36
	3	8.86 ± 0.09	3.21 ± 0.44	3.24 ± 0.41	3.25 ± 0.41	3.25 ± 0.41	3.25 ± 0.41
	4	8.96 ± 0.23	2.33 ± 0.18	2.40 ± 0.16	2.40 ± 0.16	2.42 ± 0.16	2.42 ± 0.16
	5	8.87 ± 0.03	2.23 ± 0.01	2.47 ± 0.19	2.54 ± 0.26	2.55 ± 0.25	2.55 ± 0.25
	6	8.89 ± 0.10	2.26 ± 0.06	2.31 ± 0.12	2.34 ± 0.11	2.34 ± 0.11	2.34 ± 0.11
	7	8.76 ± 0.25	1.24 ± 0.25	1.28 ± 0.30	1.28 ± 0.30	1.28 ± 0.30	1.28 ± 0.30
	8	8.84 ± 0.19	2.66 ± 0.59	2.66 ± 0.59	2.66 ± 0.59	2.66 ± 0.59	2.66 ± 0.59
	9	8.91 ± 0.13	2.92 ± 0.13	2.92 ± 0.13	2.92 ± 0.13	2.92 ± 0.13	2.92 ± 0.13
	10	8.56 ± 0.32	1.71 ± 0.07	1.73 ± 0.05	1.73 ± 0.05	1.73 ± 0.05	1.73 ± 0.05
2065 MA	1	8.31 ± 0.01	2.84 ± 0.38	2.85 ± 0.37	2.85 ± 0.37	2.85 ± 0.37	2.85 ± 0.37
	2	8.58 ± 0.26	2.90 ± 0.46	2.90 ± 0.46	2.90 ± 0.46	2.90 ± 0.46	2.90 ± 0.46
	3	8.89 ± 0.18	2.26 ± 0.19	2.27 ± 0.18	2.29 ± 0.15	2.30 ± 0.15	2.30 ± 0.15
	4	8.95 ± 0.26	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]
	5	-	-	-	-	-	-
	6	-	-	-	-	-	-
	7	-	-	-	-	-	-
	8	-	-	-	-	-	-
	9	-	-	-	-	-	-
	10	-	-	-	-	-	-
2153 MA	1	9.19 ± 0.22	4.54 ± 0.10	4.55 ± 0.10	4.55 ± 0.10	4.55 ± 0.10	4.55 ± 0.10
	2	8.09 ± 0.08	4.31 ± 0.38	4.33 ± 0.37	4.34 ± 0.37	4.34 ± 0.37	4.34 ± 0.37
	3	8.53 ± 0.69	4.23 ± 0.07	4.24 ± 0.07	4.24 ± 0.07	4.24 ± 0.07	4.25 ± 0.06
	4	8.52 ± 0.39	4.57± 0.07	4.58 ± 0.07	4.58 ± 0.07	4.58 ± 0.07	4.58 ± 0.07
	5	8.78 ± 0.07	4.48 ± 0.55	4.49 ± 0.54	4.50 ± 0.55	4.51 ± 0.53	4.52 ± 0.53
	6	8.87 ± 0.50	2.12 ± 0.60	2.20 ± 0.54	2.21 ± 0.55	2.21 ± 0.56	2.21 ± 0.56
	7	8.47 ± 0.16	3.53 ± 0.36	4.03 ± 0.40	4.05 ± 0.38	4.06 ± 0.37	4.06 ± 0.37
	8	8.46 ± 0.28	3.85 ± 0.48	3.87 ± 0.48	3.87 ± 0.48	3.87 ± 0.48	3.87 ± 0.48
	9	8.75 ± 0.32	3.79 ± 0.62	3.83 ± 0.58	3.84 ± 0.58	3.84 ± 0.58	3.84 ± 0.58
	10	8.39 ± 0.19	2.71 ± 0.33	3.26 ± 0.05	3.31 ± 0.02	3.32 ± 0.01	3.32 ± 0.01

[a] N_0, Before HHP.

[b] N.D. – Not Detected; below the limit of detection of the method (< 1 UFC mL^{-1}).

two strains, as the strain with three toxins, do not develop resistance against HPP. The bacteria that were not inactivated after the first 2 HPP cycles were not subsequently inactivated in the following cycles. The presence of viable but non-cultivable (VBNC) forms or the existence of dormant persistent cells, less sensitive to HPP treatment than their cultivable counterparts, might be an explanation for the results obtained (130,370–374). However, the discrimination between these two possibilities (non-cultivable forms or dormant persistent cells) cannot be done in light of the methods used, since no microscopy or molecular analyses were performed. Moreover, the different patterns of inactivation displayed by the three strains after consecutive HPP cycles might suggest differences in the mechanism of transmission of resistance to the inactivation induced by HPP treatments.

The multi-target nature of HPP suggests also that the effect of treatment should have irreversible effects on the bacterial viability. As the three bacterial strains were treated to obtain a sizeable number of survivor colonies it was possible to evaluate if these survivor bacteria were viable after treatment but also if potential HPP injured bacteria were able to recover their viability. During 14 days of incubation in a non-specific medium after HPP treatment, the surviving bacteria were maintained in the necessary conditions to recover from the treatment. If new colonies would appear in the petri plates during the 14 days of incubation it would mean that the bacteria, previously not able to form colonies, after the “injury period” become able to recover from the treatment. However, none of the three bacterial strains were able to recover their viability in the 14 days post-treatment period. This indicates that inactivated bacterial cells cannot recover from the treatment effect. Using 600MPa for 15 or 30 min, respectively, for enterotoxic and non-enterotoxic strains, the enterotoxic strains with 3 toxins was inactivated to the limits of detection only after 4 consecutive cycles and did not recover within 14 days after each cycle but the other two strains were not completely inactivated after the ten consecutives cycles but after each cycle they do not recover the viability within the 14 days of incubation. This result indicates that, although one cycle of treatment is not enough to inactivate all the bacteria in the suspension, the bacteria inactivated after one cycle did not recover their viability.

However, recovery of pathogenic and spoilage bacteria in different substrates after HPP treatment has been described for several species at 128—550 MPa and 20—50 °C (375–378). This recovery has been explained as the result of repairing of the bacterial injuries during food storage (361,375). Koseki et al. (2008) (378), showed that *L. monocytogenes* in milk recovers viability after 28 days of storage at 4 °C and 25 °C, but not at 37 °C, when a HPP treatment at 550 MPa for min using combined with a mild-heat treatment (30—50 °C) was applied. Bozoglu

et al. (2004) (379), observed also that *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *S. enteritidis* treated at 350, 450 and 550 MPa at 45 °C for 10 min in milk and stored at 4, 22 and 30 °C recovered after 1—15 days of HPP treatment. In both studies, the time of HPP processing was lower (5—10 min) than those used in our study (15—30 min) but in the two studies, higher temperatures were applied, thus not enabling straightforward comparison with the results not reported. However, in these other studies, bacteria recovered viability when foods were stored at 4 °C, 22 °C, 25 °C and 30 °C but not at 37 °C which was the temperature used in our studies during the recovery assays. More studies are necessary to elucidate the importance of temperature during the bacterial recovery assays in buffer solutions. Though, the differences between our results relatively to these two studies from the literature, can be, in part, explained by the protective effect of the food constituents.

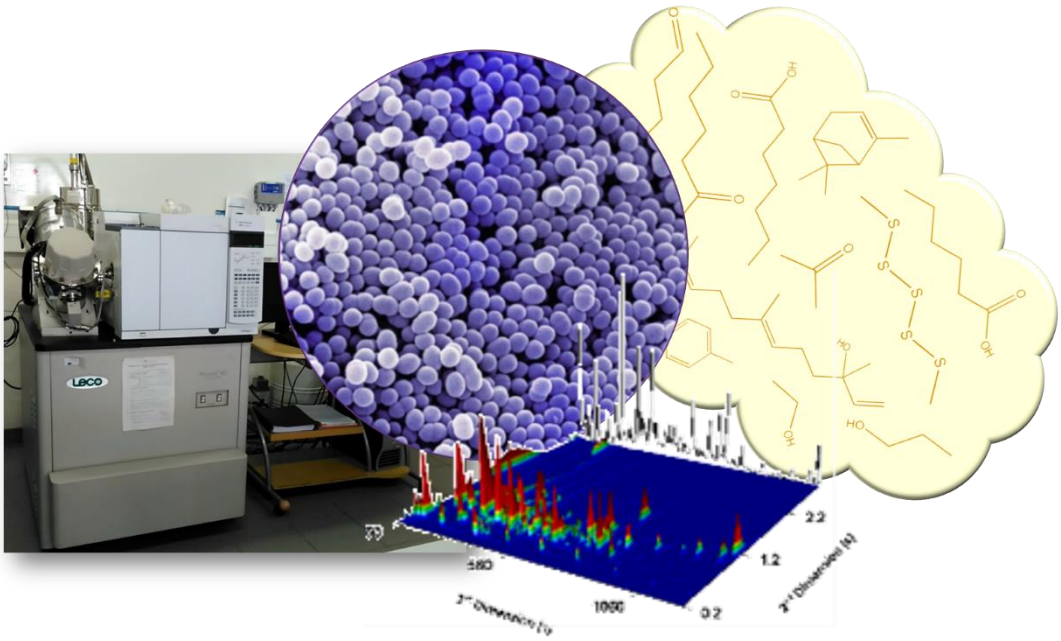
It is well demonstrated that several matrixes, such as milk, poultry meat, cream of chicken can have a baroprotective effect against HPP (380–384). For example Gervilla et al. (2000) (381), showed that several *Pseudomonas fluorescens*, *E. coli*, *L. innocua*, *S. aureus* and *L. helveticus* in ovine milk were more resilient to HPP than in buffer. Also Styles et al. (1991) (384), found that raw milk appeared to confer a protective effect to *L. monocytogenes* and *V. parahaemolyticus* against HPP treatment when compared with HPP treatment in PBS (361).

In the present study the HPP treatment was also performed in PBS, with bacteria lacking the baroprotective effect of food matrices, which rendered those most probably more sensitive to HPP and, consequently their recovery was not observed during the 14 days of incubation. On the other hand, it has been shown that VBNC forms resulting from HPP treatments are not able to growth directly in specific media but they can grow directly in non-specific media or in specific media after a first passage in a non-specific medium (370). Nevertheless, in this study a non-selective medium was used to evaluate bacterial viability after HPP treatment, differently from other studies in which specific growth medium, supplemented with different concentrations of NaCl, were used to recover injured bacterial cells and to assess the type of injuries inflicted on those cells (385). Furthermore, as *S. aureus* has no particular nutritional and environmental requirements to grow, being able to grow in a wide range of temperatures, pH and NaCl concentrations (18,363,364), the recovery in a selective medium would probably be similar. Further studies using a selective medium might be necessary in order to obtain a deeper helpful information about the types of mechanisms of recovery and if these mechanisms can be different for the enterotoxic and non-enterotoxic strains.

4.5. Conclusion

In this study, it was demonstrated that HPP treatments (600 MPa for 30 min or 15 min, depending on the strain) effectively inactivate *S. aureus* (reduction of ≈ 5 log) after a single treatment. The bacteria that survived the treatments did not develop resistance after repeating cycles of HPP, considering the conditions tested and the inactivated bacteria do not recover their viability after 14 days of incubation. The next logical step is to perform studies using *S. aureus* inoculated in food to confirm the nonappearance of resistance and the absence of viability recover after consecutive cycles of HPP.

CHAPTER 5. A comprehensive look into the volatile exometabolome of enterotoxic and non-enterotoxic *Staphylococcus aureus* strains



Abstract

Staphylococcal food poisoning is a disease that originates significant health and economic losses and is caused by *Staphylococcus aureu*s 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.

5.1. Introduction

Foodborne diarrheal diseases are responsible for 550 million people to fall ill per year and result in 230 thousands deaths (386). *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 (18). It can live as a commensal microorganism in humans and other animals, being responsible for a wide range of diseases and producing several virulence factors, such as SE which are responsible for SFP (387). *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 locals where food is prepared (53). According to WHO, in 2014, the median 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 (386).

From a health point of view, the symptoms of SFP are usually self-limiting and are solved on their own, or can be sporadically intense enough to cause hospitalization. Also, SFP may represent relevant economic losses for agro food industries among others, and elevated health costs (53,386). Thus, although SFP is not a high mortality foodborne disease, it is important enough due to the related morbidity and economic impact, requiring alternative methods of identification to the conventional ones in order to detect S. aureus enterotoxic strains in food, which might take several days. The detection of enterotoxic strains of S. aureus, using a pattern of biomarkers to indicate toxins presence, would be a convenient approach to monitor/control its presence.

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 (224). Several studies of S. aureus volatile metabolome employing different methodologies were 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) (270). Afterwards, several metabolomics studies of S. aureus have been performed. They were mainly directed to clinical applications, i.e. to early detection of different species in different clinical specimens, distinction of MSSA from MRSA strains, the evaluation of growth conditions and detection of mastitis in cows, among others (282,297,302,303,388). A wide range of analytical methods was

used in these studies including one dimensional gas and liquid chromatography, mass spectrometry (270,282,302,303,388) and NMR (297,388). Furthermore, there are already some applications using e-nose (299,300) and more advanced gas chromatographic methodology, such as comprehensive two-dimensional gas chromatography-time of flight mass spectrometry (GC×GC-ToFMS) (389), which have been reported.

GC×GC-ToFMS, a high throughput and highly sensitive methodology, has proven to be a powerful tool for microbial metabolomics, specifically for the study of microbial volatile organic compounds (MVOCs) (257,315,390). There is only one study in which GC×GC-ToFMS was applied to compare of the fatty acids methyl esters from different strains of four foodborne bacterial species, including *S. aureus* strains ATCC 25923 and ATCC 6538, with results reporting separation of species and strains (389). 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 the volatiles from S. aureus is fragmentary and only few analytes are commonly reported, which allow to infer that the growth conditions, strains, instrumental analysis, among others, may have impact on the reported volatiles. Consistently reported volatiles 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 (270,282,297,302,303,388).

Due to the importance of *S. aureus* enterotoxic strains as a source of SFP and to the lack of a detailed characterization of its volatile metabolome, the present study aims to add further insights to the volatile fraction of *S. aureus* exometabolome. To achieve this goal, fresh cultures of three strains (one non-enterotoxic and two enterotoxic) of S. aureus were in-depth studied using HS-SPME combined with GC×GC-ToFMS. Furthermore, resorting to hierarchical clustering, this study also examined whether enterotoxic strains can be distinguished from non-enterotoxic. Finally, it was also intended to see if it was possible to find a pattern of biomarkers for *S. aureus*.

5.2. 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 MSI (272–274). Experimental procedure was performed accordingly to Figure 5.1, representing the

main stages for *S. aureus* exometabolome determination, which will be described in detail in the following sub-sections.

5.2.1. Bacterial parameters

5.2.1.1. 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, catalase positive, able to do 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 (CHAPTER 3).

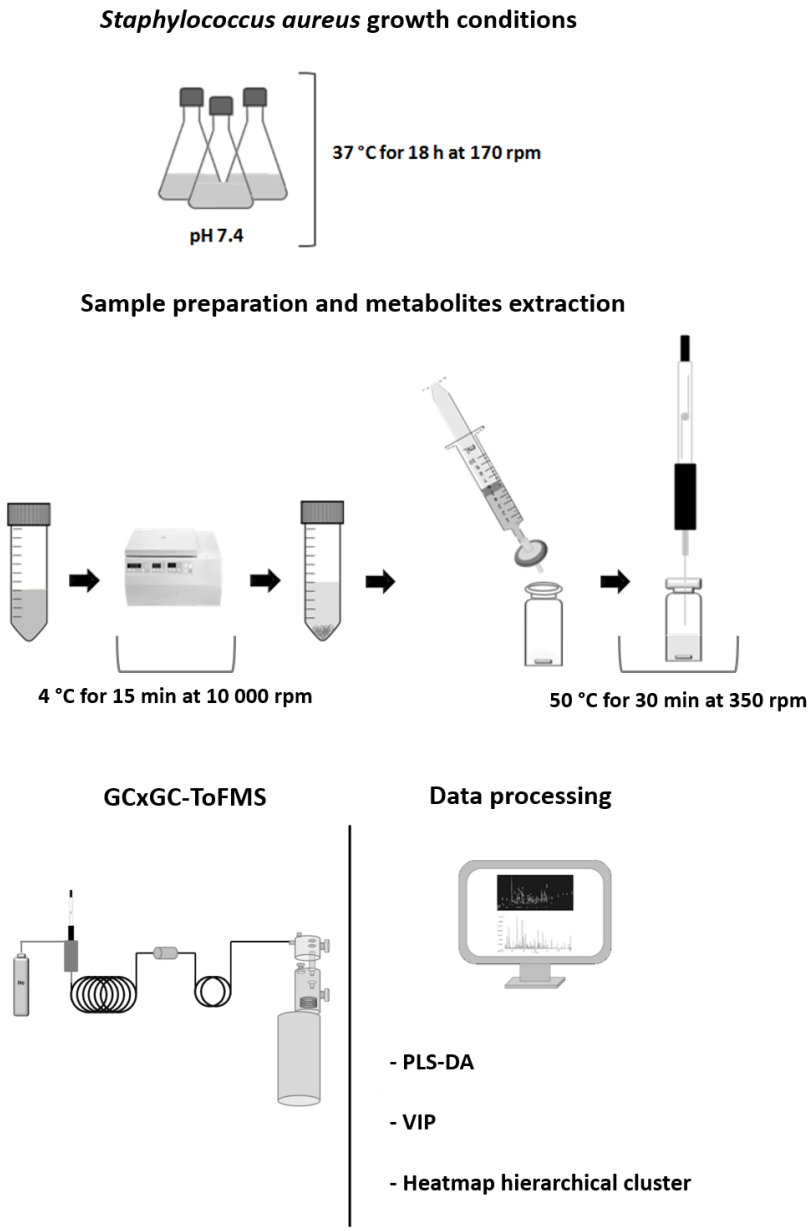


Figure 5.1 - Main stages for *Staphylococcus aureus* determination of exometabolome volatile profile: growth conditions, sample preparation, metabolites extraction, GC×GC analysis and data processing. Three independent assays were performed for each strain.

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 (391) 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 PCA_m (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 through 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.

5.2.1.2. 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.

5.2.2. *Staphylococcus 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. Also, in order 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.

The SPME and GC×GC–ToFMS experimental parameters were previously defined (257). 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.

For analytes extraction, the vials with the defrosted samples (*S. aureus* cultures and BHI blank media) were placed in a thermostated water bath at 50.0± 0.1 °C and under constant agitation at 350 rpm. Then, the SPME fiber was inserted into the headspace for 30 min. Then, the SPME fiber with the sorbed 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, using splitless injection mode. 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. 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 were 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/), and allows performing the 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.

Compounds identification 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 A5.3, Annexes). 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.

5.2.3. Statistical analysis

From the 315 volatile organic compounds released from the *S. aureus* headspace (Table A5.3., Annexes), 240 were selected for statistical purposes (for an explanation please see 5.3.2), thus a full data matrix was constructed with 9 observations (three strains, in three independent assays) and 240 variables (volatiles) (Table B5.3, Annexes). The following strategy was used to extract relevant information from the S. aureus headspace volatiles:

i. 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.
ii. 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 B5.3, Annexes) was computed, as well as classification rate and Q2 (quality-of-fit criterion) were estimated by cross-validation.
iii. A set of volatiles with VIP values higher than 1.5 was defined (Table A5.3, Annexes), and a heatmap hierarchical cluster visualization was constructed, using the chromatographic data previously normalized by $CFU.mL^{-1}$ and autoscaled.
iv. 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 conducted using MetaboAnalyst 3.0 (web interface). PLS was implemented with MATLAB, v. 7.12 (release 2011).

5.3. Results and discussion

5.3.1. 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 5.2 A), as reported before for the same strains and similar growth conditions (CHAPTER 3).

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 5.2 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 are generally also not buffered. After 8 h of incubation onwards the pH remained relatively constant. These observations can be explained by changes in the exometabolome which will be discussed further ahead.

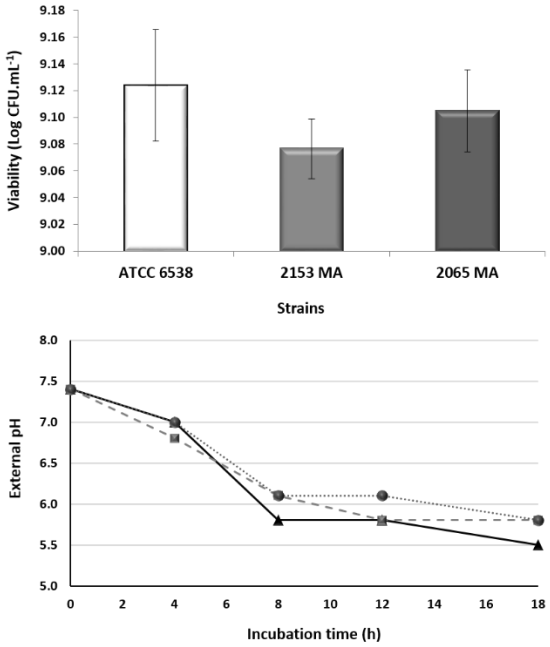


Figure 5.2 - A) Cell viability of the three strains of *Staphylococcus aureus* under study, at 18 h of incubation (log CFU.mL^{-1} ± SD): ATCC 6538 (white), 2153 MA (light grey) and 2065 MA (dark grey). No significant differences were observed between strains. **B)** Extracellular pH values over 18 h 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).

5.3.2. *Staphylococcus 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, including *S. aureus*, from a variety of non-clinical and clinical specimens, such as food products. This "meaty" culture medium contains brain heart infusion and peptone, as nitrogen (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 consists in the condensation of sugars with amino acids (AA) (392), both present in BHI.

Approximately 1400 instrumental signals were detected using HS-SPME/GC×GC-ToFMS and, after the removal of column associated artefacts, 315 VOCs were retained (Table A5.3., Annexes), including acids, alcohols, aldehydes, esters, hydrocarbons, ketones, terpenes,

norisoprenoids, N-compounds, S-compounds. Pyrroles, pyridines, furan-like compounds, pyrazines and thiazoles were removed as they are most likely formed during culture medium sterilization (392,393). Further volatiles from the remaining families were excluded due to similar areas in both samples and blank controls, even though some of them were of metabolic significance. 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), or products of leucine and phenylalanine metabolism, respectively (394). 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 (323).

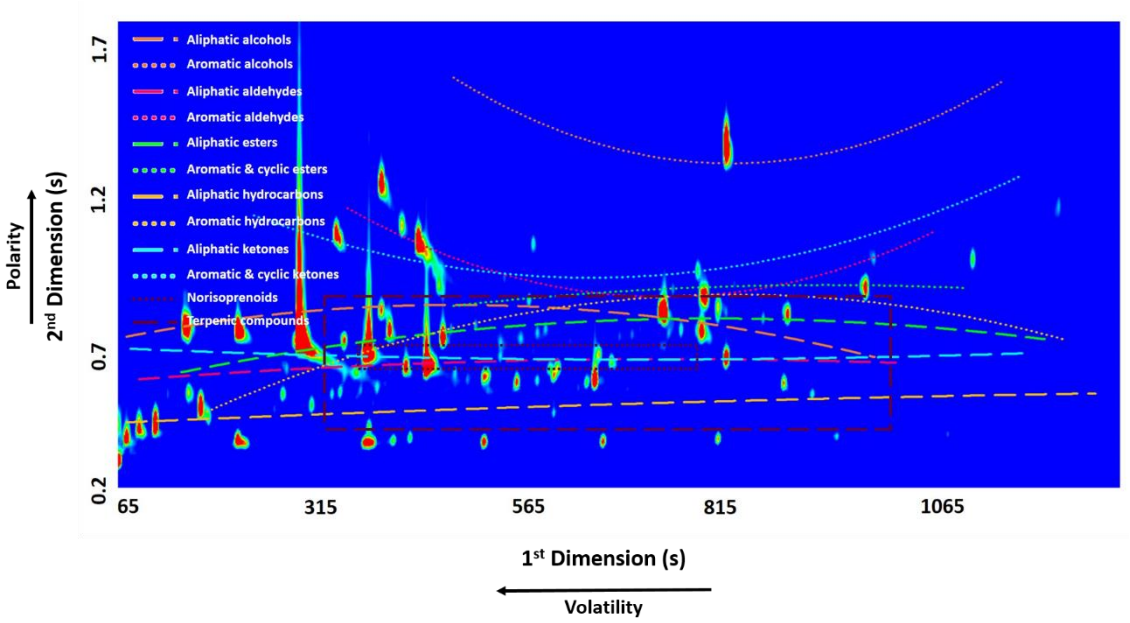


Figure 5.3 - GC×GC-ToFMS total ion chromatogram contour plot of the *Staphylococcus aureus* ATCC 6538 culture headspace volatile components. Volatiles chemical families used for statistical analysis are represented by lines and clusters. The increase in volatility (low $^{1}t_R$) is mainly related to 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.

Subsequently, 240 volatiles (Table B5.3, Annexes) were used to build the dataset, which was composed by 10 chemical families as shown in the contour plot in Figure 5.3 and in Figure 5.4: 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 dataset was considered the exometabolome of S. aureus, by the analytical procedure used in this work.

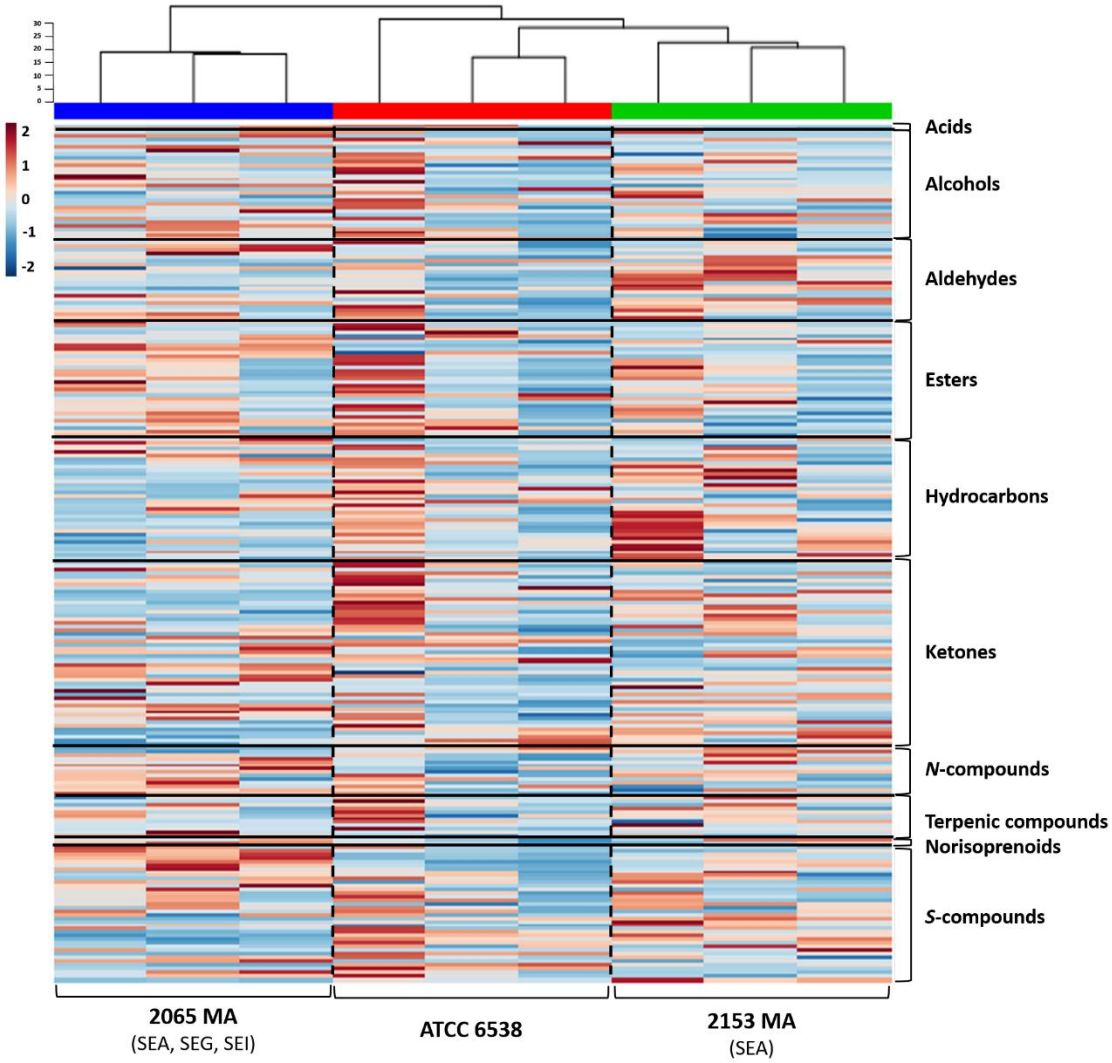


Figure 5.4 - Hierarchical clustered heatmap visualization of the three strains of *Staphylococcus aureus* cultures headspace volatiles, organized by chemical families: ATCC 6538, 2153 MA and 2065 MA. The chromatographic area of each metabolite was normalized by $CFU.mL^{-1}$ followed by autoscaling. Each line corresponds to one metabolite and each column corresponds to each independent assay.

None of the previous published studies for *S. aureus* was able to accomplish the putative identification of 240 volatiles at once. This confirms the high sensitivity and high throughput of HS-SPME/GCxGC-ToFMS in the detection of bacterial volatiles, previously demonstrated (257,315,390).

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 (270,282,297,302,303,388). This last volatile (Table A5.3, Annexes), although identified, was removed as previously explained. It is possible that after proper validation these volatiles can be used as biomarkers for S. aureus. Finding a specific

pattern of volatiles is of paramount importance for a quick and specific detection of S. aureus in food and clinical samples.

Most of the previously mentioned volatiles 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 BCAAs degradation, phenylalanine metabolism (either from degradation or from cyanoamino acid metabolism), degradation of methionine, carotenoid cleavage, mevalonate pathway (via geranyl diphosphate or via farnesyl diphosphate), or in the degradation of toluene and ethylbenzene. However some of the above mentioned volatiles are not yet associated with any pathway and no definite prove exist, as to their origin in *S. aureus*. Some of the volatiles detected play key roles in pH homeostasis while others are the result of oxidative stress, including lipid peroxidation.

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 deiaminase (395). 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 its cells. These volatiles, generated in the diacetyl pathway, contribute to pH homeostasis by decreasing the levels of pyruvate, thus avoiding the formation of acids (291). The content of these two volatiles is higher in ATCC 6538 (Table B5.3, Annexes). 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* (362).

A low extracellular pH can be responsible for oxidative stress in *S. aureus* (362). Several volatiles generated by oxidative stress were found: 1-butanol, hexanal, heptanal, octanal, nonanal, octane and nonane (396). Of these volatiles (Table B5.3, Annexes), the content of aldehydes was higher in 2153 MA, the content of 1-butanol and octane was higher in 2153 MA and equal to ATCC 6538, and nonane content was higher in 2065 MA. However, none of these volatiles have yet been described as oxidative stress markers in *S. aureus*.

The strain 2153 MA also had the highest content of volatiles related to lipid peroxidation, namely hexanal, 2-heptenal, heptanal, octanal, nonanal, decanal, undecanal, dodecanal and tridecanal (396). Octane, another lipid peroxidation product (396), was detected in equal content for 2153 MA and ATCC 6538. 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 (equal contents in both strains) (396). These results suggest that all strains suffered some degree of oxidative stress, especially 2153 MA (Table B5.3, Annexes).

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 (397). Oxidative stress causes carotenoid degradation via oxidation by non-specific enzymes, including lipoxygenases and peroxidases, which cleave non-specific double bonds, resulting in norisoprenoids (318,398). The norisoprenoid 6-methyl-5-hepten-2-one (Table B5.3, Annexes), already described for Staphylococcus spp., is the product of such sort of cleavage in γ-carotene (399). Contents of 6-methyl-5-hepten-2-one and geranylacetone (two out of three norisoprenoids detected) were higher in strain 2153 MA, suggesting the use of antioxidant role of carotenoids (which are found in the cellular membrane) as a protection against oxidative stress, mainly by this strain.

Though methyl ketones were detected in S.aureus exometabolome in this work and are reported in the literature (248,270,278,282), there is no explanation for the presence of these compounds since *S. aureus* lacks fatty acids β-oxidation pathway (324) and, for many bacteria, methyl ketones are considered products of fatty acids β-oxidation. However, 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 (324). Thus, as this subject has not yet been studied intensively it is impossible to assert the origin of the methyl ketones detected in the current strains of *S. aureus*.

5.3.3. Using the volatile exometabolome to distinguish strains

A careful observation of Figure 5.4 (and of Table B5.3, Annexes), which is a hierarchical clustered heatmap containing the dataset of 240 volatiles, allows to examine the predominance of each chemical family by strain and the similarity between strains. Thus, it is possible to observe the presence of two main clusters, the first containing the 2065 MA strain and the second containing the strains ATCC 6538 and 2153 MA. Hence, 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 stain 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 B5.3, Annexes). Among 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.

Table 5.1 - Sub-data set of volatiles with VIP (Variable Importance in Projection) and their values. Metabolic pathways from which each VIP putatively originated from is given.

