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Metabolomic profiling and molecular characterization of food matrices: identification of potential markers of microbial contamination

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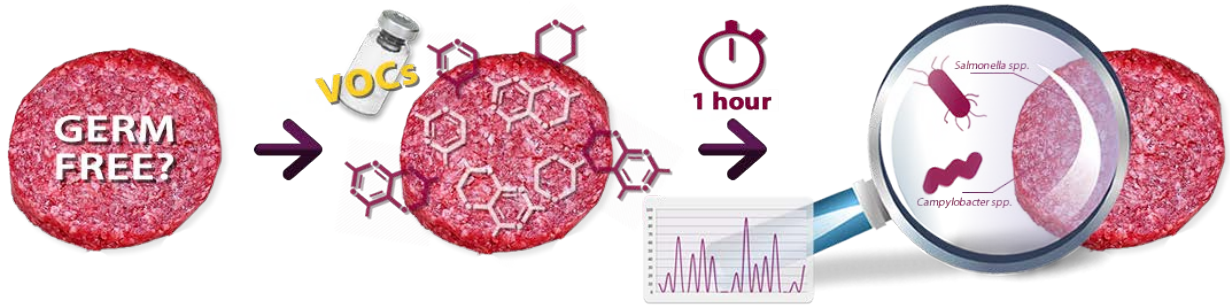
ABSTRACT

The research aims to generate an early warning system able to highlight, in real time, bacterial contamination of meat matrices and to provide information which could support companies in accepting or rejecting batches. Current microorganisms detection methods rely on techniques (plate counting), which provide retrospective values for microbial contamination. The disposal of fast headspace air measurement, using gas chromatography-mass spectrometry analysis, able to accurately and rapidly (30 min per sample) detect microbial spoilage in raw meat, could result a valid replacement to traditional and time-consuming (3 to 4 days) standardized microbiological analysis required by regulations. The experiments focused on the qualitative and quantitative evaluation of volatile organic compounds (VOCs) produced by *Salmonella* Typhimurium, *Campylobacter jejuni* and *Staphylococcus aureus* in different types of raw meat (beef, pork, chicken).

The reduction of analysis times represents the strength of the alternative method to ISO protocols, although these are currently the official procedures provided by International regulations. The applied method requires smaller sample aliquots and does not need any sample processing, thus consenting its application on different food matrices (not only meat) and for the detection of a wide variety of pathogens. The validation of the suggested analytical approach would therefore result innovative, by evaluating further samples in order to demonstrate the benefits of the technique in terms of times, costs and preservation of consumers health.

Data analysis allowed the characterization of unique VOC profiles and possible marker compounds of meat contamination due to certain pathogens. The identification of marker volatile compounds resulted essential to outline specific metabolic profiles for each microorganism responsible of meat spoilage.



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Early Warning System
for detection of microbial contamination of food

Detection of specific pathogens in meat

Beef

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S. Typhimurium

C. jejuni

S. aureus

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Single Bacteria

Bacterial Inocula Mixes

Meat with single Bacteria

Meat with 2 or 3 Bacteria

Meat with decreasing bacterial loads

Microbiology

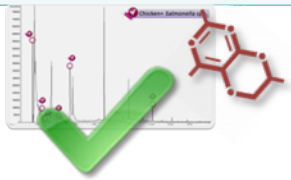
Standardized ISO protocols

- Long **sample treatments**
- Results in **3-4 days**
- **Need confirmation** tests
- Fair costs

Analytical chemistry

SPME HS-GC-MS – VOCs analysis

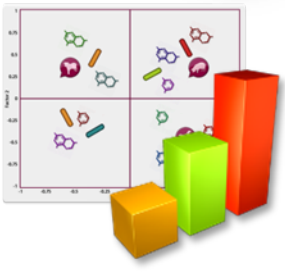
- No sample treatment
- Results in **1 hour**
- No confirmation needed
- **Same costs of microbiology**



Molecular biology

DNA extraction and PCR

- Needs sample treatment
- Results in **1 day**
- No need of confirmations
- **Very expensive** technique



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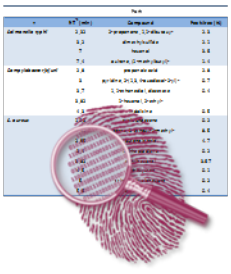
- Generation of typical profiles (**fingerprints**)
- Identification of **marker volatile organic compounds**
- Creation of dedicated **databases**



Rapid identification of the most common foodborne pathogens

SCOPE OF THE WORK

Detection of pathogens and their by-products in food spoiled by microorganisms is a significant aspect of food safety. Inadequate storage conditions of food or improper processing steps can promote the growth of pathogenic microorganisms, rapidly spreading to human outbreaks, if not detected in time. According to European Food Safety Authority (EFSA), *Campylobacter* spp., *Salmonella* spp. and bacterial toxins, including *Staphylococcus aureus* toxins, represent the main causative agents of foodborne outbreaks reported in Europe (European Food Safety Authority 2015).

Food industries are constantly looking for **rapid, cheap and valid methods** to put in evidence **microbial contaminations along the production chain**, without the need to request sophisticated and time-consuming analysis, nevertheless asking for the same results reliability and reproducibility.

Molecular methods have been considered useful approaches pointing to the characterization and rapid identification of microorganisms (Yost and others 2002); quantification of microbial loads is however difficult to obtain, unless quantitative PCR (qPCR) is employed and a standard curve is produced. Such methods, although being accurate and precise, are expensive, require complex and long sample preparations steps, in the majority of cases the raw sample needs an extraction stage before being amplified for the subsequent characterization, and results are available within one day.

Several studies have tested novel and faster protocols to detect the contamination of a meat sample within a few minutes, using real-time meat monitorings (Jääskeläinen and others 2016; Mikš-Krajnik and others 2015; Ercolini and others 2011). **The aim of the present research** was to evaluate the ability of the **HS-SPME-GC/MS** methodologies to **detect VOCs** which may be associated with a particular microbiological contamination of food.

The **three microbiological descriptors of the study**, based on European data on foodborne pathogens health risks, were ***Salmonella* Typhimurium**, ***Campylobacter jejuni***, and ***Staphylococcus aureus***.
Considering **that bacterial spoilage potential is highly related to their proteolytic activity on food**, the **choice of meat matrices** (**beef**, **chicken**, **pork**) resulted functional to metabolomics analysis; furthermore, meat has a low shelf-life, which is function of its faster spoilage, compared to other food products.
Hence, the suggested scientific approach aims to the **rapid determination and association of selected bacterial pathogens and their metabolites on specific meat samples**.

Analyses were performed employing **headspace gas chromatography-mass spectrometry** (HS-GC-MS), in order to detect specific pathogens and determine relative loads of spoilage microorganisms in meat.
GC-MS **costs are similar to microbiological evaluations**, but **do not require sample preparations**, such as pre-sampling, pre-concentration, or sample enrichment/dehydration, and the **contemporary analysis of huge amounts** of samples can be evaluated: the sample is directly analyzed and the **results are available in 20-30 minutes**. GC-MS has been showed to be useful to the **detection of volatile compounds – produced by bacteria during spoilage – as markers of meat contamination**.

1. INTRODUCTION

Fresh meat is one of the most perishable foods, especially because of its high nutrients content, which outlines the matrix as an ideal reservoir for the growth and replication of foodborne pathogens. Excepting infected cases, the muscle of live and healthy animals is generally sterile. Contamination of meat carcasses may depend on animals belly content, hide and feces. During slaughtering phases, microbial contamination can be due to workers, tools, or contact with other infected carcasses (Ghafir and others 2007; Wheatley and others 2014). Large meat animals undergo a significant amount of cutting and boning in order to result finished products. The process of cutting carcasses causes the exposition of fresh surfaces, which are highly susceptible to bacterial contamination (Satin 2002; Maharjan and others 2006; Sharma and others 2015; Holds and others 2007). To prevent foodborne outbreaks linked to harmful pathogens, the official control of meat is crucial in order to verify and ensure the conformity of products, and, therefore, protect the interests of consumers.

Meat spoilage is mostly determined by microbial activity. Bacteria are known to produce a range of volatile organic compounds (VOCs), which are thought to evolve as products or by-products of metabolic pathways (Schultz and others 2007). Pathogens exploit meat matrices muscle tissues for their enzymatic activities. The resulting metabolites consist of alcohols, aldehydes, ammines, esters, ketones, organic acids, sulfur compounds, etc., which play a substantial role in the characteristic off-odors and flavors, as an outcome of the ongoing spoilage. Current gold standard techniques for pathogens identification in meat consist of microbiological analysis, based on ISO protocols, which require from 3 to 4 days for the complete confirmation of microorganism presence. The analysis of bacterial metabolites – therefore the detection of marker bacterial metabolites and/or combinations fingerprints – produced during food spoilage, would indeed fasten the identification of pathogens contamination.

1.1. Gascromatography/mass spectrometry

Gascromatography mass spectrometry (GC-MS) is an analytical technique based on the use of a gascromatograph coupled with a mass spectrometer. It has been employed to evaluate the volatile organic compounds produced by prepared samples. Compounds are separated by gascromatograph along a column and are subsequently identified – or “revealed” – through the mass spectrometer. Such technique is able to characterize the pool of substances produced by a specific sample, allowing the correspondent quantification, as a spectral output.

Sample is injected into the injection port of the GC. GC device vaporizes the compounds, by separating them on a column, and analyzing all the components. The migration of the components along the column is

consented by carrier gases (usually helium): maintaining a constant gas flow, substances are propelled down the column. Ideally, volatile organic compounds tend to separate from the column before eluting from its end, registering a specific peak at a certain retention time. Retention time helps differentiating compounds eluting closely. It may happen that two substances share the same retention time: this indicates the possibility that those are the same substance.

The several compounds produce spectral peaks, recorded by mass spectrometer (MS) instrument. Peaks are connected to retention time, which represents the time elapsed between the injection and the elution into the column. The quantification of the components is able through the calculation of the peak area, which is indeed proportional to the concentration of the relative compounds in the evaluated sample. Migration on the GC column depends on several physical and chemical characteristics (e.g. molecule mass, shape or affinity for the carrier gas and/or column).

MS characterizes the compounds by electrically charging the sample molecules, accelerating them through a magnetic field, breaking the components into charged fragments and detecting the different charges. A spectral plot is produced, showing the mass of each fragment: mass spectra aid in qualitatively describing the substances, building up the fragment masses into the parent mass. Differently from the retention time values, each molecule mass spectrum is unique and can therefore support the final identification of the substances, by comparing masses with known compounds, whose masses are available on online and offline libraries.

The combination of GC and MS, the MS use of GC output, is a crucial tool in the qualitative analysis of volatiles: for this reason GC/MS analysis is considered the gold standard of such determinations. Despite this, some limitations need to be taken into account: for example, when GC does not separate sample compounds completely, MS analysis can result impure (Douglas and others 2016).

1.2. Practical application: the role of HS-SPME-GC/MS in food analysis

The use of a gas chromatography mass spectrometry (GC–MS) for the analysis of food can vary in the scope. For example, it can be employed for the qualitative/quantitative analysis of untargeted volatiles organic compounds, defining aroma profiles, or targeted substances (e.g. pesticides). An interesting and recent approach consists on using the GC/MS analysis to generate **fingerprints of food products in the shape of chromatography profiles**: such evaluation helps discriminating between food samples of the same type and to detect **contaminations showed by the detection of different compounds compared to the reference sample** (considered as negative control). GC–MS technique needs to be sensitive and selective, and must possess a fair separation power and speed (Tranchida and others 2012).

The advantages of **HS-SPME-GC/MS** are several: **SPME** is commercially available, **solventless, rapid, inexpensive, and portable analytical technique enabling VOCs sampling** in the headspace of a sample. **Extraction and concentration are integrated into one step** without interfering with the chemical composition of the studied food matrix. SPME sampling followed by GC/MS analysis can be a powerful tool to assess not only the spoilage potential (qualitative measurements), but also the spoilage activity (quantitative measurements). Besides the fact that **sample processing is not required**, the absolute headspace **concentrations** can be **calculated without calibration or use of standards**.
GC-MS measurements are rapid, nondestructive, and quantitative: for this reason, this method represents an **elective tool for fast bacterial analysis of meat**.

Apart from researches aimed to reduce analysis times, a wide range of applications is available:

- quality control in food markets
- hygiene monitoring in slaughterhouses
- national controls of meat distribution.

As a **practical application**, following the **identification of marker compounds of bacterial contaminations**, the method can be employed to **check the quality of the open retailed meat** at the distribution phase, by on-line measuring of the emissions in the storage area and for **identifying contaminated meat batches** and/or pieces. The advantages lay in the fact that **samples do not need any processing steps** (e.g. meat samples does not need to be cut in pieces), and the methodology allows to obtain rapid information about the bacteriological contamination. Furthermore, the simplified analytical process allows the **contemporary evaluation of a huge number of samples**, and, after optimized the technique, the early warning technique could allow the shops to sell or sort out samples in real time. The approach would be essential for **meat-processing facilities** like slaughterhouses, where ensuring high meat quality standards is essential: the possibility to monitor the incoming meat employing GC-MS, could support the selection of safe meat within production chain and guarantee money saving for industries. (Mayr and others 2003). Moreover, it is essential to underline that the analytical approach, detecting bacterial spoilage through specific volatile organic compounds production, is **not restricted to meat but can also be applied to food in general, from vegetables to cereals, passing by water and beverages**.

1.3. Foodborne pathogens and microbiological hazards

Foodborne and waterborne bacterial pathogens represent a substantial cause of mortality worldwide. The most important bacterial pathogens transmitted through contaminated food and water include species or strains of *Salmonella* spp., *Escherichia coli*, *Staphylococcus* spp. and *Campylobacter* spp.

The increased incidence of foodborne illnesses caused by microbiological hazards represents the result of several factors, all connected to our rapidly changing world. Due to the development of resistance to available antibiotics, the proportion of people who are more susceptible to microorganisms in food has substantially increased. Modified lifestyles play a heavy role in the alterations in the environment: changes in agricultural practices, wider distribution systems for food, need of an intensive production which could meets the growing demand, increasing preference of meat and poultry in developing countries, a dense food distribution in a short time; such dynamics result in the substantial increase of the risk of producing food hosting less common, antibiotic resistant or more virulent pathogens.
Alterations in consumption patterns (e.g. preference for fresh and minimally cooked foods), increasingly longer interval between processing and consumption of food and the increasing prevalence of eating food prepared outside home, all contribute to the higher incidence of foodborne illnesses.

1.4. *Salmonella* spp. and Salmonellosis

Salmonella spp. is a Gram-negative, no capsulated, nonsporulating, anaerobic bacillus, which synthetizes typical flagellar, somatic, and outer coat antigens characterized (O, H, and Vi antigens). Salmonellae are ubiquitous human and animal pathogens: there are over 2500 known serovars, which current classification indicates as separate species. (Shahane and others 2007). Contaminated food and water constitute the ideal reservoir for the pathogen. After the ingestion, the microorganisms are able to spread, through the blood flow, from the intestine to the intestinal lymph nodes, the liver, and the spleen where they start multiplying. Salmonellosis is a type of food poisoning associated with high morbidity and mortality and it is estimated that the pathology affects almost 100 million people globally each year (Majowicz and others 2010). *Salmonella* enterica serotype Typhimurium and *Salmonella enterica* serotype Enteritidis are two of the most common strains isolated from food.

Salmonella is generally divided into two categories: non-typhoid *Salmonella*, the most common form, carried by both humans and animals. Typhoid *Salmonella*, which causes typhoid fever, is rare, and is carried only by humans. The most pathogenic strain is represented by *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium), a facultative intracellular pathogen which is the responsible of typhoid fever in humans (which represent the only known natural hosts and Salmonellosis reservoir) (Mweu and others 2008). The minimum infectious dose of *Salmonella* Typhimurium, able to give rise to the infection, varies between 1000 and 1 million organisms (Hornick and others 1970).

As for clinical manifestations, Salmonellosis ranges from the common *Salmonella* gastroenteritis, presenting diarrhea, abdominal cramps, septicemia, and fever, to enteric fevers, such as typhoid fever,

caused by *Salmonella* Typhimurium: enteric fevers can be life-threatening febrile systemic illnesses, which require adequate and immediate antibiotic therapy. The most common form of Salmonellosis is a self-limited, uncomplicated gastroenteritis. Symptoms can develop from 12 to 72 hours after infection, and the illness usually lasts 4 to 7 days. Most people recover without treatment. But diarrhea and dehydration may be so to require hospitalization. Patients with impaired immune systems are at highest risk. A small number of people who are infected with Salmonellosis develop reactive arthritis, a disease that can last for months or years and can lead to chronic arthritis. (Giannella 1996).

Salmonellosis infection occurs by eating food contaminated with *Salmonella* spp. Food contamination sources are mainly represented by contaminations during food processing or food handling (e.g. unwashed employees hands). *Salmonella* may also be found in the feces of some pets, especially those with diarrhea. Reptiles, baby chicks and ducklings, and small rodents such as hamsters are particularly likely to carry *Salmonella*. Beef, poultry, milk, and eggs and products thereof are most often infected with *Salmonella* species. Vegetables may also be contaminated. (WebMD 2016).

1.5. *Campylobacter* spp. and Campylobacteriosis

Campylobacter spp. is a non-fermenting, curved, motile, and narrow rod shaped bacterium. The Gram-negative microorganism grows in microaerobic conditions, requiring 3-5% oxygen and 2-10% CO_2, at a temperature comprised from 37°C to 41°C (the majority of strains are thermo-tolerant). *Campylobacter* genus presents single-polar flagella, conferring its capability of a screw motility. The motility increases the virulence of the microorganisms, allowing the easier penetration of the mucus layer of the intestinal epithelium, and therefore colonization of the intestine of the host. The penetration is consented even through the production of chemotactic factors; *Campylobacter* additionally produces adhesins, which, together with flagella, adhere to host epithelial cells and is internalized in the host tissue through phagocytosis (Perez-Perez and others 1996).

Campylobacter virulence is consented even through the production of toxic factors, mainly an enterotoxin similar to cholera toxin and several cytotoxins.

Campylobacter is part of the normal intestinal flora of a wide variety of animals, either wild or domesticated. The pathogen is able to infect animal carcasses at different stages of the slaughtering process: poultry meat (broiler and turkey) is the most frequently contaminated food matrix, followed by cattles, pigs, and sheeps; less frequently in fish and fishery products, molluscs and fresh vegetables. Human infection is usually acquired through the consumption of undercooked contaminated meat and cross-contaminated food products during the preparation of food at home (van Vliet and others 2001). The bacterium may also be found in not pasteurized cow milk and is commonly isolated from sewage and

surface untreated waters (Ketley 1997). The constant rise in incidence of Campylobacteriosis in industrialized countries is thought to be connected to changes in eating habits rather than reflecting increased awareness or better diagnostic tools (van Vliet and others 2001).
Based on lipopolysaccharide (O) and protein (H) antigens (e.g. porin and flagellin), *Campylobacter* species gather several serogroups, although, only a few serogroups are responsible for human infection. Species most commonly involved in human intestinal infectious diseases are: *Campylobacter jejuni* subspecies *jejuni*, *Campylobacter jejuni* subspecies *doylei*, *Campylobacter coli*, *Campylobacter lari*, *Campylobacter upsaliensis*, and *Campylobacter mucosalis* (Dasti and others 2010).