Peak number	Volatiles	VIP value	Pathways	Ref.
164	2-Nonanone	2.11	-	-
234	Dimethyl trisulfide	2.06	Methionine degradation	(318)
237	Dimethyl tetrasulphide	2.03	Methionine degradation	-
7	3-Methylbutanol	2.00	Leucine degradation	(303)
63	3-Methyl-3-butenyl acetate	1.98	-	-
207	3-Methylbutanal oxime (isomer)	1.94	Leucine degradation	(400)
206	3-Methylbutanal oxime (isomer)	1.94	Leucine degradation	(400)
10	2-Methyl-3-hexanol	1.92	-	-
4	2-Methylpropanol	1.92	Valine degradation	(394)
29	2-Phenylethanol	1.90	Phenylalanine degradation	(394)
228	Methanethiol	1.90	Methionine degradation	(394)
208	Benzonitrile	1.87	Phenylalanine degradtion	(394)
181	1-Phenyl-1,2-propanedione	1.84	-	-
77	Methyl benzoate	1.82	Phenylalanine or biphenyl or toluene degradation	(401)
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	(394)

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	(318)
238	Dimethyl pentasulfide	1.69	Methionine degradation	(318)
176	2-Hexadecanone	1.69	-	

In order to examine the potential presence of groups (or clusters) among the strains a PLS-DA was applied and VIP values (Table 5.1) were assessed, and used to perform a hierarchical clustered heatmap (Figure 5.5 A). Twenty five volatiles with highest VIP values (above 1.73) were achieved. This sub-data set comprising alcohols, aldehydes, esters, ketones, N-compounds and S-compounds, allowed to see 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). Most VIP volatiles were detected in allstrains, 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. Although this hierarchical clustered heatmap (Figure 5.5 A) is similar to the one achieved for the whole dataset (Figure 5.4), the Euclidian distance is different, being lower with the sub-data set of VIP, thus indicating a higher similarity between the strains than when using the whole dataset. 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 also cyanoamino acid metabolism.

Metabolic pathways most active at the end of growth involving selected volatiles are shown in Figure 5.5 B with the aim 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 resultant 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, isomers of 3-

methylbutanal oxime, 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 are 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.

Furthermore, an exploratory study was done using the sub-set of 25 metabolites, which was used to construct a calibration model ($y = 0.9866x + 0.0178$, $R^2 = 0.9866$) for prediction of number of enterotoxins. Satisfactory correlation coefficients (above 0.9 for both calibration and validation) and low prediction root-mean-square errors (0.1293 and 0.2250 number of toxins, for calibration and validation, respectively), suggests that this sub-set of volatiles is a good candidate for a pattern of biomarkers to identify enterotoxic strains of *S. aureus* and to estimate the number of SE that they produce. To see if this pattern: higher amounts of byproducts of the degradation of BCAAs higher and lower levels of degradation of methionine in the strain with three enterotoxins than in the other two; byproducts of the degradation of methionine higher and degradation of BCAAs lower in the strain without enterotoxins than in the other two; intermedium contents of both BCAAs and methionine degradation in the strain with one enterotoxin when compared with the other two strains; PLS was applied and showed a positive correlation ($R^2 = 0.9866$) between the number of enterotoxins and the content of degradation products of BCAAs and methionine.

The higher content of BCAAs degradation volatiles in the enterotoxic strains can be explained by the degradation of their enterotoxins (monomeric proteins). The degradation of proteins has already been described for *S. aureus* when it needs to raise the extracellular pH to consequently raise the internal pH, fact that was attributed to the upregulation of genes involved in the degradation of proteins, peptides and glycopeptides (291,362). The enterotoxic strains have in their SEs constitution 20% of BCAAs, with 46 residues and 81 residues of leucine, 20 residues and 52 residues of isoleucine, and 26 and 50 residues of valine, for 2153 MA and 2065 MA, respectively (127,402,403). This seems consistent with the content of volatiles released by the enterotoxic strains from BCAAs degradation. As ATCC 6538 lacks SE, it does not have these extra AAs, which explains the lower content of BCAAs degradation volatiles. 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 is possible to examine the predominance of several chemical families in each

strain (Table B5.3, Annexes, seen in the Subtotal cells; and Figure 5.4). 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, overall, 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).

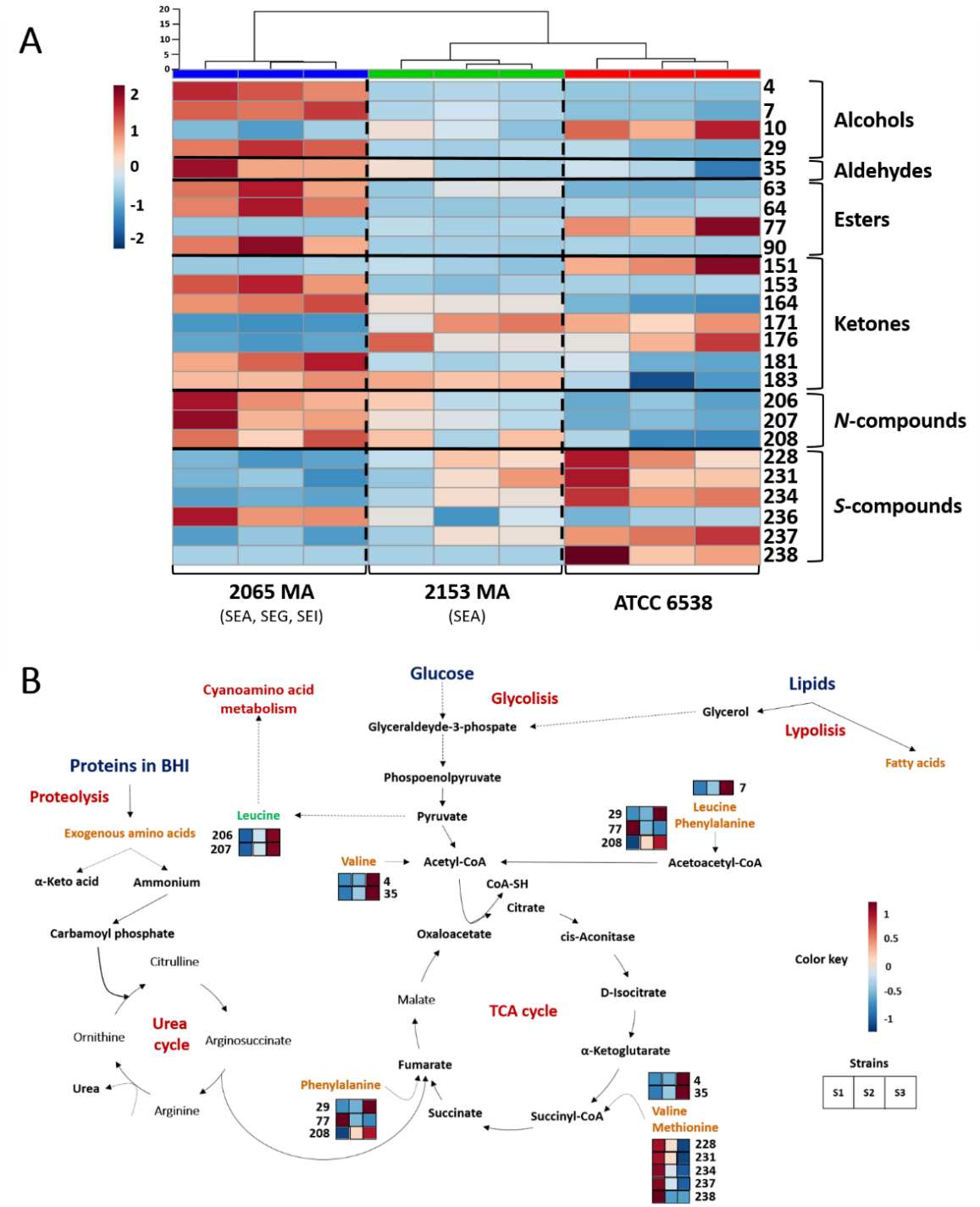


Figure 5.5 - A) Hierarchical clustered heatmap visualization of the volatiles with VIP values (Variable importance in Projection from PLS-DA) higher than 1.5 from the three strains of *Staphylococcus aureus* cultures headspace volatiles, organized by chemical families: ATCC 6538, 2153 MA and 2065 MA. 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.

Fourteen volatile sulfur volatiles had the highest content in ATCC 6538 and thirteen had the highest content in the enterotoxic strains. 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 (404). 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 (405), can be less active in the ATCC 6538 strain. Some of the other volatile sulfur volatiles shown in Table B5.3 (Annexes) have been associated with bacteria while others have been described only for fungi.

The differences between volatiles, mainly in content, in the three strains with origin in the degradation of AAs (leucine, isoleucine, valine, phenylalanine and methionine) can be the result of different contents of precursor biomolecules, including virulence factors.

5.4. Conclusion

Because enterotoxic strains of *S. aureus* are the causing agents of SFP, it is highly important to study the metabolome of *S. aureus* and to see if a distinction between non-enterotoxic strains and enterotoxic strains is possible.

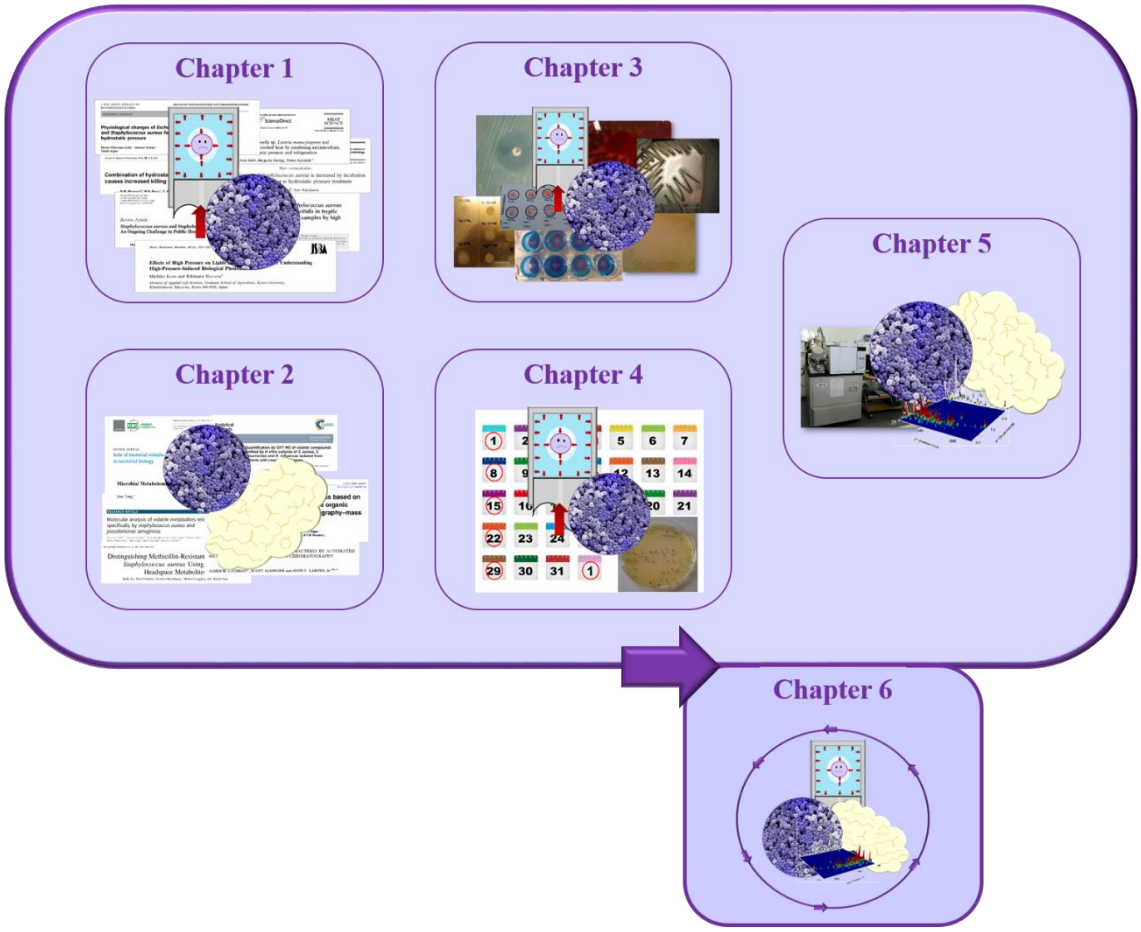
It can be concluded that *S. aureus* volatile exometabolome is more complex (240 volatiles) than it was previously reported, consisting in 10 chemical families: acids, alcohols, aldehydes, esters, hydrocarbons, ketones, terpenes, norisoprenoids, N-compounds, and S-compounds, with volatiles originating 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.

By using PLS-DA it was possible to achieve twenty five VIP (2-nonanone, dimethyl trisulfide, dimethyl tetrasulphide, 3-methylbutanol, 3-methyl-3-butenyl acetate, two isomers of 3-methyl-butyl aldoxime, 2-methylpropanol, 2-phenylethanol, methanethiol, benzonitrile, 1-phenyl-1,2-propanedione, methyl benzoate, 4-phenyl-2-butanone, 6-(methylthio)hexa-1,5-dien-3-ol, 2−methylpropanal, methyl 2-hydroxy-4-methylpentanoate, butyl cyclopropanecarboxylate, 2,3-heptanedione, 3-hydroxy-2-pentanone, 2-tridecanone, dimethyl disulfide, fimethyl pentasulfide and 2-hexadecanone) which were mainly responsible for the

distinction between the enterotoxic strain with three SE (2065 MA) and the strains with lower (2153 MA) or none (ATCC 6538) enterotoxins. Furthermore, PLS regression was used in these twenty five VIP and it was also possible to observe, that enterotoxic strain 2065 MA (three SE) produced higher contents of 3-methylbutanol, isomers of 3-methylbutanal oxime, 2-methylpropanol and 2-methylpropanal and that the strains 2153 MA and ATCC 6538 (with one or none SE, respectively) produced higher contents of of methanethiol, dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide and dimethyl pentasulfide. Thus, by using PLS regression, tt was possible to associate the contents of the VIP obtained to the number of enterotoxins. Using these results, it is possible to see 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.

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. Although the volatiles allowing its distinction must be validated, this is the beginning of a path which can be potentially applied in the food industry using simple and cheaper technologies. This would result in higher food safety for the consumer and a reduction of costs for the food industry and health services. More importantly, further works must be done to determine assure the pattern of biomarkers for *S. aureus* as a species and stains, such as using *S. aureus* in a mixture of foodborne microorganisms and also by using different culture media culture and food products.

CHAPTER 6. General discussion



General discussion

In this work it was investigated the control and detection of different strains of *S. aureus*. Specifically, HPP was used as an approach for the inactivation of microorganisms, in particular as a way of controlling enterotoxic strains of *S. aureus* that can be spread by food. As a new approach to distinguish different strains, the volatile exometabolome of *S. aureus* was characterized.

The choice of bacteria to work followed some criteria which included being: i) a foodborne pathogen (18,50,53,55); ii) of significance from the food safety point of view (18,50,53,55,60,61,91,94,327,331,406); iii) described as a challenge for HPP; (135,136,328); iv) considered a worldwide major health problem, such having the capability of causing a wide range of diseases easy to transfer, possessing a large number of virulence factors, adaptable to different sorts of environments and multi-resistant to antibiotics (8,329,330,406);

HPP, as a fairly new food processing technique has already be applied all over the world in a variety of food products with success (99,103,110). Although several studies have already been done concerning HPP, only 57 were published between 1985 and 2014 for *S. aureus*. HPP is dependent of many factors and this work was focused on three: pressurization values, holding times and strains. Furthermore, as *S. aureus* is a barotolerant species (326), in this work was evaluated if the tested strains were able to develop resistance and if they were able to recover their viability after the treatments, as seen in other HPP studies (111,208).

The results of this work are in line with results obtained for other works (177,205,326,345), showing that higher pressure values and higher holding times are more effective against the tested strains. HPP affected differently the tested strains, inactivating completely the two enterotoxic strains (2153 MA and 2065 MA) at 600 MPa for 30 min, but causing only a reduction of 5.11 log $CFU.mL^{-1}$ in the non-enterotoxic strain (ATCC 6538). The explanation for this lies most certainly in the genotypic and phenotypic traits that are different in these strains, with σ^B factor likely conferring the barotolerance or barosensitivity to the strains, since this factor controls genes related with the production of carotenoids and also the production of SE (188,201). In fact, the most barosensitive strains not only had SE but also had lower carotenoid content and the barotolerant strain have not SE and had higher carotenoid content.

The results of this study also showed that HPP has no effect on the virulence factors of none of the strains, even though the enterotoxic strains were more easily inactivated. The following step was to test if *S. aureus* was capable of developing resistance to HPP treatments.

The results showed a reduction in the survival for the ATCC 6538 and the 2153 MA (SEA) strains from the first to the second cycle. In the remaining cycles viability remained relatively constant. On the other hand, for the strain with three SE (2065 MA), the viability decreased over the HPP cycles until complete inactivation at the fourth cycle. Thus, the first strains were both barotolerant over the successive cycles while the latter was barosensitive. Although the probability for a bacterium to develop HPP resistance is low due to the fact that HPP treatment causes damages in several components of the cell, part of the population of the first two strains remain constantly barotolerant across the other 9 cycles. Independently of the strains and cycles, there was no recovery from HPP treatments with viability remaining the same.

The fact that HPP inactivates *S. aureus* enterotoxic strains, along with the inability to create resistance to HPP treatments and to recover even after 14 days, shows that this food processing method has great potential concerning food safety. A positive outcome is that by applying HPP treatments in early stages of food processing, enterotoxic strains will be totally inactivated which will result in the absence of SE and consequently, SFP is avoided.

In the second part of this work, the characterization of *S. aureus* volatile exometabolome was used in order to see if differences between enterotoxic and non-enterotoxic strains could be detected. Even though conventional methods are usually effective for the detection of *S. aureus*, with both conventional and molecular methods distinguishing *S. aureus* strains, they are contrariwise time consuming and, if molecular methods are used, it can exist an interference of food components with the activity of the polymerase enzyme. Thus, to detect differences between strains of *S. aureus*, microbial metabolomics, specifically the volatile exometabolome, was used. The use of the volatile exometabolome approach has the advantage of giving good results with the minimum sample manipulation. This procedure was used not only to have a clear picture of *S. aureus* volatile exometabolome, but also to see if it was possible, by this means, to detect if differences between enterotoxic and non-enterotoxic strains exist and if so, at what level. Furthermore, it was also intended to see, if possible, a pattern of biomarkers that could identify *S. aureus*.

The use of the volatile exometabolome has already given positive proofs for the characterization, detection and identification of species and strains not only of *S. aureus,* but of other species as well and in many different matrices [from medium culture to blood, beer, and exhaled breath, among others (12,247,257,267,274,304–306)]. Comparing the results obtained in other studies for *S. aureus* volatile exometabolome, none was able to detect as many volatile compounds as in this study, probably because HS-SPME/GC×GC-ToFMS is a sensitive approach. The 240 volatiles identified in the volatile exometabolome of *S. aureus* belong to ten different

chemical families (acids, alcohols, aldehydes, esters, hydrocarbons, ketones, terpenes, norisoprenoids, N-compounds and S-compounds). Some of these volatiles play key roles in pH homeostasis [like 3-hydroxy-2-butanone and 2,3-butanediol which raise the internal pH of cells (291)] while others are the result of oxidative stress, caused by expected decrease of pH due to the growth of the bacteria in a non-buffered medium culture (362). Furthermore, many of the volatiles associated with oxidative stress were higher in the strain 2153 MA, indicating that this strain experienced higher levels of oxidative stress although still maintaining similar viability to the other two strains. On the other hand, the volatiles 3-hydroxy-2-butanone and 2,3-butanediol were higher in the ATCC 6538 strain, indicating that this strain coped with the lower external pH by diverting the pyruvate metabolism, thus avoiding the development of intracellular acids (291,362,407).

Additionally, it was possible to detect differences in the volatile exometabolome compounds of the non-enterotoxic strain from the enterotoxic strains. By using PLS-DA, a supervised multivariate statistical analysis, twenty five VIP volatiles (Table 5.1) were extracted which were responsible for the distinction of the three strains into two main clusters: the first containing the strains ATCC 6538 and 2153 MA and the second containing solely the strain 2065 MA. Hence, it was possible to see that the strain with higher SE number was less similar than the strains with one or none SE. Analyzing in detail the twenty five volatiles (Table 5.1), it was possible to see that, although these volatiles were present in all strains, their content was different according to each strain, and that the metabolic pathway in their genesis was different, *i.e.*, the ATCC 6538 strain had higher contents of the volatiles derived from the degradation of methionine while the 2065 MA strain showed higher contents of the volatiles derived from the degradation of BCAA. The strain 2153 MA (only with one SE) had intermediate contents but more similar to the contents of the ATCC 6538 strain. When PLS regression was applied, a positive correlation (R^2 = 0.9866) between the number of enterotoxins and the content of degradation products of BCAAs and methionine was observed. Thus, by using the volatile exometabolome, it was possible to distinguish strains by the number (or absence) of SE, clustering the strains by the content of the 25 VIP volatiles and the metabolic pathway in the origin of those volatiles.

Furthermore, as a result from the analysis of the volatile exometabolome, it was also possible to see that in this study ten volatiles in common with other previous work that were highly described as volatiles of *S. aureus* were detected. Thus, these volatiles (ethanol, 3-hydroxy-2-butanone, 2-propanone, acetic acid, 3-methyl-1-butanol, 3-methylbutanoic acid, dimethyl disulfide, methanethiol, acetaldehyde and 3-methylbutanal) can be considered as

good candidates to form a pattern of biomarkers of *S. aureus*. In fact, as with this work, this is the objective of many microbial metabolomics assays, which search for a pattern of biomarkers that characterize a species responsible for a specific disease in a non-invasive way, but in the present case it would be used to detect the presence of *S. aureus* in food. Ideally, in a near future, patterns of biomarkers could be used in portable and cheaper equipments that would allow the detection of microorganisms at the species level, or even strain, in food, clinical specimens and different environments. As consequence, contaminated food can be removed from shelves or rejected in food industry lines and infections can be treated earlier and with proper antibiotics.

Limitations of the research

Despite the novelty of the presented results in the CHAPTER 3, CHAPTER 4 and CHAPTER 5, this work is not free of some limitations.

The first concern was the use of PBS as the matrix in the HPP assays, since it is well known that the use of buffers causes the overestimation of HPP effects, since in general food matrices concede a protective effect to the bacteria being tested (361,386). Furthermore, longer holding times were used in this study in comparison to the currently applied by the food industry (3—5 min) (169,326). However, the levels of bacteria used in the experiments were also much higher (between 10^8 and 10^9 CFU mL^{-1}) than those found in food. Thus if lower levels of bacterial concentrations had been used in these studies, holding times could also be lower (368). Another limitation can be found in the studies of HPP in *S. aureus* virulence factors. These results should be confirmed by direct quantification of the virulence factors, particularly in the supernatant, obtained by centrifuging the samples after HPP treatments.

Furthermore, concerning the general inactivation of *S. aureus* by HPP, it is impossible to state that there was a total inactivation based only in cultivation methods. Molecular methods should have been applied to detect the presence of VBNC forms that could still resist HPP treatments.

Although it might not be considered a limitation the information acquired from the volatile exometabolome of *S. aureus*, enabled to gain some knowledge of how *S. aureus* cells behave during growth in a non-buffered medium culture, with the last becoming more acidic with the growth of the culture. Although it is impossible to establish a comparison with food, the use of non-buffered BHI, a complex meaty medium, gives a hint as how this bacteria might behave in meat food products. Using a buffered BHI could probably give different results, concerning both types and contents of volatiles, but this is only an hypothesis.

Finally, although the use of HS-SPME/GC×GC-ToFMS allows the extraction and identification of large quantities of volatiles, confirming the identity of those volatiles requires an extensive knowledge of mass spectrometry that is also time consuming. Furthermore, the exclusion of pyrazines or furans or halogenated compounds might not be totally correct since such compounds have already been described for some bacterial species (318). However this is controversial, since most of this compounds have their origin in the culture media and not in the bacterial metabolism (392,393). Nonetheless, for example, in the study of Rode et al. (2010) (291), an increase in the levels of pyrazine is described as a means to reduce the levels of organic acids and increasing the external pH of the media culture in which *S. aureus* is growing.

Finally, an interesting finding that remains without explanation was the fact that the cluster attained in the volatile exometabolome, *i.e.*, ATCC 6538 and 2153 MA *versus* 2065 MA, was similar to the results achieved in the HPP resistance assays, in which ATCC 6538 and 2153 MA were not inactivated after ten consecutive cycles, while the strain 2065 MA was fully inactivated by the fourth cycle.

Conclusions

The main conclusions of the work developed in this thesis are:

- *S. aureus* strains enterotoxic strains were more easily inactivated by HPP treatments
- *S. aureus* strains with lower carotenoids content more easily inactivated by HPP treatments.
- Cells surviving HPP treatment did not lose their ability to produce SE and other factors/characteristics of virulence.
- HPP treatments effectively inactivate *S. aureus* (reduction of ≈ 5 log) after a single treatment.
- The strains ATCC 6538 and 2153 MA with no SE and one SE, respectively, were able to endure ten consecutive cycles of HPP treatments, while the strain with three SE, 2065 MA, was completely inactivated by the fourth cycle.
- None of the surviving cells were able to develop resistance even after repeating cycles of HPP.
- HPP can be an efficient food processing treatment for *S. aureus* depending on the initial number of CFU. This is especially important for enterotoxic strains since HPP is unable to destroy SE.

- *S. aureus* volatile exometabolome is more complex (240 volatiles) than it was previously reported in other studies, most likely due to the use of HS-SPME/GCxGC-ToFMS as an extraction and identification approach.
- The main active pathways found in this work for *S. aureus* were: degradation of BCAAs, degradation of methionine, cyanoamino acid metabolism, pyruvate metabolism and diacetyl pathway, oxidative stress (including lipid peroxidation) and carotenoid cleavage.
- Using the volatile exometabolome of the three strains of *S. aureus* it was possible to detect differences between the strain with most SE (2065 MA) from the two strains with none or one SE shown by the positive correlation (R^2 = 0.9866) between the number of enterotoxins and the content of degradation products of BCAAs and methionine.
- Finally, a possible pattern of biomarkers for *S. aureus* species, consisting of 10 volatiles, previously reported in other studies, was also found in this work.

Future work

This study demonstrated the capability of HPP to control *S. aureus*, causing either full inactivation of enterotoxic strains or a reduction of the non-enterotoxic. Further work should be done in order to confirm if enterotoxic strains are indeed more susceptible to HPP inactivation, while studying if activity of the genes controlled by the σ^B factor. Additionally, experiments should be done in food products with different features that either protect the cells or enhance inactivation. The assay concerning the resistance and recovery should also be repeated detecting not only the VBNC, but also studying the molecular profile of the bacterial population present in the samples over the ten consecutive cycles (negative controls, *i.e.*, suspensions without pressurization, and the pressurized suspensions), in order to understand the existence of barotolerant cells. Finally, the effect of HPP on virulence factors should be performed directly on the virulence factors and not on the virulence factors with origin in survivor cells.

It is also needed much more work concerning the proposed volatile biomarkers of *S. aureus*. These ones will have to be validated in a series of assays. Such assays will include metabolite target analysis, in which ethanol, 3-hydroxy-2-butanone, 2-propanone, acetic acid, 3-methyl-1-butanol, 3-methylbutanoic acid, dimethyl disulfide, methanethiol, acetaldehyde and 3-methylbutanal will be searched using other strains with different backgrounds (environmental, food or clinical strains), with different characteristics (such as antibiotic resistance and sensitivity) in different media cultures. Afterwards, if these ten volatiles are present in all the tested conditions, assays including other microorganisms will also be important

to confirm the value of the selected potential biomarkers for *S. aureus*. The microorganisms chosen should belong to the same genus, but also to different phylum. Ideally, biomarkers for those microorganisms chosen should also be known which could be bypassed with an extensive research concerning the volatiles reported for the species which will be chosen. Such assay should mingle all tested species in one single medium culture and, afterwards, other media culture, food and clinical specimens would be tested.

Moreover, in order to get an accurate picture of the volatile exometabolome of *S. aureus*, other conditions such as growth temperature, growth phase, presence of NaCl, presence/absence of oxygen, presence/absence of glucose, presence/absence of antibiotics should also be evaluated.

Additionally, in order to get a wider picture of the volatile exometabolome of *S. aureus* and also still aiming for the volatile biomarkers, conditions such as growth temperature, growth phase, different NaCl concentrations, presence/absence of oxygen, presence/absence of glucose, presence/absence of antibiotics among many others factors should also be included in future works. Furthermore, the metabolic pathways of *S. aureus* should be studied in order to avoid the attribution of incorrect origins of its volatiles. Finally, because *S. aureus* strains are frequently multi-resistant, the study of its pathways would be primary to know how a determined antibiotic acts in a sensitive and in a resistant *S. aureus* cell.

REFERENCES

1. Forbes B, Sahm D, Weissfeld A. Gastrointestinal Tract Infections. In: Forbes BA, Sahm DF, Weissfeld AS, editors. Bailey & Scott's Diagnostic Microbiology. 12th ed. St. Louis: Elsevier Mosby; 2007. p.873–90.

2. Word Health Organisation (WHO). Foodborn Disease Outbreaks: Guidelines for Investigation and Control. WHO. 2008. p.1-146

3. Devlieghere F, Vermeiren L, Debevere J. New preservation technologies: Possibilities and limitations. Int Dairy J. 2004;14(4):273–85.

4. Lado BH, Yousef AE. Alternative food-preservation technologies: efficacy and mechanisms. Microbes Infect. 2002;4(4):433–40.

5. Rastogi N, Raghavarao K, Balasubramaniam V, Niranjan K, Knorr D. Opportunities and challenges in high pressure processing of foods. Crit Rev Food Sci Nutr. 2007;47(1):69–112.

6. Kadariya J, Smith T, Thapaliya D. *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public health. Biomed Res Int. 2014;2014:827965.

7. Tang Y-W, Stratton CW. *Staphylococcus aureus*: An old pathogen with new weapons. Clin Lab Med. 2010;30(1):179–208.

8. Chambers HF, Deleo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol. 2009;7(9):629–41.

9. Harbarth S, Kahlmeter G, Kluytmans J, Mendelson M, Hospital GS, Town C, et al. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Geneva, Switzerland; 2017.

10. Ippolito G, Leone S, Lauria FN, Nicastri E, Wenzel RP. Methicillin-resistant *Staphylococcus aureus*: the superbug. Int J Infect Dis. 2010;14(SUPPL. 4):7–11.

11. Costa CP, Gonçalves Silva D, Rudnitskaya A, Almeida A, Rocha SM. Shedding light on *Aspergillus niger* volatile exometabolome. Sci Rep. 2016; 6(October 2015):27441.

12. Alves Z, Melo A, Figueiredo AR, Coimbra MA, Gomes AC, Rocha SM. Exploring the *Saccharomyces cerevisiae* volatile metabolome: Indigenous versus commercial strains. PLoS One. 2015; 10(11):e0143641.

13. Soares JC, Marques MR, Tavaria FK, Pereira JO, Malcata FX, Pintado MM. Biodiversity and characterization of Staphylococcus species isolated from a small manufacturing dairy

plant in Portugal. Int J Food Microbiol. 2011;146(2):123–9.

14. Lamers RP, Muthukrishnan G, Castoe TA, Tafur S, Cole AM, Parkinson CL. Phylogenetic relationships among Staphylococcus species and refinement of cluster groups based on multilocus data. BMC Evol Biol. 2012;12(1):171.

15. Rosenstein R, Götz F. What Distinguishes Highly Pathogenic Staphylococci from Medium- and Non-pathogenic? In: Dobrindt U, Hacker JH, Svanborg C, editors. Between Pathogenicity and Commensalism. Berlin: Springer Berlin Heidelberg; 2013.p. 33–89.

16. Brown S, Cornforth D, Mideo N. Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. Trends Microbiol. 2012;20(7):336–42.

17. Feng Y, Chen C-J, Su L-H, Hu S, Yu J, Chiu C-H. Evolution and pathogenesis of *Staphylococcus aureus*: lessons learned from genotyping and comparative genomics. FEMS Microbiol Rev. 2008;32(1):23–37.

18. Normanno G, Firinu A, Virgilio S, Mula G, Dambrosio A, Poggiu A, et al. Coagulase-positive Staphylococci and *Staphylococcus aureus* in food products marketed in Italy. Int J Food Microbiol. 2005;98(1):73–9.

19. Sannasiddappa T, Costabile A, Gibson G, Clarke S. The influence of *Staphylococcus aureus* on gut microbial ecology in an in vitro continuous culture human colonic model system. PLoS One. 2011;6(8):e23227.

20. Sigler V, Hensley S. Persistence of mixed staphylococci assemblages following disinfection of hospital room surfaces. J Hosp Infect. 2013;83(3):253–6.

21. Wertheim H, Melles D, Vos MC, Leeuwena W van, Belkum A van, Verbrugh HA, et al. The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect Dis. 2005:751–62.

22. Fitzgerald JR. Livestock-associated *Staphylococcus aureus*: origin, evolution and public health threat. Trends Microbiol. 2012;20(4):192–8.

23. Moran GJ, Krishnadasan A, Gorwitz RJ, Fosheim GE, McDougal LK, Carey RB, et al. Methicillin-Resistant *S. aureus* Infections among Patients in the Emergency Department. N Engl J Med. 2006;355(7):666–74.

24. Popovich KJ, Hayden MK. Delineating the Epidemiology-Host-Microbe Relationship for Methicillin-Resistant *Staphylococcus aureus* Infection. J Infect Dis. 2015;211(12):1857–9.

25. van Belkum A, Melles DC, Nouwen J, van Leeuwen WB, van Wamel W, Vos MC, et al. Co-

evolutionary aspects of human colonisation and infection by *Staphylococcus aureus*. Infect Genet Evol. 2009;9(1):32–47.

26. Wertheim H, Vos M, Ott A, Belkum A van. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. Lancet. 2004;364:703–5.

27. Schmidt T, Kock MM, Ehlers MM. Antimicrobial Resistance in Staphylococci at the Human-Animal Interface. In: Ossiprandi MC, editor. Antimicrobial Resistance - An Open Challenge. InTech; 2015. p.85–129.

28. Golding GR, Bryden L, Levett PN, McDonald RR, Wong A, Wylie J, et al. Livestock-associated methicillin-resistant *Staphylococcus aureus* sequence type 398 in humans, Canada. Emerg Infect Dis. 2010;16(4):587–94.

29. Monaco M, Pedroni P, Sanchini A, Bonomini A, Indelicato A, Pantosti A. Livestock-associated methicillin-resistant *Staphylococcus aureus* responsible for human colonization and infection in an area of Italy with high density of pig farming. BMC Infect Dis. 2013;13(1):258.

30. Köck R, Schaumburg F, Mellmann A, Köksal M, Jurke A, Becker K, et al. Livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) as causes of human infection and colonization in Germany. PLoS One. 2013;8(2):e55040.

31. van der Mee-Marquet N, François P, Domelier-Valentin A-S, Coulomb F, Decreux C, Hombrock-Allet C, et al. Emergence of unusual bloodstream infections associated with pig-borne-like *Staphylococcus aureus* ST398 in France. Clin Infect Dis. 2011;52(1):152–3. A

32. Lowder B V, Guinane CM, Ben NL, Weinert LA, Conway-morris A, Fitzgerald JR, et al. Recent human-to-poultry host jump , adaptation , and pandemic spread of *Staphylococcus aureus*. Proc Natl Acad Sci U.S.A. 2009;106(46):19545–19550.

33. Price LB, Stegger M, Hasman H, Aziz M, Larsen J, Andersen S, et al. *Staphylococcus aureus* CC398 : Host Adaptation and Emergence of Methicillin Resistance in Livestock. MBio. 2012;3(1):1–7.

34. Sakwinska O, Giddey M, Moreillon M, Morisset D, Waldvogel A, Moreillon P. *Staphylococcus aureus* host range and human-bovine host shift. Appl Environ Microbiol. 2011;77(17):5908–15.

35. Al-ghamdi AY. Incidence of *Staphylococcus aureus* contamination of marketed Processed

Chicken products with special reference to its antibiotics sensitivity collected from Al Baha city markets, Saudi Arabia. Pakistan J Food Sci. 2012;22(3):168–70.

36. Wendlandt S, Schwarz S, Silley P. Methicillin-resistant *Staphylococcus aureus*: a food-borne pathogen? Annu Rev Food Sci Technol. 2013;4:117–39.

37. Moellering RC. MRSA: the first half century. J Antimicrob Chemother. 2012;67(1):4–11.

38. Rutherford ST, Bassler BL. Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harb Perspect Med. 2012;2(11):1–26.

39. Dinges M, Orwin P, Schlievert P. Exotoxins of *Staphylococcus aureus*. Clin Microbiol Rev. 2000;13(1):16–34.

40. Foster TJ. *Staphylococcus aureus*. J Clin Invest. 2004; 114(12):1693–6.

41. Plata K, Rosato A, Wegrzyn G. *Staphylococcus aureus* as an infectious agent: overview of biochemistry and molecular genetics of its pathogenicity. Acta Biochim Pol. 2009;56(4):597–612.

42. Rooijakkers SHM, van Kessel KPM, van Strijp JAG. Staphylococcal innate immune evasion. Trends Microbiol. 2005;13(12):596–601.

43. Bien J, Sokolova O, Bozko P. Characterization of Virulence Factors of *Staphylococcus aureus*: Novel Function of Known Virulence Factors That Are Implicated in Activation of Airway Epithelial Proinflammatory Response. J Pathog. 2011;2011:601905.

44. Vandenesch F, Lina G, Henry T. *Staphylococcus aureus* hemolysins, bi-component leukocidins, and cytolytic peptides: a redundant arsenal of membrane-damaging virulence factors? Front Cell Infect Microbiol. 2012;2(February):12.

45. Costa AR, Batistão DWF, Ribas RM, Sousa AM, Pereira O, Botelho CM. *Staphylococcus aureus* virulence factors and disease. In: Méndez-Vilas A, editor. Microbial pathogens and strategies for combating them: science, technology and education. FORMATEX; 2013. p.702–10.

46. Foster TJ, Geoghegan J a, Ganesh VK, Höök M. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. Nat Rev Microbiol. 2014;12(1):49–62.

47. Otto A, van Dijl JM, Hecker M, Becher D. The *Staphylococcus aureus* proteome. Int J Med Microbiol; 2014;304(2):110–20.

48. Vandenbergh M, Verbrugh H. Carriage of *Staphylococcus aureus*: Epidemiology and

clinical relevance. J Lab Clin Med. 1999;133(6):525–34.

49. Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev. 1997;10(3):505–20.

50. Pinchuk IV, Beswick EJ, Reyes VE. Staphylococcal enterotoxins. Toxins. 2010;2(8):2177–97.

51. Robinson D, Monk A, Cooper J, Feil E, Enright M. Evolutionary genetics of the accessory gene regulator (agr) locus in *Staphylococcus aureus*. J Bacteriol. 2005;187(24):8312–21.

52. Kérouanton A, Hennekinne JA, Letertre C, Petit L, Chesneau O, Brisabois A, et al. Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. Int J Food Microbiol. 2007;115(3):369–75.

53. Argudín M, Mendoza M, Rodicio M. Food poisoning and *Staphylococcus aureus* enterotoxins. Toxins. 2010;2(7):1751–73.

54. Capita R, Alonso-calleja C, García-Fernández M, Moreno B. Characterization of *Staphylococcus aureus* isolated from poultry meat in Spain. Poult Sci. 2002;81:414–21.

55. Waters AE, Contente-Cuomo T, Buchhagen J, Liu CM, Watson L, Pearce K, et al. Multidrug-Resistant *Staphylococcus aureus* in US Meat and Poultry. Clin Infect Dis. 2011; 52(10):1227–30.

56. Ostyn A, Guillier F, Prufer A-L, Papinaud I, Messio S, Krys S, et al. Intra-laboratory validation of the Ridascreen® SET Total kit for detecting staphylococcal enterotoxins SEA to SEE in cheese. Lett Appl Microbiol. 2011;52(5):468–74.

57. Le Loir Y, Baron F, Gautier M. *Staphylococcus aureus* and food poisoning. Genet Mol Res. 2003;2(1):63–76.

58. Hennekinne J-A, Ostyn A, Guillier F, Herbin S, Prufer A-L, Dragacci S. How should staphylococcal food poisoning outbreaks be characterized? Toxins. 2010;2(8):2106–16.

59. Bao K, Letellier A, Beaudry F. Analysis of Staphylococcus enterotoxin B using differential isotopic tags and liquid chromatography quadrupole ion trap mass spectrometry. Biomed Chromatogr. 2012;26(9):1049–57.

60. Medveďová A, Valík Ľ. *Staphylococcus aureus* : Characterisation and Quantitative Growth Description in Milk and Artisanal Raw Milk Cheese Production. Prof. Ayman Amer Eissa editor, Struct Funct food Eng. InTech. 2012

61. Arvidson S, Tegmark K. Regulation of virulence determinants in *Staphylococcus aureus*. Int J Med Microbiol. 2001;291(2):159–70.

62. Fetsch A, Contzen M, Hartelt K, Kleiser A, Maassen S, Rau J, et al. *Staphylococcus aureus* food-poisoning outbreak associated with the consumption of ice-cream. Int J Food Microbiol. 2014;187C(April 2013):1–6.

63. Lekhraj Rampal. A food poisoning outbreak due to *Staphylococcus aureus*, Kapar, Malaysia, 1983. Med J Malaysia. 1983;38(4):294–8.

64. Evenson ML, Hinds MW, Bernstein RS, Bergdoll MS. Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of staphylococcal food poisoning involving chocolate milk. Int J Food Microbiol. 1988;7(4):311–6.

65. Levine WC, Bennett RW, Choi Y, Henning KJ, Rager JR, Hendricks KA, et al. Staphylococcal food poisoning caused by imported canned mushrooms. J Infect Dis. 1996;173(5):1263–7.

66. Danielsson-Tham M-L. Staphylococcal Food Poisoning. In: Tham W, Danielsson-Tham M-L, editors. Food Associated Pathogens. Boca Raton, FL, USA: CRC Press; 2014. p.250–268.

67. Do Carmo LS, Cummings C, Linardi VR, Dias RS, De Souza JM, De Sena MJ, et al. A case study of a massive staphylococcal food poisoning incident. Foodborne Pathog Dis. 2004;1(4):241–6.

68. Asao T, Kumeda Y, Kawai T, Shibata T, Oda H, Haruki K, et al. An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. Epidemiol Infect. 2003;130(1):33–40.

69. Ikeda T, Tamate N, Yamaguchi K, Makino S. Mass outbreak of food poisoning disease caused by small amounts of staphylococcal enterotoxins A and H. Appl Environ Microbiol. 2005;71(5):2793–5.

70. Al-Maghderi YS, Al-Mazroa LA. *Staphylococcus aureus* food poisoning out- break in Al-Madinah, Al-Monawarah, March 2003. Saudi Epidemiol Bull. 2003;10:9–13.

71. Jørgensen HJ, Mathisen T, Løvseth A, Omoe K, Qvale KS, Loncarevic S. An outbreak of staphylococcal food poisoning caused by enterotoxin H in mashed potato made with raw milk. FEMS Microbiol Lett. 2005;252(2):267–72.

72. Waite JG, Yousef AE. Overview of food safety. In: Ortega-Rivas E, editor. Processing effects on safety and quality of foods. Boca Raton, FL, USA: CRC Press; 2009. p.11–65.

73. Huang A, Ocfemia CB, Hunt DC. Staphylococcal Food Poisoning Outbreak in Southeast Kansas. 2005;(877).

74. Schmid D, Gschiel E, Mann M, Huhulescu S, Ruppitsch W, Böhm G, et al. Outbreak acute gastroenteritis in an austrian boarding school, September 2006. Eurosurveillance. 2007;12(1–3):54–6.

75. Weiler N, Leotta GA, Zarate MN, Manfredi E, Alvarez ME, Rivas M. Brote de intoxicación alimentaria asociado al consumo de leche ultrapasteurizada en la República del Paraguay. Rev Argent Microbiol. 2011;43(1):33–6.

76. Johler S, Tichaczek-Dischinger PS, Rau J, Sihto H-M, Lehner A, Adam M, et al. Outbreak of Staphylococcal food poisoning due to SEA-producing *Staphylococcus aureus*. Foodborne Pathog Dis. 2013;10(9):777–81.

77. Ostyn A, de Buyser ML, Guillier F, Groult J, Félix B, Salah S, et al. First evidence of a food poisoning outbreak due to staphylococcal enterotoxin type E, France, 2009. Eurosurveillance. 2010;15(13):10–3.

78. Hait J, Tallent S, Melka D, Keys C, Bennett R. *Staphylococcus aureus* Outbreak Investigation of an Illinois Bakery. J Food Saf. 2012;32(4):435–44.

79. Pillsbury A, Chiew M, Bates J, Sheppeard V. An outbreak of staphylococcal food poisoning in a commercially catered buffet. Commun Dis Intell. 2013;37(2):144–8.

80. CDC. Outbreak of Staphylococcal Food Poisoning from a Military Unit Lunch Party — United States, July 2012. MMWR. Morbidity and mortality weekly report. Atlanta; 2013.

81. Basavegowda M, Bettappa P, Rajegowda R, Shivanna U, Channabasappa A, Vijaykumar G. An outbreak of staphylococcal food poisoning in a nursing hostel, Mysore, Karnataka. Int J Med Public Heal.2014; 4(3):257.

82. EFSA. Trends and Sources of Zoonoses and Zoonotic Agents and Food-borne Outbreaks in 2011. EFSA Journal.2014. 1-378 p.

83. Balaban N, Rasooly A. Staphylococcal enterotoxins. Int J Food Microbiol. 2000; 61(1):1–10.

84. Munson S, Tremaine M, Betley M, Welch R. Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus*. Infect Immun. 1998;66(7):3337–48.

85. Dinges MM, Orwin PM, Schlievert PM. Exotoxins of *Staphylococcus aureus*. Clin Microbiol

Rev. 2000;13(1):16–34.

86. Sospedra I, Soriano J, Mañes J. Enterotoxinomics: The omic sciences in the study of staphylococcal toxins analyzed in food matrices. Food Res Int. 2013;54(1):1052–60.

87. Schelin J, Wallin-Carlquist N, Cohn MT, Lindqvist R, Barker GC, Rådström P. The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. Virulence. 2011;2(6):580–92.

88. Derzelle S, Dilasser F, Duquenne M, Deperrois V. Differential temporal expression of the staphylococcal enterotoxins genes during cell growth. Food Microbiol. 2009;26(8):896–904.

89. Monday SR, Bohach GA. Use of Multiplex PCR To Detect Classical and Newly Described Pyrogenic Toxin Genes in Staphylococcal Isolates. 1999;37(10):3411–4.

90. Ortega E, Abriouel H, Lucas R, Gálvez A. Multiple roles of *Staphylococcus aureus* enterotoxins: pathogenicity, superantigenic activity, and correlation to antibiotic resistance. Toxins. 2010;2(8):2117–31.

91. Ler SG, Lee F., Gopalakrishnakone P. Trends in detection of warfare agents. Detection methods for ricin, staphylococcal enterotoxin B and T-2 toxin. J Chromatogr A. 2006;1133(1–2):1–12.

92. Yang B, Shi Y, Xia X, Xi M, Wang X, Ji B, et al. Inactivation of foodborne pathogens in raw milk using high hydrostatic pressure. Food Control. 2012;28(2):273–8.

93. Hennekinne J-A, De Buyser M-L, Dragacci S. *Staphylococcus aureus* and its food poisoning toxins: characterization and outbreak investigation. FEMS Microbiol Rev. 2012;36(4):815–36.

94. Soriano J, Font G, Moltó J, Mañes J. Enterotoxigenic staphylococci and their toxins in restaurant foods. Trends Food Sci Technol. 2002;13:60–7.

95. Carfora V, Caprioli a., Marri N, Sagrafoli D, Boselli C, Giacinti G, et al. Enterotoxin genes, enterotoxin production, and methicillin resistance in *Staphylococcus aureus* isolated from milk and dairy products in Central Italy. Int Dairy J. 2015;42:12–5.

96. Jones TF, Kellum ME, Susan S, Bell M, Schaffner W. An Outbreak of Foodborne Illness Caused by *Staphylococcus aureus*. Emerg Infect Dis. 2002;8(1):82–4.

97. Normanno G, Corrente M, La Salandra G, Dambrosio A, Quaglia NC, Parisi A, et al. Methicillin-resistant *Staphylococcus aureus* (MRSA) in foods of animal origin product in

Italy. Int J Food Microbiol. 2007;117(2):219–22.

98. Spanu V, Scarano C, Cossu F, Pala C, Spanu C, De Santis EPL. Antibiotic resistance traits and molecular subtyping of *Staphylococcus aureus* isolated from raw sheep milk cheese. J Food Sci. 2014;79(10):M2066-71.

99. Rastogi NK, Raghavarao KSMS, Balasubramaniam VM, Niranjan K, Knorr D. Opportunities and challenges in high pressure processing of foods. Crit Rev Food Sci Nutr. 2007;47(1):69–112.

100. Smelt JPP. Recent advances in the microbiology of high pressure processing. Trends Food Sci Technol. 1998;9(4):152–8.

101. Ramirez R, Saraiva J, Lamela CP, Torres JA. Reaction kinetics analysis of chemical changes in pressure-assisted thermal processing. Food Eng Rev. 2009;1(1):16–30.

102. Balasubramaniam VM, Farkas D, Turek EJ. Preserving foods through high-pressure processing. Food Technol. 2008;62(11):32–8.

103. Considine KM, Kelly AL, Fitzgerald GF, Hill C, Sleator RD. High-pressure processing--effects on microbial food safety and food quality. FEMS Microbiol Lett. 2008;281(1):1–9.

104. Yordanov DG, Angelova GV. High Pressure Processing for Foods Preserving. Biotechnol Biotechnol Equip. 2014;24(3):1940–5.

105. Patterson MF. Microbiology of pressure-treated foods. J Appl Microbiol. 2005;98(6):1400–9.

106. Yaldagard M, Mortazavi SA, Tabatabaie F. The principles of ultra high pressure technology and its application in food processing/preservation: A review of microbiological and quality aspects. African J Biotechnol. 2008;7(16):2739–67.

107. Royer H. No Title. Arch Physiol Norm Pathol. 1895;7:12.

108. Hite BH. The effect of pressure in the preservation of milk. West Virginia Agric Exp Stn Bull. 1899;58:15−35.

109. Demazeau G, Rivalain N. High hydrostatic pressure and biology: a brief history. Appl Microbiol Biotechnol. 2011;89(5):1305–14.

110. Daryaei H, Balasubramaniam VM. Microbial decontamination of food by high pressure processing. In: Demirci A, Ngadi MO, editors. Microbial Decontamination in the Food Industry: Novel methods and applications. Cambridge, UK: Woodhead Publishing Limited; 2012. p.370–406.

111. Alpas H, Bozoglu F. Inactivation of *Staphylococcus aureus* and *Listeria monocytogenes* in milk and cream of chicken soup by high hydrostatic pressure and bacteriocins. Int J High Press. 2002;22(3):681-684.

112. Alpas H, Lee J, Bozoglu F, Kaletunç G. Evaluation of high hydrostatic pressure sensitivity of *Staphylococcus aureus* and *Escherichia coli* O157:H7 by differential scanning calorimetry. Int J Food Microbiol. 2003;87(3):229–37.

113. Jofré A, Garriga M, Aymerich T. Inhibition of *Salmonella* sp. *Listeria monocytogenes* and *Staphylococcus aureus* in cooked ham by combining antimicrobials, high hydrostatic pressure and refrigeration. Meat Sci. 2008;78:53–9.

114. Hayakawa I, Furukawa S, Midzunaga a, Horiuchi H, Nakashima T, Fujio Y, et al. Mechanism of inactivation of heat-tolerant spores of *Bacillus stearothermophilus* IFO 12550 by rapid decompression. J Food Sci. 1998;63(3):371–4.

115. Daryaei H, Coventry MJ, Versteeg C, Sherkat F. Effect of high pressure treatment on starter bacteria and spoilage yeasts in fresh lactic curd cheese of bovine milk. Innov Food Sci Emerg Technol. 2008;9(2):201–5.

116. Ferreira EHDR, Rosenthal A, Calado V, Saraiva J, Mendo S. *Byssochlamys nivea* inactivation in pineapple juice and nectar using high pressure cycles. J Food Eng. 2009;95(4):664–9.

117. Zook CD, Parish ME, Braddock RJ, Balaban MO. High Pressure Inactivation Kinetics of *Saccharomyces cerevisiae* Ascospores in Orange and Apple Juices. J Food Sci. 1999;64(3):533–5.

118. Calci KR, Meade GK, Tezloff RC, Kingsley DH. High-Pressure Inactivation of Hepatitis A Virus within Oysters. 2005;71(1):339–43.

119. Grove SF, Forsyth S, Wan J, Coventry J, Cole M, Stewart CM, et al. Inactivation of hepatitis A virus, poliovirus and a norovirus surrogate by high pressure processing. Innov Food Sci Emerg Technol. 2008; 9(2):206–10.

120. Kingsley DH, Holliman DR, Calci KR, Chen H, Flick GJ. Inactivation of a norovirus by high-pressure processing. Appl Environ Microbiol. 2007;73(2):581–5.

121. Wilkinson N, Kurdziel a. S, Langton S, Needs E, Cook N. Resistance of poliovirus to inactivation by high hydrostatic pressures. Innov Food Sci Emerg Technol. 2001;2(2):95–8.

122. San Martín MF, Barbosa-Cánovas GV, Swanson BG. Food processing by high hydrostatic pressure. Crit Rev Food Sci Nutr. 2002;42(6):627–45.

123. Yuste J, Capellas M, Fung DYC, Mor-Mur M. Inactivation and sublethal injury of foodborne pathogens by high pressure processing: Evaluation with conventional media and thin agar layer method. Food Res Int. 2004;37:861–6.

124. Yuste J, Capellas M, Pla R, Fung DYC. High Pressure Processing for Food Safety and Preservation : a Review. J Rapid Methods Autom Microbiol. 2001;9(1401):1–10.

125. Oger PM, Jebbar M. The many ways of coping with pressure. Res Microbiol. 2010; 161(10):799–809.

126. Liu Y, Manna AC, Pan C-H, Kriksunov I a, Thiel DJ, Cheung AL, et al. Structural and function analyses of the global regulatory protein SarA from *Staphylococcus aureus*. Proc Natl Acad Sci U.S.A. 2006;103(7):2392–7.

127. Schad EM, Zaitseva I, Zaitsev VN, Dohlsten M, Kalland T, Schlievert PM, et al. Crystal structure of the superantigen staphylococcal enterotoxin type A. EMBO J. 1995; 14(14):3292–301.

128. Campus M. High Pressure Processing of Meat, Meat Products and Seafood. Food Eng Rev. 2010;2(4):256–73.

129. Mañas P, Mackey B. Morphological and physiological changes induced by high hydrostatic pressure in exponential-and stationary-phase cells of *Escherichia coli*: relationship with cell. Appl Environ Microbiol. 2004;70(3):1545–54.

130. Rendueles E, Omer MK, Alvseike O, Alonso-Calleja C, Capita R, Prieto M. Microbiological food safety assessment of high hydrostatic pressure processing: A review. LWT - Food Science and Technology. 2011. p.1251–60.

131. Winter R, Jeworrek C. Effect of pressure on membranes. Soft Matter. 2009;5(January):3157.

132. Hauben KJA, Wuytack EY, Soontjens CCF, Michiels CW. High-Pressure Transient Sensitization of *Escherichia coli* to Lysozyme and Nisin by Disruption of Outer-Membrane Permeability. J Food Prot. 1996;59(4):350–5.

133. Russell NJ. Bacterial membranes: the effects of chill storage and food processing. An overview. Int J Food Microbiol. 2002;79(1–2):27–34.

134. Patterson MF, Ledward DA, Leadley C, Rogers N. High Pressure Processing. In: Brennan

JG, Grandison AS, editors. Food Processing Handbook. 2nd ed. Weinheim, Germany: Wiley-VCH; 2012. p.179–204.

135. Pilavtepe-Çelik M, Yousef A, Alpas H. Physiological changes of *Escherichia coli* O157:H7 and *Staphylococcus aureus* following exposure to high hydrostatic pressure. J fur Verbraucherschutz und Leb. 2013;8(2013):175–83.

136. Wang L, Pan J, Xie H, Yang Y, Lin C. Inactivation of *Staphylococcus aureus* and *Escherichia coli* by the synergistic action of high hydrostatic pressure and dissolved CO2. Int J Food Microbiol. 2010;144(1):118–25.

137. Shigehisa T, Ohmori T, Saito A, Taji S, Hayashi R. Effects of high hydrostatic pressure on characteristics of pork slurries and inactivation of microorganisms associated with meat and meat products. Int J Food Microbiol. 1991;12(2–3):207–15.

138. Hartmann C, Mathmann K, Delgado A. Mechanical stresses in cellular structures under high hydrostatic pressure. Innov Food Sci Emerg Technol. 2006;7(1–2):1–12.

139. Kato M, Hayashi R. Effects of high pressure on lipids and biomembranes for understanding high-pressure-induced biological phenomena. Biosci Biotechnol Biochem. 1999;63(8):1321–8.

140. Pilavtepe-Çelik M, Balaban MO, Alpas H, Yousef AE. Image analysis based quantification of bacterial volume change with high hydrostatic pressure. J Food Sci. 2008;73(9):423–9.

141. Alpas H, Kalchayanand N, Bozoglu F, Sikes A, Dunne CP, Ray B. Variation in resistance to hydrostatic pressure among strains of food-borne pathogens. Appl Environ Microbiol. 1999;65(9):4248–51.

142. Mota MJ, Lopes RP, Delgadillo I, Saraiva JA. Microorganisms under high pressure--adaptation, growth and biotechnological potential. Biotechnol Adv. 2013;31(8):1426–34.

143. Niven GW, Miles CA, Mackey BM. The effects of hydrostatic pressure on ribosome conformation in *Escherichia coli*: and in vivo study using differential scanning calorimetry. Microbiology. 1999;145:419–25.

144. Gross M, Jaenicke R. Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes. Eur J Biochem. 1994 221:617–30.

145. Alpas H, Lee J, Bozoglu F, Kaletunç G. Evaluation of high hydrostatic pressure sensitivity of *Staphylococcus aureus* and *Escherichia coli* O157:H7 by differential scanning

calorimetry. Int J Food Microbiol. 2003;87(3):229–37.

146. Alpas H, Kalchayanand N, Bozoglu F, Ray B. Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. Int J Food Microbiol. 2000;60:33–42.

147. Alpas H, Bozoglu F. The combined effect of high hydrostatic pressure, heat and bacteriocins on inactivation of foodborne pathogens in milk and orange juice. World J Microbiol Biotechnol. 2000;16:387–92.

148. Morgan SM, Ross RP, Beresford T, Hill C. Combination of hydrostatic pressure and lacticin 3147 causes increased killing of Staphylococcus and Listeria. J Appl Microbiol. 2000;88:414–20.

149. Masschalck B, Van Houdt R, Michiels CW. High pressure increase bactericidal activity and spectrum of lactoferrin,lactoferricin and nisin. Int J Food Microbiol. 2001;64:325–32.

150. Noma S, Hayakawa I. Barotolerance of *Staphylococcus aureus* is increased by incubation at below 0 degrees C prior to hydrostatic pressure treatment. Int J Food Microbiol. 2003;80:261–4.

151. Nakimbugwe D, Masschalck B, Atanassova M, Zewdie-Bosüner A, Michiels CW. Comparison of bactericidal activity of six lysozymes at atmospheric pressure and under high hydrostatic pressure. Int J Food Microbiol. 2006;108:355–63.

152. Jofré A, Aymerich T, Garriga M. Improvement of the food safety of low acid fermented sausages by enterocins A and B and high pressure. Food Control. 2009;20:179–84.

153. Malinowska-Pańczyk E, Kołodziejska I. Effect of lysozyme or nisin on survival of some bacteria treated with high pressure at subzero temperature. Brazilian J Microbiol. 2009;40:767–77.

154. Malinowska-Pańczyk E, Kołodziejska I. Effect of pH and high pressure at sub-zero temperature on the viability of some bacteria. High Press Res. 2009;29(January 2015):443–8.

155. Ananou S, Garriga M, Jofré A, Aymerich T, Gálvez A, Maqueda M, et al. Combined effect of enterocin AS-48 and high hydrostatic pressure to control food-borne pathogens inoculated in low acid fermented sausages. Meat Sci. 2010; 84(4):594–600.

156. Pérez Pulido R, Toledo del árbol J, Grande Burgos MJ, Gálvez A. Bactericidal effects of high hydrostatic pressure treatment singly or in combination with natural antimicrobials

on *Staphylococcus aureus* in rice pudding. Food Control. 2012;28(1):19–24.

157. Tabla R, Martínez B, Rebollo JE, González J, Ramírez MR, Roa I, et al. Bacteriophage performance against *Staphylococcus aureus* in milk is improved by high hydrostatic pressure treatments. Int J Food Microbiol. 2012;156(3):209–13.

158. Silva JL, Oliveira AC, Vieira TCRG, de Oliveira G a P, Suarez MC, Foguel D. High-pressure chemical biology and biotechnology. Chem Rev. 2014;114:7239–67.

159. Margosch D, Moravek M, Gänzle MG, Märtlbauer E, Vogel RF, Ehrmann MA. Effect of high pressure and heat on bacterial toxins. Food Technol Biotechnol. 2005;43(3):211–7.

160. René-Trouillefou M, Benzaria A, Marchal S, Lange R, Caporiccio B, Dumay E. Staphylococcal enterotoxin A: Partial unfolding caused by high pressure or denaturing agents enhances superantigenicity. Biochim Biophys Acta. 2010;1804(6):1322–33.

161. Rivalain N, Roquain J, Boiron J-M, Maurel J-P, Largeteau A, Ivanovic Z, et al. High hydrostatic pressure treatment for the inactivation of *Staphylococcus aureus* in human blood plasma. N Biotechnol. 2012;29(3):409–14.

162. Medina-Meza IG, Barnaba C, Barbosa-Cánovas G V. Effects of high pressure processing on lipid oxidation: A review. Innov Food Sci Emerg Technol. 2013;22(September 2015):1–10.

163. Winter R, Dzwolak W. Exploring the temperature-pressure configurational landscape of biomolecules: from lipid membranes to proteins. Philos Trans A Math Phys Eng Sci. 2005;363(1827):537-563.

164. Rivalain N, Roquain J, Demazeau G. Development of high hydrostatic pressure in biosciences: pressure effect on biological structures and potential applications in biotechnologies. Biotechnol Adv. 2010;28(6):659–72.

165. Mentré P, Hui Bon Hoa G. Effects of high hydrostatic pressures on living cells: a consequence of the properties of macromolecules and macromolecule-associated water. Int Rev Cytol. 2001;201:1–84.

166. Heremans K. High pressure effects on biomolecules. In: Ledward DA, Johnston DE, Earnshaw RG, Hasting APM, editors. High Pressure Processing of Foods. Loughborough: Nottingham University Press; 1995. p.81–98.

167. Cheftel JC. Review : High-pressure, microbial inactivation and food preservation. Food Sci Technol Int. 1995;1(2–3):75–90.

168. Chilton P, Isaacs NS, Mackey B, Stenning R. The effects of high hydrostatic pressure on bacteria. In: Heremans K, editor. High pressure research in the biosciences and biotechnology. Belgium: Leuven University Press; 1997.p. 225–8.

169. Hugas M, Garriga M, Monfort JM. New mild technologies in meat processing: high pressure as a model technology. Meat Sci. 2002;62(3):359–71.

170. Chamberlain NR, Mehrtens BG, Xiong Z, Kapral FA, Boardman JL, Rearick JI. Correlation of carotenoid production, decreased membrane fluidity, and resistance to oleic acid killing in *Staphylococcus aureus* 18Z. Infect Immun. 1991;59(12):4332–7.

171. Clauditz A, Resch A, Wieland K-P, Peschel A, Götz F. Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. Infect Immun. 2006;74(8):4950–3.

172. Mishra NN, Liu GY, Yeaman MR, Nast CC, Proctor RA, McKinnell J, et al. Carotenoid-related alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. Antimicrob Agents Chemother. 2011;55(2):526–31.

173. Erkmen O, Karataş Ş. Effect of high hydrostatic pressure on *Staphylococcus aureus* in milk. J Food Eng. 1997; 33(3–4):257–62.

174. Gervilla R, Sendra E, Ferragut V, Guamis B. Sensitivity of *Staphylococcus aureus* and *Lactobacillus helveticus* in ovine milk subjected to high hydrostatic pressure. J Dairy Sci. 1999; 82(6):1099–107.

175. Gervilla R, Ferragut V, Guamis B. High pressure inactivation of microorganisms inoculated into ovine milk of different fat contents. J Dairy Sci. 2000;83(4):674–82.

176. O'Reilly CE, O'Connor PM, Kelly a L, Beresford TP, Murphy PM. Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. Appl Environ Microbiol. 2000;66(11):4890–6.

177. Fioretto F, Cruz C, Largeteau A, Sarli TA, Demazeau G, El Moueffak A. Inactivation of *Staphylococcus aureus* and *Salmonella enteritidis* in tryptic soy broth and caviar samples by high pressure processing. Brazilian J Med Biol Res. 2005;38:1259–65.

178. Guan D, Chen H, Ting EY, Hoover DG. Inactivation of *Staphylococcus aureus* and *Escherichia coli* O157:H7 under isothermal-endpoint pressure conditions. J Food Eng. 2006; 77:620–7.

179. Bayindirli A, Alpas H, Bozoglu F, Hizal M. Efficiency of high pressure treatment on

inactivation of pathogenic microorganisms and enzymes in apple, orange, apricot and sour cherry juices. Food Control. 2006;17:52–8.

180. Rigaldie Y, Largeteau A, Demazeau G, Lemagnen G, Grislain L. Inactivation of *Staphylococcus aureus* using high hydrostatic pressure. High Press Res. 2007;27(1):125–128.

181. Viazis S, Farkas B, Jaykus L. Inactivation of bacterial pathogens in human milk by high-pressure processing. J Food Prot. 2008;71(1):109–18.

182. Jofré A, Aymerich T, Grèbol N, Garriga M. Efficiency of high hydrostatic pressure at 600MPa against food-borne microorganisms by challenge tests on convenience meat products. LWT - Food Sci Technol. 2009;42(5):924–8.

183. Nemeth C, Dalmadi I, Friedrich L. Pasteurization of liquid egg by high hydrostatic pressure (HHP) treatment. African J. 2012;6(3):660–4.