Infection with *Campylobacter jejuni* results from the ingestion of contaminated food or water. The infectious dose is really low and generally ranges between 500–800 organisms (Black and others 1988). The infection starts shortly after gut penetration: ingested microorganisms, passing through the gut barrier, reaches the ileum and colon, and causes a non-specific acute inflammatory reaction with neutrophils, monocytes and eosinophils, the degeneration of glands, microabscesses of the crypts and mucosal lesions. At this stage of the illness erythrocytes and leukocytes can be found in the host stool. The symptoms of the Campylobacteriosis are initially common to all bacterial enteritis, presenting fever and diarrhea: while in mild cases the symptoms are indistinguishable from a viral gastroenteritis, in more serious cases, severe forms of colitis very similar to ulcerative colitis or Crohn's disease have been described (Wallis 1994).

Campylobacteriosis in humans is caused by thermotolerant species of the genus *Campylobacter* spp. The illness can be caused by a low infectious dose. *Campylobacter jejuni*, followed by *Campylobacter coli* and *Campylobacter lari* constitute the most risky species associated with human Campylobacteriosis. The incubation period of the infection ranges from two to five days. The symptoms generally consist of fever, headache, inflammatory diarrhea, abdominal pain, nausea, and general malaise. Infections are self-limiting and last a few days: in rare cases, pathology can evolve in extra intestinal infections that can cause harmful effects such as arthritis and neurological disorders. In addition, infection with *Campylobacter jejuni* predisposes to Guillain-Barré syndrome, a pathology causing paralysis that can lead to respiratory failure and severe neurological dysfunction, in some cases, even to death (van Vliet and others 2001).

Campylobacter pathogenic species were indicated as the most common cause of zoonoses in Europe and in the world, and the trend is confirmed since 2005, although the incidence of the microorganism is not properly monitored, unlike other pathogenic bacteria such as *Salmonella* spp. and *Escherichia coli* verocytotoxic (European Food Safety Authority 2015).

1.6. *Staphylococcus aureus* and SFP

Staphylococcus aureus is a Gram-positive coccus that grows in aggregates, belonging to the family of Micrococcaceae. Currently 32 species of the Genus *Staphylococcus* are known and some of these are pathogenic to humans and animals. *Staphylococcus aureus* is capable of synthesizing several toxins, which help the colonization of the host organism from sites of infection, besides consenting the microorganism to survive extreme conditions within the human host (Liu 2010).

In the context of food poisoning, the hazard connected to *Staphylococcus aureus* resides in the capability of synthesizing thermostable toxins in contaminated food, indicated as "staphylococcal enterotoxins" (SE), which, when present in adequate quantity, cause a common form of food poisoning, named staphylococcal intoxication (SI). Staphylococcal enterotoxins are thermostable and it is therefore difficult to turn them off by employing the standard food cooking treatments. *Staphylococcus aureus* synthesizes its enterotoxins (SEs) throughout the logarithmic growth phase or during the transition from the exponential to the stationary phase. Five different enterotoxins have been currently identified: A, B, C, D, and E. The toxin A is the most implicated in cases of intoxication (about 80%), while E is very rare (Liu 2010).

Staphylococcal food poisoning (SFP) is intoxication subsequent to the consumption of foods containing sufficient amounts of one (or more) preformed staphylococcal enterotoxins (Dinges and others 2000; Le Loir and others 2003). Symptoms of poisoning consist on a very rapid onset after ingestion of contaminated food, between 2 and 8 hours, and include nausea, headache, violent vomiting, abdominal pain, with or without diarrhea; fever is usually rare; on the other hand, the illness is self-limiting and usually resolves within 24–48 h after its onset, until the patients infected were particularly susceptible cases, such as infants, elderly or debilitated people, that are considered at higher risk (Balaban and others 2000; Murray 2005; Tranter 1990). The staphylococcal food poisoning is globally widespread and extremely common, although the real incidence is probably underestimated for improper sample collection and laboratory evaluation, misdiagnosis, unreported minor outbreaks (Chiang and others 2008; Mead and others 1999).

The main source of food contamination is represented by food handlers carrying enterotoxin-producing *Staphylococcus aureus* in their noses or on their hands, transferring the microorganisms through manual contact or respiratory secretions, considering that the bacterium is commensal of the skin and mucosal membranes of humans (Kluytmans and others 2005).

Although *Staphylococcus aureus* does not compete well with microbiota of raw food, contamination is mainly associated with improper handling of processed foods, together with improper storage. Food products most frequently contaminated with staphylococcal enterotoxins mainly include meat and meat products, poultry and egg products, milk and products thereof, salads, bakery products containing cream. *Staphylococcus aureus* has been isolated even from salted food (e.g. ham), because of the capability of the pathogen of growing at low water activity conditions (Tamarapu and others 2001).

1.7. Zoonoses and food-borne outbreaks in Europe

The European Food Security Authority (EFSA) and the European Centre for Disease Prevention and Control reported that, in 2014, 5.251 food-borne outbreaks were registered in the EU. In 592 outbreaks it was possible to connect the human cases to the food vehicles. The main causative agents of 2014 outbreaks were viruses (20.4%), followed by *Salmonella* spp. (20.0%). Bacterial toxins caused human outbreaks in 16.1% cases, while outbreaks caused by *Campylobacter* spp. were reported in 8.5% of the outbreaks. Salmonella food-borne outbreaks sensibly decreased in the 2008-2014 7 years-period, while *Campylobacter* outbreaks increased compared to 2013 report (**Figure 1**).

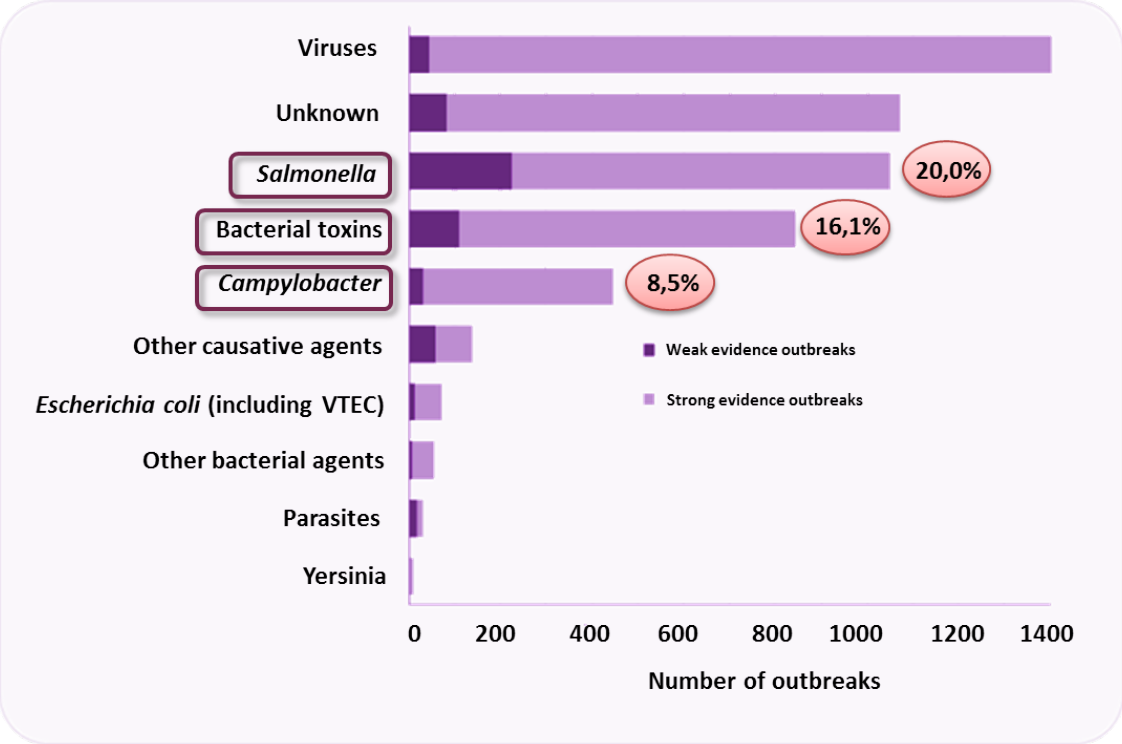


Figure 1 Distribution of food-borne outbreaks per causative agent in the Euopean Union in 2014, extracted from EFSA-ECDC report. Bacterial toxins include toxins produced by *Bacillus*, *Clostridium* and by *Staphylococcus* genera. Other causative agents include chemical agents, histamine, lectin, marine biotoxins, mushroom toxins, and wax esters. (European Food Safety Authority 2015).

The most recent **EFSA-ECDC Report**, conducted in 32 European countries, indicated Campylobacteriosis as the most commonly reported zoonosis, registering a substantial increase starting from 2008: Campylobacter infection occurrence was mainly reported in broiler meat. *Salmonella* evidences in fresh and processed poultry meat was instead lower than the past years (**Figure 2**).

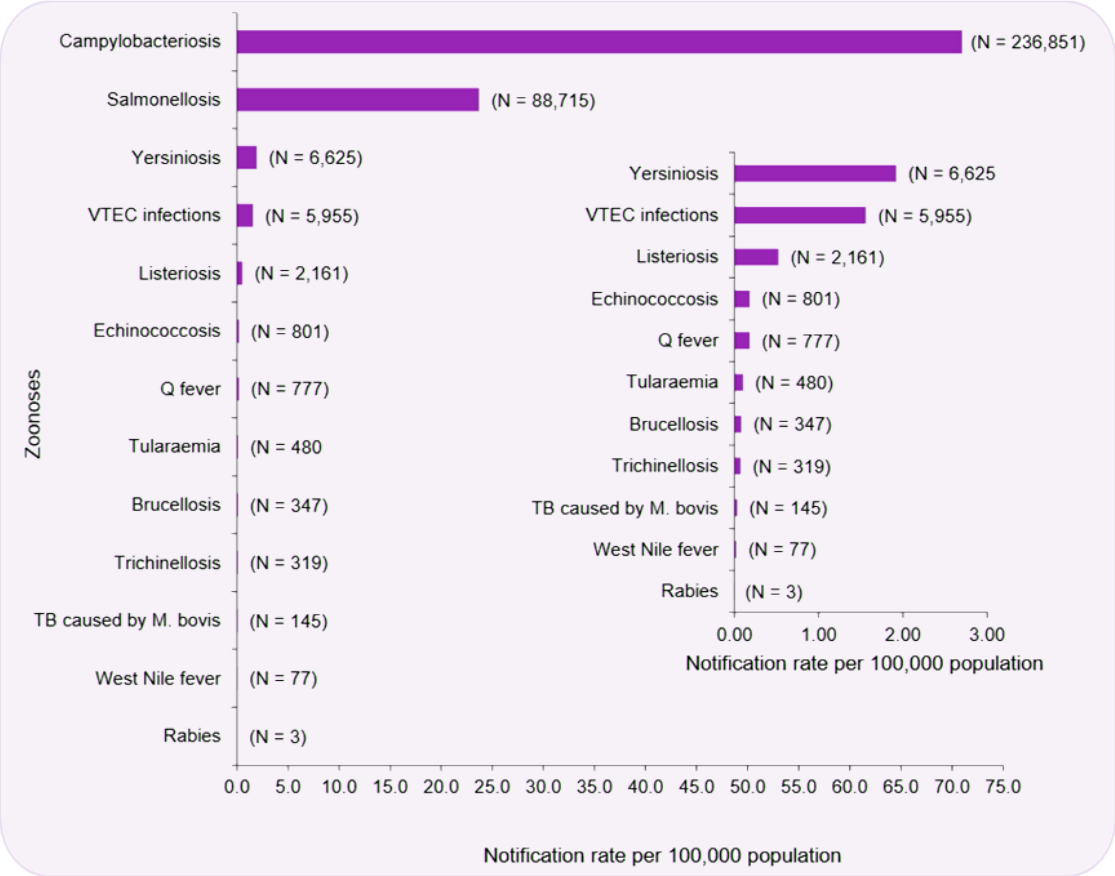


Figure 2 EFSA-ECDC reported numbers and notification rates of human zoonoses cases in the European Union in 2014. The total confirmed cases are indicated in parenthesis at the end of each bar. For West Nile fever total number of cases is indicated (European Food Safety Authority 2015).

1.7.1. *Salmonella* spp. prevalence in food

When animals are slaughtered because of their meat, *Salmonella* is transferred from their gut to the meat, causing infection. In 2014, 88.715 confirmed cases of *Salmonella* infections were reported in Europe, with a 23.4 cases per 10.000 population notification rate. The percentage registered a 15.3% increase since 2013. There was a statistically significant decreasing trend of Salmonellosis in the 7-year period of 2008-2014. With regards to serovars, the two most commonly reported *Salmonella* serovars in 2014 were *Salmonella* Enteritidis (44.4%), increased from 2013, and *Salmonella* Typhimurium (17.4%). *Salmonella* is most frequently detected in poultry meat, and less often in pig or bovine meat (Upadhyaya and others 2011; Maharjan and others 2006; Antunes and others 2003; Soltan and others 2009). EFSA reported the highest proportions of *Salmonella*-positive samples for fresh turkey meat (3.5%), followed by broiler, pig and bovine meat (European Food Safety Authority 2015). At lower levels, the microorganism was also detected in ready-to-eat (RTE) foods.

In particular, over the 7.482 units of fresh turkey meat tested in Europe in 2014, mostly sampled at the slaughterhouse or processing plant (87%), 3.5% resulted *Salmonella*-positive. The 4 *Salmonella*-positive

ready-to-eat turkey samples were analyzed in Italy; outcomes were lower compared with 2011–2013. *Salmonella* monitoring programs in fresh broiler meat consist on sampling at the slaughterhouse, at processing plants and at retail levels. In broiler meat, *Salmonella* was isolated from 2.8% of the 125.922 units tested: 2.3% at retail, 4.7% at the slaughterhouse, and 2.4% at the processing plant level, 0.6% of the over 2.000 ready-to-eat broiler meat products tested. Pig meat and products thereof are generally sampled at the slaughterhouse. In 2014, 68.134 fresh pig meat samples were analyzed, and a 0.5% rate was positive to *Salmonella.* Data from the testing of fresh bovine meat mainly originate from surveillance programs conducted at slaughterhouses and/or at processing plants: over the almost 46.000 samples of fresh bovine meat evaluated, a 0.1% rate was found to be *Salmonella* positive. With regards to eggs (animal derived food), in 2014 only 0.4% of the 13.394 tested table egg units were found positive to *Salmonella* (mostly reported by Germany and Poland). Positive units have been very low for the last couple of years.
In United States, according to the last Centers for Disease Control and Prevention report, outbreaks caused by *Salmonella* increased by 39% from 2012 to 2013 and hospitalizations caused by *Salmonella* grew by 38% between 2012 and 2013. *Salmonella* resulted the second most common cause of confirmed, single-etiology foodborne outbreaks in USA, after *Norovirus.* The most contaminated food matrices resulted chicken (700 cases) and pork (436) (Centers for Disease Control and Prevention 2015).

Several studies described the occurrence and epidemiology of *Salmonella* species mostly in slaughterhouses, less frequently at retail level. A study conducted in Europe in 2006-2007 stated a prevalence of *Salmonella* in 23.7% of poultry flocks, 30.7% turkey flocks, 13.9% pigs and 0-1% of cattle samples (Devine and others 2014). Worldwide studies focused on broiler meat reported *Salmonella* contaminations with positivity rates ranging from 10% (Käsbohrer and others 2013) to 45% (Bai and others 2015). Surveys on pig slaughterhouses registered percentages from 5.3% (Hald and others 2013) to 19.5% (Wales and others 2013), reporting, in some cases, a sensible *Salmonella* positivity growth, by analyzing samples before and after slaughtering processes (Rasschaert and others 2013).

1.7.2. *Campylobacter* spp. prevalence in food

EFSA-ECDC 2015 Report shows that *Campylobacter* represents the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union (EU). Over 236.000 confirmed cases of human Campylobacteriosis were registered in the last available report and an unexpected 71.0 per 100.000 population notification rate was indicated, stating a statistically significant 9.6% increase compared to the 2008–2013 trend.

The main food source of human Campylobacteriosis in 2014 is broiler meat: of the 6.703 fresh broiler meat samples analyzed, 38.4% were *Campylobacter* positive, reproducing the trend reported in 2013 (31.4% positivity rate). Of the Campylobacter-positive broiler meat units, 35.5% were sampled at retail and 44.4% at slaughterhouse level. From slaughterhouse, passing through processing plant and arriving to retail, the proportion of positive samples resulted gradually lower between the stages. In raw cow milk products *Campylobacter* was detected in up to 16.7% of the tested units (single or batch) (European Food Safety Authority 2015).

1.7.3. Staphylococcal enterotoxins prevalence in food

EFSA-ECDC 2015 report indicated 393 food-borne outbreaks caused by staphylococcal toxins (7.5% of all outbreaks), increased compared to 2013 data. Overall, in Europe, 0.12 cases per 100,000 were registered: the majority of outbreaks were notified from France (89.6%) and Switzerland.

The most common food category responsible for Staphylococcal enterotoxins strong-evidence outbreaks in 2014 was mixed foods (29.0%), followed by pig meat and products thereof and broiler meat and products thereof (both around 9.7%).

Regarding Staphylococcal enterotoxins weak-evidence outbreaks, the most common food vehicles reported were other foods and mixed foods (overall, 195 outbreaks), followed by red meat products, eggs, bovine meat, pig meat, vegetables, crustaceans, shellfish, and molluscs (European Food Safety Authority 2015).

1.7.4. Surveillance of food and beverages in Italy

The official monitoring of food and beverages is crucial to prevent consumer health risk and to ensure the conformity of food products. In Italy, surveillance activities are performed on both Italian products and other origin sources, generally exported to other EU Member States or to additional international destinations. Official controls are conducted at every production stage, at processing, storage, transport, distribution. Monitoring includes inspections at various stages of production: inspection, sampling, laboratory analysis of samples, employees hygiene control, examination of documents, check of verification systems operated by the company and the produced results.

The official control takes into account:

- status, hygiene conditions and uses of the production plants, equipment, tools, facilities, structures and transportations;
- raw materials, ingredients, additives and any other product employed;
- production or preparation for human consumption;
- semi-finished goods;

- finished goods;
- materials and articles in contact with food;
- disinfection, cleaning and maintenance procedures;
- production and food processing;
- labeling and presentation of foodstuffs;
- preservation measures.

Italian Health Ministry yearly delivers to Parliament an annual report indicating the results of supervisory activities and analytical controls on food and drinks in Italy. The activities allow a constant monitoring aimed to health protection to reduce the trend of fraud and adulteration in the country.

In Europe, the official control of food and beverages is established by CE Regulation n. 882/2004, which provides a Multi-annual National Control Plans (MANCP), through which authorities dispose special programs defining the nature and frequency of inspections regularly carried out within the manufacture, packaging and distribution stages. Each Member State develops monitoring programs defining the nature and frequency of the inspections, which are regularly conducted during a specific period. In Italy, D.P.R. 14 July 1995 summarizes the indications to direct and coordinate the states/provinces establishing specific criteria for official controls of food and beverages. According to art. 10, official controls include the examination of the control systems set by company employees; inspections of the products; sampling and analysis of the samples, performed by the laboratories of ASL, ARPA, IZS, ICQRF and additional laboratories indicated by the Health Ministry; check of documents presented by company; evaluation of control systems adopted by the company (HACCP), including the employees training.
Overall, in 2014, 472.856 inspections have been conducted and 66.628 non compliances were reported. The majority of non-compliances involve restaurants, distribution, production and packaging stages (Ministero della Salute 2015).