184. Gayán E, Condón S, Álvarez I, Nabakabaya M, Mackey B. Effect of pressure-induced changes in the ionization equilibria of buffers on inactivation of *Escherichia coli* and *Staphylococcus aureus* by high hydrostatic pressure. Appl Environ Microbiol. 2013;79(13):4041–7.

185. Denavaut M, Verret C, El Moueffak A, Largeteau A, Quéveau D, de Lamballerie M. Effect of high pressure treatment on the characteristics of a model emulsion. High Press Res. 2014;34:434–8.

186. Scheinberg JA, Svoboda AL, Cutter CN. High-pressure processing and boiling water treatments for reducing *Listeria monocytogenes*, *Escherichia coli* O157: H7, *Salmonella* spp., and *Staphylococcus aureus* during beef jerky processing. Food Control. 2014;39:105–10.

187. Cebrián G, Michiels CW, Mañas P, Condón S. Biological approach to modeling of *Staphylococcus aureus* high-hydrostatic-pressure inactivation kinetics. Appl Environ Microbiol. 2010; 76(21):6982–90.

188. Cebrián G, Sagarzazu N, Aertsen A., Pagán R, Condón S, Mañas P. Role of the alternative sigma factor σ^B on *Staphylococcus aureus* resistance to stresses of relevance to food preservation. J Appl Microbiol. 2009;107:187–96.

189. Black EP, Huppertz T, Fitzgerald GF, Kelly AL. Baroprotection of vegetative bacteria by milk constituents: A study of *Listeria innocua*. Int Dairy J. 2007;17(2):104–10.

190. Dogan C, Erkmen O. High pressure inactivation kinetics of *Listeria monocytogenes* inactivation in broth, milk, and peach and orange juices. J Food Eng. 2004;62(1):47–52.

191. Hauben KJ, Bernaerts K, Michiels CW. Protective effect of calcium on inactivation of *Escherichia coli* by high hydrostatic pressure. J Appl Microbiol. 1998;85(4):678–84.

192. Tassou CC, Galiatsatou P, Samaras FJ, Mallidis CG. Inactivation kinetics of a piezotolerant *Staphylococcus aureus* isolated from high-pressure-treated sliced ham by high pressure in buffer and in a ham model system: Evaluation in selective and non-selective medium. Innov Food Sci Emerg Technol. 2007;8(4):478–84.

193. Tassou CC, Panagou EZ, Samaras FJ, Galiatsatou P, Mallidis CG. Temperature-assisted high hydrostatic pressure inactivation of *Staphylococcus aureus* in a ham model system: evaluation in selective and nonselective medium. J Appl Microbiol. 2008;104(6):1764–73.

194. Syed QA, Buffa M, Guamis B, Saldo J. Effect of Compression and Decompression Rates of High Hydrostatic Pressure on Inactivation of *Staphylococcus aureus* in Different Matrices. Food Bioprocess Technol. 2014;7:1202–7.

195. Noma S, Shimoda M, Hayakawa I. Inactivation of Vegetative Bacteria by Rapid Decompression Treatment. JFS Food Eng Phys Prop. 2002;67(9):3408–11.

196. Rademacher B, Werner F, Pehl M. Effect of the pressurizing ramp on the inactivation of *Listeria innocua* considering thermofluiddynamical processes. Innov Food Sci Emerg Technol. 2002;3(1):19–24.

197. McMahon MAS, Xu J, Moore JE, Blair IS, McDowell DA. Environmental stress and antibiotic resistance in food-related pathogens. Appl Environ Microbiol. 2007;73(1):211–7.

198. Karatzas K a G, Zervos A, Tassou CC, Mallidis CG, Humphrey TJ. Piezotolerant small-colony variants with increased thermotolerance, antibiotic susceptibility, and low invasiveness in a clonal *Staphylococcus aureus* population. Appl Environ Microbiol. 2007;73(6):1873–81.

199. Karatzas K a G, Valdramidis VP, Wells-Bennik MHJ. Contingency locus in ctsR of *Listeria monocytogenes* Scott A: A strategy for occurrence of abundant piezotolerant isolates within clonal populations. Appl Environ Microbiol. 2005;71(12):8390–6.

200. Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol. 2003;48(6):1429–49.

201. Bischoff M, Dunman P, Kormanec J, Macapagal D, Murphy E, Mounts W, et al. Microarray-Based Analysis of the *Staphylococcus aureus* σ^B Regulon. J Bacteriol. 2004;186(13):4085–99.

202. Bozoglu F, Alpas H, Kaletunç G. Injury recovery of foodborne pathogens in high hydrostatic pressure treated milk during storage. FEMS Immunol Med Microbiol. 2004;40:243–7.

203. Bowman JP, Bittencourt CR, Ross T. Differential gene expression of *Listeria monocytogenes* during high hydrostatic pressure processing. Microbiology. 2008;154(Pt 2):462–75.

204. Syed QA, Buffa M, Guamis B, Saldo J. Effect of Compression and Decompression Rates of High Hydrostatic Pressure on Inactivation of *Staphylococcus aureus* in Different Matrices. Food Bioprocess Technol. 2014;7:1202–7.

205. Jofré A, Aymerich T, Bover-Cid S, Garriga M. Inactivation and recovery of *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus* after high hydrostatic pressure treatments up to 900 MPa. Int Microbiol. 2010;13(3):105–12.

206. Yao J, Zhou B, Wang R, Wang T, Hu X, Liao X, et al. Inactivation of *Staphylococcus aureus* by high hydrostatic pressure in saline solution and meat slurry with different initial inoculum levels. Food Bioprod Process. 2015;94:592–600.

207. Black EP, Stewart CM, Hoover DG. Microbiological Aspects of High-Pressure Food Processing. In: Zhang HQ, Barbosa-Cánovas G V., Balasubramaniam VM, Dunne CP, Farkas DF, Yuan JTC, editors. Nonthermal Processing Technologies for Food. Oxford, UK: Wiley-Blackwell; 2010.p. 51–71.

208. Chen H, Hoover DG. Modeling the combined effect of high hydrostatic pressure and mild heat on the inactivation kinetics of *Listeria monocytogenes* Scott A in whole milk. Innov Food Sci Emerg Technol. 2003;4(1):25–34.

209. Klotz B, Pyle DL, Mackey BM. New mathematical modeling approach for predicting microbial inactivation by high hydrostatic pressure. Appl Environ Microbiol. 2007;73(8):2468–78.

210. Chen H. Use of linear, Weibull, and log-logistic functions to model pressure inactivation of seven foodborne pathogens in milk. Food Microbiol. 2007;24:197–204.

211. Van Boekel MAJS. On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. Int J Food Microbiol. 2002;74(1–2):139–59.

212. Peleg M, Normand MD, Corradini MG. Generating microbial survival curves during thermal processing in real time. J Appl Microbiol. 2005;98(2):406–17.

213. Marks BP. Status of microbial modeling in food process models. Compr Rev Food Sci Food Saf. 2008;7(1):137–43.

214. Panagou EZ, Tassou CC, Manitsa C, Mallidis C. Modelling the effect of high pressure on the inactivation kinetics of a pressure-resistant strain of *Pediococcus damnosus* in phosphate buffer and gilt-head seabream (*Sparus aurata*). J Appl Microbiol. 2007;102(6):1499–507.

215. Peleg M, Cole MB. Estimating the survival of *Clostridium botulinum* spores during heat treatments. J Food Prot. 2000;63(2):190–5.

216. Pilavtepe-Çelik M, Buzrul S, Alpas H, Bozoğlu F. Development of a new mathematical model for inactivation of *Escherichia coli* O157:H7 and *Staphylococcus aureus* by high hydrostatic pressure in carrot juice and peptone water. J Food Eng. 2009;90:388–94.

217. Peleg M. Isothermal Microbial Heat Inactivation. In: Peleg M, editor. Advanced Quantitative Microbiology for Foods and Biosystems: Models for Predicting Growth and Inactivation. Boca Raton, FL, USA: CRC Press; 2006.p. 1–48.

218. Metrick C, Hoover DG, Farkas DF. Effects of High Hydrostatic Pressure on Heat-Resistant and Heat-Sensitive Strains of Salmonella. J Food Sci. 1989;54(6):1547–9.

219. Peleg M, Cole MB. Reinterpretation of microbial survival curves. Crit Rev Food Sci Nutr. 1998;38(5):353–80.

220. Bhaduri S, Smith PW, Palumbo SA., Turner-Jones CO, Smith JL, Marmer BS, et al. Thermal destruction of *Listeria monocytogenes* in liver sausage slurry. Food Microbiol. 1991;8(1):75–8.

221. Cole MB, Davies KW, Munro G, Holyoak CD, Kilsby DC. A vitalistic model to describe the thermal inactivation of *Listeria monocytogenes*. J Ind Microbiol. 1993;12(3):232–9.

222. Baranyi J, Roberts TA. A dynamic approach to predicting bacterial growth in food. Int J Food Microbiol. 1994;23(3–4):277–94.

223. Vaidyanathan S. Profiling microbial metabolomes: what do we stand to gain? Metabolomics. 2005;1(1):17–28.

224. Tang J. Microbial Metabolomics. Curr Genomics. 2011;12(6):391–403.

225. Oliver SG, Winson MK, Kell DB, Baganz F. Systematic functional analysis of the yeast

genome. Trends Biotechnol. 1998;16(9):373–8.

226. Mathew AK, Padmanaban VC. Metabolomics: The apogee of the omics trilogy. Int J Pharm Pharm Sci. 2013;5(SUPPL. 2):45–8.

227. Agin A, Heintz D, Ruhland E, Chao de la Barca JM, Zumsteg J, Moal V, et al. Metabolomics - an overview. From basic principles to potential biomarkers (part 1). Med Nucl [Internet]. Elsevier Masson SAS; 2016;40(1):4–10.

228. Fiehn O. Metabolomics - The link between genotypes and phenotypes. Plant Mol Biol. 2002;48(1–2):155–71.

229. Villas-Boas SG. Introduction to Microbial Metabolomics. In: Beale DJ, Palombo EA, Kouremenos KA, editors. Microbial Metabolomics: Applications in Clinical, Environmental, and Industrial Microbiology. 1st ed. Cham, Switzerland: Springer; 2016.p. 1–12.

230. Monciardini P, Iorio M, Maffioli S, Sosio M, Donadio S. Discovering new bioactive molecules from microbial sources. Microb Biotechnol. 2014;7(3):209–20.

231. Nai C, Magrini B, Offe J. Let microorganisms do the talking, let us talk more about microorganisms. Fungal Biol Biotechnol. 2016;3(1):5.

232. Krastanov A. Metabolomics—The State of Art. Biotechnol Biotechnol Equip. 2010;24(1):1537–43.

233. Schlegel V. Metabolomic Foot Printing and Microbial Pathogens. Metabolomics. 2015; 5(2):4172.

234. Cevallos-Cevallos JM. Microbial Metabolomics: towards Pathogen Detection and Biological Prospecting. J Postgenomics Drug Biomark Dev. 2013;3(1):4172.

235. Zhou J, Ma Q, Yi H, Wang L, Song H, Yuan YJ. Metabolome profiling reveals metabolic cooperation between *Bacillus megaterium* and *Ketogulonicigenium vulgare* during induced swarm motility. Appl Environ Microbiol. 2011;77(19):7023–30.

236. Filipiak W, Sponring A, Filipiak A, Baur M, Ager C, Wiesenhofer H, et al. Volatile Organic Compounds (VOCs) Released by Pathogenic Microorganisms in vitro: Potential Breath Biomarkers for Early-Stage Diagnosis of Disease. In: Amann A, Smith D, editors. Volatile Biomarkers: Non-Invasive Diagnosis in Physiology and Medicine. Oxford, UK: Elsevier B.V.; 2013.p. 463–512.

237. Audrain B, Farag MA., Ryu C-M, Ghigo J-M. Role of bacterial volatile compounds in

bacterial biology. FEMS Microbiol Rev. 2015:39(2):222-33.

238. Merlo M, Jankevics A, Takano E, Breitling R. Exploring the metabolic state of microorganisms using metabolomics. Bioanalysis. 2011; 3(21):2443–58.

239. Azizan KA, Baharum SN, Noor NM. Metabolic profiling of *Lactococcus lactis* under different culture conditions. Molecules. 2012;17(7):8022–36.

240. Tait E, Perry JD, Stanforth SP, Dean JR. Identification of Volatile Organic Compounds Produced by Bacteria Using HS-SPME-GC – MS. J Chromatogr Sci. 2014;52(4):363–73.

241. Putri SP, Nakayama Y, Matsuda F, Uchikata T, Kobayashi S, Matsubara A, et al. Current metabolomics: Practical applications. J Biosci Bioeng. 2013;115(6):579–89.

242. McMillan A, Rulisa S, Sumarah M, Macklaim JM, Renaud J, Bisanz JE, et al. A multi-platform metabolomics approach identifies highly specific biomarkers of bacterial diversity in the vagina of pregnant and non-pregnant women. Sci Rep. 2015;5:14174.

243. Chippendale TWE, Gilchrist FJ, Španěl P, Alcock A, Lenney W, Smith D. Quantification by SIFT-MS of volatile compounds emitted by in vitro cultures of *S. aureus*, *S. pneumoniae* and *H. influenzae* isolated from patients with respiratory diseases. Anal Methods. 2014;6(8):2460.

244. Nizio KD, Perrault KA, Troobnikoff AN, Ueland M, Shoma S, Iredell JR, et al. *In vitro* volatile organic compound profiling using GC×GC-TOFMS to differentiate bacteria associated with lung infections: a proof-of-concept study. J Breath Res. 2016;10(2):26008.

245. Raamsdonk LM, Teusink B, Broadhurst D, Zhang N, Hayes A, Walsh MC, et al. A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. Nat Biotechnol. 2001;19(1):45–50.

246. Liebeke M, Meyer H, Donat S, Ohlsen K, Lalk M. A metabolomic view of *Staphylococcus aureus* and its ser/thr kinase and phosphatase deletion mutants: involvement in cell wall biosynthesis. Chem Biol. 2010;17(8):820–30.

247. Abdelnur P V, Caldana C, Martins MCM. Metabolomics applied in bioenergy. Chem Biol Technol Agric. 2014;1(1):22.

248. Bos LDJ, Sterk PJ, Schultz MJ. Volatile Metabolites of Pathogens: A Systematic Review. PLoS Pathog. 2013;9(5):1–8.

249. Korpi A, Järnberg J, Pasanen A-L. Microbial volatile organic compounds. Critic Rev Toxicol. 2009;39(2):139-93.

250. Lemfack MC, Nickel J, Dunkel M, Preissner R, Piechulla B. MVOC: A database of microbial volatiles. Nucleic Acids Res. 2014;42(D1):1–5.

251. Schmidt R, Cordovez V, de Boer W, Raaijmakers J, Garbeva P. Volatile affairs in microbial interactions. ISME J. 2015;9:2329–2335.

252. Tait E, Perry JD, Stanforth SP, Dean JR. Use of volatile compounds as a diagnostic tool for the detection of pathogenic bacteria. TrAC - Trends Anal Chem. 2014;53:117–25.

253. Oldiges M, Noack S, Paczia N. Metabolomics in Biotechnology (Microbial Metabolomics). In: Lämmerhofer M, Weckwerth W, editors. Metabolomics in Practice: Successful Strategies to Generate and Analyze Metabolic Data. Weinheim, Germany: Wiley-VCH Verlag & Co. KGaA; 2013.p. 379–92.

254. Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB. Metabolomics by numbers: Acquiring and understanding global metabolite data. Trends Biotechnol. 2004;22(5):245–52.

255. Murray JA. Qualitative and quantitative approaches in comprehensive two-dimensional gas chromatography. J Chromatogr A. 2012;1261:58–68.

256. Gao P, Xu G. Mass-spectrometry-based microbial metabolomics: Recent developments and applications. Anal Bioanal Chem. 2015;407(3):669–80.

257. Costa CP, Gonçalves Silva D, Rudnitskaya A, Almeida A, Rocha SM. Shedding light on *Aspergillus niger* volatile exometabolome. Sci Rep. 2016;6:27441.

258. Mashego MR, Rumbold K, De Mey M, Vandamme E, Soetaert W, Heijnen JJ. Microbial metabolomics: past, present and future methodologies. Biotechnol Lett. 2007;29(1):1–16.

259. Sasidharan K, Soga T, Tomita M, Murray DB. A Yeast Metabolite Extraction Protocol Optimised for Time-Series Analyses. PLoS One. 2012;7(8):1–10.

260. Tian J, Sang P, Gao P, Fu R, Yang D, Zhang L, et al. Optimization of a GC-MS metabolic fingerprint method and its application in characterizing engineered bacterial metabolic shift. J Sep Sci. 2009;32(13):2281–8.

261. Hiller J, Franco-Lara E, Papaioannou V, Weuster-Botz D. Fast sampling and quenching procedures for microbial metabolic profiling. Biotechnol Lett. 2007;29(8):1161–7.

262. Risticevic S, DeEll JR, Pawliszyn J. Solid phase microextraction coupled with comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry

for high-resolution metabolite profiling in apples: implementation of structured separations for optimization of sample preparation . J Chromatogr A. 2012;1251:208–18.

263. Risticevic S, Carasek E, Pawliszyn J. Headspace solid-phase microextraction-gas chromatographic-time-of-flight mass spectrometric methodology for geographical origin verification of coffee. Anal Chim Acta. 2008;617(1–2):72–84.

264. Kataoka H, Lord HL, Pawliszyn J. Applications of solid-phase microextraction in food analysis. J Chromatogr A. 2000;880(1–2):35–62.

265. Koek MM, Muilwijk B, van Stee LLP, Hankemeier T. Higher mass loadability in comprehensive two-dimensional gas chromatography-mass spectrometry for improved analytical performance in metabolomics analysis. J Chromatogr A. 2008;1186(1–2):420–9.

266. Dallüge J, Beens J, Brinkman UAT. Comprehensive two-dimensional gas chromatography: A powerful and versatile analytical tool. J Chromatogr A. 2003;1000(1–2):69–108.

267. Bean HD, Dimandja J-MD, Hill JE. Bacterial volatile discovery using solid phase microextraction and comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2012;901:41–6.

268. Zhu J, Bean HD, Jimenez-Diaz J, Hill JE. Secondary electrospray ionization-mass spectrometry (SESI-MS) breathprinting of multiple bacterial lung pathogens, a mouse model study. J Appl Physiol. 2013;114(11):1544–9.

269. O'Hara M, Mayhew CA. A preliminary comparison of volatile organic compounds in the headspace of cultures of *Staphylococcus aureus* grown in nutrient, dextrose and brain heart bovine broths measured using a proton transfer reaction mass spectrometer. J Breath Res. 2009;3(2):027001.

270. Zechman JM, Aldinger S, Labows JN. Characterization of pathogenic bacteria by automated headspace concentration—gas chromatography. J Chromatogr B Biomed Sci Appl. 1986;377:49–57.

271. Yusuf N, Omar MI, Zakaria A, Jeffree AI, Thriumani R, Abdullah AA, et al. Evaluation of E-nose technology for detection of the causative bacteria in different culture media on diabetic foot infection. IECBES 2014, Conf Proc-2014 IEEE Conf Biomed Eng Sci "Miri, Where Eng Med Biol Humanit Meet.2015:67–70.

272. Goodacre R, Broadhurst D, Smilde AK, Kristal BS, Baker JD, Beger R, et al. Proposed minimum reporting standards for data analysis in metabolomics. Metabolomics.

2007;3(3):231–41.

273. Fiehn O, Robertson D, Griffin J, vab der Werf M, Nikolau B, Morrison N, et al. The metabolomics standards initiative (MSI). Metabolomics. 2007; 3(3):175–8.

274. Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA., et al. Proposed minimum reporting standards for chemical analysis. Metabolomics. 2007;3(3):211–21.

275. Sugimoto M, Kawakami M, Robert M, Soga T, Tomita M. Bioinformatics Tools for Mass Spectroscopy-Based Metabolomic Data Processing and Analysis. Curr Bioinform. 2012;7(1):96–108.

276. Worley B, Powers R. Multivariate Analysis in Metabolomics. Curr Metabolomics. 2013; 1(1):92–107.

277. Chagoyen M, Pazos F. Tools for the functional interpretation of metabolomic experiments. Brief Bioinform. 2013; 14(6):737–44.

278. Filipiak W, Sponring A, Baur MM, Ager C, Filipiak A, Wiesenhofer H, et al. Characterization of volatile metabolites taken up by or released from *Streptococcus pneumoniae* and *Haemophilus influenzae* by using GC-MS. Microbiology. 2012; 158(Pt 12):3044–53.

279. Filipiak W, Beer R, Sponring A, Filipiak A, Ager C, Schiefecker A, et al. Breath analysis for *in vivo* detection of pathogens related to ventilator-associated pneumonia in intensive care patients: a prospective pilot study. J Breath Res. 2015;9(1):16004.

280. Scotter JM, Allardyce RA, Langford VS, Hill A, Murdoch DR. The rapid evaluation of bacterial growth in blood cultures by selected ion flow tube-mass spectrometry (SIFT-MS) and comparison with the BacT/ALERT automated blood culture system. J Microbiol Methods. 2006;65(3):628–31.

281. Allardyce RA, Hill AL, Murdoch DR. The rapid evaluation of bacterial growth and antibiotic susceptibility in blood cultures by selected ion flow tube mass spectrometry. Diagn Microbiol Infect Dis. 2006;55(4):255–61.

282. Jia B, Sohnlein B, Mortelmans K, Coggiola M, Oser H. Distinguishing Methicillin-Resistant and Sensitive *Staphylococcus aureus* Using Volatile Headspace Metabolites. IEEE Sens J. 2010;10(1):71–5.

283. Dryahina K, Sovova K, Nemec A, Spanel P. Differentiation of pulmonary bacterial pathogens in cystic fibrosis by volatile metabolites emitted by their in vitro cultures: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia* and

the *Burkholderia cepacia* complex. J Breath Res. 2016;10(3):37102.

284. Birkenstock T, Liebeke M, Winstel V, Krismer B, Gekeler C, Niemiec MJ, et al. Exometabolome analysis identifies pyruvate dehydrogenase as a target for the antibiotic triphenylbismuthdichloride in multiresistant bacterial pathogens. J Biol Chem. 2012;287(4):2887–95.

285. Wiesner K, Jaremek M, Pohle R, Sicard O Von, Stuetz E. Monitoring of Bacterial Growth and Rapid Evaluation of Antibiotic Susceptibility by Headspace Gas Analysis. Procedia Eng. 2014;87:332–5.

286. Zhu J, Bean HD, Kuo Y-M, Hill JE. Fast detection of volatile organic compounds from bacterial cultures by secondary electrospray ionization-mass spectrometry. J Clin Microbiol. 2010;48(12):4426–31.

287. Jünger M, Vautz W, Kuhns M, Hofmann L, Ulbricht S, Baumbach JI, et al. Ion mobility spectrometry for microbial volatile organic compounds: a new identification tool for human pathogenic bacteria. Appl Microbiol Biotechnol. 2012;93(6):2603–14.

288. Dolch ME, Hornuss C, Klocke C, Praun S, Villinger J, Denzer W, et al. Volatile organic compound analysis by ion molecule reaction mass spectrometry for Gram-positive bacteria differentiation. Eur J Clin Microbiol Infect Dis. 2012;31(11):3007–13.

289. Zhu J, Hill JE. Detection of *Escherichia coli* via VOC profiling using secondary electrospray ionization-mass spectrometry (SESI-MS). Food Microbiol. 2013;34(2):412–7.

290. Elgaali H, Hamilton-Kemp TR, Newman MC, Collins RW, Yu K, Archbold DD. Comparison of long-chain alcohols and other volatile compounds emitted from food-borne and related Gram positive and Gram negative bacteria. J Basic Microbiol. 2002;42(6):373–80.

291. Rode TM, Møretrø T, Langsrud S, Langsrud O, Vogt G, Holck A. Responses of *Staphylococcus aureus* exposed to HCl and organic acid stress. Can J Microbiol. 2010;56:777–92.

292. Thorn RMS, Reynolds DM, Greenman J. Multivariate analysis of bacterial volatile compound profiles for discrimination between selected species and strains in vitro. J Microbiol Methods. 2011;84(2):258–64.

293. Boots AW, Smolinska A, van Berkel JJBN, Stobberingh EE, Boumans MLL, Moonen M, et al. Identification of microorganisms based on gas chromatography-mass spectrometric analysis of volatile organic compounds in headspace gases. J Biomed Biotechnol. 2014;8(2):1752.

294. Sun J, Zhang S, Chen J, Han B. Metabolic profiling of *Staphylococcus aureus* cultivated under aerobic and anaerobic conditions with $^{(1)}$H-NMR-based nontargeted analysis. Can J Microbiol. 2012;58(6):709–18.

295. Mader D, Liebeke M, Winstel V, Methling K, Leibig M, Götz F, et al. Role of N-terminal protein formylation in central metabolic processes in *Staphylococcus aureus*. BMC Microbiol. 2013;13(7):1–9.

296. Lechner M, Fille M, Hausdorfer J, Dierich MP, Rieder J. Diagnosis of bacteria *in vitro* by mass spectrometric fingerprinting: a pilot study. Curr Microbiol. 2005;51(4):267–9.

297. Liebeke M, Dörries K, Zühlke D, Bernhardt J, Fuchs S, Pané-Farré J, et al. A metabolomics and proteomics study of the adaptation of *Staphylococcus aureus* to glucose starvation. Mol Biosyst. 2011;7:1241–53.

298. Dutta R, Hines EL, Gardner JW, Boilot P. Bacteria classification using Cyranose 320 electronic nose. Biomed Eng Online. 2002;1(4).

299. Yusuf N, Omar MI, Zakaria A, Abdullah AA, Kamarudin LM, Shakaff AYM, et al. Diagnosis of bacteria for diabetic foot infection using electronic nose technology. 2013 IEEE Conf Wirel Sensor, ICWISE 2013. Sarawak, Malaysia. 2013;114–8.

300. Yusuf N, Abdullah AA, Omar MI, Zakaria A, Kamarudin LM, Shakaff AYM, et al. Comparison of various pattern recognition techniques based on e-nose for identifying bacterial species in diabetic wound infections. WIT Trans Inf Commun Technol. 2014;53:43–59.

301. Abdullah AA, Yusuf N, Omar MI, Zakaria A, Kamarudin LM, Shakaff AY, et al. Multivariate prediction model for early detection and classification of bacterial species in diabetic foot ulcers. Int Conf Adv Intell Syst Bioinformatics, Chem-Informatics, Bus Intell Soc Media Cybern (IntelSys). 2014; Kuala Lumpur, Malaysia;27–34.

302. Allardyce RA, Langford VS, Hill AL, Murdoch DR. Detection of volatile metabolites produced by bacterial growth in blood culture media by selected ion flow tube mass spectrometry (SIFT-MS). J Microbiol Methods.2006;65(2):361–5.

303. Filipiak W, Sponring A, Baur MM, Filipiak A, Ager C, Wiesenhofer H, et al. Molecular analysis of volatile metabolites released specifically by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. BMC Microbiol. 2012;12(1):113.

304. Preti G, Thaler E, Hanson CW, Troy M, Eades J, Gelperin A. Volatile compounds characteristic of sinus-related bacteria and infected sinus mucus: analysis by solid-phase

microextraction and gas chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877(22):2011–8.

305. Syhre M, Scotter JM, Chambers ST. Investigation into the production of 2-Pentylfuran by *Aspergillus fumigatus* and other respiratory pathogens in vitro and human breath samples. Med Mycol. 2008;46(May):209–15.

306. Zhu J, Jimenez-Diaz J, Bean HD, Daphtary NA, Aliyeva MI, Lundblad LKA, et al. Robust detection of *P. aeruginosa* and *S. aureus* acute lung infections by secondary electrospray ionization-mass spectrometry (SESI-MS) breathprinting: from initial infection to clearance. J Breath Res. 2013;7(3):37106.

307. Kramer R, Sauer-Heilborn A, Welte T, Guzman CA, Höfle MG, Abraham W-R. A rapid method for breath analysis in cystic fibrosis patients. Eur J Clin Microbiol Infect Dis. 2015;34(4):745–51.

308. Neerincx AH, Geurts BP, van Loon J, Tiemes V, Jansen JJ, Harren FJM, et al. Detection of *Staphylococcus aureus* in cystic fibrosis patients using breath VOC profiles. J Breath Res. 2016;10(4):46014.

309. Julák J, Stránská E, Procházková-Francisci E, Rosová V. Blood cultures evaluation by gas chromatography of volatile fatty acids. Med Sci Monit Int Med J Exp Clin Res. 2000;6(3):605–10.

310. Julák J, Procházková-Francisci E, Stránská E, Rosová V. Evaluation of exudates by solid phase microextraction-gas chromatography. J Microbiol Methods. 2003;52(1):115–22.

311. Hettinga KA, van Valenberg HJF, Lam TJGM, van Hooijdonk ACM. Detection of mastitis pathogens by analysis of volatile bacterial metabolites. J Dairy Sci. Elsevier; 2008;91(10):3834–9.

312. Storer MK, Hibbard-Melles K, Davis B, Scotter J. Detection of volatile compounds produced by microbial growth in urine by selected ion flow tube mass spectrometry (SIFT-MS). J Microbiol Methods. 2011;87(1):111–3.

313. Monteiro MS, Carvalho M, Bastos ML, Guedes de Pinho P. Metabolomics analysis for biomarker discovery: advances and challenges. Curr Med Chem. 2013;20(2):257–71.

314. Editorial. Biomarkers on a roll. Nat Biotechnol. 2010;28(5):431–431.

315. Alves Z, Melo A., Figueiredo A. R, Coimbra MA., Gomes AC, Rocha SM. Exploring the *Saccharomyces cerevisiae* volatile metabolome: indigenous versus commercial strains.

PLoS One. 2015;10(11):e0143641.

316. Wilson AD, Baietto M. Advances in electronic-nose technologies developed for biomedical applications. Sensors. 2011;11(1):1105–76.

317. Peng G, Hakim M, Broza YY, Billan S, Abdah-Bortnyak R, Kuten A, et al. Detection of lung, breast, colorectal, and prostate cancers from exhaled breath using a single array of nanosensors. Br J Cancer. 2010;103(4):542–51.

318. Schulz S, Dickschat JS. Bacterial volatiles: the smell of small organisms. Nat Prod Rep. 2007;24(4):814–42.

319. Landaud S, Helinck S, Bonnarme P. Formation of volatile sulfur compounds and metabolism of methionine and other sulfur compounds in fermented food. Appl Microbiol Biotechnol. 2008;77(6):1191–205.

320. Vallet A, Lucas P, Lonvaud-Funel A, De Revel G. Pathways that produce volatile sulphur compounds from methionine in *Oenococcus oeni*. J Appl Microbiol. 2008;104(6):1833–40.

321. Kanehisa M, Goto S. KEGG: Kyoto Encyclopaedia of Genes and Genomes. Nucl Acids Res. 2000; 28(1):27–30.

322. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 2016;44(D1):D457–62.

323. Holt JG, Krieg NR, Sneath PH, Safety JT, Williams ST. Bergey's Manual of Determinative Bacteriology. 9th Ed. Williams K, Wilkins O, editors. Baltimore, U.S.A: William & Wilkins; 1993. 532 p.

324. Cronan JE. A new pathway of exogenous fatty acid incorporation proceeds by a classical phosphoryl transfer reaction. Mol Microbiol. 2014;92(2):217–21.

325. Bien J, Sokolova O, Bozko P. Characterization of Virulence Factors of *Staphylococcus aureus*: Novel Function of Known Virulence Factors That Are Implicated in Activation of Airway Epithelial Proinflammatory Response. J Pathog. 2011 Jan;2011:601905.

326. Cebrián G, Michiels CW, Mañas P, Condón S. Biological approach to modeling of *Staphylococcus aureus* high-hydrostatic-pressure inactivation kinetics. Appl Environ Microbiol. 2010; 76(21):6982–90.

327. Clarisse T, Michèle S, Olivier T, Valérie E, Vincent LM, Jacques-Antoine H, et al. Detection and quantification of staphylococcal enterotoxin A in foods with specific and sensitive

polyclonal antibodies. Food Control. 2013 Jul;32(1):255–61.

328. EFSA, ECDC. Trends and Sources of Zoonoses and Zoonotic Agents and Food-borne Outbreaks in the European Union in 2008. EFSA J. 2010;8(1):1496.

329. Paulin S, Horn B, Hudson JA. Factors influencing staphylococcal enterotoxin production in Dairy Products. 2012.1-79 p.

330. DeGrasse JA. A single-stranded DNA aptamer that selectively binds to *Staphylococcus aureus* enterotoxin B. PLoS One. 2012;7(3):e33410.

331. Medina M. A Biosensor Method For Detection of Staphylococcal Enterotoxin A in Raw Whole Egg. J Rapid Meth Auto Microbio. 2006;14(215):119–32.

332. Rasooly, R., Friedman, M. 2012. Food compounds inhibit *Staphylococcus aureus* bacteria and the toxicity of Staphylococcus Enterotoxin A (SEA) associated with atopic dermatitis. In: Esparza-Gordillo, J., editor. Atopic Dermatitis-Disease Etiology and Clinical Management. Croatia: Intech Europe. p. 387-404.

333. Barretti P, Montelli AC, Batalha JEN, Caramori JCT, Cunha MDLRS. The role of virulence factors in the outcome of staphylococcal peritonitis in CAPD patients. BMC Infect Dis. 2009;9:212.

334. Liu GY, Nizet V. Color me bad: microbial pigments as virulence factors. Trends Microbiol. 2009;17(9):406–13.

335. Declercq P, Petré D, Gordts B, Voss A. Complicated community-acquired soft tissue infection by MRSA from porcine origin. Infection. 2008;36(6):590–2.

336. O'Brien AM, Hanson BM, Farina SA, Wu JY, Simmering JE, Wardyn SE, et al. MRSA in conventional and alternative retail pork products. PLoS One. 2012;7(1):e30092.

337. Neetoo H, Nekoozadeh S, Jiang Z, Chen H. Application of high hydrostatic pressure to decontaminate green onions from Salmonella and *Escherichia coli* O157:H7. Food Microbiol. 2011;28(7):1275–83.

338. Pulido RP, Toledo del árbol J, Burgos MJG, Gálvez A. Bactericidal effects of high hydrostatic pressure treatment singly or in combination with natural antimicrobials on *Staphylococcus aureus* in rice pudding. Food Control. 2012;28(1):19–24.

339. Karatzas KAG, Bennik MHJ. Characterization of a *Listeria monocytogenes* Scott A Isolate with High Tolerance towards High Hydrostatic Pressure Appl Environ Microbiol. 2002;68(7):3183-9.

340. Perrett S, Zhou JM. Expanding the pressure technique: Insights into protein folding from combined use of pressure and chemical denaturants. Biochim Biophys Acta - Protein Struct Mol Enzymol. 2002;1595:210–23.

341. Margosch D, Moravek M. Effect of high pressure and heat on bacterial toxins. Food Technol. Biotechnol. 2005;43(3):211–217.

342. Lachica R V, Genigeorgis C, Hoeprich PD. Metachromatic agar-diffusion methods for detecting staphylococcal nuclease activity. Appl Microbiol. 1971;21(4):585–7.

343. Morikawa K, Maruyama A, Inose Y, Higashide M, Hayashi H, Ohta T. Overexpression of sigma factor, sigma(B), urges *Staphylococcus aureus* to thicken the cell wall and to resist beta-lactams. Biochem Biophys Res Commun. 2001;288(2):385–9.

344. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement. Clinical and Laboratory Standards Institute. 2011.

345. O'Reilly CE, O'Connor PM, Kelly AL, Beresford TP, Murphy PM. Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. Appl Environ Microbiol. 2000; 66(11):4890–6.

346. Alpas H, Kalchayanand N, Bozoglu F, Sikes A, Dunne CP, Ray B. Variation in resistance to hydrostatic pressure among strains of food-borne pathogens. Appl Environ Microbiol. 1999; 65(9):4248–51.

347. Cebrián G, Sagarzazu N, Pagán R, Condón S, Mañas P. Heat and pulsed electric field resistance of pigmented and non-pigmented enterotoxigenic strains of *Staphylococcus aureu*s in exponential and stationary phase of growth. Int J Food Microbiol. 2007; 118(3):304–11.

348. Liu GY, Essex A, Buchanan JT, Datta V, Hoffman HM, Bastian JF, et al. *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. J Exp Med. 2005 Jul 18; 202(2):209–15.

349. Wang L, Pan J, Xie H, Yang Y, Lin C. Inactivation of *Staphylococcus aureus* and *Escherichia coli* by the synergistic action of high hydrostatic pressure and dissolved CO_2. Int J Food Microbiol. 2010; 144(1):118–25.

350. Marjanović D, Jovanović S, Baars A, Barać M. Effects of high hidrostatic pressure on the viscosity of β-lactoglobulin solution. Mljekarstvo. 2011;61(2):135–44.

351. Winter R. Synchrotron X-ray and neutron small-angle scattering of lyotropic lipid mesophases, model biomembranes and proteins in solution at high pressure. Biochim Biophys Acta - Protein Struct Mol Enzymol. 2002; 1595:160–84.

352. Sharma A, Scott JH, Cody GD, Fogel ML, Hazen RM, Hemley RJ, et al. Microbial activity at gigapascal pressures. Science. 2002; 295(5559):1514–6.

353. Syed Q-A, Reineke K, Saldo J, Buffa M, Guamis B, Knorr D. Effect of compression and decompression rates during high hydrostatic pressure processing on inactivation kinetics of bacterial spores at different temperatures. Food Control. 2012; 25(1):361–7.

354. Fonberg-Broczek M, Windyga B, Szczawiński J, Szczawińska M, Pietrzak D, Prestamo G. High pressure processing for food safety. Acta Biochim Pol. 2005; 52(3):721–4.

355. Karatzas K, Bennik M. Characterization of a *Listeria monocytogenes* Scott A isolate with high tolerance towards high hydrostatic pressure. Appl Environ Microbiol. 2002; 68(7):3183–3189.

356. Rodriguez E, Arques J. Combined effect of high-pressure treatments and bacteriocin-producing lactic acid bacteria on inactivation of *Escherichia coli* O157: H7 in raw-milk cheese. Appl Environ Microbiol. 2005; 71(7):3399–404.

357. Garriga M, Grèbol N, Aymerich MT, Monfort JM, Hugas M. Microbial inactivation after high-pressure processing at 600 MPa in commercial meat products over its shelf life. Innov Food Sci Emerg Technol. 2004; 5(4):451–7.

358. Van Opstal I, Vanmuysen SCM, Wuytack EY, Masschalck B, Michiels CW. Inactivation of *Escherichia coli* by high hydrostatic pressure at different temperatures in buffer and carrot juice. Int J Food Microbiol. 2005; 98(2):179–91.

359. Hauben K, Bartlett D. *Escherichia coli* mutants resistant to inactivation by high hydrostatic pressure. Appl Environ Microbiol. 1997; 63(3):945–950.

360. Ritz M, Tholozan JL, Federighi M, Pilet MF. Physiological damages of *Listeria monocytogenes* treated by high hydrostatic pressure. Int J Food Microbiol. 2002; 79(1–2):47–53.

361. Koseki S, Yamamoto K. Recovery of *Escherichia coli* ATCC 25922 in phosphate buffered saline after treatment with high hydrostatic pressure. Int J Food Microbiol. 2006; 110(1):108–11.

362. Bore E, Langsrud S, Langsrud Ø, Rode TM, Holck A. Acid-shock responses in

Staphylococcus aureus investigated by global gene expression analysis. Microbiology. 2007; 153:2289–303.

363. Dengremont E, Membré JM. Statistical approach for comparison of the growth rates of five strains of *Staphylococcus aureus*. Appl Environ Microbiol. 1995; 61(12):4389–95.

364. Medveďová A, Valík Ľ. *Staphylococcus aureus*: Characterisation and Quantitative Growth Description in Milk and Artisanal Raw Milk Cheese Production. Structure and Function of Food Engineering. Prof. Ayman Amer Eissa, editor, InTech. 2012; Chapter 4:75.

365. Heinz V, Buckow R. Food preservation by high pressure. J für Verbraucherschutz und Leb [Internet]. 2009; 5(1):73–81.

366. Muñoz-Cuevas M, Guevara L, Aznar A, Martínez A, Periago PM, Fernández PS. Characterisation of the resistance and the growth variability of *Listeria monocytogenes* after high hydrostatic pressure treatments. Food Control. 2013; 29(2):409–15.

367. Seo KS, Bohach GA. *Staphylococcus aureus*. In: Doyle MP, Buchanan RL, editors. Food Microbiology: Fundamentals and Frontiers. 4th ed. Washington, DC: ASM Press; 2013. p. 547–73.

368. Chapleau N, Ritz M, Delépine S, Jugiau F, Federighi M, de Lamballerie M. Influence of kinetic parameters of high pressure processing on bacterial inactivation in a buffer system. Int J Food Microbiol. 2006; 106(3):324–30.

369. Jofré A, Garriga M, Aymerich T. Inhibition of *Salmonella* sp. *Listeria monocytogenes* and *Staphylococcus aureus* in cooked ham by combining antimicrobials, high hydrostatic pressure and refrigeration. Meat Sci. 2008; 78(1–2):53–9.

370. Berlin DL, Herson DS, Hicks DT, Hoover G. Response of Pathogenic Vibrio Species to High Hydrostatic Pressure Appl Environ Microbiol. 1999; 65(6):2776-80.

371. Conlon BP, Nakayasu ES, Fleck LE, LaFleur MD, Isabella VM, Coleman K, et al. Activated ClpP kills persisters and eradicates a chronic biofilm infection. Nature. 2013; 503(7476):365-70

372. Pasquaroli S, Zandri G, Vignaroli C, Vuotto C, Donelli G, Biavasco F. Antibiotic pressure can induce the viable but non-culturable state in *Staphylococcus aureus* growing in biofilms. J Antimicrob Chemother. 2013; 68(8):1812-7

373. Wen J, Anantheswaran RC, Knabel SJ. Changes in barotolerance, thermotolerance, and cellular morphology throughout the life cycle of *Listeria monocytogenes*. Appl Environ

Microbiol. 2009; 75(6):1581–8.

374. Zandri G, Pasquaroli S, Vignaroli C, Talevi S, Manso E, Donelli G, et al. Detection of viable but non-culturable staphylococci in biofilms from central venous catheters negative on standard microbiological assays. Clin Microbiol Infect. 2012; 18(7):E259-61.

375. Bozoglu F, Alpas H, Kaletunç G. Injury recovery of foodborne pathogens in high hydrostatic pressure treated milk during storage. FEMS Immunol Med Microbiol. 2004; 40(3):243–7.

376. Chilton P, Isaacs NS, Manias P, Mackey BM. Biosynthetic requirements for the repair of membrane damage in pressure-treated *Escherichia coli*. Int J Food Microbiol. 2001; 71(1):101–4.

377. Ellenberg L, Hoover DG. Injury and survival of *Aeromonas hydrophila* 7965 and *Yersinia enterocolitica* 9610 from high hydrostatic pressure. J Food Saf. 1999; 19: 263–276.

378. Koseki S, Mizuno Y, Yamamoto K. Use of mild-heat treatment following high-pressure processing to prevent recovery of pressure-injured *Listeria monocytogenes* in milk. Food Microbiol. 2008; 25(2):288–93.

379. Lee J, Alpas H, Bozoglu F. Evaluation of Structural Changes Induced by High Hydrostatic Pressure in *Leuconostoc mesenteroides*. 2004; 70(2):1116–22.

380. Alpas H, Bozoglu F. Inactivation of *Staphylococcus Aureus* and *Listeria Monocytogenes* in Milk and Cream of Chicken Soup by High Hydrostatic Pressure and Bacteriocins. High Press Res. 2002; 22(November 2011):681–4.

381. Gervilla R, Ferragut V, Guamis B. High pressure inactivation of microorganisms inoculated into ovine milk of different fat contents. J Dairy Sci. 2000; 83(4):674-82.

382. Nema V, Agrawal R, Kamboj DV, Goel AK, Singh L. Isolation and characterization of heat resistant enterotoxigenic *Staphylococcus aureus* from a food poisoning outbreak in Indian subcontinent. Int J Food Microbiol. 2007; 117(1):29–35.

383. Patterson MF, Quinn M, Simpson R, Gilmour A. Sensitivity of Vegetative Pathogens to High Hydrostatic Pressure Treatment in Phosphate-Buffered Saline and Foods. J Food Prot. 1995; 58(5): 524-529.

384. Styles MF, Hoover DG, Farkas DF. Response of *Listeria monocytogenes* and *Vibrio parahaemolyticus* to high hydrostatic pressure. J Food Sci. 1991; 56(5):1404–7.

385. Bozoglu F, Alpas H, Kaletunç G. Injury recovery of foodborne pathogens in high

hydrostatic pressure treated milk during storage. FEMS Immunol Med Microbiol. 2004; 40(3):243–7.

386. WHO. The World Health Organization Estimates of the Global Burden of Foodborne Diseases: FERG Project Report. 2015.

387. Grumann D, Nübel U, Bröker BM. *Staphylococcus aureus* toxins -their functions and genetics. Infect Genet Evol. 2014; 21:583–92.

388. Dörries K, Lalk M. Metabolic footprint analysis uncovers strain specific overflow metabolism and D-isoleucine production of *Staphylococcus aureus* COL and HG001. PLoS One. 2013; 8(12):e81500.

389. Gardner JY, Brillhart DE, Benjamin MM, Dixon LG, Mitchell LM, Dimandja JMD. The use of GCxGC/ToF-MS with multivariate analysis for the characterization of foodborne pathogen bacteria profiles. J Sep Sci. 2011; 34(2):176–85.

390. Loots DT. An altered *Mycobacterium tuberculosis* metabolome induced by katG mutations resulting in isoniazid resistance. Antimicrob Agents Chemother. 2014; 58(4):2144–9.

391. ISO/TC 34/SC 9. ISO 6888-3:2003 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) - Part 3: Detection and MPN technique for low numbers. 2003.

392. Jousse F, Jongen T, Agterof W, Russell S, Braat P. Simplified Kinetic Scheme of Flavor Formation by the Maillard Reaction. J Food Sci. 2002; 67(7):2534–42.

393. van Boekel MAJS. Formation of flavour compounds in the Maillard reaction. Biotechnol Adv. 2006; 24(2):230–3.

394. Hazelwood LA, Daran J-M, van Maris AJA, Pronk JT, Dickinson JR. The Ehrlich Pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl Environ Microbiol. 2008; 74(8):2259–66.

395. Stavropoulou DA, Borremans W, De Vuyst L, De Smet S, Leroy F. Amino acid conversions by coagulase-negative staphylococci in a rich medium: Assessment of inter- and intraspecies heterogeneity. Int J Food Microbiol. 2015; 212:34–40.

396. Calenic B, Miricescu D, Greabu M, Kuznetsov A V., Troppmair J, Ruzsanyi V, et al. Oxidative stress and volatile organic compounds: Interplay in pulmonary, cardio-

vascular, digestive tract systems and cancer. Open Chem. 2015; 13(1):1020–30.

397. Marshall JH, Wilmoth GJ. Pigments of *Staphylococcus aureus*, a series of triterpenoid carotenoids. J Bacteriol. 1981; 147(3):900–13.

398. Hammond RAYK, White DC. Carotenoid Formation by *Staphylococcus aureus*. J Bacteriol. 1970; 103(1):191–8.

399. Barbieri E, Gioacchini AM, Zambonelli A, Bertini L, Stocchi V. Determination of microbial volatile organic compounds from *Staphylococcus pasteuri* against *Tuber borchii* using solid-phase microextraction and gas chromatography/ion trap mass spectrometry. Rapid Commun Mass Spectrom. 2005;19(22):3411-5.

400. Ganjewala D, Kumar S, Devi S A, Ambika K. Advances in cyanogenic glycosides biosynthesis and analyses in plants: A review. Acta Biol Szeged. 2010;54(1):1–14.

401. Møller JKS, Hinrichsen LL, Andersen HJ. Formation of amino acid (L-leucine, L-phenylalanine) derived volatile flavour compounds by *Moraxella phenylpyruvica* and *Staphylococcus xylosus* in cured meat model systems. Int J Food Microbiol. 1998;42(1–2):101–17.

402. Fernández MM, Cho S, De Marzi MC, Kerzic MC, Robinson H, Mariuzza R a., et al. Crystal structure of Staphylococcal Enterotoxin G (SEG) in complex with a mouse T-cell receptor β chain. J Biol Chem. 2011;286(2):1189–95.

403. Fernández MM, Guan R, Swaminathan CP, Malchiodi EL, Mariuzza RA. Crystal Structure of Staphylococcal Enterotoxin I (SEI) in Complex with a Human Major Histocompatibility Complex Class II Molecule. J Biol Chem. 2006;281(35):25356–25364.

404. Landaud S, Helinck S, Bonnarme P. Formation of volatile sulfur compounds and metabolism of methionine and other sulfur compounds in fermented food. Appl Microbiol Biotechnol. 2008;77(6):1191–205.

405. Pang YY, Schwartz J, Bloomberg S, Boyd JM, Horswill AR, Nauseef WM. Methionine sulfoxide reductases protect against oxidative stress in staphylococcus aureus encountering exogenous oxidants and human neutrophils. J Innate Immun. 2014;6(3):353–64.

406. Ortega E, Abriouel H, Lucas R, Gálvez A. Multiple roles of *Staphylococcus aureus* enterotoxins: pathogenicity, superantigenic activity, and correlation to antibiotic resistance. Toxins. 2010;2(8):2117–31.

407. Boots AW, Smolinska A, Van Berkel BN, Fijten RRR, Stobberingh EE, Boumans MLL, et al. Identification of microorganisms based on headspace analysis of volatile organic compounds by gas chromatography–mass spectrometry. J Breath Res J Breath Res J Breath Res [Internet]. 2014;8. Available from: http://iopscience.iop.org/1752-7163/8/2/027106

408. Dörries K, Schlueter R, Lalk M. Impact of antibiotics with various target sites on the metabolome of *Staphylococcus aureus*. Antimicrob Agents Chemother. 2014;58(12):7151–63.

409. Chingin K, Liang J, Hang Y, Hu L, Chen H. Rapid recognition of bacteremia in humans using atmospheric pressure chemical ionization mass spectrometry of volatiles emitted by blood. RSC Adv. 2015;5:13952–7.

410. Saranya R, Aarthi R, Sankaran K. Simple and specific colorimetric detection of Staphylococcus using its volatile 2-[3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl] propanoic acid in the liquid phase and head space of cultures. Appl Microbiol Biotechnol. 2015;99(10):4423–33.

411. Engel E, Ratel J. Correction of the data generated by mass spectrometry analyses of biological tissues: application to food authentication. J Chromatogr A. 2007;1154(1–2):331–41.

412. Santos MC, Nunes C, Rocha MAM, Rodrigues A, Rocha SM, Saraiva JA, et al. High pressure treatments accelerate changes in volatile composition of sulphur dioxide-free wine during bottle storage. Food Chem. 2015;188:406–14.

413. Colahan-Sederstrom PM, Peterson DG. Inhibition of key aroma compound generated during ultrahigh-temperature processing of bovine milk via epicatechin addition. J Agric Food Chem. 2005;53(2):398–402.

414. Forero MD, Quijano CE, Pino JA. Volatile compounds of chile pepper (*Capsicum annuum* L. var. *glabriusculum*) at two ripening stages. Flavour Fragr J. 2009;24(1):25–30.

415. Berdagué JL, Tournayre P, Cambou S. Novel multi-gas chromatography-olfactometry device and software for the identification of odour-active compounds. J Chromatogr A. 2007;1146(1):85–92.

416. Rodríguez-Burruezo A, Kollmannsberger H, Prohens J, Nitz S, Nuez F. Analysis of the volatile aroma constituents of parental and hybrid clones of pepino (*Solanum muricatum*). J Agric Food Chem. 2004;52(18):5663–9.

417. Pino JA, Mesa J, Muñoz Y, Martí MP, Marbot R. Comment on volatile components from mango (*Mangifera indica* L.) cultivars. J Agric Food Chem. 2005;53(21):2213–2223.

418. Salvador ÂC, Baptista I, Barros AS, Gomes NCM, Cunha Â, Almeida A, et al. Can Volatile Organic Metabolites Be Used to Simultaneously Assess Microbial and Mite Contamination Level in Cereal Grains and Coffee Beans? PLoS One. 2013; 8(4):e59338.

419. Silva I, Rocha SM, Coimbra MA, Marriott PJ. Headspace solid-phase microextraction combined with comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry for the determination of volatile compounds from marine salt. J Chromatogr A. 2010;1217(34):5511–21.

420. Silva I, Rocha SM, Coimbra MA. Headspace solid phase microextraction and gas chromatography-quadrupole mass spectrometry methodology for analysis of volatile compounds of marine salt as potential origin biomarkers. Anal Chim Acta. 2009;635(2):167–74.

421. Pino JA, Marbot R, Rosado A, Vázquez C. Volatile constituents of Malay rose apple [*Syzygium malaccense* (L.) Merr. & Perry]. Flavour Fragr J. 2004;19:32–5.

422. Splivallo R, Bossi S, Maffei M, Bonfante P. Discrimination of truffle fruiting body versus mycelial aromas by stir bar sorptive extraction. Phytochemistry. 2007;68(20):2584–98.

423. Aaslyng MD, Elmore JS, Mottram DS. Comparison of the aroma characteristics of acid-hydrolyzed and enzyme-hydrolyzed vegetable proteins produced from soy. J Agric Food Chem. 1998;46(12):5225–31.

424. Zhao C, Li X, Liang Y, Fang H, Huang L-F, Guo F. Comparative analysis of chemical components of essential oils from different samples of Rhododendron with the help of chemometrics methods. Chemom Intell Lab Syst. 2006;82(1–2):218–28.

425. Rembold H, Wallner P, Nitz S, Kollmannsberger H, Drawert F. Volatile components of chickpea (*Cicer arietinum* L.) seed. J Agric Food Chem. 1989;37(3):659–62.

426. Martins C, Brandão T, Almeida A, Rocha SM. Insights on beer volatile profile: Optimization of solid-phase microextraction procedure taking advantage of the comprehensive two-dimensional gas chromatography structured separation. J Sep Sci. 2015;38(12):2140–8.

427. Loureiro CC, Duarte IF, Gomes J, Carrola J, Barros AS, Gil AM, et al. Urinary metabolomic changes as a predictive biomarker of asthma exacerbation. J Allergy Clin Immunol. 2014;133(1).

428. Miyazaki T, Plotto A, Goodner K, Gmitter FG. Distribution of aroma volatile compounds in tangerine hybrids and proposed inheritance. J Sci Food Agric [Internet]. 2011;91(3):449–60.

429. Rocha SM, Caldeira M, Carrola J, Santos M, Cruz N, Duarte IF. Exploring the human urine metabolomic potentialities by comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry. J Chromatogr A. 2012;1252:155–63.

430. Caldeira M, Perestrelo R, Barros AS, Bilelo MJ, Morête A, Câmara JS, et al. Allergic asthma exhaled breath metabolome: A challenge for comprehensive two-dimensional gas chromatography. J Chromatogr A. 2012;1254(7):87–97.

431. Gkinis G, Tzakou O, Iliopoulou D, Roussis V. Chemical Composition and Biological Activity of *Nepeta parnassica* Oils and Isolated Nepetalactones. Zeitschrift fur Naturforsch - Sect C J Biosci. 2003;58:681–6.

432. Solina M, Baumgartner P, Johnson RL, Whitfield FB. Volatile aroma components of soy protein isolate and acid-hydrolysed vegetable protein. Food Chem. 2005;90:861–73.

433. Zhao Y, Li J, Xu Y, Duan H, Fan W, Zhao G. Extraction, preparation and identification of volatile compounds in Changyu XO brandy. Chinese J Chromatogr. 2008;26(2):212–22.

434. Methven L, Tsoukka M, Oruna-Concha MJ, Parker JK, Mottram DS. Influence of sulfur amino acids on the volatile and nonvolatile components of cooked salmon (*Salmo salar*). J Agric Food Chem. 2007;55(4):1427–36.

435. Pino J, Marbot R, Rosado A. Volatile constituents of star apple (*Chrysophyllum cainito* L.) from Cuba. Flavour Fragr J. 2002;17(5):401–3.

436. Bredie WLP, Mottram DS, Guy R. Aroma volatiles generated during extrusion cooking of maize flour. J Agric Food Chem. 1998;46(4):1479–87.

437. Xu X, Stee LLP, Williams J, Beens J, Adahchour M, Vreuls RJJ, et al. Comprehensive two-dimensional gas chromatography (GC × GC) measurements of volatile organic compounds in the atmosphere. Atmos Chem Phys. 2003;3(3):665–82.

438. Hazzit M, Baaliouamer A, Faleiro ML, Miguel MG. Composition of the essential oils of Thymus and Origanum species from Algeria and their antioxidant and antimicrobial activities. J Agric Food Chem. 2006;54(17):6314–21.

439. Sampaio TS, Nogueira PCL. Volatile components of mangaba fruit (*Hancornia speciosa* Gomes) at three stages of maturity. Food Chem. 2006;95(4):606–10.

440. Adams RP. Identification of essential oil components by gas chromatography/mass spectrometry. 4th Edition. Carol Stream, IL: Allured Publishing Corporation; 2007. 804 p.

441. Radulovic N, Blagojevic P, Palic R. Comparative study of the leaf volatiles of *Arctostaphylos uva-ursi* L. (Spreng). and *Vaccinium vitis-idaea* L. (Ericaceae). Molecules. 2010;15:6168–85.

442. Andriamaharavo NR. Retention Data. 2014.

443. Mondello L, Costa R. A new generation of GC capillary columns: SLB-5ms. Report. 2006;20:17–9.

444. Rocha SM, Freitas R, Cardoso P, Santos M, Martins R, Figueira E. Exploring the potentialities of comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry to distinguish bivalve species: Comparison of two clam species (*Venerupis decussata* and *Venerupis philippinarum*). J Chromatogr A. 2013;1315(8):152–61.

445. Whitfield FB, Mottram DS. Heterocyclic volatiles formed by heating cysteine or hydrogen sulfide with 4-hydroxy-5-methyl-3(2H)-furanone at pH 6.5. J Agric Food Chem. 2001;49:816–22.

446. Elmore JS, Mottram DS, Enser M, Wood JD. Effect of the Polyunsaturated Fatty Acid Composition of Beef Muscle on the Profile of Aroma Volatiles. J Agric Food Chem. 1999;47(4):1619–25.

447. Zaikin VG, Borisov RS. Chromatographic-mass spectrometric analysis of Fischer-Tropsch synthesis products. J Anal Chem. 2002;57(6):653–60.

448. Xie J, Sun B, Yu M. Constituents of top fragrance from fresh flowers of *Robinia pseudoacacia* L. occurring in China. Flavour Fragr J. 2006;21(5):798–800.

449. Buchin S, Salmon JC, Carnat AP, Berger T, Bugaud C, Bosset JO. Identification de composés monoterpéniques, sesquiterpéniques et benzéniques dans un lait d'alpage très riche en ces substances. Mitteilungen aus Leb und Hyg. 2002;93:199–216.

450. Adams RP. Systematics of Juniperus section Juniperus based on leaf essential oils and random amplified polymorphic DNAs (RAPDs). Biochem Syst Ecol. 2000;28(6):515–28.

451. Zeng Y-X, Zhao C-X, Liang Y-Z, Yang H, Fang H-Z, Yi L-Z, et al. Comparative analysis of volatile components from Clematis species growing in China. Anal Chim Acta. 2007;595(1–2):328–39.

452. Landy P, Nicklaus S, Sémon E, Mielle P, Guichard E. Representativeness of extracts of offset paper packaging and analysis of the main odor-active compounds. J Agric Food Chem. 2004;52(8):2326–34.

453. Song C, Lai W-C, Reddy KM, Wei B. Temperature-Programmed Retention Indices for GC and GC-MS of Hydrocarbon Fuels and Simulated Distillation GC of Heavy Oils. In: Hsu CS, editor. Analytical Advances for Hydrocarbon Research. 1st ed. New York: Springer US; 2003. p. 147–210.

454. Shapi MM, Hesso A. Thermal decomposition of polystyrene: Volatile compounds from large-scale pyrolysis. J Anal Appl Pyrolysis. 1990;18(2):143–61.

455. Kotowska U, Żalikowski M, Isidorov VA. HS-SPME/GC–MS analysis of volatile and semi-volatile organic compounds emitted from municipal sewage sludge. Environ Monit Assess. 2012;184(5):2893–907.

456. Saraiva MJ, Salvador ÂC, Fernandes T, Ferreira JP, Barros AS, Rocha SM, et al. Three mammal species distinction through the analysis of scats chemical composition provided by comprehensive two-dimensional gas chromatography. Biochem Syst Ecol. 2014;55:46–52.

457. Cerny C, Guntz-Dubini R. Role of the solvent glycerol in the Maillard reaction of d-fructose and l-alanine. J Agric Food Chem. 2006;54(2):574–7.

458. Owens JD, Allagheny N, Kipping G, Ames JM. Formation of Volatile Compounds During *Bacillus subtilis* Fermentation of Soya Beans. J Sci Food Agric. 1997;74:132–40.

459. Dickschat JS, Wenzel SC, Bode HB, Müller R, Schulz S. Biosynthesis of volatiles by the myxobacterium *Myxococcus xanthus*. ChemBioChem. 2004;5(6):778–87.

460. Caldeira M, Barros AS, Bilelo MJ, Parada A, Câmara JS, Rocha SM. Profiling allergic asthma volatile metabolic patterns using a headspace-solid phase microextraction/gas chromatography based methodology. J Chromatogr A. 2011;1218(24):3771–80.

461. Rostad CE, Pereira WE. Kovats and Lee retention indices determined by gas chromatography/mass spectrometry for organic compounds of environmental interest. J High Resolut Chromatogr. 1986;9(6):328–34.

462. Salido S, Altarejos J, Nogueras M, Sánchez A, Pannecouque C, Witvrouw M, et al. Chemical studies of essential oils of *Juniperus oxycedrus* ssp. *badia*. J Ethnopharmacol. 2002;81(1):129–34.

463. Ádámová M, Oriňák A, Halás L. Retention indices as identification tool in pyrolysis-capillary gas chromatography. J Chromatogr A. 2005;1087(1–2):131–41.

464. Radulovic N, Blagojevic P, Palic R, Zlatkovic B, Stevanovic B. Volatiles from vegetative organs of the palaeoendemic resurrection plants *Ramonda serbica* Panč. and *Ramonda nathaliae* Panč. et Petrov. J Serbian Chem Soc. 2009;74(1):35–44.

465. Dickschat JS, Wagner-Döbler I, Schulz S. The chafer pheromone buibuilactone and ant pyrazines are also produced by marine bacteria. J Chem Ecol. 2005;31(4):925–47.

466. Mevy JP, Bessiere JM, Pelissier Y, Masotti V, Ruzzier M, Rabier J, et al. Composition of the volatile constituents of the aerial parts of an endemic plant of Ivory Coast, *Monanthotaxis capea* (E.G. & A. Campus) verdc. Flavour Fragr J. 2004;19(6):526–8.

467. Su Y, Ho C, Wang I, Chang T. Antifungal activities and chemical compositions of essential oils from leaves of four eucalypts. Taiwan J Sci. 2006;21(1):49–61.

468. Yoo SJ, Pace G V., Khoo BK, Lech J, Hartman TG. Mass spectrometry and gas chromatographic retention indices of selected UV/EB-curable monomers and photoinitiators commonly used in food packaging print and coating formulations. Radtech Rep. 2004;18(3):60–8.

469. Kallio M, Jussila M, Rissanen T, Anttila P, Hartonen K, Reissell A, et al. Comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry in the identification of organic compounds in atmospheric aerosols from coniferous forest. J Chromatogr A. 2006;1125(2):234–43.

470. Ansorena D, Gimeno O, Astiasarán I, Bello J. Analysis of volatile compounds by GC-MS of a dry-fermented sausage: Chorizo de Pamplona. Food Res Int. 2001;34(1):67–75.

471. Ames JM, Guy RCE, Kipping GJ. Effect of pH, Temperature, and Moisture on the Formation of Volatile Compounds in Glycine/Glucose Model Systems. J Agric Food Chem. 2001;49(9):4315–4323.

472. Jarunrattanasri A, Theerakulkait C, Cadwallader KR. Aroma components of acid-hydrolyzed vegetable protein made by partial hydrolysis of rice bran protein. J Agric Food Chem. 2007;55(8):3044–50.

473. Rout PK, Rao YR, Sree A, Naik SN. Composition of essential oil, concrete, absolute, wax and headspace volatiles of *Murrarya paniculata* (Linn.) Jack flowers. Flavour Fragr J. 2007;22(3):352–357.

474. Bredie WLP, Mottram DS, Guy RCE. Effect of temperature and pH on the generation of flavor volatiles in extrusion cooking of wheat flour. J Agric Food Chem. 2002;50:1118–25.

475. Ames JM, Defaye AB, Bates L. The effect of pH on the volatiles formed in an extruded starch-glucose-lysine model system. Food Chem. 1997;58(4):323–7.

476. Oruna-Concha MJ, Craig Duckham S, Ames JM. Comparison of volatile compounds isolated from the skin and flesh of four potato cultivars after baking. J Agric Food Chem. 2001;49(5):2414-21

477. Matsui T, Guth H, Grosch W. A comparative study of potent odorants in peanut, hazelnut, and pumpkin seed oils on the basis of aroma extract dilution analysis (AEDA) and gas chromatography-olfactometry of headspace samples (GC-OH). Fett/Lipid. 1998;100(2):51–6.

478. Rodrigues CII, Hanson CM, Nogueira JMF. Coffees and industrial blends aroma profile discrimination according to the chromatic value. Coffee Sci. 2012;7(2):167–76.

479. Moon SY, Cliff M a., Li-Chan ECY. Odour-active components of simulated beef flavour analysed by solid phase microextraction and gas chromatography-mass spectrometry and -olfactometry. Food Res Int. 2006;39(3):294–308.

480. Petronilho S, Rocha SM, Ramírez-Chávez E, Molina-Torres J, Rios-Chavez P. Assessment of the terpenic profile of *Callistemon citrinus* (Curtis) Skeels from Mexico. Ind Crops Prod. 2013;46:369–79.

481. Rocha SM, Coelho E, Zrostlíková J, Delgadillo I, Coimbra MA. Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry of monoterpenoids as a powerful tool for grape origin traceability. J Chromatogr A. 2007;1161(1–2):292–9.