1.7.5. Italian PNI Annual Report on food and beverages

The Annual PNI (Piano Nazionale Integrato) Report for 2015, the plan of surveillance and control on food and beverages, prepared according to Regulation (CE) n. 882/2004 and Regulation (CE) 654/2008, gathers all the data regarding official controls performed during the year on food and beverages. Annual Report provides a dual operational tool: to test the effectiveness of official controls and to guide future work, aiming to improve and optimize the entire system of controls.
The official controls on food production activities, food and beverages distribution in 2015, were conducted on 107.247 samples. Foods of animal origins registered the highest microbial non compliances (84%). Over

the 424 non-compliances regarding food of animal origin, 359 were due to microorganisms, of which 162 contaminated samples were meat products, thus representing half the non-compliances. In 2015, 107.247 contaminants were researched, a 63% rate represented by pathogens and workplace contamination indicator microorganisms: the highest percentage of pathogenic microorganisms was related to *Salmonella* spp. (29.29%), *Listeria monocytogenes* (22.2%), and *Escherichia coli* (14.15%) (Ministero della Salute 2016). With regards to *Salmonella* spp., the majority of non-compliances were mainly observed on meat and eggs and products thereof, in vegetables, and in ready-to-eat food products. *Listeria* spp., instead, was mainly isolated from ready-to-eat food products, meat and products thereof, fish and milk products (Ministero della Salute 2015).

2. MATERIALS AND METHODS

2.1. Study design and sampling

The research was conducted from October 2014 and July 2016 and was performed on raw meat sampled from 7 slaughterhouses and 7 butcher shops in Regione Campania (Italy). Sampling was based on random selection of sampling days and batches, to ensure the maximum samples representativeness. Sampling ensured the analysis of the same amount and type of samples for each slaughterhouse.

Samples (storage temperature: 4°C; storage time: 1-2 days) were prepared starting from 3 different types of meat: beef, poultry, pork. The experiments were based on the identification of chemical markers and metabolomics profiles of meat contamination, through the analysis of volatile organic compounds (VOCs).

The samples underwent contemporary microbiological and molecular biology analysis for the research and identification of spiked *Salmonella* Typhimurium, *Campylobacter jejuni*, and *Staphylococcus aureus*. The samples were kept cold during transport to the laboratory (taking care of shortening the times as much as possible). Microbiological analyses were conducted using standardized procedures, by weighing and homogenizing sample aliquots to obtain the desired solution. Afterwards, sub-aliquots were inoculated in liquid and solid enrichment growth media, to allow subsequent identification and enumeration of metabolic germs.

Selected food matrices, when microbiological evaluations proved the absence of specific pathogens, were analyzed by HS-GC-MS. The evaluations work flow consisted on several stages, based on the analysis of profiles produced by:

1. inoculums of ATCC strains of *Salmonella* Typhimurium (ATCC 14028), *Campylobacter jejuni* (ATCC 33291), *Staphylococcus aureus* (ATCC 26923).
2. Mixed inoculums of the three ATCC strains (4 combinations: S+C, S+A, C+A, S+C+A; **Table 1**).
3. Meat matrices (beef, chicken, pork), all presenting the basic spoilage flora, but not the target pathogens (**Table 2**).
4. Meat matrices spiked with a 10^6 UFC/mL concentration of the three ATCC strains (9 combinations: B+S, B+C, B+A, P+S, P+C, P+A, Ch+S, Ch+C, Ch+A; **Table 3**).
5. Meat matrices spiked with the 4 combinations (see 2.) of the ATCC strains (4 mixes for 3 meat samples: 12 combinations; **Table 4**).
6. Meat matrices spiked with a 10^3 and 10^2 UFC/mL concentration of the three ATCC strains (18 combinations: 9 each bacterial cell concentration; **Table 5**).

A replicate of each sample was evaluated with standard cultural methods for *Salmonella* spp. *Campylobacter* spp. and *Staphylococcus aureus* identification, to confirm the proper contamination procedure and the adequate inoculum concentration.

Furthermore, additional replicates of samples spiked with *Salmonella* Typhimurium (ATCC 14028), *Campylobacter jejuni* (ATCC 33291), *Staphylococcus aureus* (ATCC 26923) were submitted for molecular biology analysis: employing the CTAB- chloroform-isoamyl alcohol method for the extraction of DNA and amplifying the samples with primers able to select the three pathogens, bacterial DNA was detected by gel electrophoresis through band productions. The presence of bands on the gel electrophoresis indicates the proper contamination of each sample due to the pathogens. Such multi-field approach aid in the overall analysis of the data aimed to the identification of typical compounds for each contamination.

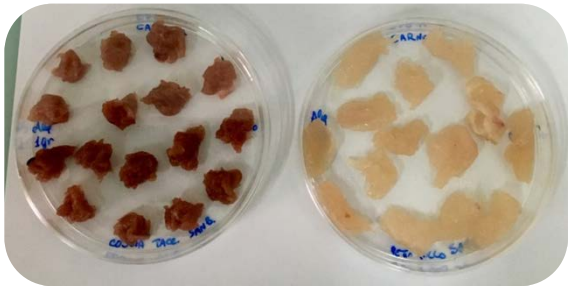


Figure 3 Preparation of meat samples for molecular biology and analytical chemistry protocols.

2.2. Microbiological analysis

2.2.1. *Salmonella* spp. analysis

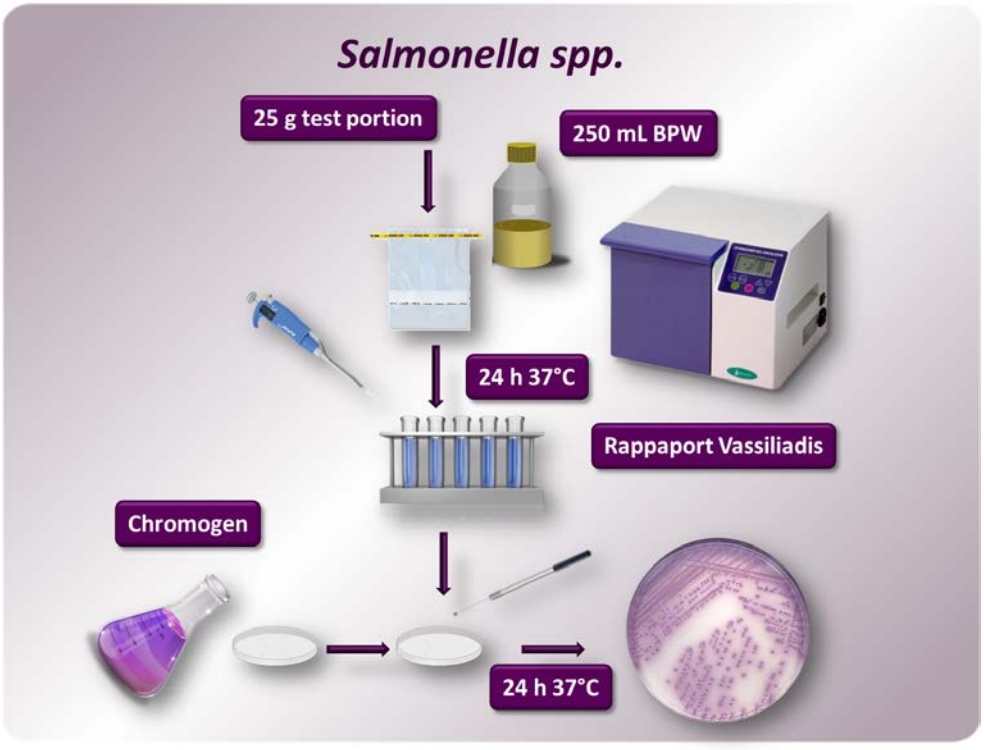


Figure 4 Flow chart of microbiological technique for *Salmonella* spp. detection.

According to **UNI EN ISO 6579:2008**, an aliquot of 25 g meat sample and 225 mL BPW (Buffered Peptone Water – OXOID) were added to a Stomacher bag. Bags were homogenized and incubated at 37±1°C for 24 h. Homogenate underwent a subsequent enrichment step: 100 µL from the sample were added to 10 mL RPV broth (Rappavort Vassilliadis Broth – OXOID). The enrichment tryptone provides the carbon and the nitrogen necessary for growth; high magnesium chloride concentration increases the osmotic pressure; low medium (5.1 ± 0.2) and malachite green inhibits the growth of other microorganisms ensuring the broth selectivity for *Salmonella* spp. Samples were incubated at 41±1 °C for further 24 h and then surface plated on selected media, XLD and Salmonella Chromogenic Agar Base (OXOID), incubated at 37±1 °C for 24 h (International Organization for Standardization 2008).

Salmonella Chromogenic Agar Base (OXOID) represents the unique medium consenting the characterization of lactose-positive strains of *Salmonella*: such selectivity is ensured by a mixture of inhibitory substances such as a cephalosporin mainly active in the suppression of *Pseudomonas* spp., sodium deoxycholate and sodium cholate, active in the suppression of Gram-positives and Gram-negatives, tergitol 4, inhibiting *Proteus* spp. The differentiation of *Salmonella* strains is possible through the presence in plates of a chromogenic substrate acting on a specific *Salmonella* esterase, with the release of a magenta-red metabolite; the presence of a chromogenic glucopyranoside derivative, hydrolyzed by *Salmonella* beta-glucosidases, with the release of a green-blue metabolite (**Figure 4**).
Salmonella spp. evaluation is qualitative (presence/absence): agar plates showing typical growth characteristics (red-magenta) undergo biochemical and serological confirmations. Serological evaluations use latex tests (OXOID – Salmonella Latex Test): a wide range of *Salmonella* flagella antigens have been used to produce polyvalent antisera in rabbits. The purified antibodies are used to sensitize the latex particles. Presumptive *Salmonella* colonies are selected from an agar plate, mixed with the latex test reagent and agglutination occurs when *Salmonella* spp. is present.

Biochemical analysis helps revealing the identity of presumptive *Salmonella* species and is based on the use of RapID ONE System (REMEL), consisting of 19 biochemical reactions. To each available strip, bacterial suspensions prepared from Salmonella Shigella Agar (OXOID) and inoculation fluid were inoculated and then incubated at 37±1 °C for 4 h. Metabolism produces color changes: the reactions – positive and negative test scores – were interpreted according to ERIC® software (REMEL).
The absence of typical colonies on selective media (black colonies on XLD, red-magenta colonies on Salmonella Chromogenic) allowed the preparation of *Salmonella*-free meat samples.

2.2.2. *Campylobacter* spp. analysis

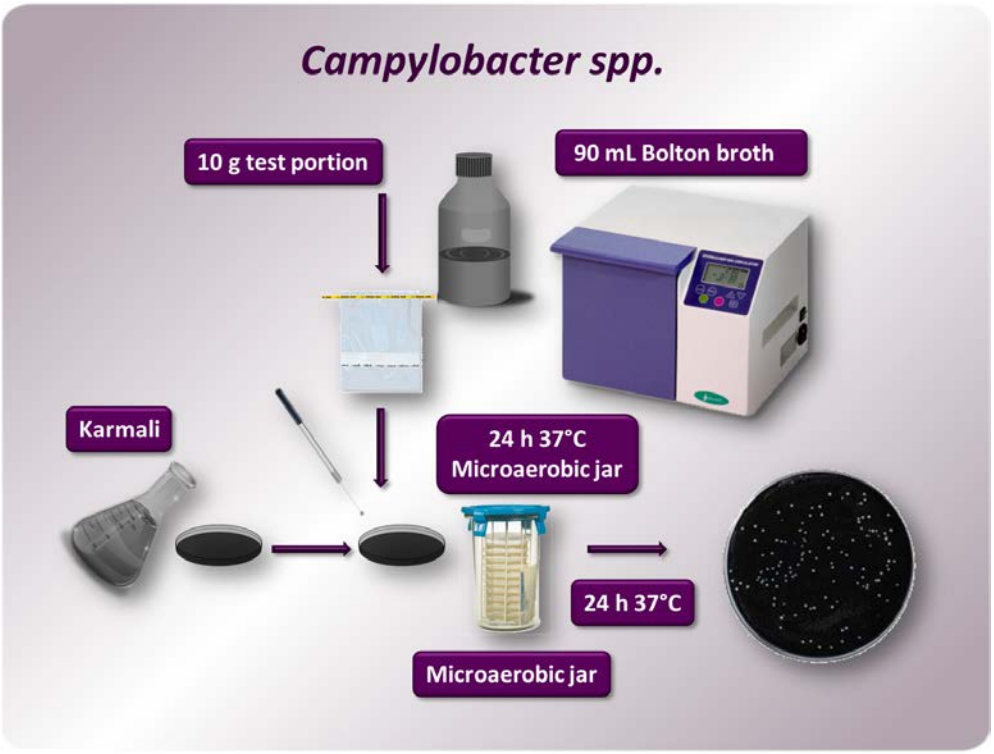


Figure 5 Flow chart of microbiological technique for *Campylobacter* spp. detection.

An aliquot of 10 g meat sample and 90 mL Bolton Broth Selective Enrichment Broth (OXOID) was added to a Stomacher bag. The broth was formulated to revitalize damaged cells, allowing their multiplication. It is supplemented with Laked Horse Blood (OXOID SR0048) and Bolton Broth Selective Supplement (OXOID SR0183), containing several antibiotics, vancomycin (inhibiting the growth of Gram-positive microorganisms), cefoperazone (inhibiting Gram-negatives), trimethoprim (against several Gram-negative and Gram-positive) and cicloheximide (antifungal). Stomacher bag was first homogenized, and then incubated at 37±1 °C for 4 h and 41±1 °C for 48 h, under microaerophilic conditions (5% O_2). The change in growing temperatures is crucial in order to increase the selective pressure on the microorganisms competitors, considering that the majority of species of Campylobacter genus are thermotolerant. After the incubation, using a loop, an aliquot from the homogenate was surface plated on mCCD (OXOID) and Campylobacter Agar Base (Karmali, OXOID) and incubated at 41±1 °C for 24 hours, under microaerophilic conditions (5% O_2) **(ISO 10272:2006)** (International Organization for Standardization 2006).

Campylobacter spp. evaluation is qualitative (presence/absence): colonies result grey, flat and swarming (**Figure 5**). Suspect colonies are subjected to further biochemical tests as a confirmation. In particular, *Campylobacter* spp. is positive to oxidase and catalase texts. Oxidase test evaluates the presence of cytochrome oxidase enzyme: employing Microbact Oxidase Reaction Strips (OXOID), a positive reaction is

evident when a blue-violet color develops within a few seconds, after the suspect colony is put in contact with the reaction strip. Catalase reaction test consists on picking up a colony and suspending it on a drop of hydrogen peroxide (H_2O_2): the formation of bubbles (O_2) indicates positivity to the test. Karmali agar plates with growth characteristics (small grey colonies) are subjected to serological confirmation, using Dryspot Campylobacter Test Kit (OXOID). Purified antibodies are used to sensitize the latex particles. Suspect *Campylobacter* spp. colonies mixed are with the latex test reagent and agglutination occurs when *Campylobacter* spp. is present. It is additionally possible to precede with the identification of *Campylobacter jejuni* strains through antibiotic susceptibility tests employing cephalotin and nalidixic acid BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs (BD - Biomerieux Diagnostics). MICs were determined by agar dilution using a modification of Tryptone Soy Agar (OXOID) agar supplemented with 7% Lysed Horse Blood (OXOID): antibiotics discs are put on agar plates with swab-streaked bacterial suspension; plates are incubated at 41±1 °C for 24 hours, under microaerophilic conditions. The resistance to cephalotin and sensitivity to nalidixic acid – through the formation of an inhibiting halo around the disk - confirms the strain identification as *Campylobacter jejuni*.

The absence of typical colonies on selective media (greyish colonies on both) allowed the preparation of *Campylobacter*-free meat samples.

2.2.3. *Staphylococcus aureus* analysis

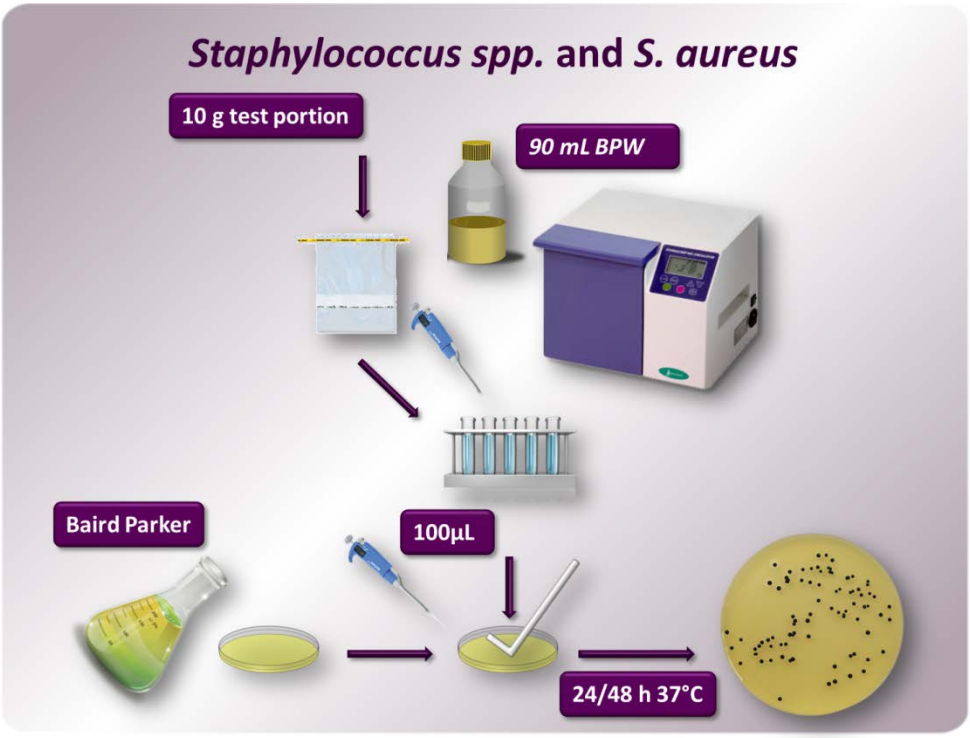


Figure 6 Flow chart of microbiological technique for *Campylobacter* spp. detection.

An aliquot of 10 g meat sample and 90 mL Buffered Peptone Water (BPW – OXOID) were homogenized into a Stomacher bag and an 100 μL aliquot is spread plated on Baird Parker selective agar (OXOID). Selective medium, is supplemented with Egg Yolk Tellurite Emulsion (OXOID); agar plates are then incubated at 37±1°C for 24-48 h. Baird Parker is used for the isolation of coagulase positive *Staphylococcus* spp. from food: lithium chloride and potassium tellurite inhibit the flora, glycine and sodium pyruvate allow *Staphylococcus* spp. growth, tellurite reduction to tellurium (black precipitate) and clarification of the egg yolk around the colony allow the presumptive identification of colonies on Baird Parker agar; furthermore, the formation of an opaque halo on Baird Parker RPF Agar (OXOID) ensures the definitive identification of coagulase positive Staphylococci. The growth of a variety of other strains (Streptococci, Micrococci, Corynebacteria and Enterobacteria) is observable, but the typical reaction does not develop. Plates are incubated for 48 hours, and the presence of typical colonies is verified after 24 and 48 hours. Two dilutions, containing up to 150 typical and/or atypical colonies, are counted **(UNI EN ISO 6888:1999)**.
Staphylococcus aureus analysis is quantitative: colonies are black, shiny, convex, 1-1.5 mm in diameter after 24 hours of 1.5-2.5 mm after 48 hours, surrounded by an opaque egg clarification halo. After 48 hours, *Staphylococcus aureus* colonies evidence a double halo, an inner opaque and an outer transparent. Black colonies, not presenting halos, are counted separately and classified as *Staphylococcus* spp. (**Figure 6)**
Presumptive *Staphylococcus aureus* undergoes a serological investigation with Staphytect plus (OXOID). The test employs blue latex particles coated with fibrinogen of porcine origin and rabbit IgG including specific polyclonal antibodies directed against *Staphylococcus aureus* capsular polysaccharides.
The absence of typical colonies on selective medium, black colonies indicating *Staphylococcus* spp., black colonies surrounded by a halo of egg clarification indicating *Staphylococcus aureus*, allowed the preparation of *Staphylococcus*-free meat samples.