482. Jalali HT, Petronilho S, Villaverde JJ, Coimbra M a., Domingues MRM, Ebrahimian ZJ, et al. Assessment of the sesquiterpenic profile of *Ferula gummosa* oleo-gum-resin (galbanum) from Iran. Contributes to its valuation as a potential source of sesquiterpenic compounds. Ind Crops Prod. 2013;44:185–91.

483. Stashenko EE, Jaramillo BE, Martínez JR. Comparación de la composición química y de la actividad antioxidante *in vitro* de los metabolitos secundarios volátiles de plantas de la familia Verbenaceae. Rev Acad Colomb Ciencias. 2003;27(105):579–97.

484. Su Y-C, Ho C-L, Wang E. Analysis of leaf essential oils from the indigenous five conifers of Taiwan. Flavour Fragr J. 2006;21(3):447–52.

485. Petronilho S, Maraschin M, Delgadillo I, Coimbra M a., Rocha SM. Sesquiterpenic composition of the inflorescences of Brazilian chamomile (*Matricaria recutita* L.): Impact of the agricultural practices. Ind Crops Prod. 2011;34(3):1482–90.

486. Adams RP, Morris J a., Pandey RN, Schwarzbach AE. Cryptic speciation between J*uniperus deltoides* and *Juniperus oxycedrus* (Cupressaceae) in the Mediterranean. Biochem Syst Ecol. 2005;33(8):771–87.

487. Mondello L, Sciarrone D, Casilli A, Tranchida PQ, Dugo P, Dugo G. Fast gas chromatography-full scan quadrupole mass spectrometry for the determination of allergens in fragrances. J Sep Sci. 2007;30(12):1905–11.

488. Bonaïti C, Irlinger F, Spinnler HE, Engel E. An iterative sensory procedure to select odor-active associations in complex consortia of microorganisms: application to the construction of a cheese model. J Dairy Sci. 2005;88(5):1671–84.

489. Insausti K, Goñi V, Petri E, Gorraiz C, Beriain MJ. Effect of weight at slaughter on the volatile compounds of cooked beef from Spanish cattle breeds. Meat Sci. 2005;70(1):83–90.

490. Verdier-Metz. I, Coulon J-B, Pradel P, Viallon C, Berdague J-L. Effect of forage conservation (hay or silage) and cow breed on the coagulation properties of milks and on the characteristics of ripened cheeses. J Dairy Res. 1998;65(1):9–21.

491. Al-Qudaha MA, Muhaidat R, Trawenhc IN, Jaberd HI Al, Zargae MHA, Orabi STA. Volatile Constituents of Leaves and Bulbs of *Gynandriris sisyrinchium* and their Antimicrobial activity. Jordan J Chem. 2012;7(3):287–95.

492. Mottram D, Whitfield F. Volatile compounds from the reaction of cysteine, ribose, and phospholipid in low-moisture systems. J Agric Food Chem. 1995;43:984–8.

ANNEXES

Annex from Chapter 2.2

Table A2.2 - Volatiles of *Staphylococcus aureus* detected and reported by different studies. Most volatiles reported were produced by this species. However, few of the reported were detected but not produced (for example, 2-propanone and butanal).

Name (Color from Figure *2.3*)	Formula	CAS Number	Techniques	Refs.	Pathway
Acids					
Acetic acid	$C_2H_4O_2$	64-19-7	GC-FID	(309)	NA
			GC-FID	(310)	NA
			GC-MS	(311)	NA
			GC-MS	(282)	NA
			SESI-MS	(286)	NA
			SIFT-MS	(292)	NA
			GC-MS	(303)	Pyruvate metabolism
			GC-MS	(279)	NA
			GC-MS	(236)	Pyruvate metabolism
			SIFT-MS	(283)	NA
			LC-MS	(291)	NA
Acetate	$C_2H_3O_2$	71-50-1	^{1}H-NMR	(297)	Glycolysis
			^{1}H-NMR	(294)	Pyruvate metabolism
			^{1}H-NMR	(284)	Glycolysis
			^{1}H-NMR	(408)	NA
Propanoic acid	$C_3H_6O_2$	79-09-4	GC-MS	(282)	NA
			SIFT-MS	(283)	NA
2-Methylpropanoate	$C_4H_7O_2$	-	^{1}H-NMR	(294)	NA
			^{1}H-NMR	(408)	NA
2-Methylpropanoic acid	$C_4H_8O_2$	79-31-2	GC-MS	(270)	NA
			GC-MS and GC/FPD	(304)	NA
Butanoic acid	$C_4H_8O_2$	107-92-6	GC-MS	(311)	NA
			GC-MS and GC/FPD	(304)	NA
			SIFT-MS	(292)	NA
			APCI-MS	(409)	NA
			SIFT-MS	(283)	NA
2-hydroxybutanoic acid	$C_4H_8O_3$	600-15-7	^{1}H-NMR	(294)	NA

2-Hydroxy-2-methylpropanoic acid	$C_4H_8O_3$	594-61-6	^{1}H-NMR	(294)	NA
2-Methylbutanoate	$C_5H_9O_2$	-	GC-MS	(311)	NA
			^{1}H-NMR	(408)	NA
3-Methylbutanoate	$C_5H_9O_2$	108-21-4	GC-MS	(311)	NA
			^{1}H-NMR	(294)	NA
			^{1}H-NMR	(408)	NA
2-Methylbutanoic acid	$C_5H_{10}O_2$	116-53-0	GC-MS and GC/FPD	(304)	NA
			^{1}H-NMR	(297)	NA
3-Methylbutanoic acid	$C_5H_{10}O_2$	503-74-2	GC-MS	(270)	NA
			GC-FID	(309)	NA
			GC-FID	(310)	NA
			GC-MS and GC/FPD	(304)	NA
			GC-MS	(282)	NA
			GC-MS	(303)	Leucine metabolism
			GC-MS	(240)	NA
			GC-MS	(279)	NA
			APCI-MS	(409)	NA
			GC-MS	(236)	Leucine metabolism
			GC-MS	(291)	NA
2-Hydroxy-3-methylbutanoic acid	$C_5H_{10}O_3$	4026-18-0	^{1}H-NMR	(294)	NA
3-Hydroxy-3-methylbutanoic acid	$C_5H_{10}O_3$	625-08-1	^{1}H-NMR	(294)	NA
4-methyl-2-oxopentanoic acid	$C_6H_{10}O_2$	816-66-0	^{1}H-NMR	(294)	NA
Benzoic acid	$C_7H_6O_2$	65-85-0	GC-MS	(282)	NA
4-Methylhexanoic acid	$C_7H_{14}O_2$	1561-11-1	GC-MS and GC/FPD	(304)	NA
2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-propanoic acid [a]	$C_{27}H_{42}O_4$	-	GC-MS	(410)	NA
Alcohols					
Methanol	CH_4O	67-56-1	SIFT-MS	(243)	NA
			SIFT-MS	(283)	NA

Ethanol	C_2H_6O	64-17-5	SIFT-MS	(302)	NA
			SIFT-MS	(281)	NA
			GC-MS	(282)	NA
			SESI-MS	(286)	NA
			^{1}H-NMR	(297)	NA
			SIFT-MS	(292)	NA
			GC-MS	(303)	Pyruvate metabolism
			MCC-IMS and GC-MS	(287)	NA
			^{1}H-NMR	(294)	Pyruvate metabolism
			^{1}H-NMR	(284)	Pyruvate metabolism
			^{1}H-NMR	(295)	Pyruvate metabolism
			SIFT-MS	(243)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	Pyruvate metabolism
			GC-MS	(308)	NA
			SIFT-MS	(283)	NA
			LC-MS and GC-MS	(291)	NA
Ethane-1,2-diol	$C_2H_6O_2$	107-21-1	GC-MS	(282)	NA
1-Propanol	C_3H_8O	71-23-8	GC-MS	(282)	NA
			SIFT-MS	(243)	NA
			SIFT-MS	(283)	NA
2-Propanol	C_3H_8O	67-63-0	^{1}H-NMR	(294)	NA
Glycerol	$C_3H_8O_3$	56-81-5	^{1}H-NMR	(294)	NA
1-Butanol	$C_4H_{10}O$	71-36-3	GC-MS	(282)	NA
			SESI-MS	(286)	NA
			SIFT-MS	(292)	NA
			GC-MS	(303)	Pyruvate metabolism
			GC-MS	(279)	NA
			GC-MS	(236)	Pyruvate metabolism
			SIFT-MS	(283)	NA
2-Butanol	$C_4H_{10}O$	78-92-2	GC-MS	(270)	NA
			GC-MS	(303)	NA

2-Methyl-1-propanol	$C_4H_{10}O$	78-83-1	GC-FID	(270)	NA
			GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
1,1-Butanediol	$C_4H_{10}O_2$	25265-75-2	^{1}H-NMR	(295)	Pyruvate metabolism
2,3-Butanediol	$C_4H_{10}O_2$	513-85-9	^{1}H-NMR	(297)	NA
			^{1}H-NMR	(284)	Pyruvate metabolism
			^{1}H-NMR	(408)	NA
2-Methylbut-3-en-2-ol	$C_5H_{10}O$	115-18-4	GC-FID	(270)	NA
1-Pentanol	$C_5H_{12}O$	71-41-0	SIFT-MS	(292)	NA
			SIFT-MS	(283)	NA
3-Methyl-1-butanol	$C_5H_{12}O$	123-51-3	GC-FID	(270)	NA
			GC-MS	(311)	NA
			GC-MS	(282)	NA
			SESI-MS	(286)	NA
			GC-MS	(303)	Leucine catabolism
			MCC-IMS and GC-MS	(287)	NA
			GC-MS	(240)	NA
			E-nose and GC-MS	(300)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	
Phenol	C_6H_6O	108-95-2	SIFT-MS	(283)	NA
(E)-2-methylpent-2-en-1-ol	$C_6H_{12}O$	1610-29-3	GC-FID	(270)	NA
4-Methylphenol	C_7H_8O	106-44-5	GC-MS	(282)	NA
			SESI-MS	(286)	
2-Methyl-3-hexanol	$C_7H_{16}O$	617-29-8	GC-MS	(282)	NA
4-Methyl-1-hexanol	$C_7H_{16}O$	818-49-5	GC-MS	(282)	NA
2-Phenylethanol	$C_8H_{10}O$	60-12-8	GC-MS and GC/FPD	(304)	NA
2-Ethyl-1-hexanol	$C_8H_{18}O$	104-76-7	E-nose and GC-MS	(300)	NA
2,4,7,9-tetramethyldec-5-yne-4,7-diol	$C_{14}H_{26}O_2$	126-86-3	GC-MS	(282)	NA

Tetradecanol	$C_{14}H_{30}O$	112-72-1	GC-MS	(282)	NA
2,6-Di-tert-butyl-4-methylphenol	$C_{15}H_{24}O$	128-37-0	GC-MS	(282)	NA
Aldehydes					
Formaldehyde	CH_2O	50-00-0	SIFT-MS	(292)	NA
			SIFT-MS	(312)	NA
			SIFT-MS	(283)	NA
Acetaldehyde	C_2H_4O	75-07-0	SIFT-MS	(302)	NA
			SIFT-MS	(281)	NA
			GC-MS	(311)	NA
			GC-MS	(282)	NA
			SIFT-MS	(292)	NA
			GC-MS	(303)	Pyruvate metabolism
			SIFT-MS	(243)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	Pyruvate metabolism
Propanal	C_3H_6O	123-38-6	GC-MS	(282)	NA
			GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
			SIFT-MS	(283)	NA
Propanedial	$C_3H_4O_2$	542-78-9	SIFT-MS	(283)	NA
Methacrolein	C_4H_6O	78-85-3	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
2-Butenal	C_4H_6O	4170-30-3	SIFT-MS	(283)	NA
Butanal	C_4H_8O	123-72-8	SIFT-MS	(243)	NA
2−Methylpropanal	C_4H_8O	78-84-2	SIFT-MS	(292)	NA
			GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA

(Z)-2-Methyl-2-butenal	C_5H_8O	1115-11-3	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
(E)-2-Methyl-2-butenal	C_5H_8O	497-03-0	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
3-Methyl-2-butenal	C_5H_8O	107-86-8	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
2-Ethylacrolein	C_5H_8O	922-63-4	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
2-Pentenal	C_5H_8O	764-39-6	SIFT-MS	(283)	NA
Pentanal	$C_5H_{10}O$	110-62-3	SIFT-MS	(243)	NA
			SIFT-MS	(283)	NA
2-Methylbutanal	$C_5H_{10}O$	96-17-3	GC-MS	(270)	NA
			GC-MS	(311)	NA
			GC-MS	(282)	NA
			SIFT-MS	(292)	NA
			SIFT-MS	(312)	NA
			GC-MS	(293)	NA
3-Methylbutanal	$C_5H_{10}O$	590-86-3	GC-MS	(311)	NA
			GC-MS	(282)	NA
			GC-MS	(303)	Leucine catabolism
			MCC-IMS and GC-MS	(287)	NA
			GC-MS	(240)	NA
			GC-MS	(293)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	Leucine catabolism
2-Hexenal	$C_6H_{10}O$	505-57-7	SIFT-MS	(283)	NA
Hexanal	$C_6H_{12}O$	66-25-1	SIFT-MS	(302)	NA
			GC-MS	(308)	NA
			SIFT-MS	(283)	NA

Benzaldehyde	C_7H_6O	100-52-7	GC-MS	(282)	NA
			GC-MS	(293)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
2-Heptenal	$C_7H_{12}O$	2463-63-0	SIFT-MS	(283)	NA
2-Octenal	$C_8H_{14}O$	2363-89-5	SIFT-MS	(283)	NA
Octanal	$C_8H_{16}O$	124-13-0	SIFT-MS	(283)	NA
Nonanal	$C_9H_{18}O$	124-19-6	GC-MS	(282)	NA
			SIFT-MS	(283)	NA
Decanal	$C_{10}H_{20}O$	112-31-2	GC-MS	(282)	NA
Esters					
Ethyl formate	$C_3H_6O_2$	109-94-4	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
Ethyl acetate	$C_4H_8O_2$	141-78-6	GC-MS	(311)	NA
			GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
Methyl methacrylate	$C_5H_8O_2$	80-62-6	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
Ethyl butanoate	$C_6H_{12}O_2$	105-54-4	GC-MS	(311)	NA
			SIFT-MS	(292)	NA
Butyl acetate	$C_6H_{12}O_2$	123-86-4	GC-MS	(303)	NA
			GC-MS	(240)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
Ethyl 2-methylbutanoate	$C_7H_{14}O_2$	7452-79-1	GC-MS	(311)	NA
			GC-MS	(240)	NA
Ethyl 3-methylbutanoate	$C_7H_{14}O_2$	108-64-5	GC-MS	(311)	NA

			GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
3-Methylbutyl acetate	$C_7H_{14}O_2$	123-92-2	GC-MS	(303)	NA
			GC-MS	(240)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
Butyl butanoate	$C_8H_{16}O_2$	109-21-7	GC-MS	(311)	NA
Ethyl hexanoate	$C_8H_{16}O_2$	123-66-0	GC-MS	(311)	NA
Butyl 2-methylbutanoate	$C_9H_{18}O_2$	15706-73-7	GC-MS	(240)	NA
4-Isopropyl-1-methyl-3-oxocyclohexyl acetate	$C_{12}H_{20}O_3$	-	GC-MS	(282)	NA
Decyl acetate	$C_{12}H_{24}O_2$	112-17-4	GC-MS	(240)	NA
Phenyl benzoate	$C_{13}H_{10}O_2$	93-99-2	GC-MS	(282)	NA
Isopropyl tetradecanoate	$C_{17}H_{34}O_2$	110-27-0	GC-MS	(308)	NA
Furan-like compounds					
3-Phenylfuran	$C_{10}H_8O$	13679-41-9	GC-MS	(282)	NA
Halogenated compounds					
1,1,2,2-tetrachloroethane	$C_2H_2Cl_4$	79-34-5	GC-MS	(293)	NA
1,4-dichloroacetic acid	C2H2Cl2O2	-	GC-MS	(293)	NA
Trichloroacetic acid	$C_2HCl_3O_2$	76-03-9	GC-MS	(293)	NA
N,N-dimethylsulfamoyl chloride	$C_2H_6ClNO_2S$	13360-57-1	GC-MS	(282)	NA
2,4,6-Triphenyl-1,3,5-tripropylborazine	$C_{27}H_{36}B_3N_3$	-	GC-MS	(282)	NA
Hydrocarbons					
Methane	CH_4	74-82-8	SIFT-MS	(283)	NA

Propane	C_3H_8	74-98-6	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
1,3-Butadiene	C_4H_6	106-99-0	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
2-Methylpropene	C_4H_8	115-11-7	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
(Z)-2-butene	C_4H_8	590-18-1	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
(E)-2-butene	C_4H_8	624-64-6	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
Butane	C_4H_{10}	106-97-8	GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
1,4-Pentadiene	C_5H_8	591-93-5	GC-MS	(308)	NA
Isoprene	C_5H_8	78-79-5	SIFT-MS	(283)	NA
Pentane	C_5H_{12}	109-66-0	SIFT-MS	(283)	NA
Benzene	C_6H_6	71-43-2	GC-MS	(282)	NA
1-Heptene	C_7H_{14}	592-76-7	GC-MS	(282)	NA
Styrene	C_8H_8	100-42-5	GC-MS	(282)	NA
1,4-Dimethylbenzene	C_8H_{10}	106-42-3	GC-MS	(282)	NA
2,3,3-trimethylpentane	C_8H_{16}	560-21-4	GC-MS	(293)	NA
1-methyl-4-(1-methylethenyl)cyclohexane	C10H18	6252-33-1	GC-MS	(293)	NA
2-Methylnaphthalene	$C_{11}H_{10}$	91-57-6	GC-MS	(308)	NA

Undecane	$C_{11}H_{24}$	1120-21-4	GC-MS	(308)	NA
2,6,10,14-tetramethylhexadecane	$C_{20}H_{42}$	638-36-8	GC-MS	(282)	NA
Ketones					
2-Propanone	C_3H_6O	67-64-1	SIFT-MS	(302)	NA
			SIFT-MS	(281)	NA
			SIFT-MS	(280)	NA
			SESI-MS	(286)	NA
			^{1}H-NMR	(294)	NA
			SIFT-MS	(243)	NA
			GC-MS	(293)	NA
			GC-MS	(308)	NA
			SIFT-MS	(283)	NA
1-Hydroxy-2-propanone	$C_3H_6O_2$	116-09-6	GC-MS and GC/FPD	(304)	NA
			GC-MS	(303)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
1,3-Dihydroxy-2-propanone	$C_3H_6O_3$	96-26-4	^{1}H-NMR	(294)	NA
			^{1}H-NMR	(295)	NA
2,3-Butadione	$C_4H_6O_2$	431-03-8	GC-MS	(311)	NA
			GC-MS	(303)	NA
			GC-MS	(293)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
			GC-MS	(291)	NA
2-Butanone	C_4H_8O	78-93-3	GC-MS	(270)	NA
			GC-MS	(311)	NA
			GC-MS	(308)	NA
			SIFT-MS	(283)	NA
3-Hydroxy-2-butanone	$C_4H_8O_2$	513-86-0	GC-FID	(270)	NA
			GC-MS	(311)	NA
			GC-MS and GC/FPD	(304)	NA
			^{1}H-NMR	(297)	NA

			GC-MS	(303)	Pyruvate metabolism
			^{1}H-NMR	(284)	Pyruvate metabolism
			^{1}H-NMR	(295)	Pyruvate metabolism
			^{1}H-NMR	(408)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	Pyruvate metabolism
			GC-MS	(308)	NA
			SIFT-MS	(283)	NA
2-Pentanone	$C_5H_{10}O$	107-87-9	GC-MS	(270)	NA
			GC-MS	(311)	NA
			SESI-MS	(286)	NA
			SIFT-MS	(283)	NA
2-Hexanone	$C_6H_{12}O$	591-78-6	SIFT-MS	(283)	NA
2-Heptanone	$C_7H_{14}O$	110-43-0	GC-MS	(270)	NA
			GC-MS	(311)	NA
			SIFT-MS	(283)	NA
5-Methyl-2-hexanone	$C_7H_{14}O$	110-12-3	GC-MS	(282)	NA
2-Octanone	$C_8H_{16}O$	111-13-7	SIFT-MS	(283)	NA
2-methylacetophenone	$C_9H_{10}O$	577-16-2	SIFT-MS	(283)	NA
2-Nonanone	$C_9H_{18}O$	821-55-6	GC-MS	(270)	NA
			SESI-MS	(286)	NA
			SIFT-MS	(283)	NA
3-Methyl-1-phenyl-1-butanone	$C_{11}H_{14}O$	582-62-7	GC-MS	(282)	NA
1-(4-methylphenyl)-1-Pentanone	$C_{12}H_{16}O$	1671-77-8	GC-MS	(282)	NA
2,10,10-Trimethyl-6-methylene-1-oxaspiro[4.5]decan-7-one	$C_{13}H_{20}O_2$	-	GC-MS	(282)	NA
(Z)-6,10-dimethyl-5,9-undecadien-2-one	$C_{13}H_{22}O$	3879-26-3	GC-MS	(282)	NA
2-Tridecenone	$C_{13}H_{24}O$	-	GC-MS	(290)	NA

***N*-Compounds**

Ammonia	H_3N	7664-41-7	SIFT-MS	(281)	NA
			SIFT-MS	(280)	NA
			SIFT-MS	(312)	NA
			SIFT-MS	(243)	NA
			SIFT-MS	(283)	NA
			LC-MS	(291)	NA
Acetonitrile	C_2H_3N	75-05-8	SESI-MS	(286)	NA
2-Aminoacetic acid	$C_2H_5NO_2$	56-40-6	GC-MS	(282)	NA
2-Hydrazinyl-2-oxoacetamide	$C_2H_5N_3O_2$	515-96-8	GC-MS	(282)	NA
Trimethylamine	C_3H_5N	75-50-3	SIFT-MS	(302)	NA
			SIFT-MS	(292)	NA
Pyrimidine	$C_4H_4N_2$	289-95-2	SESI-MS	(286)	NA
Pyrazine	$C_4H_4N_2$	290-37-9	GC-MS	(291)	NA
1H-Pyrrole	C_4H_5N	109-97-7	GC-MS	(282)	NA
			SIFT-MS	(292)	NA
1,4,5,6-Tetrahydropyridazine	$C_4H_8N_2$	-	GC-MS	(282)	NA
2-Aminobutanoic acid	$C_4H_9NO_2$	2835-81-6	^{1}H-NMR	(294)	NA
Pyridine	C_5H_5N	110-86-1	GC-MS	(270)	NA
N,N-Dimethyl-4-pyridinamine	$C_7H_{10}N$	1122-58-3	GC-MS	(282)	NA
Indole	C_8H_7N	120-72-9	GC-MS	(270)	NA
			SIFT-MS	(302)	NA
			SIFT-MS	(292)	NA
			SIFT-MS	(243)	NA
2-Aminoacetophenone	C_8H_9NO	551-93-9	SIFT-MS	(302)	NA
			SIFT-MS	(281)	NA
			SIFT-MS	(292)	NA
			SIFT-MS	(283)	NA

Quinoline	C_9H_7N	91-22-5	GC-MS	(282)	NA
S-Compounds					
Hydrogen cyanide	CHN	74-90-8	SIFT-MS	(283)	NA
Hydrogen sulfide	H_2S	7783-06-4	SIFT-MS	(302)	NA
			SIFT-MS	(281)	NA
			SIFT-MS	(292)	NA
			SIFT-MS	(283)	NA
Methanethiol	CH_4S	74-93-1	SIFT-MS	(302)	NA
			SIFT-MS	(281)	NA
			SIFT-MS	(292)	NA
			SIFT-MS	(312)	NA
			GC-MS	(303)	NA
			IMR-MS	(285)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
			SIFT-MS	(283)	NA
Methyl thiocyanate	C_2H_3NS	556-64-9	SIFT-MS	(283)	NA
Dimethyl sulfide	C_2H_6S	75-18-3	SIFT-MS	(302)	NA
			SIFT-MS	(281)	NA
			SIFT-MS	(283)	NA
Dimethyl disulfide	$C_2H_6S_2$	624-92-0	GC-MS	(270)	NA
			GC-MS	(290)	NA
			SIFT-MS	(302)	NA
			SIFT-MS	(281)	NA
			GC-MS	(303)	NA
			GC-MS	(293)	NA
			GC-MS	(279)	NA
			GC-MS	(236)	NA
			SIFT-MS	(283)	NA
Dimethyl trisulfide	$C_2H_6S_3$	3658-80-8	GC-MS	(293)	NA
			SIFT-MS	(283)	NA

S-Methyl methanethiosulfonate	$C_2H_6O_2S_2$	2949-92-0	GC-MS	(282)	NA
2-Methylsulfonylethanol	$C_3H_8O_3S$	15205-66-0	MCC-IMS and GC-MS	(287)	NA
Benzothiazole	C_7H_5NS	95-16-9	GC-MS	(282)	NA
Methyl benzyl sulfide	$C_8H_{10}S$	766-92-7	GC-MS	(282)	NA

NA – Not addressed

[a] absent from figure (just one volatile described)

Annex from Chapter 5.3

Table A5.3 - Chromatographic data of the 315 analytes putatively identified from the three *Staphyloccus aureus* strains under study: ATCC 6538 (without enterotoxins), 2153 MA (with enterotoxin A) and 2065 MA (with enterotoxin A, G, I), using HS-SPME/GC×GC-ToFMS. Grey background colour lines represent volatiles used for statistical analysis.

^1t_R (s)[a]	^2t_R (s)[a]	Name	Formula	CAS Number	RI calc[b]	RI GCxGC lit[c]	Ref.
		Acids					
		Aliphatics					
120	2.420	Acetic acid	$C_2H_4O_2$	64-19-7	658	602	(411)
175	2.950	2-Methylpropanoic acid	$C_4H_8O_2$	79-31-2	775	762	(412)
250	2.790	3-Methylbutanoic acid	$C_5H_{10}O_2$	503-74-2	874	875	(413)
355	1.190	4-Methyl-3-pentenoic acid	$C_6H_{10}O_2$	504-85-8	985	1011	(414)
425	2.650	4-Methyl-2-oxopentanoic acid	$C_6H_{10}O_3$	816-66-0	1064	-	(412)
		Alcohols					
		Aliphatics					
70	0.460	Ethanol	C_2H_6O	64-17-5	548	577	(415)
100	0.610	2-Methyl-1-propanol	$C_4H_{10}O$	78-83-1	612	602	(412)
115	0.670	1-Butanol	$C_4H_{10}O$	71-36-3	644	637	(412)
145	0.740	3-Methyl-1-butanol	$C_5H_{12}O$	123-51-3	707	731	(412)
145	0.860	3-Methyl-3-buten-1-ol	$C_5H_{10}O$	763-32-6	707	716	(416)
180	0.970	3-Methyl-2-buten-1-ol	$C_5H_{10}O$	556-82-1	781	778	(417)
200	1.030	2,3-Butanediol	$C_4H_{10}O_2$	513-85-9	812	789	(411)
270	1.010	2-Methyl-3-hexanol	$C_7H_{16}O$	617-29-8	895	-	
345	0.790	1-Heptanol	$C_7H_{16}O$	111-70-6	975	974	(418)
350	0.770	1-Octen-3-ol	$C_8H_{16}O$	3391-86-4	980	985	(418)
360	0.760	6-Methyl-1-heptanol	$C_8H_{18}O$	1653-40-3	990	-	
395	0.740	2-Ethyl-1-hexanol	$C_8H_{18}O$	104-76-7	1029	1031	(418)

400	0.790	4-Methyl-1-heptanol	$C_8H_{18}O$	817-91-4	1034	-	
420	0.900	2,3-Octanediol	$C_8H_{18}O_2$	20653-90-1	1057	-	
435	0.770	1-Octanol	$C_8H_{18}O$	111-87-5	1073	1071	(418)
450	0.930	4-Methyl-1-hepten-4-ol	$C_8H_{16}O$	1186-31-8	1090	-	
525	0.740	1-Nonanol	$C_9H_{20}O$	143-08-8	1173	1179	(419)
575	0.570	2-Methyl-2-nonanol	$C_{10}H_{22}O$	10297-57-1	1230	-	
685	0.610	2,4-Undecadien-1-ol	$C_{11}H_{22}O$	59376-58-8	1363	-	
735	0.690	2-Methyl-1-undecanol	$C_{12}H_{26}O$	10522-26-6	1426	-	
775	0.730	1-Dodecanol	$C_{12}H_{26}O$	112-53-8	1476	1480	(420)
905	0.600	5,9-Dimethyl-1-decanol	$C_{12}H_{26}O$	91482-38-1	1630	-	
950	0.730	1-Tetradecanol	$C_{14}H_{30}O$	112-72-1	1683	1686	(420)
		Aromatics					
415	2.260	Benzyl alcohol	C_7H_8O	100-51-6	1053	1043	(412)
455	1.730	2-Methoxyphenol	$C_7H_8O_2$	90-05-1	1096	1087	(421)
460	3.670	4-Methylphenol	C_7H_8O	106-44-5	1104	1077	(411)
485	1.740	2-Phenylethanol	$C_8H_{10}O$	60-12-8	1130	1132	(412)
595	1.280	2-Phenylbutane-1-ol	$C_{10}H_{14}O$	2035-94-1	1254	1261	(422)
675	1.700	2-tert-Butyl-4-methylphenol	$C_{11}H_{16}O$	2409-55-4	1352	1387	(423)
705	1.050	2,4-Dimethyl-6-tert-butylphenol	$C_{12}H_{18}O$	1879-09-0	1389	-	
735	1.590	3,5-Diisopropylphenol	$C_{12}H_{18}O$	26886-05-5	1427	-	
805	1.380	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	96-76-4	1513	1513	(424)
1100	1.700	2,3-Dihydro-3-phenyl-1H-inden-1-ol	$C_{15}H_{14}O$	79454-17-4	1861	-	
		Aldehydes					
		Aliphatics					
85	0.350	2−Methylpropanal	C_4H_8O	78-84-2	580	554	(425)
110	0.420	3-Methylbutanal	$C_5H_{10}O$	590-86-3	632	643	(426)
115	0.390	2−Methylbutanal	$C_5H_{10}O$	96-17-3	643	635	(427)

180	0.680	3-Methyl-2-butenal	C_5H_8O	107-86-8	780	783	(428)
190	0.500	Hexanal	$C_6H_{12}O$	66-25-1	801	801	(412)
250	0.540	2-Methyl-2-hexenal	$C_7H_{12}O$	28467-88-1	871	884	(429)
275	0.520	Heptanal	$C_7H_{14}O$	111-71-7	901	904	(430)
325	0.650	2-Heptenal	$C_7H_{12}O$	2463-63-0	953	962	(418)
370	0.540	Octanal	$C_8H_{16}O$	124-13-0	1001	1006	(418)
465	0.530	Nonanal	$C_9H_{18}O$	124-19-6	1106	1101	(418)
550	0.540	Decanal	$C_{10}H_{20}O$	112-31-2	1201	1207	(418)
635	0.540	Undecanal	$C_{11}H_{22}O$	112-44-7	1301	1307	(418)
720	0.550	Dodecanal	$C_{12}H_{24}O$	112-54-9	1407	1415	(412)
800	0.570	Tridecanal	$C_{13}H_{26}O$	10486-19-8	1507	1512	(427)
890	0.580	Tetradecanal	$C_{14}H_{28}O$	124-25-4	1612	1613	(427)
975	0.590	Pentadecanal	$C_{15}H_{30}O$	2765-11-9	1713	1711	(417)
		Aromatics					
330	1.070	Benzaldehyde	C_7H_6O	100-52-7	959	959	(412)
410	1.100	2-Phenylacetaldehyde	C_8H_8O	122-78-1	1046	1043	(426)
410	1.230	2-Hydroxybenzaldehyde	$C_7H_6O_2$	90-02-8	1046	1041	(417)
565	0.980	3,5-Dimethylbenzaldehyde	$C_9H_{10}O$	5779-95-3	1219	-	
570	0.820	4-(1-Methylethyl)-benzaldehyde	$C_{10}H_{12}O$	122-03-2	1225	1226	(431)
640	0.830	4-tert-Butylbenzaldehyde	$C_{11}H_{14}O$	939-97-9	1307	-	
755	0.810	2-Phenyl-4-pentenal	$C_{13}H_{14}O$	24401-36-3	1451		
790	0.890	5-Methyl-2-phenyl-2-hexenal	$C_{13}H_{16}O$	21834-92-4	1495	1486	(432)
1020	1.110	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	$C_{15}H_{22}O_2$	1620-98-0	1766	1774	(433)
		Azoles					
150	0.870	Thiazole	C_3H_3NS	288-47-1	718	735	(434)
165	2.410	Pyrrole	C_4H_5N	109-97-7	752	751	(434)
200	0.710	2-Methylthiazole	C_4H_5NOS	3581-87-1	813	808	(434)