2.3. Gas chromatography – mass spectroscopy analysis

2.3.1. Bacterial strains, medium, and growth conditions

The strains employed in the current research were *Salmonella* Typhimurium ATCC 14028, *Campylobacter jejuni* ATCC 33291, *and Staphylococcus aureus* ATCC 26923. Monocultures of all strains were cultured according to each microorganism's growth protocols for 24 h at 37°C with constant shaking at 150-200 rpm in 30 mL of Tryptic Soy Broth (TSB – OXOID) in 50 mL sterilized tubes. The final optical densities, measured employing spectrophotometer at 600 nm, in order to obtain a 10^8 cell/mL concentration, were of 0.1 O.D.: samples were then serially diluted to obtain the desired concentrations (10^6, 10^3, and 10^2 cell/mL). Mixed cultures of the 3 pathogens were prepared by individually culturing each microorganism in 30 mL TSB for 23 h at 37°C, shaking at 150-200 rpm. The monocultures were then mixed in same proportions of

Salmonella Typhimurium, *Campylobacter jejuni*, and *Staphylococcus aureus*, to obtain a final volume of 30 mL and then incubated for an additional hour at 37°C and 150-200 rpm, followed by GC-MS spectrum acquisition, as described below.

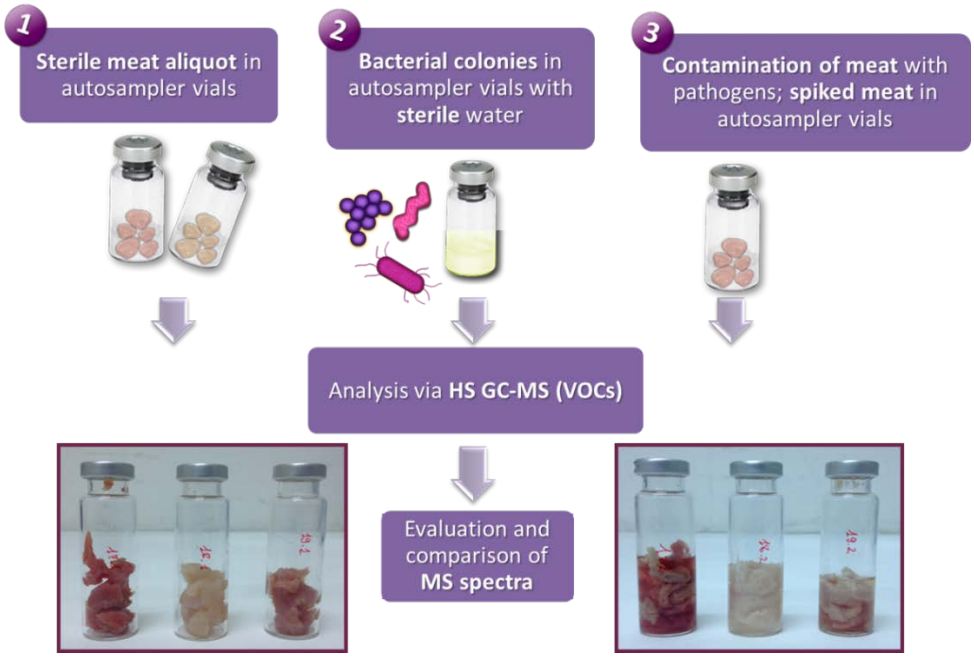


Figure 7 Graphical design of the sample preparations steps for chemical analysis (SPME HS-GC/MS).

2.3.2. Determination of VOCs produced by bacterial inoculate

Pure colonies of ATCC strains from *Salmonella* Typhimurium (ATCC 14028), *Campylobacter jejuni* (ATCC 33291), *and Staphylococcus aureus* (ATCC 26923) were surface plated on Plate Count Agar (PCA – OXOID). Colonies from each plate were inoculated in 5 mL sterile water, poured in duplicate in sterile glass autosampler vials and incubated at 37±1°C for 24 hours, in order to allow the production of VOCs. Aliquots from bacterial inocula were furthermore combined to verify the contemporary growth of pathogens in the same environment. All inocula contained a bacterial concentration of 10^6 UFC/mL (**Table 1**).

ABBREVIATION	DESCRIPTION
S	*Salmonella* Typhimurium in 5 mL H_2O_S
C	*Campylobacter jejuni* in 5 mL H_2O_S
A	*Staphylococcus aureus* in 5 mL H_2O_S
S+C	*Salmonella* Typhimurium + *Campylobacter jejuni* in 5 mL H_2O_S
S+A	*Salmonella* Typhimurium + *Staphylococcus aureus* in 5 mL H_2O_S
C+A	*Campylobacter jejuni* + *Staphylococcus aureus* in 5 mL H_2O_S
S+C+A	*Salmonella* Typhimurium + *Campylobacter jejuni* + *S. aureus* in 5 mL H_2O_S

Table 1 List of bacterial inocula (single or mixes) analyzed. Left coloumn shows the abbreviations chosen for each sample. Right column describes the samples preparations protocols.

2.3.3. Determination of VOCs produced in not contaminated raw meat (negative control)

Meat chops for each meat type, whose microbiology proved the absence of *Salmonella* Typhimurium, *Campylobacter jejuni* and *Staphylococcus aureus*, weighing 5 g each, were placed in sterile glass autosampler vials (20 mL, Agilent), and stored for 24h at 4°C (**Table 2**).

ABBREVIATION	DESCRIPTION
B	Beef (5.0 g)
Ch	Chicken (5.0 g)
p	Pork (5.0 g)

Table 2 List of negative control samples analyzed. Left coloumn indicates the abbreviation chosen for each sample. Right column describes the samples weight evaluated.

2.3.4. Determination of VOCs produced in meat spiked by each bacterium

Meat chops for each meat type, weighing 5 g each, were placed in sterile glass autosampler vials. An aliquot of 5mL each bacterial inoculum (with a **10^6 UFC/mL concentration**) was poured into the vials containing meat, in order to obtain meat samples contaminated by each pathogen (**Table 3**). Vials were incubated at 37±1°C for 24 hours.

ABBREVIATION	DESCRIPTION
B+S	5.0 g beef + *Salmonella* Typhimurium 10^6 UFC/mL in 5 mL H_2O_S
B+C	5.0 g beef + *Campylobacter jejuni* 10^6 UFC/mL in 5 mL H_2O_S
B+A	5.0 g beef + *Staphylococcus aureus* 10^6 UFC/mL in 5 mL H_2O_S
Ch+S	5.0 g chicken + *Salmonella* Typhimurium 10^6 UFC/mL in 5 mL H_2O_S
Ch+C	5.0 g chicken + *Campylobacter jejuni* 10^6 UFC/mL in 5 mL H_2O_S
Ch+A	5.0 g chicken + *Staphylococcus aureus* 10^6 UFC/mL in 5 mL H_2O_S
P+S	5.0 g pork + *Salmonella* Typhimurium 10^6 UFC/mL in 5 mL H_2O_S
P+C	5.0 g pork + *Campylobacter jejuni* 10^6 UFC/mL in 5 mL H_2O_S
P+A	5.0 g pork + *Staphylococcus aureus* 10^6 UFC/mL in 5 mL H_2O_S

Table 3 List of combination of meat matrices and single bacteria under analysis. Left coloumn shows the abbreviations for each sample. Right column indicates the protocols to prepare each contaminated sample.

2.3.5. Determination of VOCs produced in meat spiked by bacterial mixtures

5g meat chops for each meat type were weighed in sterile autosampler vials and spiked with 5 mL bacterial inocula, prepared combining two or three bacteria. Samples were incubated for 24h at 37±1°C. Samples replicates were analyzed to detect the contemporary presence of the different bacteria in each spiked meat (**Table 4**).

ABBREVIATION	DESCRIPTION
B+S+C	5.0 g beef + *Salmonella* Typhimurium + *Campylobacter jejuni* in 5 mL H_2O_S
B+S+A	5.0 g beef + *Salmonella* Typhimurium + *Staphylococcus aureus* in 5 mL H_2O_S
B+C+A	5.0 g beef + *Campylobacter jejuni* + *Staphylococcus aureus* in 5 mL H_2O_S
B+S+C+A	5.0 g beef + *Salmonella* Typhimurium + *Campylobacter jejuni* + *S. aureus* in 5 mL H_2O_S
P+S+C	5.0 g pork + *Salmonella* Typhimurium + *Campylobacter jejuni* in 5 mL H_2O_S
P+S+A	5.0 g pork + *Salmonella* Typhimurium + *Staphylococcus aureus* in 5 mL H_2O_S
P+C+A	5.0 g pork + *Campylobacter jejuni* + *Staphylococcus aureus* in 5 mL H_2O_S
P+S+C+A	5.0 g pork + *Salmonella* Typhimurium + *Campylobacter jejuni* + *S. aureus* in 5 mL H_2O_S
Ch.+S+C	5.0 g chicken + *Salmonella* Typhimurium + *Campylobacter jejuni* in 5 mL H_2O_S
Ch+S+A	5.0 g chicken + *Salmonella* Typhimurium + *Staphylococcus aureus* in 5 mL H_2O_S
Ch+C+A	5.0 g chicken + *Campylobacter jejuni* + *Staphylococcus aureus* in 5 mL H_2O_S
Ch+S+C+A	5.0 g chicken + *Salmonella* Typhimurium + *Campylobacter jejuni* + *S. aureus* in 5 mL H_2O_S

Table 4 List of combination of meat matrices and mixed bacterial inocula (2 or 3 pathogens) under analysis. Left coloumn indicates the abbreviations for each sample. Right column describes methods employed to prepare each contaminated sample.

2.3.6. Determination of VOCs from meat spiked by bacteria at decreasing concentrations

Meat chops for each meat type, weighing 5 g each, were placed in sterile glass autosampler vials. An aliquot of 5mL each bacterial inoculum with 10^3 UFC/mL and 10^2 UFC/mL bacterial concentrations were poured into the vials containing meat, in order to obtain meat samples contaminated by each pathogen (**Table 5**). Vials were incubated at 37±1°C for 24 hours.

ABBREVIATION	DESCRIPTION
B+S2	5.0 g beef + *Salmonella* Typhimurium 10^2 UFC/mL in 5 mL H_2O_S
B+C2	5.0 g beef meat + *Campylobacter jejuni* 10^2 UFC/mL in 5 mL H_2O_S
B+A2	5.0 g beef + *Staphylococcus aureus* 10^2 UFC/mL in 5 mL H_2O_S
B+S3	5.0 g beef + *Salmonella* Typhimurium 10^3 UFC/mL in 5 mL H_2O_S
B+C3	5.0 g beef meat + *Campylobacter jejuni* 10^3 UFC/mL in 5 mL H_2O_S
B+A3	5.0 g beef + *Staphylococcus aureus* 10^3 UFC/mL in 5 mL H_2O_S
Ch+S2	5.0 g chicken + *Salmonella* Typhimurium 10^2 UFC/mL in 5 mL H_2O_S
Ch+C2	5.0 g chicken + *Campylobacter jejuni* 10^2 UFC/mL in 5 mL H_2O_S
Ch+A2	5.0 g chicken + *Staphylococcus aureus* 10^2 UFC/mL in 5 mL H_2O_S
Ch+S3	5.0 g chicken + *Salmonella* Typhimurium 10^3 UFC/mL in 5 mL H_2O_S
Ch+C3	5.0 g chicken + *Campylobacter jejuni* 10^3 UFC/mL in 5 mL H_2O_S
Ch+A3	5.0 g chicken + *Staphylococcus aureus* 10^3 UFC/mL in 5 mL H_2O_S
P+S2	5.0 g pork + *Salmonella* Typhimurium 10^2 UFC/mL in 5 mL H_2O_S
P+C2	5.0 g pork + *Campylobacter jejuni* 10^2 UFC/mL in 5 mL H_2O_S
P+A2	5.0 g pork + *Staphylococcus aureus* 10^2 UFC/mL in 5 mL H_2O_S
P+S3	5.0 g pork + *Salmonella* Typhimurium 10^3 UFC/mL in 5 mL H_2O_S
P+C3	5.0 g pork + *Campylobacter jejuni* 10^3 UFC/mL in 5 mL H_2O_S
P+A3	5.0 g pork + *Staphylococcus aureus* 10^3 UFC/mL in 5 mL H_2O_S

Table 5 List of combination of meat matrices and single bacteria, spiked at decreasing concentrations. Left coloumn shows the abbreviations for each sample. Right column indicates the protocols to prepare the spiked samples.

All samples were prepared in triplicate and analyzed via HS-GC/MS, to detect volatiles patterns. A replicate from each sample underwent microbiological analysis, according to ISO protocols, to confirm the presence of pathogens every combination and inoculum.

2.3.7. SPME-GC/MS settings

The gas chromatographic analyses were performed with an Agilent 6890 Series GC, coupled to a MS 5973 detector. The column used is a DB-5ms capillary column (30 m × 0.25mm ID, 0.25µm film, 5% phenyl 95% polydimethylsiloxane). It has been used Helium as carrier gas, with a flow-rate of 1.0 mL/min. For SPME analyses, 5 g meat sample in 10 mL vials were used.

Agilent GC 6890 with MSD 5973

Column: 30 m DB-5ms, 0.25mm ID, 0.25µm film

Oven: 45°C for 3 min, 150°C to 12°C/min, 230°C to 18°C/min, 250 °C to 19°C/min

Injection: 1 mL gas phase at room temperature

Figure 8 GC/MS settings and useful information regarding analytical protocol employed for VOCs detection.

Solid phase micro extractions were performed by using DVB/CAR/PDMS 50/30 µm fibers, exposed for 30 min in the headspace of the flask, under constant stirring, at a temperature of 70 °C (adsorption). Subsequently, the fiber was exposed in the injector of the GC injector, maintained at a temperature of 230°C for 3 min (desorption). The gradient used for analysis was as follows: 45°C for 3 min, 150°C to 12°C/min, 230°C to 18°C/min, 250°C to 19°C/min (**Figure 9**). The analyzer of the GC was maintained at 250°C. The collision energy in the source was set to a value of 70 eV and the resulting; fragment ions generated were analyzed in the mass range of 30-450 mass m/z. For the evaluation of volatile components, the NIST (National Institute of Standards and Technology) Mass Spectral library and comparison with the electron impact mass spectra and retention times of standards were used.

2.4. Molecular biology analysis

2.4.1. Bacterial direct DNA extraction

A molecular biology analysis, performed in parallel with GC-MS and cultural determinations, analyzing the samples prepared for microbiological and chemical evaluations, was conducted. A replicate of the meat samples contaminated with each single microorganisms where evaluated through DNA extraction and PCR amplification.

2.4.2. DNA extraction from meat

A 5 g meat aliquot contaminated with *Salmonella* Typhimurium ATCC 14028, *Campylobacter jejuni* ATCC 33291, and *Staphylococcus aureus* ATCC 26923 was homogenized with 10 mL 0.9% NaCl solution; 2 mL from

the sample was transferred to 2 mL fresh tubes and vigorously vortexed (for 10-20 seconds) liquid portion was recovered in new tubes. Sample was centrifuged at 6000 g for 5 minutes and supernatant was discarded. A modified CTAB-phenol–chloroform-isoamyl alcohol method was employed to extract bacterial DNA from meat. Pellet was reconstituted with 700 µL of CTAB extraction buffer (200-mM Tris–HCl (pH 8.0), 25-mM EDTA (pH 8.0), 250-M NaCl, 10 % CTAB) (Doyle and others 1990).

Samples were kept in a water bath for 15 min at 65 °C and then put on ice for 10 min (at -20° C). One volume of chloroform: isoamyl alcohol (24:1) was added; solution was mixed and later centrifuged at 10.000 rpm for 10 min. The aqueous phase was recovered in new tubes and one volume of chloroform: isoamyl alcohol (24:1) was added; tubes were centrifuged at 10.000 rpm for 10 min, and the aqueous phase was recovered. A 0.7 volume of ice-cold isopropanol was added and samples were stored for 30 minutes at −20 °C, allowing DNA precipitation. Samples were centrifuged at 13.300 rpm for 15 min, supernatant was discarded and pellet was rinsed twice with 500 µL of 80% ethanol. The DNA was then resuspended in 40 µL Milli-Q Type 1 Ultrapure Water.

2.4.3. Bacterial DNA polymerase chain reaction

PCR reactions were carried out in a TECHNE Prime Thermal Cycler. A typical 25 µl PCR reaction contained 50 µM of each primer from the three bacteria primers set (**Table 6**), 0.2 µM of each dNTP (VWR Chemicals), PCR Key Buffer Triton Free (Tris-HCl pH 8.5, KCl, 15 mM $MgCl_2$ – VWR Chemicals), 1u VWR Taq DNA polymerase (VWR Chemicals) and 2 µL sample DNA.

PCR Target and Gene	Primer	Size (bp)	Reference/source
***Salmonella* spp.** **invA gene**	**f-invA** 139: 5'-GTG AAA TTA TCG CCA CGT TCG GGC AA-3' **r-invA** 141: 5'-TCA TCG CAC CGT CAA AGG AAC C-3'	284	Rahn and others 1992 Galàn and others 1992
***Campylobacter* spp.** **ceuE gene**	**COL3Upper** : 5'-ATTTGAAAATTGCTCCAACTATG-3' **MDCOL2Lower:** 5'-TGATTTTATTATTTGTAGCAGCG-3'	462	Gonzales and others 1997 Denis and others 1999
Staphylococcus aureus **nuc gene**	**NF1** : 5'-GCGATTGATGGTGATACGGTT-3' **NR2** : 5'-AGCCAAGCCTTGAACGAACTAAAGC-3'	270	Ali and others 2014

Table 6 List of specific primers sets employed to amplify DNA extracts and to discriminate from the 3 pathogens analyzed (*Salmonella* Typhimurium*, Campylobacter jejuni, Staphylococcus aureus*).

The incubation conditions for *Salmonella* Typhimurium amplification were 95°C for 2 min (initial denaturation), followed by 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s. A final extension of 72°C for 5 minutes was employed. *Campylobacter jejuni* settings for primary amplification were 1 cycle at 95°C for 2 min; 25 cycles of 30 s at 94°C, 90 s at the annealing temperature, 60 s at 72°C; and a final

extension for 10 min at 72°C (Douglas Inglis and others 2003). For *Staphylococcus aureus* amplification, initial denaturation at 95 °C for 2 min was followed by 35 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 60 s) and terminated with final extension at 72 °C for 10 min (Ali and others 2014). The amplified products were visualized and analyzed for size on agarose gel (1.5%), stained with GelRed (Nucleic Acid Gel Stain – BIOTIUM) using DNA 100 bp ladder as a reference.

Periodically, to confirm the identification of the ATCC strains, the bacterial DNA was amplified using Universal PCR primers, complementary to V3 and V6 regions (16S rDNA) (Chakravorty and others 2007). The selected oligo were V3_F (5'-CCAGACTCCTACGGGAGGCAG-3') and V6_R (5'-TCGATGCAACGCGAAGAA-3'). PCR products (≈700 bp) were purified with PEG (polyethylene glycol) precipitation protocol and used in sequencing reactions with the ABI PRISM Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). The sequence data obtained were compared to those available in the NCBI Sequence Database.

2.5. Statistical analysis

In order to simplify the visualization of the combined results, to emphasize the associations between the components, and to better evaluate the wide pool of data, **multivariate analysis** was employed. The relationships between variables and the variation present in the dataset matrix were accounted by biplotting both the ordination component scores and the variable loading coefficients through **Principal Component Analysis (PCA)** based on the Pearson's correlation matrix, in order to identify the major discriminating variables associated with a given principal component. Normality of data and homogeneity of variance were previously checked. **XLSTAT software**, version 2016.18.7.01, a data analysis and statistical application available for Microsoft Excel®, was used for data elaboration.

3. RESULTS

The analysis of individual volatile species produced by inoculation of *Salmonella* Typhimurium, *Campylobacter jejuni,* and *Staphylococcus aureus* in different meat samples was performed by using the highly sensitive Solid-Phase Micro Extraction (SPME) technique in order to investigate at molecular level on pathogenic bacteria causing commune foodborne diseases. Rapid methods of detecting bacteria species are required to prevent outbreaks of food poisoning. Solid-phase microextraction (SPME) is an innovative, solvent-free sample preparation technology that is fast, cheap, and versatile and reduce sample manipulation at minimum. It uses a fused-silica fiber coated with a polymer to extract analytes from liquid and gas matrices.

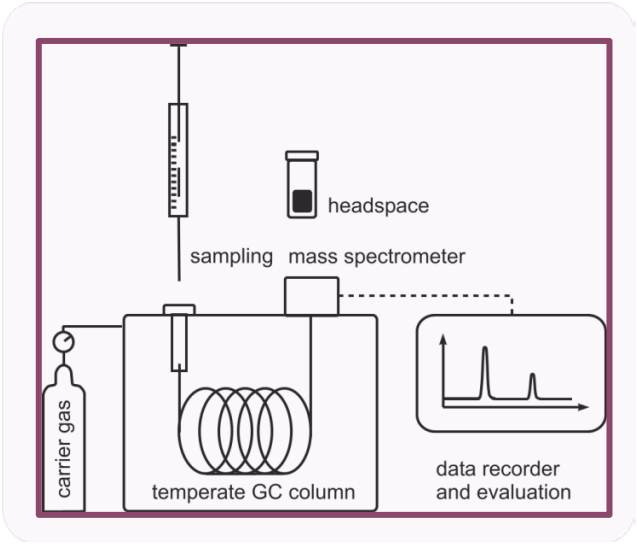


Figure 9 Example of a GC/MS system. VOCs pass through the column and are registered by mass spectrometer.

All the samples were subjected to head space SPME. Different SPME fibers were tested, being the DVB/CAR/PDMS 50/30 µm fiber the most suitable in terms of signal-to-noise ratio and number of analytes adsorbed. Headspace SPME was used for sample collection and then the extracts were injected directly into the GC system (**Figure 9**).

Applying SPME to mass spectral GC-MS technique, the mass spectra of the headspace volatiles produced by *Salmonella* Typhimurium, *Campylobacter jejuni* and *Staphylococcus aureus* were collected.

SPME mass spectral analyses were performed on *S.* Typhimurium, *C.jejuni* and *S. aureus* samples analyzed as single and as mixtures at two and three components and considered as blanks. Blank extractions of bacterial cells using SPME had only a few chromatographic peaks at very short retention times and different from species detected in inoculated samples. Similar analyses were carried out on diverse meat samples (beef, chicken, and pork) without the spiking of the bacteria; subsequently, the meat samples were inoculated with the same bacteria and bacterial mixes at different concentrations.

The volatile compounds were analysed by **SPME-GC/MS**. Percentages indicated in the quantitative data describe the **Peak Area** values of the chromatograms: the area is proportional to the amount of the compound that has been detected. The area refers to the mean relative abundance registered by each volatile compound within the measurements. The peak aria values described in the Tables and Figures indicate the mean between 5 different samples each considered evaluation, analyzed in triplicate by SPME-GC/MS.

Qualitatively, these spectra are all unique, possessing distinctive features that can be used to distinguish these bacterial groups from one another strictly by their volatile profiles. The mass spectrum captures more information about the bacterial volatiles than just the smell of the culture (Zhu J. and others 2010). It is important to specify that **no naturally occurring *Salmonella* spp., *Campylobacter* spp. and/or *Staphylococcus* spp. were present in the control samples.**

In order to ensure a better data interpretation, abbreviations have been employed to describe the samples. **Table 7** summarizes the list of abbreviations referred to each sample evaluated.

DESCRIPTION	ABBREVIATION
S. Typhimurium 10^6 UFC/mL	**S**
C. jejuni 10^6 UFC/mL	**C**
S. aureus 10^6 UFC/mL	**A**
S. Typhimurium + *C. jejuni* 10^6 UFC/mL	**S+C**
S. Typhimurium + *S. aureus* 10^6 UFC/mL	**S+A**
C. jejuni + *S. aureus* 10^6 UFC/mL	**C+A**
S. Typhimurium + *C. jejuni* + *S. aureus* 10^6 UFC/mL	**S+C+A**
Beef (5.0 g)	**B**
Chicken (5.0 g)	**Ch**
Pork (5.0 g)	**P**
5.0 g beef + *S.* Typhimurium 10^6 UFC/mL	**B+S**
5.0 g beef + *C. jejuni* 10^6 UFC/mL	**B+C**
5.0 g beef + *S. aureus* 10^6 UFC/mL	**B+A**
5.0 g chicken + *S.* Typhimurium 10^6 UFC/mL	**Ch+S**
5.0 g chicken + *C. jejuni* 10^6 UFC/mL	**Ch+C**
5.0 g chicken + *S. aureus* 10^6 UFC/mL	**Ch+A**
5.0 g pork + *S.* Typhimurium 10^6 UFC/mL	**P+S**
5.0 g pork + *C. jejuni* 10^6 UFC/mL	**P+C**
5.0 g pork + *S. aureus* 10^6 UFC/mL	**P+A**
5.0 g beef + *S.* Typhimurium + *C. jejuni*	**B+S+C**
5.0 g beef + *S.* Typhimurium + *S. aureus*	**B+S+A**
5.0 g beef + *C. jejuni* + *S. aureus*	**B+C+A**
5.0 g beef + *S.* Typhimurium + *C. jejuni* + *S. aureus*	**B+S+C+A**
5.0 g pork + *S.* Typhimurium + *C. jejuni*	**P+S+C**
5.0 g pork + *S.* Typhimurium + *S. aureus*	**P+S+A**
5.0 g pork + *C. jejuni* + *S. aureus*	**P+C+A**
5.0 g pork + *S.* Typhimurium + *C. jejuni* + *S. aureus*	**P+S+C+A**
5.0 g chicken + *S.* Typhimurium + *C. jejuni*	**Ch.+S+C**
5.0 g chicken + *S.* Typhimurium + *S. aureus*	**Ch+S+A**
5.0 g chicken + *C. jejuni* + *S. aureus*	**Ch+C+A**
5.0 g chicken + *S.* Typhimurium + *C. jejuni* + *S. aureus*	**Ch+S+C+A**
5.0 g beef + *S.* Typhimurium 10^2 UFC/mL	**B+S2**
5.0 g beef meat + *C. jejuni* 10^2 UFC/mL	**B+C2**
5.0 g beef + *S. aureus* 10^2 UFC/mL	**B+A2**
5.0 g beef + *S.* Typhimurium 10^3 UFC/mL	**B+S3**
5.0 g beef meat + *C. jejuni* 10^3 UFC/mL	**B+C3**
5.0 g beef + *S. aureus* 10^3 UFC/mL	**B+A3**
5.0 g chicken + *S.* Typhimurium 10^2 UFC/mL	**Ch+S2**
5.0 g chicken + *C. jejuni* 10^2 UFC/mL	**Ch+C2**
5.0 g chicken + *S. aureus* 10^2 UFC/mL	**Ch+A2**
5.0 g chicken + *S.* Typhimurium 10^3 UFC/mL	**Ch+S3**

5.0 g chicken + *C. jejuni* 10^3 UFC/mL	**Ch+C3**
5.0 g chicken + *S. aureus* 10^3 UFC/mL	**Ch+A3**
5.0 g pork + *S.* Typhimurium 10^2 UFC/mL	**P+S2**
5.0 g pork + *C. jejuni* 10^2 UFC/mL	**P+C2**
5.0 g pork + *S. aureus* 10^2 UFC/mL	**P+A2**
5.0 g pork + *S.* Typhimurium 10^3 UFC/mL	**P+S3**
5.0 g pork + *C. jejuni* 10^3 UFC/mL	**P+C3**
5.0 g pork + *S. aureus* 10^3 UFC/mL	**P+A3**

Table 7 List of samples analyzed in the study. Left column describes the samples; right column indicates the abbreviations for each samples, used in the text to simplify the descriptions.

3.1. Single bacterial inocula

Table 8 describes the volatile organic compounds produced by the single bacteria inocula, with a **10^6 concentration**:

- As for ***S.* Typhimurium** (code: **S**), **ether, tert-butyl ethyl** represents the compound with the higher peak area (2.46%), followed by **dichloroacetaldehyde** (1.56 %) and **1,2-dichloropentane** (1.69%).
- ***C. jejuni*** (code: **C**) produced **ether, tert-butyl ethyl**, with a 27% concentration, **1,2-dichloropentane** (6.64%), **methylene chloride** (5.99%), **hexane** (5.46%).
- ***S. aureus*** profile (code: **A**) evidences the presence, with really low concentrations, of **methylene chloride** (0.86%), **hexane** (0.83%) and **dimethylbenzamide.**

Salmonella Typhimurium 10^6		
RT^{-1} (min)	**Compound**	**PA (%)**
1,98	dichloroacetaldehyde	1.56
1,56	hexane	0.87
2,44	ether, tert-butyl ethyl	2.46
2,53	dichloropentane	1.69
2,97	benzene	0.55

Campylobacter jejuni 10^6		
RT^{-1} (min)	**Compound**	**PA (%)**
2,27	hexane	5.46
2,41	ether, tert-butyl ethyl	27.5
2,53	dichloropentane	6.64
1,92	methylene chloride	5.99

Staphylococcus aureus 10^6		
RT^{-1} (m)	**Compound**	**PA (%)**
1,95	methylene chloride	0.86
2,08	ethylthioacetone	4.21
2,27	hexane	0.83
2,43	ter- butyl formiate	1.13
4,99	2,5-dimethyl- 2,5-hexandiol	0.23
5,34	dimethylbenzamide	0.22

Table 8 Results of GC-MS analysis: VOCs produced by *S.*Typhimurium, *C.jejuni*, and *S. aureus* bacterial inocula. [RT^{-1} (m): Retention time; PA (%): Peak Area normalized].

3.2. Bacterial mixtures inocula

The combination between the pathogens inocula brought out 4 profiles, 3 with 2 bacteria, 1 with the mix of the three microorganisms. The most abundant compounds of the ***S.* Typhimurium** and ***C. jejuni*** combination (**S+C**) are **hexane** (4.67%), **pentane, 1,2-dichloro-** (2.01%) and **ether, tert-butyl ethyl** (1.38%). Inoculum containing ***S.* Typhimurium** and ***S. aureus*** (**S+A**) produced 4 compounds (**methylene chloride**, **hexane**, **ethylacetate**, and **3,4-dimethylbenzamide**), while the combination between ***C. jejuni*** and ***S. aureus*** (**C+A**) showed the production, in low concentrations, of **1,2-dichloropentane** (2.44%), **hexane** (2.04%), and **methylene dichloride** (0.59%). The contemporary growth of the three bacteria (**S+C+A**) produced **hexane**, **ethylacetate** and **methylene dichloride** (Table 9).

Salmonella Typhimurium + *Staphylococcus aureus* 10^6 UFC/mL		
RT^{-1} (min)	**Compound**	**PA (%)**
1,94	methylene dichloride	5.51
2,27	hexane	3.49
2,45	ethyl acetate	3.86
5,56	3,4-dimethylbenzamide	0.58

Salmonella Typhimurium + *Campylobacter jejuni* 10^6 UFC/mL		
RT^{-1} (min)	**Compound**	**PA (%)**
1,9	methylene dichloride	0.52
2,24	hexane	4.67
2,44	ether, tert-butyl ethyl	1.38
2,54	pentane, 1,2-dichloro-	2.01

Campylobacter jejuni + *Staphylococcus aureus* 10^6 UFC/mL		
RT^{-1} (min)	**Compound**	**PA (%)**
1,94	methylene dichloride	0.59
2,27	hexane	2.04
2,53	pentane, 1,2-dichloro-	2.44

S. Typhimurium + *C. jejuni* + *S. aureus* 10^6 UFC/mL		
RT^{-1} (min)	**Compound**	**PA (%)**
1,92	methylene dichloride	0.71
2,44	hexane	2.19
2,37	ethyl acetate	1.64

Table 9 Results of GC-MS analysis: VOCs produced by bacterial inocula resulting from mixes of 2 or 3 pathogens (*S.*Typhimurium, *C.jejuni*, and *S. aureus*). [RT^{-1} (m): Retention time; PA (%): Peak Area normalized].

3.3. Single meat matrices

The subsequent step consisted on evaluating the spectra produced by single meat matrices (beef, chicken, and pork) (**Table 10**):

- The sample of **beef** (code: **B**) produced a wide set of volatiles, mainly consisting of **ethanol** (20.12%), **1-buthanol 3-methyl** (12.54%), **ethyl acetate** (6.34%), **1-heptene**, **hexane**, and **benzene**.
- **Chicken** (code: **Ch**) metabolism evidences the higher presence of **ethanol** (6.13%), **ethyl acetate** (**4.31**), **cyclohexane**, and **1-buthanol 3-methyl**.
- **Pork** (code: **P**) spectrum highlights the production of 4 compounds: **ethanol** (18.57%, the most abundant), **1-buthanol 3-methyl**, **heptane**, and **octane.**

Beef		
RT^{-1} (min)	**Compounds**	**PA (%)**
1.7	ethanol	20.12
2.18	ethyl acetate	6.34
2.27	hexane	5.88
2.45	benzene	5.78
2.67	1-butanol 3-methyl	12.54
2.7	1-heptene	6.12
2.81	1-pentanol	3.48
2.92	3-methylheptane	2.98
3.03	octane	2.27
3.08	2-butanone 3-hydroxy	2.09
3.2	2-hexanone 4-methyl	3.56
4.59	cuminal	1.69

Chicken		
RT^{-1} (min)	**Compounds**	**PA (%)**
1.7	ethanol	6.13
2.18	ethyl acetate	4.31
2.48	cyclohexane	3.22
2.61	1-heptene	2.73
2.67	1-butanol 3-methyl	3.07
3.03	octane	2.99
3.56	3-hepten 1-ol	2.84

Pork		
RT^{-1} (min)	**Compounds**	**PA (%)**
1.7	ethanol	18.57
2.65	heptane	4.96
2.67	1-butanol 3-methyl	5.44
3.03	octane	2.12

Table 10 Volatile organic compounds produced by the meat matrices (beef, chicken, and pork). [RT^{-1} (m): Retention time; PA (%): Peak Area normalized].

3.4. Production of volatile profiles on meat due to single bacterial contamination

Each meat matrix was spiked with the same concentration of **the three bacteria (10^6)** and was submitted for GC-MS analysis. Output chromatograms resulted pretty diversified.

3.4.1. Beef spiked with single bacterial inocula

S.* Typhimurium on beef** (**B+S**) produced 10 volatiles, whose more representative in terms of concentration are: **hexane** (18.33%), **ether, tert-butyl ethyl** (14.12%), **ethanol** (12.45%) and pentane (11.06%). Spectra of **beef** contaminated with ***C. jejuni (**B+C**) mainly highlight the presence of **pentane** (9.63%), **benzene** (4.9%), **furan, tetrahydro- 2,2,5,5-**, and **ethyl acetate**. The most abundant compounds from the combination between **beef** and ***S. aureus*** (**B+A**) result **ethanol** (10.04%), **propane, 2-ethoxy-2-methyl-** (9.02%), **pentanol** (7.69%), and **octane** (Table 11).

Beef + *Salmonella* Typhimurium 10^6		
RT^{-1} (min)	**Compound**	**PA (%)**
1,7	ethanol	12.45
1,7	butane	3.7
1,89	pentane	11.06
2,27	hexane	18.33
2,42	ethyl acetate	1.7
2,87	furan, tetrahydro-2,2,5,5-tetramethyl-	1.9
3,08	2-butanone, 3-hydroxy-	2.2
3,52	2-heptanol	2.3
5,8	ether, tert-butyl ethyl	14.12
6,3	ciclohexane 1,1,4 trimethyl	0.2

Beef + *Campylobacter jejuni* 10^6		
RT^{-1} (min)	**Compound**	**PA (%)**
1.89	pentane	9.63
2.43	ethyl acetate	3.4
2.45	benzene	4.9
2.87	furan, tetrahydro-2,2,5,5-tetramethyl-	4.23
3.03	octane	3.67
3.22	2 propane 1,3 dichloro	0.3

Beef + *Staphylococcus aureus* 10^6		
RT^{-1} (min)	**Compound**	**PA (%)**
1.69	ethanol	10.04
2.35	propane, 2-ethoxy-2-methyl-	9.02
2.45	benzene	3.6
2.60	1-heptene	3.29
2.81	pentanol	7.69
2.87	furan, tetrahydro-2,2,5,5-tetramethyl-	5.1
3.03	octane	4.87
3.2	methylcyclohexane	2.1
3.7	oxime-, methxy-phenyl-	0.8
5.4	glyoxal, tetrabutyl acetal	1.7

Table 11 Volatile organic compounds produced by beef spiked with *S.*Typhimurium, *C.jejuni*, and *S. aureus* (10^6 UFC/mL). [RT^{-1} (m): Retention time; PA (%): Peak Area normalized].

3.4.2. Chicken spiked with single bacterial inocula

The production of volatiles organic compounds due to ***S.* Typhimurium** contamination on **chicken** (**Ch+S**), mostly consists on **ethanol** (14.89%), **pentane** (12.28%), **hexane**, **benzoic acid**, and **heptane**. ***C. jejuni***, expoilting **chicken** nutrients (**Ch+C**), produced, with a significantly high concentration, **benzyl disulfide** (38.25%) and **indole** (29.9%), followed by **dimethyltrisulfide**, **butanoic acid, ethyl ester** and additional

compounds quantitatively less concentrated. ***S. aureus*** most abundant compounds produced on **chicken** (**Ch+A**) were **isopenthyl alcohol** (20.34%), **furan, tetrahydro- 2,2,5,5-** (16.89%), and **ethanol** (13.69%) (**Table 12**).

Chicken + *Salmonella* Typhimurium 10^6		
RT^{-1} (m)	**Compound**	**PA (%)**
1.7	1-propanol	2.9
1.7	ethanol	14.89
1.89	pentane	12.28
2.27	hexane	6.03
2.3	orthoformic acid	1.3
2.65	heptane	4.5
2.7	heptene (oxirane, pentyl-)	0.8
2.7	isopenthyl alcohol	0.6
3.03	octane	0.1
4.35	benzoic acid	5.9
5.8	ether, tert-butyl ethyl	1.1
6.2	1 octyne-3-ol	2.2
14.24	isoamylchloride	0.7

Chicken + *Campylobacter jejuni* 10^6		
RT^{-1} (min)	**Compound**	**PA (%)**
1,55	methanethiol	2.57
1,67	ethanol	1.56
2	1-propanol	1.78
2,1	hexane	0.76
2,4	ethyl acetate	2.02
2,89	isovaleric acid	2.1
2,92	butanal, 2-methyl-	1.91
3,46	methyl thiolacetate	3.98
3,7	propanoic acid, ethyl ester	3.43
4,2	isopenthyl alcohol	1.09
4,36	benzyl disulfide	38.25
5,45	butanoic acid, ethyl ester	5.6
7,41	disulfide, pentyl propyl	0.9
8,28	isopropyl valerate	1.2
8,97	dimethyl trisulfide	6.05
14,24	indole	29.9

Chicken + *Staphylococcus aureus* 10^6		
RT^{-1} (min)	**Compound**	**PA (%)**
1.71	ethanol	13.69
2.62	2 chloro propane	0.6
2.87	furan, tetrahydro-2,2,5,5-tetramethyl-	16.89
4.11	undecene	7.4
4.24	isopenthyl alcohol	20.34

Table 12 Volatile organic compounds produced by chicken spiked with *S.*Typhimurium, *C.jejuni*, and *S. aureus* (10^6 UFC/mL). [RT^{-1} (m): Retention time; PA (%): Peak Area normalized].