205	0.790	4-Methylthiazole	C_4H_5NS	693-95-8	819	802	(434)
385	1.240	2-Acetylthiazole	C_5H_5NOS	24295-03-2	1018	1014	(435)
470	1.010	2-Acetyl-4-methylthiazole	C_6H_7NOS	7533-07-5	1112	1128	(436)
480	1.050	2-Propionylthiazole	C_6H_7NOS	43039-98-1	1123	1132	(434)
515	0.900	2-Butanoylthiazole	C_7H_9NOS	-	1162	-	
575	1.310	Benzothiazole	C_7H_5NS	95-16-9	1231	1223	(430)
690	0.780	2,5,6-Trimethylbenzimidazole	$C_{10}H_{12}N_2$	3363-56-2	1370	-	
750	1.080	3-Methyl-5-phenyl-1H-pyrazole	$C_{10}H_{10}N_2$	3347-62-4	1445	-	
		Esters					
		Aliphatics					
135	0.420	Ethyl propanoate	$C_5H_{10}O_2$	105-37-3	685	684	(412)
135	0.450	Methyl methacrylate	$C_5H_8O_2$	80-62-6	685	710	(437)
175	0.440	Methyl isovalerate	$C_6H_{12}O_2$	556-24-1	769	765	(438)
200	0.480	Butyl acetate	$C_6H_{12}O_2$	123-86-4	812	819	(412)
230	0.430	Ethyl 2-methylbutanoate	$C_7H_{14}O_2$	7452-79-1	848	851	(412)
260	0.540	3-Methyl-3-butenyl acetate	$C_7H_{12}O_2$	5205-07-2	883	881	(439)
320	0.930	Methyl 2-hydroxy-4-methylpentanoate	$C_7H_{14}O_3$	40348-72-9	948	-	
370	0.410	Propyl 2,2-dimethylpropanoate	$C_8H_{16}O_2$	5129-35-1	1001	-	
380	0.540	tert-Butyl isopropyl carbonate	$C_8H_{16}O_3$	30221-21-7	1012	-	
555	0.730	Dimethyl 2,4-dimethylpentanedioate	$C_9H_{16}O_4$	2121-68-8	1207	-	
560	0.790	Dimethyl isopropylidenesuccinate	$C_9H_{14}O_4$	87384-00-7	1213	-	
585	0.660	Dimethyl 2-methylhexanedioate	$C_9H_{16}O_4$	19780-94-0	1242	-	
585	0.740	1-Ethyl 6-methyl hexanoate	$C_9H_{16}O_4$	18891-13-9	1242	-	
620	0.820	Dimethyl 2-(2-methylprop-2-enyl)propanedioate	$C_9H_{14}O_4$	50598-40-8	1283	-	
625	0.680	Dimethyl 2,2-dimethylpentanedioate	$C_9H_{16}O_4$	13051-32-6	1289	-	
645	0.620	Dimethyl 2,3-dimethyl hexanodioate	$C_{10}H_{18}O_4$	58219-48-0	1313	-	
675	0.780	1-Methylhexyl butanoate	$C_{11}H_{22}O_2$	39026-94-3	1351	-	

690	0.730	2-Ethyl-3-hydroxyhexyl butyrate	$C_{12}H_{24}O_3$	18618-89-8	1370	-	
1020	0.920	Diethyl diallylmalonate	$C_{13}H_{20}O_4$	3195-24-2	1766	-	
		Aromatics					
455	0.890	Methyl benzoate	$C_8H_8O_2$	93-58-3	1095	1100	(437)
530	0.950	Methyl phenylacetate	$C_9H_{10}O_2$	101-41-7	1179	-	
545	0.780	1-Phenylethyl acetate	$C_{10}H_{12}O_2$	93-92-5	1195	1192	(440)
585	0.840	Ethyl phenylethanoate	$C_{10}H_{12}O_2$	101-97-3	1242	1244	(417)
665	0.830	2-Benzylacrylic acid methyl ester	$C_{11}H_{12}O_2$	3070-71-1	1239	-	
665	0.870	Cinnamyl methanoate	$C_{10}H_{10}O_2$	104-65-4	1339	-	
730	0.800	2-Methyl-4-phenylbutyric acid methyl ester	$C_{12}H_{16}O_2$	-	1420	-	
765	0.870	5-Phenyl-2-pentenoic acid methyl ester	$C_{12}H_{14}O_2$	26429-97-0	1464	-	
795	0.830	Ethyl 5-phenyl-2-pentenoate	$C_{13}H_{16}O_2$	55282-95-6	1501	-	
855	0.870	Dimethyl phenylethyl carbinyl acetate	$C_{13}H_{18}O_2$	103-07-1	1572	-	
890	1.350	Phenyl benzoate	$C_{13}H_{10}O_2$	93-99-2	1613	-	
950	0.810	Hexyl 2-hydroxybenzoate	$C_{13}H_{18}O_3$	6259-76-3	1683	1683	(441)
1055	0.760	2-Ethylhexyl 2-hydroxybenzoate	$C_{15}H_{22}O_3$	118-60-5	1807	1817	(442)
		Cyclics					
515	0.660	Butyl cyclopropanecarboxylate	$C_8H_{14}O_2$	54947-39-6	1162	-	
930	0.940	Methyl dihydrojasmonate	$C_{13}H_{22}O_3$	24851-98-7	1660	1650	(443)
		Furan-like compounds					
230	1.350	Furfural	$C_5H_4O_2$	98-01-1	849	840	(412)
285	1.220	1-(2-Furanyl)-ethanone	$C_6H_6O_2$	1192-62-7	912	917	(412)
360	0.480	2-Pentylfuran	$C_9H_{14}O$	3777-69-3	990	994	(444)
365	0.890	Benzofuran	C_8H_6O	271-89-6	996	996	(412)
380	1.020	1-(2-Furanyl)-1-propanone	$C_7H_8O_2$	3194-15-8	1012	1008	(445)
455	0.790	1-(2,4-Dimethyl-furan-3-yl)-ethanone	$C_8H_{10}O_2$	32933-07-6	1095	-	
545	0.950	1,3-Dihydro isobenzofuran	C_8H_8O	496-14-0	1196	-	

560	0.960	Furfuryl methyl disulfide	$C_6H_8OS_2$	57500-00-2	1213	1226	(446)
570	1.040	3-Phenylfuran	$C_{10}H_8O$	13679-41-9	1225	1228	(432)
		Furanones					
195	0.770	Dihydro-2-methyl-3(2H)-furanone	$C_5H_8O_2$	3188-00-9	807	804	(417)
335	1.400	4-Methyldihydro-2(3H)-furanone	$C_5H_8O_2$	1679-49-8	965	-	
690	1.040	Dihydro-5-pentyl-2(3H)-furanone	$C_9H_{16}O_2$	104-61-0	1370	1360	(417)
1010	0.990	Dihydro-2,2-dimethyl-5-phenyl-3(2H)-furanone	$C_{12}H_{14}O_2$	63678-00-2	1754	-	
		Hydrocarbons					
		Aliphatics					
70	0.300	2-Butene	C_4H_8	107-01-7	548	411	(447)
190	0.350	Octane	C_8H_{18}	111-65-9	800	800	(430)
275	0.360	Nonane	C_9H_{20}	111-84-2	900	900	(430)
375	0.360	2,2,4,6,6-Pentamethyl-3-heptene	$C_{12}H_{24}$	123-48-8	1006	-	
475	0.370	3-Ethyl-2,7-dimethyl-octane	$C_{12}H_{26}$	62183-55-5	1117	1180	(448)
520	0.380	6-Methyl-3-undecene	$C_{12}H_{24}$	74630-52-7	1167	-	
540	0.390	Dodecene	$C_{12}H_{24}$	112-41-4	1189	1192	(430)
615	0.370	4-Ethylundecane	$C_{13}H_{28}$	17312-59-3	1277	1281	(430)
710	0.400	Tetradecene	$C_{14}H_{28}$	1120-36-1	1394	1400	(430)
760	0.400	2-Methyltetradecane	$C_{15}H_{32}$	1560-95-8	1457	1467	(447)
835	0.410	3-Ethyl-3-methyltridecane	$C_{16}H_{34}$	-	1548	1544	(430)
875	0.420	Hexadecane	$C_{16}H_{34}$	544-76-3	1595	1600	(429)
970	0.410	Heptadecane	$C_{17}H_{36}$	629-78-7	1706	1700	(429)
1045	0.430	Octadecane	$C_{18}H_{38}$	593-45-3	1795	1800	(429)
1150	0.420	Nonadecane	$C_{19}H_{40}$	629-92-5	1919	1900	(429)
1210	0.450	Eicosane	$C_{20}H_{42}$	112-95-8	1994	1993	(429)
		Aromatics					
165	0.470	Methylbenzene	C_7H_8	108-88-3	748	776	(418)

245	0.500	1,3-Dimethylbenzene	C_8H_{10}	108-38-3	865	878	(437)
330	0.510	1-Ethyl-4-methylbenzene	C_9H_{12}	622-96-8	958	970	(437)
365	0.610	1-Propenylbenzene	C_9H_{10}	637-50-3	995	1000	(449)
390	0.570	1,2,3-Trimethylbenzene	C_9H_{12}	526-73-8	1023	1022	(450)
420	0.570	2-Phenyl-1-butene	$C_{10}H_{12}$	2039-93-2	1056	-	
440	0.530	2-Ethyl-1,3-dimethylbenzene	$C_{10}H_{14}$	2870-04-4	1078	1100	(437)
535	0.920	Naphthalene	$C_{10}H_8$	91-20-3	1184	1203	(437)
630	0.870	1-Methylnaphthalene	$C_{11}H_{10}$	90-12-0	1295	1299	(451)
645	0.890	2-Methylnaphthalene	$C_{11}H_{10}$	91-57-6	1314	1315	(451)
655	0.590	5,6,7,8,9,10-Hexahydrobenzocyclooctene	$C_{12}H_{16}$	1076-69-3	1326	-	
660	0.660	4-Phenyl-1-cyclohexene	$C_{12}H_{14}$	4994-16-5	1332	1345	(452)
695	0.950	Biphenyl	$C_{12}H_{10}$	92-52-4	1376	1377	(453)
730	0.900	1,4-Dimethylnaphthalene	$C_{12}H_{12}$	571-58-4	1420	1424	(453)
740	0.900	Diphenylmethane	$C_{13}H_{12}$	101-81-5	1432	-	
775	0.860	3-Ethyl-3-phenyl-1-pentene	$C_{13}H_{18}$	19781-34-1	1476	-	
785	0.950	3-Methylbiphenyl	$C_{13}H_{12}$	643-93-6	1489	1488	(453)
785	1.050	Acenaphthene	$C_{12}H_{10}$	83-32-9	1489	1489	(453)
790	0.810	2,2'-Dimethylbiphenyl	$C_{14}H_{14}$	605-39-0	1495	-	
795	0.790	1,1-Diethyl-1,2,3,4-tetrahydronaphthalene	$C_{14}H_{20}$	2938-66-1	1501	-	
795	1.110	(1-Ethylhexyl)benzene	$C_{14}H_{22}$	18335-15-4	1501	-	
810	0.920	1,1-Diphenyl-ethylene	$C_{14}H_{12}$	530-48-3	1519	-	
835	0.830	2-Methyl-6-phenyl-1,6-heptadiene	$C_{14}H_{18}$	51708-97-5	1613	-	
855	0.850	1,4-Diethyl-1,2,3,4-tetrahydronaphthalene	$C_{14}H_{20}$	81356-57-2	1572	-	
880	0.790	(3-Methyl-1-methylenepentyl)benzene	$C_{13}H_{18}$	74810-69-8	1601	-	
895	0.900	1,1-Diethylnaphthalene	$C_{14}H_{20}$	74710-00-2	1619	-	
925	0.930	1,3-Diphenylpropane	$C_{15}H_{16}$	1081-75-0	1654	1633	(454)
935	0.530	3-Phenyldecane	$C_{16}H_{26}$	4621-36-7	1665	-	
950	0.860	1,3-Diphenylbutane	$C_{16}H_{18}$	1520-44-1	1683	-	

975	0.890	1,2,3-Trimethyl-4-propenylnaphthalene	$C_{16}H_{18}$	26137-53-1	1713	-	
990	0.520	5-Phenyldodecane	$C_{18}H_{30}$	2719-63-3	1730	1731	(455)
1000	0.530	4-Phenyldodecane	$C_{18}H_{30}$	2719-64-4	1742	-	
1005	0.950	9-Ethyl-9,10-dihydro-10-methyl-anthracene	$C_{17}H_{18}$	36778-20-8	1748	-	
1020	0.540	3-Phenyldodecane	$C_{18}H_{30}$	2400-00-2	1765	-	
1070	0.530	4-Phenyltridecane	$C_{19}H_{32}$	4534-51-4	1824	-	
1175	0.980	(1,2-Dicyclopropyl-2-phenylethyl)benzene	$C_{20}H_{22}$	110330-90-0	1951	-	
		Ketones					
		Aliphatics					
75	0.370	2-Propanone	C_3H_6O	67-64-1	559	572	(456)
90	0.410	2-Butanone	C_4H_8O	78-93-3	590	601	(430)
120	0.430	2-Pentanone	$C_5H_{10}O$	107-87-9	654	682	(437)
130	1.130	1-Hydroxy-2-propanone	$C_3H_6O_2$	116-09-6	676	662	(457)
140	1.010	3-Hydroxy-2-butanone	$C_4H_8O_2$	513-86-0	707	697	(412)
150	0.450	4-Methyl-2-pentanone	$C_6H_{12}O$	108-10-1	717	733	(437)
180	0.470	3-Hexanone	$C_6H_{12}O$	589-38-8	780	783	(437)
185	0.500	2-Hexanone	$C_6H_{12}O$	591-78-6	791	795	(418)
190	0.550	3-Hexen-2-one	$C_6H_{10}O$	763-93-9	801	834	(422)
195	0.990	3-Hydroxy-2-pentanone	$C_5H_{10}O_2$	3142-66-3	807	838	(446)
200	1.030	2-Hydroxy-3-pentanone	$C_5H_{10}O_2$	5704-20-1	813	-	
220	0.520	2,3-Heptanedione	$C_7H_{12}O_2$	96-04-8	836	-	
235	0.510	5-Methyl-2-hexanone	$C_7H_{14}O$	110-12-3	854	862	(423)
250	0.480	4-Heptanone	$C_7H_{14}O$	123-19-3	871	869	(417)
265	0.500	3-Heptanone	$C_7H_{14}O$	106-35-4	889	887	(430)
265	0.520	2-Heptanone	$C_7H_{14}O$	110-43-0	889	892	(444)
290	0.510	5-Methyl-2-heptanone	$C_8H_{16}O$	18217-12-4	916	971	(458)
310	0.500	4-Methyl-2-heptanone	$C_8H_{16}O$	6137-06-0	937	936	(437)

325	0.520	2-Methyl-6-heptanone	$C_8H_{16}O$	928-68-7	953	962	(458)
355	0.510	3-Octanone	$C_8H_{16}O$	106-68-3	985	990	(418)
360	0.540	2-Octanone	$C_8H_{16}O$	111-13-7	990	994	(444)
445	0.520	3-Nonanone	$C_9H_{18}O$	925-78-0	1084	1091	(459)
450	0.550	2-Nonanone	$C_9H_{18}O$	821-55-6	1090	1093	(460)
470	0.650	2,6-Dimethyl-2,5-heptadien-4-one	$C_9H_{14}O$	504-20-1	1112	-	
500	0.760	2,5-Dimethyl-3,4-hexanedione	$C_8H_{14}O_2$	4388-87-8	1145	-	
540	0.540	2-Decanone	$C_{10}H_{20}O$	693-54-9	1190	1194	(437)
575	0.730	4-Methyl-3-octanone	$C_9H_{18}O$	6137-15-1	1230	-	
610	0.510	5-Undecanone	$C_{11}H_{22}O$	33083-83-9	1271	-	
625	0.550	2-Undecanone	$C_{11}H_{22}O$	112-12-9	1289	1291	(444)
790	0.570	2-Tridecanone	$C_{13}H_{26}O$	593-08-8	1495	1498	(420)
850	0.580	2-Tetradecanone	$C_{14}H_{28}O$	2345-27-9	1565	1597	(461)
875	0.590	3-Tetradecanone	$C_{14}H_{28}O$	629-23-2	1595	-	
940	0.550	6-Tetradecanone	$C_{14}H_{28}O$	6836-42-6	1671	-	
965	0.590	2-Pentadecanone	$C_{15}H_{30}O$	2345-28-0	1701	1698	(462)
1025	0.600	2-Hexadecanone	$C_{16}H_{32}O$	18787-63-8	1771	1800	(461)
1130	0.600	2-Heptadecanone	$C_{17}H_{34}O$	2922-51-2	1895	1900	(417)
		Aromatics					
430	1.050	1-Phenylethanone	C_8H_8O	98-86-2	1068	1067	(418)
485	1.040	1-Phenyl-2-propanone	$C_9H_{10}O$	103-79-7	1129	1110	(463)
520	1.120	1-(2-Hydroxyphenyl)-ethanone	$C_8H_8O_2$	118-93-4	1168	1167	(464)
525	1.100	1-Phenyl-1,2-propanedione	$C_9H_8O_2$	579-07-7	1173	1186	(465)
575	0.910	1-Phenyl-2-butanone	$C_{10}H_{12}O$	1007-32-5	1231	-	
590	0.960	4-Phenyl- 2-butanone	$C_{10}H_{12}O$	2550-26-7	1248	1218	(466)
595	0.850	1-Phenyl-1-butanone	$C_{10}H_{12}O$	495-40-9	1254	1253	(467)
650	0.960	1-(4-Ethylphenyl)-ethanone	$C_{10}H_{12}O$	937-30-4	1320	1274	(437)
700	0.950	3-Methyl-1-phenyl-2-buten-1-one	$C_{11}H_{12}O$	5650-07-7	1382	-	

750	0.870	1-(4-tert-Butylphenyl)propan-2-one	$C_{13}H_{18}O$	81561-77-5	1445	-	
915	1.460	Benzophenone	$C_{13}H_{18}O$	119-61-9	1643	1621	(417)
1065	1.070	3,5-di-tert-Butyl-4-hydroxyacetophenone	$C_{16}H_{24}O_2$	14035-33-7	1819	-	
1120	1.350	2,2-Dimethoxy-1,2-diphenyl-ethanone	$C_{16}H_{16}O_3$	24650-42-8	1884	1874	(468)
		Cyclics					
275	0.730	Cyclohexanone	$C_6H_{10}O$	1629-59-408-94-1	901	897	(437)
470	0.780	3,4,4-Trimethyl-2-cyclopenten-1-one	$C_8H_{12}O$	30434-65-2	1112	-	
480	0.780	3,5,5-Trimethyl-2-cyclohexen-1-one	$C_9H_{14}O$	78-59-1	1123	1118	(417)
490	0.710	4-Cyclopentylidene-2-butanone	$C_9H_{14}O$	51004-21-8	1134	-	
770	0.620	2,6-di-Butyl-2,5-cyclohexadiene-1,4-dione	$C_{14}H_{20}O_2$	719-22-2	1470	1472	(469)
770	0.940	2,6-di(t-Butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	$C_{15}H_{24}O_2$	10396-80-2	1470	1478	(470)
		***N*-Compounds**					
		Amines					
		Aliphatics					
140	0.540	*N*-methyleneethenamine	C_3H_5N	38239-27-9	696	-	
		Aromatics					
335	0.600	Benzenamine	C_6H_7N	62-53-3	964	971	(461)
595	0.750	2,4,6-Trimethyl-benzenamine	$C_9H_{13}N$	88-05-1	1254	1261	(461)
655	0.560	4,5-Dimethyl-ortho-phenylenediamine	$C_8H_{12}N_2$	3171-45-7	1326	-	
655	0.730	4-Ethylphenethylamine	$C_{10}H_{15}N$	64353-29-3	1326	-	
		Cyclics					
470	0.680	1-Methyl-1H-pyrrole-2-ethanamine	$C_7H_{12}N_2$	83732-75-6	1112	-	
		Amides					
		Aliphatics					
515	0.880	Dibutyramide	$C_8H_{15}NO_2$	4494-12-6	1162	-	
640	0.700	*N,N*-Dibutylformamide	$C_9H_{19}NO$	761-65-9	1307	-	
		Aromatics					

740	0.750	2-Phenylbutanamide	$C_{10}H_{13}NO$	90-26-6	1432	-	
		Oximes					
240	1.420	3-Methylbutanal oxime (isomer)	$C_5H_{11}NO$	5780-40-5	860	-	
260	1.480	3-Methylbutanal oxime (isomer)	$C_5H_{11}NO$	5775-74-6	884	-	
		Pyridines					
360	0.640	2,4,6-Trimethylpyridine	$C_8H_{11}N$	108-75-8	990	987	(461)
400	1.030	1-(2-Pyridinyl)-ethanone	C_7H_7NO	1122-62-9	1035	1057	(471)
535	0.680	4-Pyrrolidinopyridine	$C_9H_{12}N_2$	2456-81-7	1184	-	
710	0.820	2-Butyrylpyridine	$C_9H_{11}NO$	22971-32-0	1395	-	
900	1.050	4-(4-Dimethylaminophenyl)-pyridine	$C_{13}H_{14}N_2$	1137-80-0	1625	-	
		Pyrimidines					
765	1.360	2-Phenylpyrimidine	$C_{10}H_8N_2$	7431-45-0	1464	-	
980	1.030	5-Ethyl-2-(4-ethylphenyl)pyrimidine	$C_{14}H_{16}N_2$	98495-10-4	1719	-	
		Others					
355	1.240	Benzonitrile	C_7H_5N	100-47-0	986	988	(437)
500	1.700	Phenylacetonitrile	C_8H_7N	140-29-4	1146	1137	(421)
645	1.890	2-Aminoacetophenone	C_8H_9NO	551-93-9	1315	1315	(472)
650	3.460	Indole	C_8H_7N	120-72-9	1323	1320	(473)
720	2.890	3-Methylindole	C_9H_9N	83-34-1	1410	1410	(411)
		Pyrazine					
150	0.770	Pyrazine	$C_4H_4N_2$	290-37-9	717	740	(474)
210	0.750	Methylpyrazine	$C_5H_6N_2$	109-08-0	824	827	(434)
285	0.730	2,5-Dimethylpyrazine	$C_6H_8N_2$	123-32-0	911	915	(434)
290	0.730	Ethylpyrazine	$C_6H_8N_2$	13925-00-3	917	920	(434)
290	0.740	2,3-Dimethylpyrazine	$C_6H_8N_2$	5910-89-4	917	932	(475)
305	0.900	Ethenylpyrazine	$C_6H_6N_2$	4177-16-6	933	954	(474)
340	0.650	Isopropylpyrazine	$C_7H_{10}N_2$	9820-90-0	969	-	

365	0.660	2-Ethyl-5-methylpyrazine	$C_7H_{10}N_2$	13360-64-0	995	1000	(432)
370	0.680	Trimethylpyrazine	$C_7H_{10}N_2$	14667-55-1	1001	1014	(471)
385	0.810	2-Ethenyl-5-methylpyrazine	$C_7H_8N_2$	13925-08-1	1018	1034	(471)
415	0.620	2-Methyl-3-isopropylpyrazine	$C_8H_{12}N_2$	15986-81-9	1051	-	
440	0.630	3-Ethyl-2,5-dimethyl-pyrazine	$C_8H_{12}N_2$	13360-65-1	1083	1088	(471)
455	0.600	2-Ethyl-3,5-dimethyl-pyrazine	$C_8H_{12}N_2$	13925-07-0	1095	1084	(476)
460	0.720	2-Methyl-5-(1-propenyl)-pyrazine	$C_8H_{10}N_2$	18217-82-8	1101	1133	(477)
465	0.550	2,5-Dimethyl-3-propylpyrazine	$C_9H_{14}N_2$	18433-97-1	1106	1131	(432)
480	0.970	1-(6-Methyl-2-pyrazinyl)-1-ethanone	$C_7H_8N_2O$	22047-26-3	1123	1095	(478)
500	0.610	2-Isobutyl-3-methylpyrazine	$C_9H_{14}N_2$	13925-06-9	1145	1144	(263)
510	0.580	3,5-Diethyl-2-methyl-pyrazine	$C_9H_{14}N_2$	18138-05-1	1156	1172	(471)
520	0.590	2,3-Dimethyl-5-propylpyrazine	$C_9H_{14}N_2$	32262-98-9	1167	1166	(475)
525	0.790	1-(3,5-Dimethylpyrazinyl)-ethanone	$C_8H_{10}N_2O$	54300-08-2	1173	-	
530	0.650	2-Isoamylpyrazine	$C_9H_{14}N_2$	40790-22-5	1179	-	
535	0.530	2,3-Dimethyl-5-(1-methylpropyl)-pyrazine	$C_{10}H_{16}N_2$	32263-00-6	1184	-	
545	0.630	2-Pentyl-5-methylpyrazine	$C_{10}H_{16}N_2$	-	1195	-	
545	0.820	2-Methyl 5H-6,7-dihydrocyclopentapyrazine	$C_8H_{10}N_2$	-	1195	-	
550	0.570	2,5-Dimethyl-3-(2-methylpropyl)-pyrazine	$C_{10}H_{16}N_2$	32736-94-0	1201	1207	(476)
570	0.560	2,5-Diethyl-3,6-dimethylpyrazine	$C_{10}H_{16}N_2$	-	1224	1225	(479)
580	0.750	2,5-Dimethyl-3-(E-1-propenyl)pyrazine	$C_9H_{12}N_2$	55138-77-7	1236	-	
585	0.570	2,3,5-Trimethyl-6-propylpyrazine	$C_{10}H_{16}N_2$	92233-82-4	1242	1280	(471)
590	0.620	2-Isoamyl-6-methylpyrazine	$C_{10}H_{16}N_2$	91010-41-2	1248	1248	(432)
595	0.590	2-Butyl-3,5-dimethylpyrazine	$C_{10}H_{16}N_2$	50888-63-6	1254	-	
615	0.540	2-(2-Methylpropyl)-3-(1-methylethyl)pyrazine	$C_{11}H_{18}N_2$	-	1277	-	
635	0.560	2,6-Dimethyl-3(2-methyl-1-butyl)pyrazine	$C_{11}H_{18}N_2$	56617-70-0	1301	1307	(476)
645	0.580	2,5-Dimethyl-3-(3-methylbutyl)-pyrazine	$C_{11}H_{18}N_2$	18433-98-2	1313	1319	(476)
705	0.560	2,3,5-Trimethyl-6-isopentylpyrazine	$C_{12}H_{20}N_2$	10132-43-1	1388	1390	(476)
710	0.630	2-Methyl-3-octylpyrazine	$C_{13}H_{20}N_2$	71700-39-5	1395	-	

755	0.600	Hexyl-5-dimethyl-2,3-pyrazine	$C_{12}H_{20}N_2$	73570-19-1	1451	-	
		Terpenes					
		Monoterpenes					
305	0.380	α-Pinene	$C_{10}H_{16}$	7785-26-4	932	932	(412)
345	0.410	Sabinene	$C_{10}H_{16}$	3387-41-5	974	1009	(480)
375	0.420	γ-Terpinene	$C_{10}H_{16}$	99-85-4	1006	1080	(481)
395	0.440	Limonene	$C_{10}H_{16}$	5989-54-8	1028	1028	(412)
435	0.630	Dihydromyrcenol	$C_{10}H_{20}O$	18479-58-8	1073	1076	(481)
520	0.840	Isoborneol	$C_{10}H_{18}O$	124-76-5	1168	1159	(482)
525	0.720	Dihydro γ-terpineol	$C_{10}H_{20}O$	21129-27-1	1173	1206	(481)
540	0.780	α-Terpineol	$C_{10}H_{18}O$	98-55-5	1190	1195	(412)
545	0.700	Tetrahydrogeraniol	$C_{10}H_{22}O$	106-21-8	1195	1196	(483)
575	0.880	Nerol	$C_{10}H_{18}O$	106-25-2	1230	1245	(481)
670	0.580	4-Terpinenyl acetate	$C_{12}H_{20}O_2$	4821-04-9	1345	1300	(484)
		Sesquiterpenes					
760	0.650	β-Caryophyllene	$C_{15}H_{24}$	87-44-5	1457	1423	(485)
850	0.720	Nerolidol	$C_{15}H_{26}O$	7212-44-4	1566	1573	(412)
1035	0.680	Methyl farnesoate	$C_{16}H_{26}O_2$	3675-00-1	1783	1789	(442)
		Norisoprenoids					
355	0.610	6-Methyl-5-hepten-2-one	$C_8H_{14}O$	110-93-0	985	990	(418)
755	0.640	Geranylacetone	$C_{13}H_{22}O$	3796-70-1	1451	1455	(486)
780	0.630	α-iso-Methyl ionone	$C_{14}H_{22}O$	127-51-5	1482	1481	(487)

S-Compounds

		Aliphatics					
70	0.320	Methanethiol	CH_4S	74-93-1	548	464	(488)
70	2.650	Mercaptoacetic acid	C_2H_4OS	68-11-1	553	-	
80	0.320	Carbon disulfide	CS_2	75-15-0	569	544	(489)
125	0.520	Methylthiolacetate	C_3H_6OS	1534-08-3	664	699	(490)
150	0.530	Dimethyl disulfide	$C_2H_6S_2$	624-92-0	717	719	(412)
225	0.870	Methylthio-2-propanone	C_4H_8OS	14109-72-9	842	863	(423)
280	1.040	3-(methylthio)-propanal	C_4H_8OS	3268-49-3	906	904	(411)
340	0.710	Dimethyl trisulfide	$C_2H_6S_3$	3658-80-8	969	967	(430)
340	0.730	2-Methylsulfinylethanol	$C_3H_8O_2S$	21281-74-3	969	-	
445	1.230	5-Methyl-2-thiophenecarboxaldehyde	C_6H_6OS	13679-70-4	1085	1135	(434)
455	1.350	2-Acetylthiophene	C_6H_6OS	88-15-3	1096	1092	(445)
485	1.310	3-Methyl-2-thiophenecarboxaldehyde	C_6H_6OS	5834-16-2	1129	1133	(434)
510	1.050	2-Acetyl-5-methylthiophene	C_7H_8OS	13679-74-8	1157	1157	(445)
540	0.830	6-(Methylthio)hexa-1,5-dien-3-ol	$C_7H_{12}OS$	-	1190	-	
540	0.880	2,6-Dimethyl-4-thiopyrone	C_7H_8OS	1004-37-1	1190	1193	(491)
540	1.120	2-Propionylthiophene	C_7H_8OS	13679-75-9	1190	1188	(492)
545	1.000	Dimethyl formylthiophene	C_7H_7OS	-	1196	-	
565	0.850	Dimethyl tetrasulphide	$C_2H_6S_4$	5756-24-1	1219	1234	(459)
765	0.570	Dimethyl pentasulfide	$C_2H_6S_5$	7330-31-6	1463	-	
825	0.530	1-Dodecanethiol	$C_{12}H_{26}S$	112-55-0	1536	-	
860	0.730	2-Methyl-2-undecanethiol	$C_{12}H_{16}S$	10059-13-9	1577	-	
910	0.520	Dodecyl methyl sulfide	$C_{13}H_{28}S$	3698-89-3	1636	-	

[a] Retention times for first (^1t_R) and second (^2t_R) dimensions in seconds.

[b] RI,Retention Index obtained through the modulated chromatogram.

[c] RI, Retention Index reported in the literature for Equity-5 column or equivalents.

Table B5.3 - Volatiles used for statistics processing identified by HS-SPME/GC×GC-ToFMS in three *Staphylococcus aureus* strains under study: ATCC 6538 (without enterotoxins; light grey), 2153 MA (with enterotoxin A; medium grey) and 2065 MA (with enterotoxin A, G, I; dark grey).