3.4.3. Pork spiked with single bacterial inocula

VOC profiles resulted from the metabolisms of the three bacteria on pork were, in terms of produced compounds, the most numerous: a wide variety of substances were eluted. ***S.* Typhimurium** on **pork** (**P+S**) mainly produced **1-butanol, 3-methyl** (16.11%), **benzene** (14.06%), **hexane** (13.50%), **ether, tert-butyl**

ethyl (11.22%), and **pentane** (10.88%). As for ***C. jejuni*** on the same meat type (**P+C**), similarly to *Salmonella* outcomes, the most concentrated volatiles were **ether, tert-butyl ethyl** (11.32%), **heptane** (9.6%), **1-butanol, 3-methyl** (8.92%), and benzoic acid; while ***S. aureus*** on **pork** (**P+A**) result described the prevalence of **butanal, 3-methyl** (15.02%), **undecene** (9.55%), and **propane, 2-ethoxy, 2-methyl-** (8.5%) (**Table 13**).

Pork + *Salmonella* Typhimurium 10^6		
RT^{-1} (m)	Compound	PA (%)
1.6	methanethiol	1.6
1.89	pentane	10.88
2.1	propanol	1.4
2.27	hexane	13.50
2.32	2-propanone, 1,1-dibutoxy-	2.3
2.45	benzene	14.06
2.48	ether, tert-butyl ethyl	11.22
2.65	heptane	8.59
2.83	3-pentanol, 2-methyl-	7.31
3.2	dimethylsulfide	2.1
3.31	cyclopentane, 1,2-dimethyl-	4.98
3.4	nonane	4.54
3.9	1-butanol, 3-methyl-	16.11
3.94	1-hexanol, 3,5,5-trimethyl-	6.35
4.39	benzoic acid	9.2
7.0	hexanol	3.6
7.4	oxirane, (1-methylbutyl)-	1.4

Pork + *Campylobacter jejuni* 10^6		
RT^{-1} (min)	Compound	PA (%)
1.89	pentane	3.16
2.45	benzene	4.5
2.48	ether, tert-butyl ethyl	11.32
2.65	heptane	9.6
2.67	1-butanol, 3-methyl-	8.92
2.8	propanoic acid	2.8
2.81	butanal, 3 methyl	7.29
2.83	3-pentanol, 2-methyl-	6.5
3.0	pyridine, 2-(1,3,4-oxadiazol-2-yl)-	0.7
3.03	octane	1.1
3.31	cyclopentane, 1,2-dimethyl	5.43
3.4	nonane	3.98
3.7	1,1-ethanediol, diacetate	0.4
3.82	1-hexanol, 2-ethyl-	3.2
3.94	1 hexanol- 3,5,5 trimethyl	3.33
4.2	indole	0.6
4.3	indolizine	0.5
4.39	benzoic acid	8.34
4.5	dimethylbenzamide	0.3

Pork + *Staphylococcus aureus* 10^6		
RT^{-1} (min)	Compound	PA (%)
1.71	ethanol	6.02
1.89	pentane	6.5
10.2	cycloundecene	0.2
2.27	hexane	5.3
2.31	propane, 2-ethoxy-2-methyl-	8.5
2.45	benzene	6.32
2.65	heptane	5.8
2.67	1-butanol, 3-methyl-	4.2
2.95	butanoic acid	4.7
3.31	cyclopentane 1,2-dimethyl	5.59
3.4	1,4-heptadiene	0.2
3.4	nonane	4.3
3.82	ethylhexanol	3.67
4.11	undecene	9.55
4.24	butanal, 3-methyl-	15.02
4.5	hydroxyurea	0.1
5.0	trimethylcyclohexane	0.2
5.6	ethosuximide	0.4

Table 13 Volatile organic compounds produced by pork spiked with *S.*Typhimurium, *C.jejuni*, and *S. aureus* (10^6 UFC/mL). [RT^{-1} (m): Retention time; PA (%): Peak Area normalized].

3.5. Production of volatile profiles on meat due to mixed bacterial contamination

3.5.1. Beef spiked with bacterial mixes

The next phase of the research was based on the contamination of the same meat matrices with mixtures of the three microorganisms. In this case, as it is possible to observe in the tables, less compounds for each profile were released:

- from the samples of **beef** spiked with ***S.* Typhimurium** and ***C. jejuni*** (**B+S+C**) it is predominantly observable the production of **ethanol** (11.79%), **2-butanone, 3 hydroxy-** (3.24**%), butanal, 3-methyl-** (2.9%), and **pentanol** (2.84%).
- ***S.* Typhimurium** and ***S. aureus*** on **beef** (**B+S+A**) produced, with concentrations ranging from 4% and 7%, **ethanol**, **ethyl acetate**, and **2-butanone** and other compounds with lower concentrations.
- The combination between ***C. jejuni*** and ***S. aureus*** on **beef** (**B+C+A**) released mostly **2-butanone, 3-hydroxy-**, **isopenthyl alcohol**, **ethanol**, and **hexane**.
- Beef spiked with the three bacteria (**B+S+C+A**) produced, with a really high concentration, **hexane** (33.63%), followed by **ethanol** (14.37%), **2-butanone, 3-hydroxy-**, **isopenthyl alcohol**, and **1,3-cyclohexadiene** (Table 14).

Beef + *S.*Typhimurium + *C. jejuni*		
RT^{-1} (min)	Compound	PA (%)
1,77	ethanol	11.79
2,33	2,3-butanedione	2.6
2,94	butanal, 3-methyl-	2.9
4,23	2-butanone, 3-hydroxy-	3.24
4,51	1-butanol, 3-methyl-	0.6
4,56	pentanol	2.84

Beef + *S.* Typhimurium + *S. aureus*		
RT^{-1} (min)	Compound	PA (%)
1,67	ethanol	6.82
2,23	2-butanone	4.33
2,36	ethylacetate	5.18
2,93	butanal, 3-methyl-	1.34
3,91	2-butanone, 3-hydroxy-	3.22
4,32	1-butanol, 3-methyl-	2.68
5,54	butanoic acid, ethyl ester	0.38

Beef + *C. jejuni* + *S.aureus*		
RT^{-1} (min)	Compound	PA (%)
1,69	ethanol	6.36
2,24	hexane	4.78
2,4	pentane, 1,2-dichloro-	3.72
2,83	butanal, 3-methyl-	1.94
3,7	2-butanone, 3-hydroxy-	6.93
4,17	1-butanol, 3-methyl-	6.54

Beef + *S.* Typhimurium + *C. jejuni* + *S. aureus*		
RT^{-1} (min)	Compound	PA (%)
1,67	ethanol	14.37
2,24	hexane	33.63
3,77	2-butanone, 3-hydroxy-	1.65
4,2	1-butanol, 3-methyl-	0.76
5,19	1,3-cyclohexadiene	0.39

Table 14 VOCs produced by beef spiked with mixed inocula of 2 or 3 bacteria (*S.*Typhimurium, *C.jejuni*, and *S. aureus* 10^6 UFC/mL). [RT^{-1} (m): Retention time; PA (%): Peak Area normalized].

3.5.2. Chicken spiked with bacterial mixes

Samples of **chicken** contaminated with ***S.* Typhimurium** and ***C. jejuni*** (**Ch+S+C**) show the presence of **ethanol** (6.36%), **ether, tert-butyl ethyl**, and **1-butanol, 3-methyl**. ***S.* Typhimurium** and ***S. aureus*** on **chicken** (**Ch+S+A**) predominantly released **ethanol**, **n-methyltaurine** (5.94%), **butanal, 3-methyl-**, and **chloroform**. Samples of chicken spiked with ***C. jejuni*** and ***S. aureus*** (**Ch+ C+A**) highlight the emission of mainly **ethanol**, **glyceric acid**, **pentane**, and **1,2-dichloro**. The inoculum containing the three pathogens, employed to contaminate chicken samples (**Ch+S+C+A**), produced **ethanol** (12.33%), **butanal, 3-methyl-**, 1-**butanol, 3-methyl-**, **ethyl acetate**, and **pentane, 1,2.dichloro-** (**Table 15**).

Chicken + *S.* Typhimurium + *C. jejuni*		
RT^{-1} (min)	**Compounds**	**PA (%)**
1,69	ethanol	6.36
2,41	ether, tert-butyl ethyl	3.83
4,41	1-butanol, 3-methyl-	2.95

Chicken + *S.* Typhimurium + *S. aureus*		
RT^{-1} (min)	**Compounds**	**PA (%)**
1,7	ethanol	16.32
1,89	n-methyltaurine	5.94
2,45	pentane, 1,2-dichloro-	1.66
2,52	chloroform	3.46
2,88	butanal, 3-methyl-	4.2
2,99	1-butanol, 2-methyl-	1.99
3,92	2-butanone, 3-hydroxy-	0.53

Chicken + *C. jejuni* + *S.aureus*		
RT^{-1} (min)	**Compounds**	**PA (%)**
1,59	n-methyltaurine	0.35
1,69	ethanol	9.66
2,03	glyceric acid	2.5
2,41	ethylacetate	1.19
2,48	pentane, 1,2-dichloro-	1.67
2,93	butanal, 3-methyl-	1.58

Chicken + *S.* Typhimurium + *C. jejuni* + *S. aureus*		
RT^{-1} (min)	**Compounds**	**PA (%)**
1,74	ethanol	12.33
2,46	pentane, 1,2-dichloro-	1.98
2,52	ethylacetate	3
2,88	butanal, 3-methyl-	5.81
3	1-butanol, 3-methyl-	4.96

Table 15 VOCs produced by chicken spiked with mixed inocula of 2 or 3 bacteria (*S.*Typhimurium, *C.jejuni*, and *S. aureus* 10^6 UFC/mL). [RT^{-1} (m): Retention time; PA (%): Peak Area normalized].

3.5.3. Pork spiked with bacterial mixes

Profiles of pork contaminated with the bacterial combinations show a low production of volatiles, with low concentrations. ***S.* Typhimurium** and ***C. jejuni*** on **pork** (**P+S+C**) released **ethanol** (4.79%), **benzyl chloride** (4.3%), **chloroform** (3.12%), and **1-propanol** (1.26%). ***S.* Typhimurium** and ***S. aureus*** on **pork** (**P+S+A**) released **ethanol** (4.55%) and **benzyl chloride** (4.27%); ***C. jejuni*** and ***S. aureus*** spiked **pork** samples (**P+C+A**)

produced **chloroform** and **ethanol**. The three bacteria exploiting pork components (**P+S+C+A**) produced **ethanol**, **1-butanol, 3-methyl-**, and **1,2-dichloropentane** (**Table 16**).

Pork + *S.* Typhimurium + *C. jejuni*		
RT^{-1} (min)	**Compound**	**PA (%)**
1,66	ethanol	4.79
2,14	1-propanol	1.26
2,53	chloroform	3.12
11,23	benzyl chloride	4.3

Pork + *S.* Typhimurium + *S. aureus*		
RT^{-1} (min)	**Compound**	**PA (%)**
1,7	ethanol	4.55
11,36	benzyl chloride	4.27

Pork + *C. jejuni* + *S. aureus*		
RT^{-1} (min)	**Compound**	**PA (%)**
1,7	ethanol	3.58
2,53	chloroform	6.61

Pork + *S.* Typhimurium + *C. jejuni* + *S. aureus*		
RT^{-1} (min)	**Compound**	**PA (%)**
1,65	ethanol	6.7
3,01	dichloropentane	0.92
4,3	1-butanol, 3-methyl-	1.01

Table 16 VOCs produced by pork spiked with mixed inocula of 2 or 3 bacteria (*S.*Typhimurium, *C.jejuni*, and *S. aureus* 10^6 UFC/mL). [RT^{-1} (m): Retention time; PA (%): Peak Area normalized].

3.6. VOCs profiles of meat spiked with single bacteria in varying concentrations

3.6.1. Beef spiked with decreasing concentrations of the bacterial inocula

The evaluation of bacterial contamination of meat matrices with a 10^6 UFC/mL concentration was followed by the same sample preparation employing different concentrations: **10^3 and 10^2 UFC/mL**. The production of volatiles organic compounds in due to **microorganisms contamination** of **beef** (e.g. **B+S3, B+S2**), mostly consists on **ethanol**, **hexane**, **ethylacetate**, **butanal, 3-methyl**, **2-butanone, 3-hydroxy-**, which resulted common to mostly all combination of beef spiked with a specific pathogen (**Table 17)**:

- **Beef + *S.* Typhimurium 10^3** and **10^2** VOCs production registers almost the same compounds, whose concentrations slightly decreased proportionally to the reduction of bacterial contamination: **ethanol**, **hexane** (the two most abundant compounds), and **methylene dichloride**, **hexane**. Other compounds eluted report a similar abundance, considering the two different bacterial concentrations (**butanal, 3-methyl-**, **2-butanone, 3-hydroxy**, etc.).
- **Beef + *C. jejuni* 10^3** and **10^2** volatile profiles are really similar. Several compounds are common to both 10^3 and 10^2 samples, and compounds concentrations decreased in function of the reduction of bacterial content: **ethanol**, **ethyl acetate** (the two most abundant compounds),

dichloroacetaldehyde, **hexane, butanal, 3-methyl-**. The remaining compounds register a similar abundance, considering the two different bacterial concentrations. **Butanoic acid, ethyl ester**, **butanoic acid**, and **1-octyn-3-ol, 4-ethyl-** were produced only by **Beef + *C. jejuni* 10^2**.

- **Beef+ *S. aureus* 10^3** and **10^2** compounds show, apart from few common compounds (**hexane**, **1-butanol 3-methyl, butanal, 3-methyl-**), different compounds when a lower bacterial concentration is added to the meat.

Beef + *Salmonella* Typhimurium 10^3		
RT^{-1} (min)	Compounds	PA (%)
1.7	ethanol	4.96
1.93	methylene dichloride	2.37
2.27	hexane	4.81
2.85	butanal, 3-methyl-	0.62
3.08	2-butanone, 3-hydroxy-	1.07
5.99	butanoic acid	3.38

Beef + *Salmonella* Typhimurium 10^2		
RT^{-1} (min)	Compounds	PA (%)
1.72	ethanol	4.92
1.96	methylene dichloride	2.19
2.27	hexane	3.79
2.44	ethyl acetate	1.67
2.87	butanal, 3-methyl-	1.42
3.98	2-butanone, 3-hydroxy-	1.73

Beef + *Campylobacter jejuni* 10^3		
RT^{-1} (min)	Compounds	PA (%)
1.84	ethanol	3.38
1.94	dichloroacetaldehyde	3.79
2.24	hexane	5.28
2.4	ethyl acetate	7.63
2.85	butanal, 3-methyl-	1.65
2.96	2-butanone, 3-hydroxy-	2.86

Beef + *Campylobacter jejuni* 10^2		
RT^{-1} (min)	Compounds	PA (%)
1.71	ethanol	2.38
1.93	dichloroacetaldehyde	1.49
2.26	hexane	3.35
2.39	ethyl acetate	4.28
2.84	butanal, 3-methyl-	0.79
3.83	2-butanone, 3-hydroxy-	4.91
5.52	butanoic acid, ethyl ester	0.86
6.11	butanoic acid	0.63
10.89	1-octyn-3-ol, 4-ethyl-	0.17

Beef + *Staphylococcus aureus* 10^3		
RT^{-1} (min)	Compounds	PA (%)
2.27	hexane	15.17
2.86	1-butanol, 3-methyl-	0.94
4.03	2-butanone, 3-hydroxy-	2.29
4.41	butanal, 3-methyl-	0.26
5.58	butanoic acid, ethenyl ester	2.48
6.25	butanoic acid	7.43
6.48	pentanoic acid (valeric acid)	0.69
6.75	3-heptenoic acid	1.25

Beef + *Staphylococcus aureus* 10^2		
RT^{-1} (min)	Compounds	PA (%)
1.71	ethanol	0.21
1.94	methylene chloride	9.72
2.27	hexane	4.01
2.43	ethyl acetate	2.68
2.86	1-butanol, 3-methyl-	1.01
4.42	butanal, 3-methyl-	1.67

Table 17 VOCs produced by beef spiked with decreasing microbial loads of the three microorganisms (10^3 and 10^2 UFC/mL). [RT^{-1} (m): Retention time; PA (%): Peak Area normalized].

3.6.2. Chicken spiked with decreasing concentrations of the bacterial inocula

Chicken + *Salmonella* Typhimurium 10^3		
RT^{-1} (min)	Compounds	PA (%)
1.66	ethanol	2.09
2.51	isobutanol	0.42
2.79	butanal, 3-methyl-	0.53
3.53	1 sulfonylacetone	0.18
4.23	1-butanol, 3-methyl-	5.9
4.37	2,3-dithiobutane	31.1
5.22	cuminal	0.83
5.58	tetrachloroethylene	0.19
7.03	isopentyl alcohol, acetate	0.44
8.75	ethyl isocaproate	0.65
8.08	dimethyl trisulfide	18.07

Chicken + *Campylobacter jejuni* 10^3		
RT^{-1} (min)	Compounds	PA (%)
1,58	methanethiol	1.99
1,64	ethanol	0.9
2	1-propanol	0.70
2,1	hexane	0.66
2,31	ethyl acetate	0.92
2,74	isovaleric acid	0.61
2,92	butanal, 2-methyl-	0.53
3,46	methyl thiolacetate	3.6
3,7	propanoic acid, ethyl ester	1.67
4,26	isopentyl alcohol	0.88
4,37	benzyl disulfide	32.9
5,45	butanoic acid, ethyl ester	3.9
7,41	disulfide, pentyl propyl	0.46
8,31	isopropyl valerate	0.81
8,92	dimethyl trisulfide	7.06
14,32	indole	28.09

Chicken + *Staphylococcus aureus* 10^3		
RT^{-1} (min)	Compound	PA (%)
1,68	ethanol	3.23
1,91	methylene chloride	0.78
2,12	glyceric acid	0.69
2,21	hexane	1.07
2,39	butyl nitrite	0.23
2,46	chloroform	1.69
2,81	butanoic acid, 3-methyl-	1.12
2,91	2-methyl butanale	0.58
4,22	isopentyl alcohol	12.1
5,57	tetrachloroethylene	0.3

Chicken + *Salmonella* Typhimurium 10^2		
RT^{-1} (min)	Compounds	PA (%)
1.66	ethanol	1.35
2.51	isobutanol	0.13
2.79	butanal, 3-methyl-	0.14
4.23	1-butanol, 3-methyl-	6.7
4.37	2,3-dithiobutane	27.8
5.22	cuminal	0.5
7.03	isopentyl alcohol, acetate	0.23
8.75	ethyl isocaproate	0.47
8.08	dimethyl trisulfide	14.1
7.03	isopentyl alcohol, acetate	0.23

Chicken + *Campylobacter jejuni* 10^2		
RT^{-1} (min)	Compounds	PA (%)
1,58	methanethiol	1.87
1,64	ethanol	0.4
2	1-propanol	0.37
2,1	hexane	0.25
2,31	ethyl acetate	0.42
2,74	isovaleric acid	0.27
2,92	butanal, 2-methyl-	0.23
3,46	methyl thiolacetate	3.7
3,7	propanoic acid, ethyl ester	1.64
4,26	isopentyl alcohol	0.56
4,37	benzyl disulfide	29.6
5,45	butanoic acid, ethyl ester	3.1
7,41	disulfide, pentyl propyl	0.3
8,31	isopropyl valerate	0.53
8,92	dimethyl trisulfide	4.9
14,32	indole	23.1

Chicken + *Staphylococcus aureus* 10^2		
RT^{-1} (min)	Compound	PA (%)
1,68	ethanol	1.53
1,91	methylene chloride	0.46
2,21	hexane	0.55
2,46	chloroform	1.57
2,81	butanoic acid, 3-methyl-	0.73
2,91	2-methyl butanale	0.37
4,22	isopentyl alcohol	9.9

Table 18 VOCs produced by chicken spiked with decreasing loads of the three microorganisms (10^3 and 10^2 UFC/mL).

Profiles of volatiles organic compounds produced due to **bacterial spiking** of **chicken** (e.g. **Ch+S3, Ch+S2**) are showed in **Table 18**: **ethanol** and **hexane** were common to all combination of chicken spiked with a specific pathogen:

- **Chicken + *S.* Typhimurium 10^3** and **10^2** VOCs profiles are extremely similar, and all compounds concentrations slightly decreased with the diminution of *S.* Typhimurium contamination. The most abundant volatiles are **2,3-dithiobutane** (31.1% and 27.8%), and **dimethyl trisulfide** (18.7% and 14.1%). **1-sulfonylacetone** is the **only** compound produced by 10^3 and not by 10^2 samples.
- **Chicken + *C. jejuni* 10^3** and **10^2** volatile organic compounds are all the same. Substances concentrations are lower in 10^3, rather than **10^2**, excepting **methyl thiolacetate** and **propanoic acid, ethyl ester**, where similar concentrations are observed. Other compounds eluted report a similar abundance, considering the two different bacterial concentrations. The **most abundant** compounds in the two profiles are **benzyl disulfide** (32.9% and 29.6%), and **indole** (28.9% and 23.1%).
- **Chicken + *S. aureus* 10^3** and **10^2** volatiles show mostly common compounds (whose most abundant were **isopentyl alcohol** and **ethanol**), although 10^3 sample produced different compounds: **tetrachloroethylene**, **butyl nitrite**, **glyceric acid**, evidencing low concentrations.

3.6.3. Pork spiked with decreasing concentrations of the bacterial inocula

Bacterial contamination of **pork** (e.g. **P+S3, P+S2**) highlighted the lowest number of volatiles organic compounds production but the most diversified profiles, describing common substances within the same bacterial contamination (**Table 19**):

- **Pork + *S.* Typhimurium 10^3** and **10^2** VOCs profiles show the production of the same substances, and compounds concentrations are similar, excepting for **butanal, 3-methyl-**, decreased from 4.57% to 0.47% as function of the diminution of *S.* Typhimurium contamination. The most abundant volatiles are **butanal, 3-methyl-**, **1-butanol, 3-methyl-**, and **ethanol**.

- **Pork + *C. jejuni* 10^3** and **10^2** profiles, as for *S.* Typhimurium contamination, are all the same. Components concentrations are lower in 10^3, rather than **10^2**. The **most abundant** compound in the two profiles is **ethanol** (41.25% and 32.63%).

- **Pork + *S. aureus* 10^3** and **10^2** data evidence the production of different compounds. While the 10^3 sample produced **4-amino pentanol** and **ether, tert-butyl ethyl**, the 10^2 sample produced **methanethiol**, **dichloroacetaldehyde**, **butanal, 3-methyl-**, and **dimethyl sulfide**. It is interesting to highlight that **dimethyl sulfide** concentration is really high, **39.76%**, even though it was not eluted by the most concentrated sample.

Pork + *Salmonella* Typhimurium 10^3		
RT^{-1} (m)	**Compound**	**PA (%)**
1,75	ethanol	9.54
2,27	hexane	2.11
2,86	butanal, 3-methyl-	4.57
3,9	1-butanol, 3-methyl-	9.32

Pork + *Salmonella* Typhimurium 10^2		
RT^{-1} (m)	**Compound**	**PA (%)**
1,69	ethanol	10.04
2,26	hexane	3.27
2,83	butanal, 3-methyl-	0.47
4,17	1-butanol, 3-methyl-	8.12

Pork + *Campylobacter jejuni* 10^3		
RT^{-1} (min)	**Compound**	**PA (%)**
1,71	ethanol	41.25
2,45	ether, tert-butyl ethyl	5.39
2,81	butanal, 3 methyl	3.02

Pork + *Campylobacter jejuni* 10^2		
RT^{-1} (min)	**Compound**	**PA (%)**
1,69	ethanol	32.63
2,4	ether, tert-butyl ethyl	2.14
2,85	butanal, 3 methyl	1.08

Pork + *Staphylococcus aureus* 10^3		
RT^{-1} (min)	**Compound**	**PA (%)**
2,2	4-amino pentanol	2.45
2,38	ether, tert-butyl ethyl	1.83

Pork + *Staphylococcus aureus* 10^2		
RT^{-1} (min)	**Compound**	**PA (%)**
1,59	methanethiol	4.89
1,88	dichloroacetaldehyde	2.1
4,24	butanal, 3-methyl-	2.19
4,37	dimethyl sulfide	39.76

Table 19 VOCs produced by beef spiked with decreasing microbial loads of the three microorganisms (10^3 and 10^2 UFC/mL). [RT^{-1} (m): Retention time; PA (%): Peak Area normalized].

4. DISCUSSION

The metabolomics approach to food analysis allowed the identification of **typical volatile organic compounds in contaminated meat** samples: volatiles were **generated only when a particular pathogen was present**. It was also found that, not only some volatiles appeared as a result of their release as a consequence of the growth of a particular bacteria, but also that the volatile compounds profile changed significantly between the contaminated and control meat samples (Ercolini and others 2009). This may be related to changes in bacteria metabolism, due to the availability of specific nutrients in meat, compared to the negative controls, consisting in the inoculation of the single bacteria in sterile distilled water.

According to the work flow scheme planned, volatile organic **compounds profiles were compared and significant similarities and differences were evidenced**. Besides defining potential fingerprints of each sample, when unique compounds were identified, potential typical profiles were built.

4.1. Single bacteria

Pools of substances produced by each bacterial inoculum (***S.* Typhimurium**, ***C. jejuni***, and ***S. aureus***), as showed in **Figure 10**, result different.

Although some substances were produced by all the three inocula, especially by *Salmonella* Typhimurium and *Campylobacter jejuni*, some substances are indicative of a single microorganism:

- ***S.* Typhimurium**: dichloroacetaldehyde, benzene.
- ***S. aureus***: dimethylbenzamide, ter-butyl formiate, ethyl acetone.
- ***C. jejuni*** inoculum did not produce VOCs useful to distinguish the bacterium.

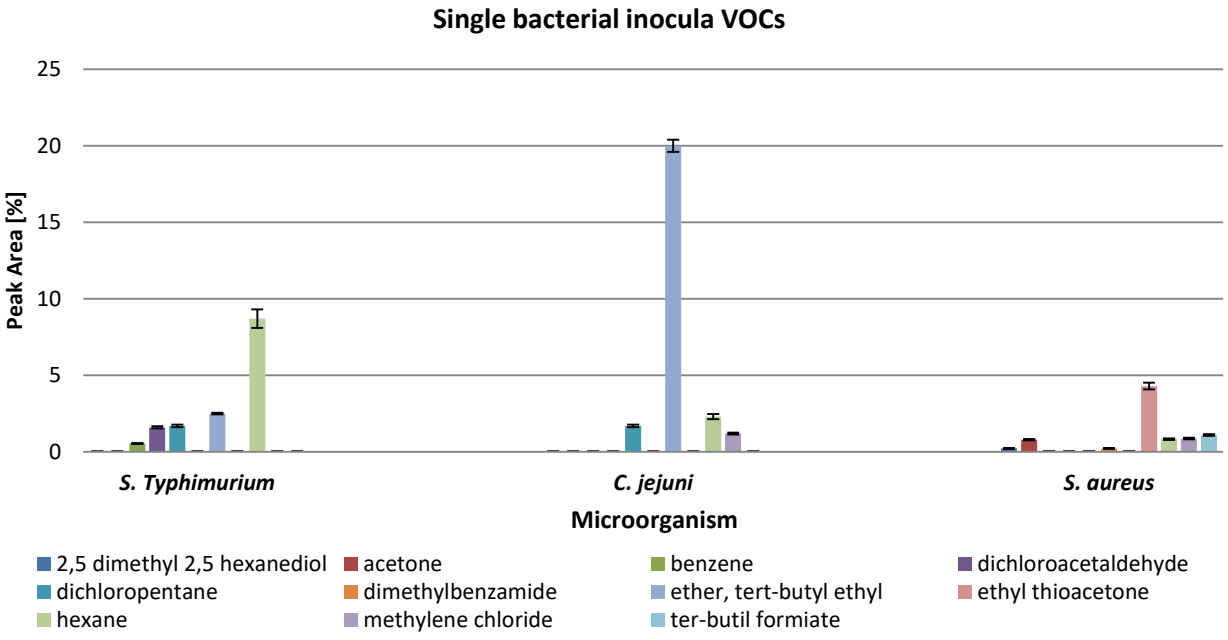


Figure 10 VOCs emitted by single bacterial inocula: analysis of similarities and differences between the three target germs.

4.2. Bacterial mixtures

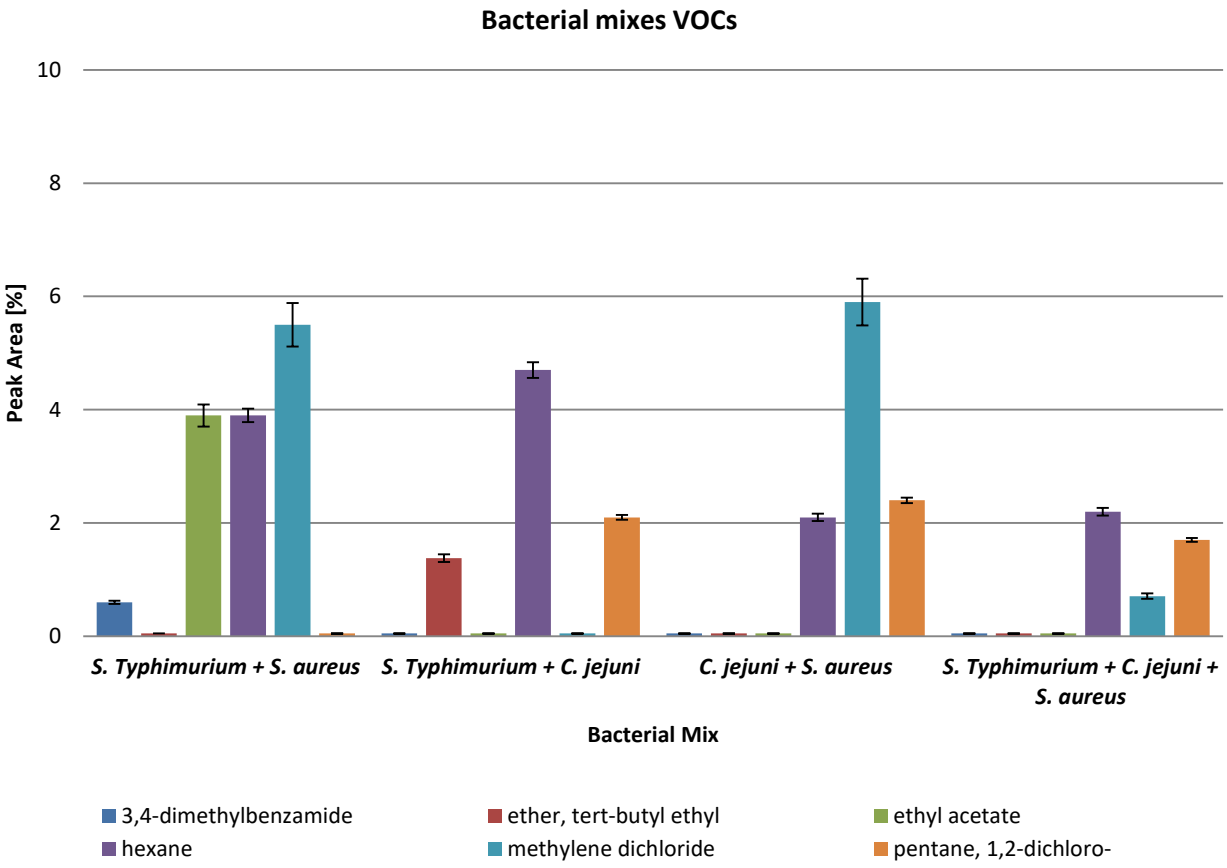


Figure 11 Analysis of VOCs emitted by mixed bacterial inocula: the comparison between the 4 combinations of 2 or 3 bacteria has been performed.

Data resulted from bacterial inocula mixtures analysis (**Figure 11**) describe that:

- Whenever ***C.jejuni*** is present in the inoculum, **pentane, 1,2-dichloro-** is produced. The compound is also produced by ***C. jejuni*** and ***S.* Typhimurium** single inocula.
- The compound **methylene dichloride**, although produced by all combinations, describes a higher peak area when ***S.* Typhimurium** and ***S. aureus*** are put together. It is also produced by ***C. jejuni*** and ***S. aureus*** single inocula.
- **Ethyl acetate** is produced by ***S.* Typhimurium** + ***S. aureus and S.* Typhimurium** + ***C. jejuni*** + ***S. aureus*,** but not by single inocula.
- In general, it is **not** possible to identify **marker compounds**, typical of a unique mixture.

From the observation of the data, it is possible to highlight that the **presence of specific molecules** in the bacterial mixtures profiles **certainly does not match identities in the VOC profiles of the single bacterial inocula**.

4.3. Meat matrices

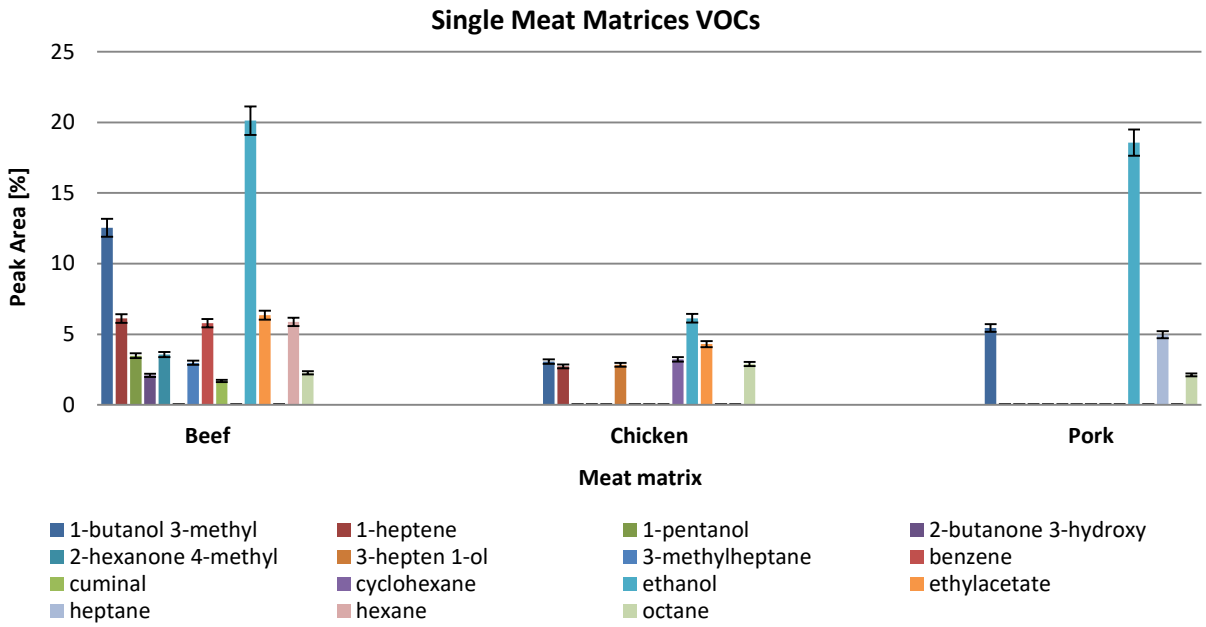


Figure 12 Analysis of volatiles emitted by metabolism of endogenous microbial flora of not spiked meat matrices.

Chromatographic outcomes of single meat matrices are indicated in **Figure 12**; it can be evidenced that:

- **All meat matrices** produce **ethanol**, **octane**, **1-butanol 3-methyl**, which can be considered spoilage indicators, as described by available researches on the same matrices (Mikš-Krajnik and others 2014).
- In general, **beef** and **chicken profiles** seem to be **different**, when the comparison is made between the matrices.
- **Beef** and **chicken** additionally produce **ethyl acetate**, a generic food spoilage marker (Flores and others 2013; Hernandez-Macedo and others 2014), and **1-heptene**, probably because they share similar metabolic pathways.
- It is possible to identify **marker volatiles profiles**, produced by a single matrix and not by others:
    - **Beef**: hexane, benzene, 1-pentanol, 3-methyl heptane, 2-butanone 3-hydroxy, 2-hexanone 4-methyl, cuminal.
    - **Chicken**: cyclohexane, 3-hepten 1-ol.
- Meat profiles, apart from **benzene** and **hexane**, produced by **beef** and the **single bacterial inocula**, are different from compounds emitted by the bacteria.
- **Volatile organic compounds, typical of each negative control meat** (produced by endogenous microbial flora), are:
    - **Beef:** 1-butanol, 3 methyl, 3-methyl heptane, 2-hexanone 4-methyl.
    - **Chicken**: cyclohexane, 1-butanol 3-methyl, 3-hepten 1-ol.
    - **Pork**: no marker compounds were identifiable.

4.4. Meat spiked with single bacterial inocula

The analysis of the results was developed following 4 steps:

1. Research of **common substances** derived by the comparison with the corresponding **meat matrix**.
2. **Relations** between the combinations and the **single bacterial inocula**.
3. Evaluation of **associations and differences between the sample and other meat+bacterium combinations** within the same meat matrix.
4. Identification of **potential marker VOCs**: unique compounds, not produced by other combinations in the current research.

It is important to specify that, despite the production of common compounds compared to negative controls, **the presence of specific molecules in the profiles of meat + microorganisms combinations** does **not find crucial identities in the VOC profiles of the single meat matrices**, neither bacterial inocula.