Compound	Name	CAS Number	ATCC 6538 (x10^2)			2153 MA (x10^2)			2065 MA (x10^2)		
			Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
	Acids										
	Aliphatics										
1	Acetic acid	64-19-7	4.42	4.84	0.18	0.1	0.06	0.07	0.07	0.09	2.68
2	3-Methylbutanoic acid	503-74-2	4.19	13.47	3.09	13.67	6.14	4.1	16.86	17.76	23.24
		Subtotal (GC Peak Area)		**10.06**			**8.05**			**20.23**	
		Subtotal (%)		**8.11**			**6.42**			**12.30**	
	Alcohols										
	Aliphatics										
3	Ethanol	64-17-5	1.29	0.04	0.14	1.9	0.23	0.21	0.42	0.21	1.53
4	2-Methyl-1-propanol	78-83-1	0.09	0.08	0.06	0.21	0.16	0.17	1.11	0.94	1.27
5	1-Butanol	71-36-3	0.04	0.03	0.02	0.03	0.03	0.03	0.02	0.02	0.02
6	3-methyl-1-butanol	123-51-3	3.06	3.08	1.74	6.09	4.7	4.72	14.05	16.49	14.91
7	3-Methyl-3-buten-1-ol	763-32-6	0.2	0.16	0.22	0.15	0.23	0.15	0.13	7.66	0.12
8	3-Methyl-2-buten-1-ol	556-82-1	0.09	0.07	0.07	0.05	0.08	0.07	0.07	0.07	0.08
9	2,3-Butanediol	513-85-9	0.04	0.05	0.1	0.01	0.02	0.01	0.02	0.02	0.02
10	2-Methyl-3-hexanol	617-29-8	0.06	0.06	0.07	0.04	0.05	0.04	0.03	0.04	0.04
11	1-Heptanol	111-70-6	0.14	0.04	0.02	0.05	0.15	0.04	0.08	0.04	0.02
12	1-Octen-3-ol	3391-86-4	0.13	0.06	0.07	0.13	0.1	0.1	0.14	0.11	0.13
13	6-Methyl-1-heptanol	1653-40-3	0.51	0.16	0.07	0.36	0.23	0.04	0.18	0.12	0.07
14	2-Ethyl-1-hexanol	104-76-7	5.14	2.07	2.05	3.68	2.61	2.53	3.07	3.08	2.65

15	4-Methyl-1-heptanol	817-91-4	0.6	0.09	0.07	0.9	0.33	0.04	12.29	1.07	0.06
16	2,3-Octanediol	20653-90-1	0.03	0.01	0.02	0.01	0.01	0.02	0.07	0.05	0.04
17	1-Octanol	111-87-5	1.77	0.23	0.19	0.56	0.62	0.4	0.65	0.36	0.4
18	4-Methyl-1-hepten-4-ol	1186-31-8	0.65	0.55	0.49	0.38	0.57	0.62	0.86	0.98	0.92
19	1-Nonanol	143-08-8	0.49	0.04	1.03	0.57	0.53	0.48	0.36	0.17	0.22
20	2-Methyl-2-nonanol	10297-57-1	0.46	0.33	0.34	0.48	0.4	0.39	0.32	0.41	0.43
21	2,4-Undecadien-1-ol	59376-58-8	0.08	0.11	0.08	0.13	0.08	0.09	0.07	0.08	0.05
22	2-Methyl-1-undecanol	10522-26-6	0.61	0.01	0.35	0.05	0.04	0.53	0.05	0.02	0.04
23	1-Dodecanol	112-53-8	8.94	7.49	4.13	7.42	4.1	4.23	4.62	7.67	4.07
24	5,9-Dimethyl-1-decanol	91482-38-1	0.04	0.03	0.02	0.03	0.03	0.02	0.04	0.03	0.04
25	1-Tetradecanol	112-72-1	0.61	0.77	0.68	0.42	0.1	0.33	1.27	0.8	2.46
	Aromatics										
26	Benzyl alcohol	100-51-6	0.87	0.79	0.78	1.38	1.83	1.55	1	1.06	1.03
27	2-Methoxyphenol	90-05-1	0.46	0.06	0.04	0.21	0.32	0.03	0.34	0.11	0.07
28	4-Methylphenol	106-44-5	1.8	1.62	1.55	1.81	2.21	2.11	1.64	2.22	1.95
29	2-Phenylethanol	60-12-8	0.15	0.05	0.04	0.11	0.13	0.15	0.6	0.53	0.48
30	2-tert-Butyl-4-methylphenol	2409-55-4	0.9	0.87	0.49	0.88	0.34	0.58	0.51	0.95	0.64
31	2,4-Dimethyl-6-tert-butylphenol	1879-09-0	0.07	0.04	0.03	0.05	0.03	0.04	0.05	0.05	0.05
32	3,5-Diisopropylphenol	26886-05-5	0.41	0.33	0.22	0.33	0.15	0.25	0.29	0.4	0.29
33	2,4-Di-tert-butylphenol	96-76-4	4.85	5.19	5.2	3.61	3.23	4.56	4.55	4.9	4.27
34	2,3-Dihydro-3-phenyl-1H-inden-1-ol	79454-17-4	0.13	0.06	0.01	0.04	0.07	0.04	0.05	0.06	0.05
		Subtotal (GC Peak Area)		**26.49**			**26.77**			**46.01**	
		Subtotal (%)		**21.36**			**21.35**			**27.98**	
	Aldehydes										
	Aliphatics										
35	2−Methylpropanal	78-84-2	0.29	0.27	0.13	0.26	0.34	0.25	0.42	0.42	0.58
36	2−Methylbutanal	96-17-3	0.69	0.88	0.42	0.85	0.84	0.36	0.53	1.32	1.72

37	3-Methyl-2-butenal	107-86-8	0.08	0.06	0	0.01	0.12	0.09	0.15	0.81	0.18
38	Hexanal	66-25-1	0.1	0.05	0.04	0.1	0.14	0.14	0.1	0.07	0.08
39	2-Methyl-2-hexenal	28467-88-1	0.02	0.06	0.06	0.03	0.07	0.05	0.03	0.02	0.03
40	Heptanal	111-71-7	0.12	0	0.04	0.11	0.16	0.08	0.11	0.02	0
41	2-Heptenal	2463-63-0	0.04	0.03	0.03	0.03	0.05	0.03	0.01	0.04	0.04
42	Octanal	124-13-0	0.17	0.1	0.06	0.31	0.51	0.19	0.23	0.18	0.06
43	Nonanal	124-19-6	1.24	0.1	0.08	2.42	2.65	1.05	1.08	0.48	0.32
44	Decanal	112-31-2	0.43	0.11	0.26	0.9	0.87	0.44	0.42	0.33	0.42
45	Undecanal	112-44-7	0.15	0.05	0.07	0.31	0.04	0.37	0.1	0.09	0.14
46	Dodecanal	112-54-9	0.29	0.11	0.06	1.07	0.53	0.55	0.19	0.17	0.11
47	Tridecanal	10486-19-8	0.03	0.02	0.04	0.05	0.04	0.04	0.02	0.02	0.03
48	Tetradecanal	124-25-4	0.13	0.05	0.03	0.06	0.06	0.03	0.04	0.04	0.03
49	Pentadecanal	2765-11-9	0.02	0.02	0.01	0.01	0.01	0.01	0.03	0.02	0.02
	Aromatics										
50	Benzaldehyde	100-52-7	5.11	4.46	3.64	8.41	10.48	10.76	6.69	7.07	7.07
51	2-Hydroxybenzaldehyde	90-02-8	0.15	0.1	0.08	0.17	0.16	0.22	0.12	0.2	0.2
52	3,5-Dimethylbenzaldehyde	5779-95-3	1.59	0.78	0.32	1.09	0.93	0.56	0.69	0.59	0.42
53	4-(1-Methylethyl)-benzaldehyde	122-03-2	0.14	0.02	0.01	0.15	0.06	0.02	0.07	0.05	0.01
54	4-tert-Butylbenzaldehyde	939-97-9	0.16	0.02	0.04	0.11	0.06	0.03	0.14	0.18	0.07
55	2-phenyl-4-pentenal	24401-36-3	0.27	0.1	0.09	0.38	0.06	0.11	0.22	0.26	0.12
56	5-Methyl-2-phenyl-2-hexenal	21834-92-4	0.4	0.26	0.24	0.15	0.2	0.18	1.78	0.77	0.85
57	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	1620-98-0	0.47	0.07	0.05	0.06	0.19	0.07	0.34	0.09	0.08
		Subtotal (GC Peak Area)		**7.84**			**16.4**			**11.92**	
		Subtotal (%)		**6.32**			**13.08**			**7.25**	
	Esters										
	Aliphatics										
58	Ethyl propanoate	105-37-3	0.15	0.08	0.02	0.05	0.06	0.05	0.04	0.04	0.05

59	Methyl methacrylate	80-62-6	0.06	0.15	0.06	0.05	0.05	0.06	0.04	0.06	0.05
60	Methyl isovalerate	556-24-1	0.24	0.54	0.48	0.36	0.43	0.29	0.39	0.38	0.49
61	Butyl acetate	123-86-4	0.21	0.16	0.12	0.11	0.08	0.15	0.14	0.14	0.19
62	Ethyl 2-methylbutanoate	7452-79-1	0.2	0.17	0.06	0.35	0.3	0.47	0.28	0.21	0.34
63	3-Methyl-3-butenyl acetate	5205-07-2	0.02	0.02	0.02	0.03	0.03	0.03	0.05	0.04	0.05
64	Methyl 2-hydroxy-4-methylpentanoate	40348-72-9	0.06	0.04	0.06	0.02	0.04	0.03	0.49	0.35	0.34
65	Propyl 2,2-dimethylpropanoate	5129-35-1	0.01	0.46	0.48	0.27	0.26	0.27	0.37	0.36	0.41
66	tert-Butyl isopropyl carbonate	30221-21-7	0.41	0.26	0.23	0.19	0.24	0.17	0.23	0.36	0.3
67	Dimethyl 2,4-dimethylpentanedioate	2121-68-8	13.37	4.4	0.47	9.67	4.67	1.01	6.74	7.84	1.04
68	Dimethyl isopropylidenesuccinate	87384-00-7	1.31	0.24	0.04	0.87	0.44	0.02	0.54	0.53	0.01
69	Dimethyl 2-methylhexanedioate	19780-94-0	0.49	0.59	0.07	3.37	1.32	0.9	0.42	1.12	0.08
70	1-Ethyl 6-methyl hexanoate	18891-13-9	3.42	0.67	0.16	2.27	1.67	0.22	2.28	1.42	0.15
71	Dimethyl 2-(2-methylprop-2-enyl)propanedioate	50598-40-8	0.36	0.08	0.02	0.27	0.15	0.1	0.27	0.18	0.03
72	Dimethyl 2,2-dimethylpentanedioate	13051-32-6	0.67	0.27	0.1	0.62	0.2	0.07	0.33	0.54	0.1
73	Dimethyl 2,3-dimethyl hexanodioate	58219-48-0	0.43	0.31	0.13	0.42	0.21	0.08	4.91	0.71	0.09
74	1-Methylhexyl butanoate	39026-94-3	0.76	0.25	0.21	0.38	0.52	0.24	0.56	0.32	0.29
75	2-Ethyl-3-hydroxyhexyl butyrate	18618-89-8	1.3	0.63	0.34	0.93	0.91	0.54	1.27	0.89	0.65
76	Diethyl diallylmalonate	3195-24-2	0.18	0.05	0.01	0.07	0.11	0.03	0.11	0.06	0.01
	Aromatics										
77	Methyl benzoate	93-58-3	0.62	0.56	0.92	0.23	0.28	0.25	0.19	0.18	0.19
78	Methyl phenylacetate	101-41-7	0.11	0.12	0.17	0.15	0.15	0.08	0.13	0.16	0.13
79	1-Phenylethyl acetate	93-92-5	0.04	0.03	0.02	0.03	0.09	0.02	0.05	0.05	0.03
80	Ethyl phenylethanoate	101-97-3	0.23	0.04	0	0.14	0.1	0.07	0.1	0.09	0.08
81	2-Benzylacrylic acid methyl ester	3070-71-1	2.62	1.32	0.4	2.37	0.78	0.67	1.46	1.7	0.62
82	Cinnamyl methanoate	104-65-4	0.06	0.08	0.03	0.1	0.06	0.02	0.08	0.11	0.06
83	2-Methyl-4-phenylbutyric acid methyl ester	-	16.36	9.59	6.49	12.71	5.99	8.19	8.99	13.39	8.37
84	5-Phenyl-2-pentenoic acid methyl ester	26429-97-0	0.07	0.09	0.04	0.01	0.03	0.01	0.1	0.12	0.09
85	Ethyl 5-phenyl-2-pentenoate	55282-95-6	2.5	2.15	0.71	1.71	0.29	1.28	1.33	2.05	1.96

86	Dimethyl phenylethyl carbinyl acetate	103-07-1	0.04	0.06	0.03	0.03	0.02	0.02	0.01	0.03	0.01
87	Phenyl benzoate	93-99-2	0.04	0.02	0	0.03	0.01	0	0.03	0.04	0
88	Hexyl 2-hydroxybenzoate	6259-76-3	0.01	0.01	0.01	0.01	0	0.01	0.01	0.01	0.01
89	2-Ethylhexyl 2-hydroxybenzoate	118-60-5	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
	Cyclics											
90	Butyl cyclopropanecarboxylate	54947-39-6	0.04	0.01	0.02	0.03	0.03	0.02	0.55	0.28	0.36
		Subtotal (GC Peak Area)		**27.26**			**24.26**			**27.62**	
		Subtotal (%)		**21.98**			**19.35**			**16.80**	
	Hydrocarbons											
	Aliphatics											
91	2-Butene	107-01-7	0.07	0.04	0.04	0.04	0.02	0.05	0.04	0.05	0.05
92	Octane	111-65-9	0.08	0.03	0.04	0.04	0.08	0.03	0.05	0.03	0.03
93	Nonane	111-84-2	0.04	0.02	0.03	0.04	0.06	0.03	0.14	0.07	0.08
94	2,2,4,6,6-Pentamethyl-3-heptene	123-48-8	1.11	1.24	0.55	0.8	1.52	0.32	0.98	1.49	0.1
95	3-Ethyl-2,7-dimethyl-octane	62183-55-5	0.24	0.12	0.06	0.03	0.2	0.05	0.03	0.16	0.24
96	6-Methyl-3-undecene	74630-52-7	0.12	0.11	0.04	0.09	0.06	0.03	0.04	0.08	0.1
97	Dodecene	112-41-4	0.48	0.2	0.12	0.59	0.39	0.31	0.27	0.29	0.23
98	4-Ethylundecane	17312-59-3	0.04	0.02	0.02	0.05	0.11	0.04	0.02	0.04	0.04
99	Tetradecene	1120-36-1	0.21	0.06	0.1	0.26	0.28	0.12	0.08	0.11	0.22
100	2-Methyltetradecane	1560-95-8	0.02	0.01	0.02	0.02	0.04	0.01	0.01	0.01	0.01
101	3-Ethyl-3-methyltridecane	-	0.09	0.05	0.03	0.05	0.02	0.02	0.03	0.02	0.01
102	Hexadecane	544-76-3	0.11	0.09	0.05	0.08	0.19	0.09	0.05	0.04	0.06
103	Heptadecane	629-78-7	0.12	0.08	0.21	0.03	0.12	0.03	0.03	0.01	0.04
104	Octadecane	593-45-3	0.05	0.04	0.03	0.03	0.03	0.03	0.02	0.02	0.04
105	Nonadecane	629-92-5	0.02	0.01	0.02	0.02	0.01	0.02	0.02	0.01	0.03
106	Eicosane	112-95-8	0.06	0.01	0.04	0.06	0.01	0.01	0.01	0.01	0.05
	Aromatics											

107	Methylbenzene	108-88-3	1.78	2.88	2.49	1.11	1.52	0.83	1.22	2.02	2.05
108	1,3-Dimethylbenzene	108-38-3	0.31	0.12	0.11	0.18	0.23	0.09	0.12	0.19	0.16
109	1-Ethyl-4-methylbenzene	622-96-8	0.11	0.11	0.06	0.12	0.1	0.07	0.08	0.16	0.13
110	1,2,3-Trimethylbenzene	526-73-8	0.13	0.08	0.05	0.18	0.09	0.08	0.06	0.13	0.07
111	2-Phenyl-1-butene	2039-93-2	0.07	0.03	0.03	0.14	0.09	0.05	0.04	0.02	0.03
112	2-Ethyl-1,3-dimethylbenzene	2870-04-4	0.04	0.04	0.02	0.04	0.04	0.01	0.02	0.02	0.02
113	Naphthalene	91-20-3	0.68	0.38	0.23	0.87	0.55	0.41	0.36	0.39	0.22
114	1-Methylnaphthalene	90-12-0	0.1	0.08	0.05	0.12	0.08	0.08	0.07	0.06	0.06
115	2-Methylnaphthalene	91-57-6	0.08	0.06	0.04	0.1	0.03	0.07	0.07	0.07	0.06
116	5,6,7,8,9,10-Hexahydrobenzocyclooctene	1076-69-3	0.18	0.08	0.08	0.19	0.07	0.13	0.01	0.08	0.11
117	4-Phenyl-1-cyclohexene	4994-16-5	0.02	0.01	0.01	0.03	0.01	0.02	0.01	0.01	0.01
118	Biphenyl	92-52-4	0.03	0.02	0.03	0.03	0.02	0.03	0.02	0.03	0.02
119	1,4-Dimethylnaphthalene	571-58-4	0.03	0.02	0.03	0.09	0.03	0.05	0.01	0.02	0.01
120	Diphenylmethane	101-81-5	0.02	0.02	0.02	0.05	0.01	0.03	0.01	0.01	0.01
121	3-Ethyl-3-phenyl-1-pentene	19781-34-1	7.77	6.69	2.9	6.25	1.98	4.16	3.96	7.71	5.07
122	3-Methylbiphenyl	643-93-6	0.02	0.02	0.02	0.08	0.04	0.09	0.01	0.03	0.02
123	Acenaphthene	83-32-9	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
124	2,2'-Dimethylbiphenyl	605-39-0	0.19	0.13	0.06	0.09	0.02	0.08	0.04	0.04	0.05
125	1,1-Diethyl-1,2,3,4-tetrahydronaphthalene	2938-66-1	0.93	0.67	0.38	0.68	0.47	0.49	0.52	0.6	0.41
126	(1-Ethylhexyl)benzene	18335-15-4	0.14	0.12	0.11	0.1	0.09	0.12	0.4	0.24	0.22
127	1,1-Diphenyl-ethylene	530-48-3	0.19	0.19	0.13	0.09	0.09	0.18	0.1	0.11	0.12
128	2-Methyl-6-phenyl-1,6-heptadiene	51708-97-5	0.75	0.51	0.34	0.49	0.55	0.4	0.41	0.6	0.48
129	1,4-Diethyl-1,2,3,4-tetrahydronaphthalene	81356-57-2	0.12	0.04	0.05	0.05	0.01	0.06	0.06	0.07	0.06
130	(3-Methyl-1-methylenepentyl)benzene	74810-69-8	5.1	2.32	1.73	2	2.6	1.78	3.25	2.26	1.96
131	1,1-Diethylnaphthalene	74710-00-2	0.09	0.07	0.22	0.06	0.03	0.07	0.05	0.07	0.06
132	1,3-Diphenylpropane	1081-75-0	0.13	0.11	0.12	0.16	0.08	0.17	0.07	0.08	0.07
133	3-Phenyldecane	4621-36-7	0.13	0.06	0.06	0.12	0.14	0.07	0.04	0.04	0.05
134	1,3-Diphenylbutane	1520-44-1	0.07	0.07	0.02	0.08	0.05	0.05	0.03	0.03	0.03

135	1,2,3-Trimethyl-4-propenylnaphthalene	26137-53-1	3.25	1.79	2.1	1.33	1.51	1.5	1.6	1.06	1.51
136	5-Phenyldodecane	2719-63-3	0.18	0.06	0.07	0.1	0.17	0.05	0.05	0.05	0.07
137	4-Phenyldodecane	2719-64-4	0.07	0.03	0.02	0.04	0.06	0.02	0.02	0.02	0.02
138	9-Ethyl-9,10-dihydro-10-methyl-anthracene	36778-20-8	0.13	0.1	0.02	0.08	0.09	0.11	0.05	0.08	0.01
139	3-Phenyldodecane	2400-00-2	0.07	0.03	0.03	0.05	0.07	0.03	0.02	0.02	0.02
140	4-Phenyltridecane	4534-51-4	0.07	0.03	0.02	0.03	0.06	0.02	0.04	0.01	0.02
141	(1,2-Dicyclopropyl-2-phenylethyl)benzene	110330-90-0	0.73	0.04	0.03	0.05	0.39	0.05	0.58	0.07	0.05
		Subtotal (GC Peak Area)		**19.61**			**14.86**			**16.23**	
		Subtotal (%)		**15.81**			**11.85**			**9.87**	
	Ketones										
	Aliphatics										
142	2-Propanone	67-64-1	2.48	1.92	1.85	2.68	2.34	2.2	2.08	2.12	2.31
143	2-Butanone	78-93-3	2.63	2.17	1.91	2.1	2.57	2.43	2.72	2.39	2.56
144	2-Pentanone	107-87-9	0.14	0.18	0.1	0.18	0.18	0.15	0.09	0.11	0.15
145	1-Hydroxy-2-propanone	116-09-6	0.34	0.66	0.75	0.49	0.42	0.52	0.47	0.99	1.04
146	3-Hydroxy-2-butanone	513-86-0	0.21	0.18	0.22	0.09	0.09	0.09	0.2	0.16	0.11
147	4-Methyl-2-pentanone	108-10-1	0.3	0.29	0.18	0.12	0.19	0.18	0.13	0.13	0.29
148	3-Hexanone	589-38-8	0.02	0.02	0.02	0.02	0.02	0.03	0.03	0.02	0.03
149	2-Hexanone	591-78-6	0.09	0.08	0.08	0.08	0.1	0.1	0.1	0.1	0.1
150	3-Hexen-2-one	763-93-9	0.03	0.03	0.02	0.02	0.04	0.03	0.02	0.02	0.03
151	3-Hydroxy-2-pentanone	3142-66-3	0.16	0.19	0.29	0.04	0.07	0.03	0.03	0.05	0.04
152	2-Hydroxy-3-pentanone	5704-20-1	0.04	0.05	0.09	0.01	0.02	0.01	0.02	0.02	0.02
153	2,3-Heptanedione	96-04-8	0.02	0.01	0.02	0.01	0.01	0.02	0.1	0.07	0.08
154	5-Methyl-2-hexanone	110-12-3	0.2	0.14	0.17	0.18	0.15	0.3	0.27	0.24	0.29
155	4-Heptanone	123-19-3	0.02	0.06	0.05	0.05	0.05	0.06	0.06	0.05	0.06
156	3-Heptanone	106-35-4	0.06	0.04	0.04	0.04	0.05	0.04	0.06	0.04	0.06
157	2-Heptanone	110-43-0	0.9	0.81	0.72	0.74	0.94	1.07	0.98	1.07	1.25

158	5-Methyl-2-heptanone	18217-12-4	0.02	0.01	0.01	0.02	0.03	0.02	0.01	0.05	0.02
159	4-Methyl-2-heptanone	6137-06-0	0.16	0.18	0.11	0.94	0.14	0.16	0.05	0.16	0.18
160	2-Methyl-6-heptanone	928-68-7	0.41	0.35	0.35	0.36	0.4	0.44	1.95	0.67	0.57
161	3-Octanone	106-68-3	0.25	0.15	0.17	0.23	0.22	0.23	0.13	0.14	0.15
162	2-Octanone	111-13-7	0.32	0.23	0.15	0.35	0.28	1.17	2	0.43	0.44
163	3-Nonanone	925-78-0	0.01	0.01	0	0.01	0.01	0.01	0.01	0.01	0.01
164	2-Nonanone	821-55-6	0.14	0.12	0.11	0.22	0.22	0.21	0.3	0.32	0.28
165	2,6-Dimethyl-2,5-heptadien-4-one	504-20-1	0.07	0.03	0.04	0.03	0.03	0.05	0.06	0.08	0.11
166	2,5-Dimethyl-3,4-hexanedione	4388-87-8	0.56	0.48	0.57	0.56	0.58	0.52	0.58	0.88	0.62
167	2-Decanone	693-54-9	0.16	0.08	0.03	0.15	0.11	0.08	0.13	0.12	0.11
168	4-Methyl-3-octanone	6137-15-1	0.65	0.48	0.41	0.51	0.57	0.45	0.53	0.68	0.51
169	5-Undecanone	33083-83-9	0.04	0.02	0.02	0.05	0.05	0.07	0.04	0.02	0.02
170	2-Undecanone	112-12-9	0.57	0.23	0.22	0.23	0.13	0.14	0.34	0.21	0.17
171	2-Tridecanone	593-08-8	0.07	0.06	0.07	0.07	0.05	0.08	0.03	0.03	0.03
172	2-Tetradecanone	2345-27-9	0.06	0.15	0.2	0.25	0.26	0.31	0.03	0.07	0.07
173	3-Tetradecanone	629-23-2	0.03	0.03	0.06	0.04	0.03	0.07	0.03	0.01	0.02
174	6-Tetradecanone	6836-42-6	0.03	0.05	0.05	0.03	0.02	0.03	0.01	0.02	0.03
175	2-Pentadecanone	2345-28-0	0.03	0.03	0.05	0.03	0.03	0.04	0.01	0.02	0.03
176	2-Hexadecanone	18787-63-8	0.08	0.12	0.16	0.08	0.15	0.08	0.02	0.03	0.03
177	2-Heptadecanone	2922-51-2	0.02	0.02	0.02	0.02	0.03	0.03	0.01	0.01	0.02
	Aromatics										
178	1-Phenylethanone	98-86-2	7.52	5.83	5.15	6.76	9.26	6.38	8.04	7.53	6.13
179	1-Phenyl-2-propanone	103-79-7	0.61	0.51	0.4	0.55	0.75	0.8	0.62	0.75	1
180	1-(2-Hydroxyphenyl)-ethanone	118-93-4	0.03	0.02	0.03	0.03	0.05	0.02	0.03	0.03	0.04
181	1-Phenyl-1,2-propanedione	579-07-7	0.18	0.07	0.06	0.12	0.15	0.15	0.39	0.46	0.32
182	1-Phenyl-2-butanone	1007-32-5	0.09	0.07	0.12	0.1	0.1	0.11	0.11	0.1	0.16
183	4-Phenyl-2-butanone	2550-26-7	0.26	0.05	0.16	0.39	0.42	0.4	0.4	0.45	0.4
184	1-Phenyl-1-butanone	495-40-9	0.61	0.24	0.11	0.47	0.39	0.12	0.42	0.3	0.13

185	1-(4-Ethylphenyl)-ethanone	937-30-4	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0
186	3-Methyl-1-phenyl-2-buten-1-one	5650-07-7	0.04	0.01	0.02	0.06	0.02	0.02	0.04	0.09	0.03
187	1-(4-tert-Butylphenyl)propan-2-one	81561-77-5	0.13	0.07	0.13	0.01	0.05	0.04	0.1	0.12	0.06
188	Benzophenone	119-61-9	0.23	0.18	0.21	0.14	0.27	0.24	0.23	0.23	0.25
189	3,5-di-tert-Butyl-4-hydroxyacetophenone	14035-33-7	0.01	0.01	0	0.01	0.01	0.01	0.02	0.01	0.01
190	2,2-Dimethoxy-1,2-diphenyl-ethanone	24650-42-8	0.03	0.02	0.02	0.03	0.04	0.03	0.01	0.02	0.02
	Cyclics										
191	Cyclohexanone	108-94-1	0.36	0.08	0.04	0.02	0.07	0.07	0.04	0.03	0.03
192	3,4,4-Trimethyl-2-cyclopenten-1-one	30434-65-2	0.08	0.07	0.02	0.01	0.13	0.03	0.01	0.13	0.06
193	3,5,5-Trimethyl-2-cyclohexen-1-one	78-59-1	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
194	4-Cyclopentylidene-2-butanone	51004-21-8	0.04	0	0	0.02	0.02	0.02	0.02	0.01	0
195	2,6-di-Butyl-2,5-cyclohexadiene-1,4-dione	719-22-2	0.75	0.12	0.59	0.56	0.6	0.4	0.71	0.49	0.34
196	2,6-di(t-Butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	10396-80-2	9.01	0.84	0.61	1.35	3.33	0.86	3.72	1.77	0.86
		Subtotal (GC Peak Area)		**22.07**			**23.04**			**24.78**	
		Subtotal (%)		**17.79**			**18.38**			**15.07**	
	_N_-Compounds										
	Amines										
	Aliphatics										
197	*N*-methyleneethenamine	38239-27-9	0.09	0.08	0.07	0.05	0.08	0.08	0.07	0.07	0.07
	Aromatics										
198	Benzenamine	62-53-3	0.01	0.01	0	0.38	0.01	0.01	0.01	0.02	0.01
199	2,4,6-Trimethyl-benzenamine	88-05-1	0.13	0.04	0.04	0.07	0.06	0.07	0.05	0.06	0.06
200	4,5-Dimethyl-ortho-phenylenediamine	3171-45-7	0.08	0.4	0.04	0.05	0.07	0.09	0.2	2.69	0.09
201	4-Ethylphenethylamine	64353-29-3	0.07	0.05	0.08	0.12	0.06	0.1	0.11	0.13	0.1
	Cyclics										
202	1-Methyl-1H-pyrrole-2-ethanamine	83732-75-6	0.06	0.05	0.04	0.05	0.07	0.07	0.06	0.06	0.07
	Amides										

	Aliphatics										
203	Dibutyramide	4494-12-6	0.11	0.1	0.08	0.08	0.1	0.12	0.13	0.16	0.14
204	*N,N*-Dibutylformamide	761-65-9	0.1	0.02	0.01	0.04	0.02	0.01	0.13	0.09	0.02
	Aromatics										
205	2-Phenylbutanamide	90-26-6	0.28	0.21	0.19	0.15	0.18	0.35	0.58	0.42	0.57
	Oximes										
206	3-Methylbutanal oxime (isomer)	5780-40-5	0.16	0.18	0.15	0.2	0.25	0.2	0.28	0.26	0.34
207	3-Methylbutanal oxime (isomer)	5775-74-6	0.1	0.11	0.1	0.14	0.15	0.12	0.17	0.18	0.23
	Others										
208	Benzonitrile	100-47-0	0.41	0.35	0.35	0.41	0.49	0.49	0.48	0.56	0.54
209	Phenylacetonitrile	140-29-4	1.17	1.06	1.03	1.15	1.29	1.37	1.2	1.63	1.35
		Subtotal (GC Peak Area)		**2.54**			**2.93**			**4.46**	
		Subtotal (%)		**2.05**			**2.34**			**2.71**	
	Terpens										
	Monoterpens										
210	α-Pinene	7785-26-4	0.08	0.09	0.05	0.08	0.13	0.06	0.06	0.08	0.08
211	Sabinene	3387-41-5	0.02	0.02	0.01	0.02	0.02	0.01	0.01	0.02	0.02
212	γ-Terpinene	99-85-4	0.06	0.03	0.01	0.05	0.04	0.01	0.02	0.02	0.05
213	Limonene	5989-54-8	0.06	0.11	0.07	0.15	0.08	0.07	0.13	0.12	0.12
214	Dihydromyrcenol	18479-58-8	0.59	0.24	0.16	0.53	0.25	0.17	0.63	0.25	1.76
215	Isoborneol	124-76-5	0.05	0.03	0.04	0.03	0.03	0.05	0.04	0.06	0.04
216	Dihydro γ-Terpineol	21129-27-1	0.48	0.36	0.08	0.54	0.19	0.15	0.28	0.41	0.14
217	α-Terpineol	98-55-5	0.73	0.25	0.08	0.5	0.24	0.13	0.33	0.5	0.13
218	Tetrahydrogeraniol	106-21-8	0.81	0.82	0.12	0.76	0.21	0.11	0.39	0.71	0.22
219	Nerol	106-25-2	0.17	0.09	0.19	0.34	0.36	0.28	0.27	0.36	0.23
220	4-Terpinenyl acetate	4821-04-9	0.13	0.08	0.04	0.12	0.03	0.09	0.05	0.12	0.09
	Sesquiterpens										

221	β-Caryophyllene	87-44-5	0.39	0.31	0.02	0.04	0.04	0.25	0.38	0.16	0.12
222	Nerolidol	7212-44-4	0.24	0.13	0.16	0.18	0.24	0.19	0.23	0.11	0.24
223	Methyl farnesoate	3675-00-1	0	0.02	0.02	0.02	0.03	0.03	0	0	0
		Subtotal (GC Peak Area)		**2.48**			**2.28**			**8.98**	
		Subtotal (%)		**2.00**			**1.82**			**5.47**	
	Norisoprenoids										
224	6-Methyl-5-hepten-2-one	110-93-0	0.52	0.17	0.4	1.14	0.59	0.35	0.19	0.19	0.27
225	Geranylacetone	3796-70-1	0.13	0.23	0.16	0.38	0.29	0.26	0.25	0.3	0.28
226	α-iso-Methyl ionone	127-51-5	0.05	0.01	0.01	0.01	0.04	0.01	0.04	0.01	0.02
		Subtotal (GC Peak Area)		**0.56**			**1.02**			**0.52**	
		Subtotal (%)		**0.45**			**0.81**			**0.32**	
	S-Compounds										
	Aliphatics										
227	Methanethiol	74-93-1	0.58	0.48	0.39	0.42	0.33	0.4	0.23	0.24	0.27
228	Carbon disulfide	75-15-0	0.24	0.24	0.24	0.29	0.22	0.22	0.15	0.17	0.16
229	Methylthiolacetate	1534-08-3	0.45	0.31	0.27	0.38	0.34	0.45	0.14	0.09	0.15
230	Dimethyl disulfide	624-92-0	1.55	1.17	1.19	1.11	0.88	1.29	0.89	0.72	0.83
231	Methylthio-2-propanone	14109-72-9	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
232	3-(methylthio)-propanal	3268-49-3	0.1	0.12	0.08	0.08	0.22	3.37	1.79	0.11	0.27
233	Dimethyl trisulfide	3658-80-8	1.29	1.06	1.13	0.84	0.62	0.8	0.47	0.44	0.43
234	2-Methylsulfinylethanol	21281-74-3	0.12	0.07	0.06	0.1	0.07	0.03	0.07	0.11	0.1
235	6-(Methylthio)hexa-1,5-dien-3-ol	-	0.01	0.02	0.02	0.01	0.02	0.02	0.03	0.03	0.04
236	Dimethyl tetrasulphide	5756-24-1	0.43	0.46	0.53	0.33	0.22	0.31	0.21	0.14	0.15
237	Dimethyl pentasulfide	7330-31-6	0.02	0.01	0.01	0	0	0	0	0	0
238	1-Dodecanethiol	112-55-0	0.29	0.17	0.02	0.07	0.1	0.02	0.15	0.4	0.54
239	2-Methyl-2-undecanethiol	10059-13-9	0.33	0.16	0.1	0.12	0.07	0.15	0.14	0.21	0.21

240	Dodecyl methyl sulfide	3698-89-3	0.57	0.45	0.39	1.46	0.71	0.91	0.14	0.18	0.12
		Subtotal (GC Peak Area)		**5.06**			**5.68**			**3.53**	
		Subtotal (%)		**4.08**			**4.53**			**2.15**	