4.4.1. Beef and single bacterial inocula

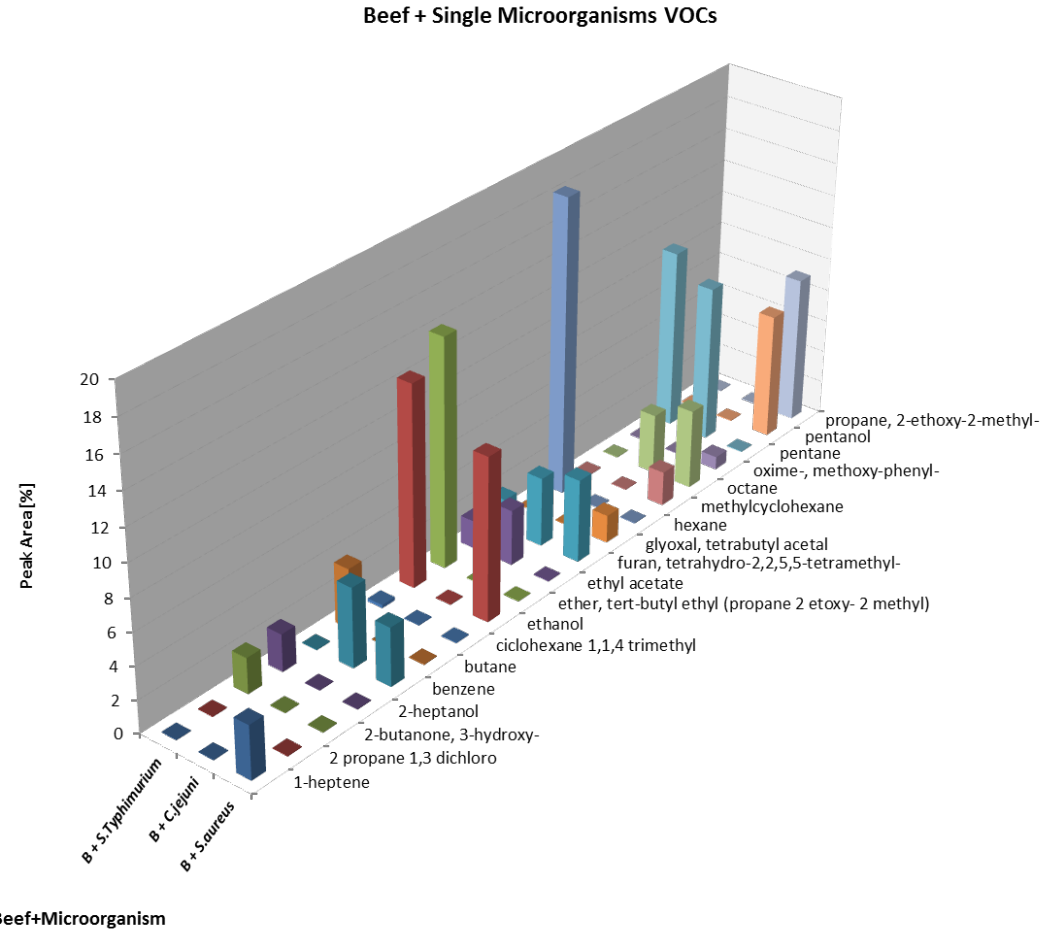


Figure 13 Analysis of similar and different volatiles emitted by beef spiked with *S.*Typhimurium, *C. jejuni*, and *S. aureus*.

Figure 13 shows the profiles of the most significant volatile organic compounds produced by each **Beef+Microorganism** combination.

1. Data **comparison** between the **three combinations** and the **beef profile** (negative control), evidences that a wide pool of **substances is produced from both spiked and not spiked beef** samples:
   - **Beef + *S.* Typhimurium:** ethanol (decreases), ethyl acetate (decreases), hexane, 2-butanone 3-hydroxy.
   - **Beef + *C. jejuni*:** ethyl acetate (decreases), benzene, octane.
   - **Beef + *S. aureus*:** ethanol (decreases), benzene, 1-heptene, pentanol, octane.

2. The research of **relations** between **bacterial inocula** (**S**, **C**, **A**, negative bacterial controls) and volatile profiles of **spiked beef samples** (**B+S**, **B+C**, **B+A**) brought out the following considerations:
   - **Hexane** was the only substance eluted by **Beef**, ***Salmonella* Typhimurium** and **Beef+*S.* Typhimurium,** and the concentration registered a high peak area value (**18.33%**): it can be hypothesized that, although the compound is also produced by beef and *Salmonella* Typhimurium alone, **when meat is spiked with *S.* Typhimurium, hexane value highly increases** (four times more concentrated).
   - **Ether, tert-butyl ethyl** is produced by ***S.* Typhimurium** and **Beef+*S.* Typhimurium**, but not by **Beef**: the value **increased** in the spiked sample by a 12% concentration, probably indicating it as a **bacterial** - but not meat - **metabolite**, whose production is incremented by the **exploitation of meat nutrients** along the beef contamination.
   - The comparisons between ***C. jejuni* vs Beef+*C. jejuni*** and ***S. aureus* vs Beef+*S. aureus*** did not evidence any significant correlation: profiles are different and this is indication of the sensibly modified bacterial metabolism, compared to the inoculation in sterile distilled water.

3. **Association** analysis between the three **spiked beef samples** (**B+S**, **B+C**, **B+A**) shows that the three samples produce **furan, tetrahydro-2,2,5,5-**, allowing to consider it as an **outcome of bacterial exploitation of beef nutrients**, presumably result of a common metabolic mechanism.

4. Substances that can be considered unique, therefore **potential marker of beef contamination due to a single microorganism** are:
   - **Beef + *S.* Typhimurium**: butane, 2-heptanol, ciclohexane 1,1,4 trimethyl.
   - **Beef + *C. jejuni***: 2 propane 1,3 dichloro.
   - **Beef + *S. aureus***: propane, 2-ethoxy-2-methyl-, methylcyclohexane, oxime- methoxy-phenyl, glyoxal tetrabutyl acetal.

4.4.2. Chicken and single bacterial inocula

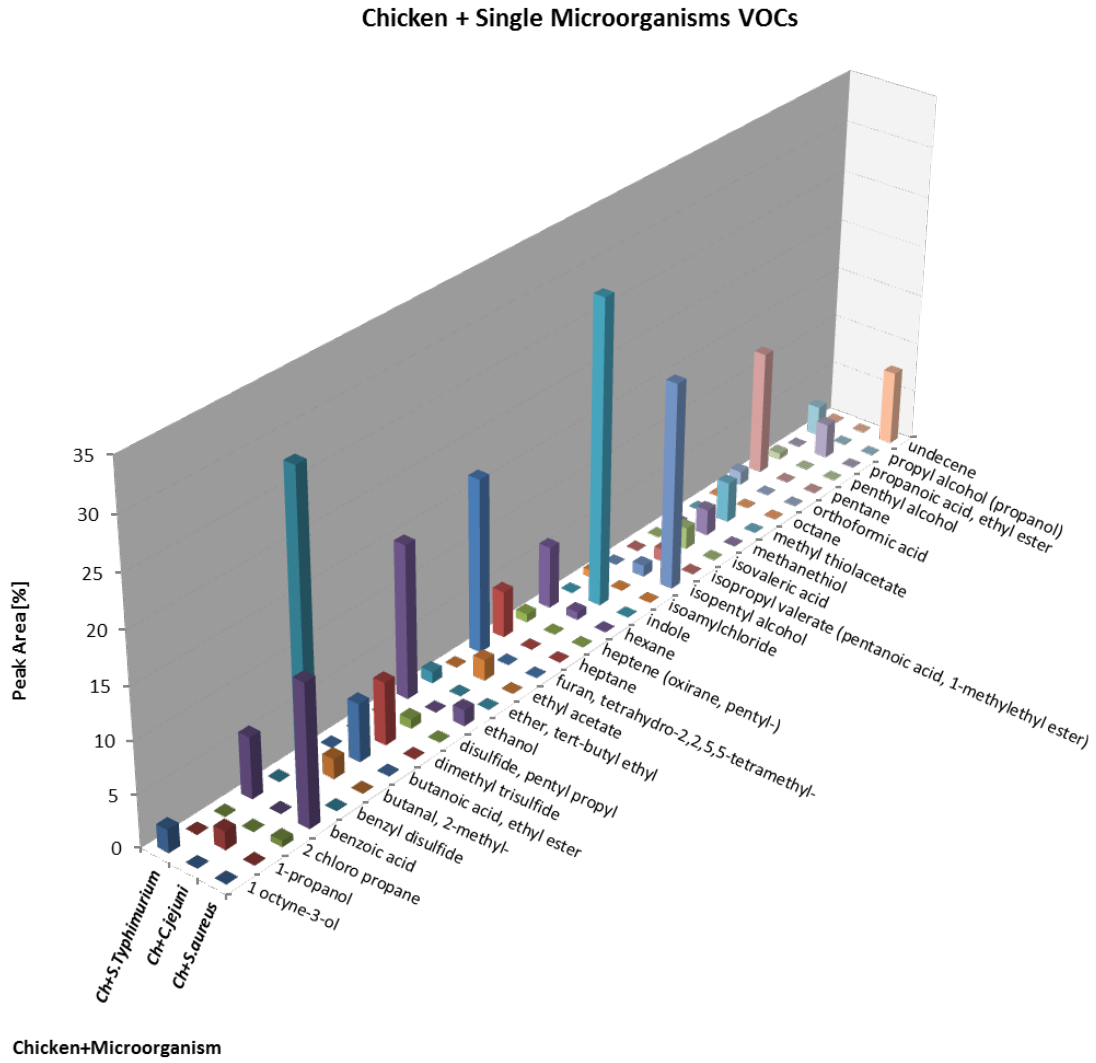


Figure 14 Analysis of similar and different volatiles emitted by chicken spiked with *S.*Typhimurium, *C. jejuni*, and *S. aureus*.

Profiles of **chicken spiked with the three pathogens under analysis**, especially for the combination with *S.* Typhimurium *and C. jejuni*, described a wide difference and larger number of volatiles registered (**Figure 14**).

1. Compared to single **chicken profile** (negative control), the **three combinations** produced, as observed also in beef, **common** volatiles:
   - **Chicken + *S.* Typhimurium:** ethanol (increases), octane.
   - **Chicken + *C. jejuni*:** ethanol (decreases), ethylacetate.
   - **Chicken + *S. aureus*:** ethanol (twice more concentrated).

This result could evidence the **similarities between *S.* Typhimurium and *S. aureus* metabolisms** as regards as **ethanol** production from chicken exploitation.

2. **Similarities** between **bacterial inocula profiles** (**S**, **C**, **A**, negative bacterial controls) and volatile profiles of **spiked chicken samples** (**Ch+S**, **Ch+C**, **Ch+A**) can be summarized as follows:

   Chicken + *S.* Typhimurium vs *S.* Typhimurium:

   - **Hexane** is present in ***S.* Typhimurium** and **Chicken+*S.* Typhimurium**, but not in Chicken. Its production is boosted (from 0.87% to 6.3%) by the use of chicken nutrients, demonstrating *Salmonella* spp. contribution in the higher values, rather than other bacteria.

   Chicken + *C. jejuni* vs *C. jejuni:*

   - **Hexane** is produced by ***C.r jejuni*** inoculum and by **Chicken+*C. jejuni***, although its **value is sensibly reduced in the combination**, suggesting that **metabolisms changes when bacterial growth substrate is modified**.

   Chicken + *S. aureus* vs *S. aureus*: although substances produced are fewer compared to other combinations, the **profiles are totally different**.

3. **Association** analysis between the three **spiked chicken samples** (**Ch+S**, **Ch+C**, **Ch+A**) highlights:

   - the common production of **ethanol**, allowing considering it as an **outcome of bacterial exploitation of chicken nutrients**, presumably result of a similar metabolic mechanism.
   - **Isopentyl alcohol** is produced by **Chicken+*C. jejuni*** and **Chicken+*S. aureus*** combination: the substance can be considered significant in **Chicken+*S. aureus*** mix, since its value (**20.34%**) represents the **most abundant volatile** emitted.

4. Substances that can be considered **potential marker of chicken contamination due to a single microorganism** are:

   - **Chicken + *S.*Typhimurium**: pentane, orthoformic acid, heptane, heptene (oxirane, pentyl-), penthyl alcohol, benzoic acid, 1 octyne-3-ol, isoamylchloride.
   - **Chicken + *C. jejuni***: isovaleric acid, butanal 2-methyl, methyl thiolacetate, propanoic acid, ethyl ester, benzyl disulfide, butanoic acid ethyl ester, disulfide pentyl propyl, isopropyl valerate, dimethyl trisulfide.
   - **Chicken + *S.aureus***: 2 chloro propane.

4.4.3. Pork and single bacterial inocula

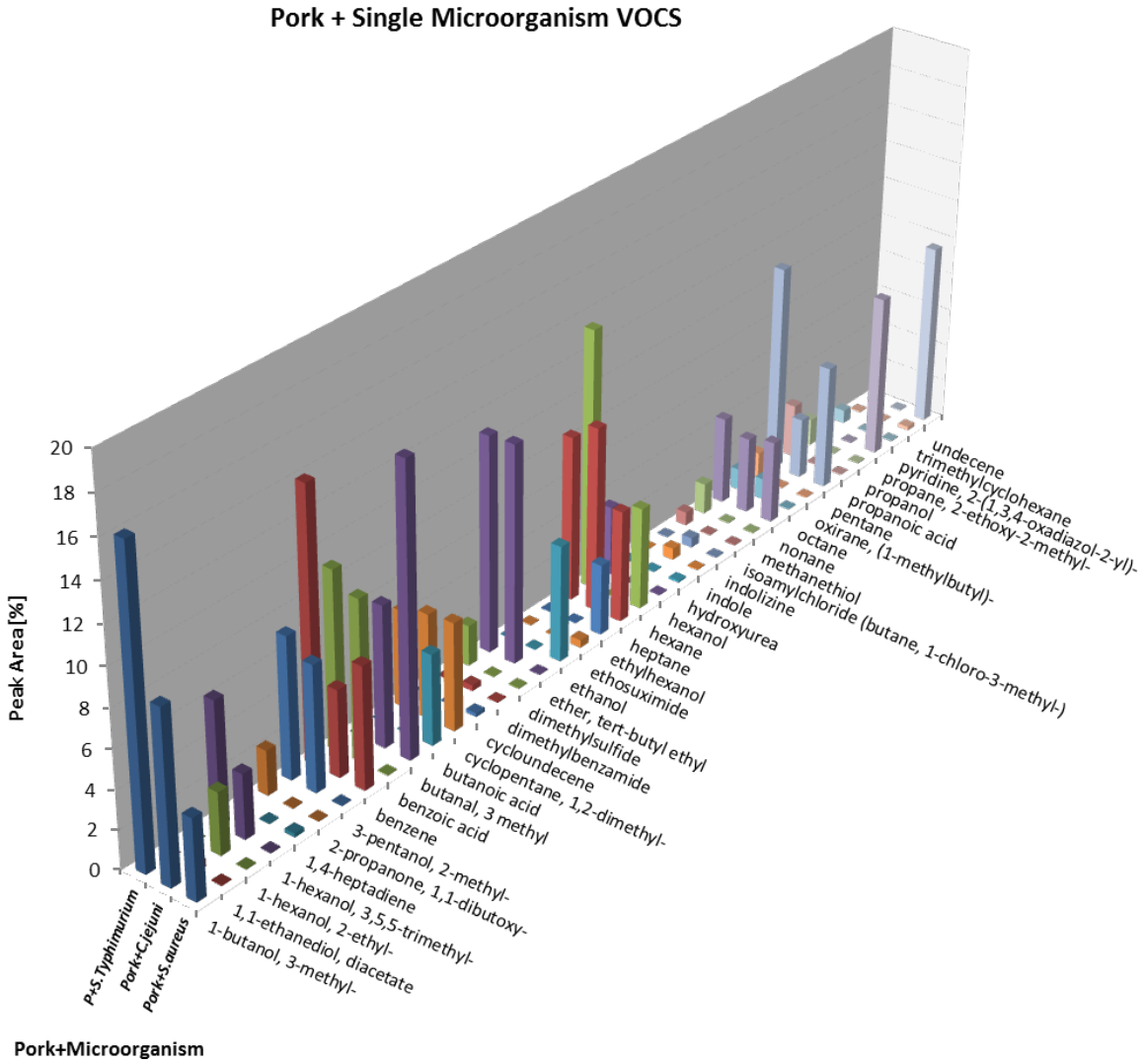


Figure 14 Analysis of similar and different volatiles emitted by pork spiked with *S.*Typhimurium, *C. jejuni*, and *S. aureus*.

Referring to volatiles profiles of **pork spiked with the three pathogens**, the wider number of the eluted substances and the largest difference between the mixes was evidenced (**Figure 15**).

1. The comparison between **pork profile** (negative control) and **three combinations** produced **common** volatiles:
   - **Pork + *S.* Typhimurium:** ethanol, heptane, 1-butanol 3 methyl.
   - **Pork + *C. jejuni*:** heptane, 1-butanol 3 methyl, octane.
   - **Pork + *S. aureus*:** ethanol, heptane, 1-butanol 3 methyl.

- **Ethanol** value between single pork and **Pork + *S. aureus*** mix **highly decreased** (3 times lower), probably because the microorganism **exploits pork nutrients to activate different metabolisms**.
- **Heptane** is **produced by the three samples** of bacterial spiked pork, but **not by single bacterial inocula**, and the abundance, compared to negative control, is doubled in **Pork+*S.* Typhimurium** and **Pork+*C. jejuni*:** this indicates that, especially in ***S.* Typhimurium** and ***C. jejuni***, **heptane-related metabolisms** from chicken exploitation **are similar**.
- **1-Butanol 3-methyl**, previously hypothesized as generic microbial spoilage indicator in meat, is produced by **the three samples of bacterial spiked pork**, but not by single bacterial inocula. As evidenced for heptane, **1-butanol 3-methyl** value is doubled in **Pork+*S.* Typhimurium** and **Pork+*C. jejuni***, indicating that especially ***S.* Typhimurium** and ***C. jejuni* metabolisms**, as regards as **1-butanol 3-methyl** production **from pork exploitation**, are **similar**.

2. **Relations** between **bacterial inocula profiles** (**S**, **C**, **A**, negative bacterial controls) and volatile profiles of **spiked pork samples** (**P+S**, **P+C**, **P+A**) show that:
   - ***S.* Typhimurium vs Pork + *S.* Typhimurium*:* hexane** and **ether, tert-butyl ethyl** are produced by ***S.* Typhimurium** and **Pork + *S.* Typhimurium**, but not in Pork. The concentrations **highly incremented** (the first 10 times more concentrated, the second 5 times) by the use of pork nutrients, demonstrating ***Salmonella* spp. contribution in the higher values**.
     - **Ether, tert-butyl ethyl** is also produced by ***C. jejuni*** inoculum and by **Pork+*C. jejuni*** mix, although its **value is sensibly reduced in the combination**, suggesting that **metabolisms changes when bacterial growth substrate is modified**.
   - ***S. aureus* vs Pork + *S. aureus:*** the only common VOC is **hexane**, whose **concentration increases**, although registering **lower values compared to Pork+*S.* Typhimurium** results.

3. Research of **connections** between the three **spiked chicken samples** (**P+S**, **P+C**, **P+A**) highlights the **common production of several substances:**
   - **P+S vs P+C**: 3-pentanol 2-methyl, **1-hexanol- 3,5,5-trimethyl** (doubled for P+S), cyclopentane 1,2-dimethyl, benzoic acid. When values in the two mixes are **similar**, the substance cannot be considered elective for one or another combination.
   - **P+S vs P+A**: **hexane** (three times more abundant in P+S), **benzene** (doubled for P+S). In general, the **two substances can be associated to Pork+*S.* Typhimurium profile**, rather than Pork+*S. aureus* one, considering the **higher concentrations** evidenced.

- **P+C vs P+A**: 1-butanol 3-methyl (higher in P+C), **butanal 3-methyl**. **Butanal 3-methyl** in **Pork+*S. aureus*** was produced with the **highest abundance** compared to other volatiles from the mix (15.02%) and the concentration is Pork+*C. jejuni* mix is half the Pork+*S. aureus* value. Although it is not a marker compound, due to its high concentration, it could be considered as **indicator of *S. aureus* contamination on pork**.
- **P+S vs P+C vs P+A**: pentane, heptane, cyclopentane, 1,2-dimethyl, 1-butanol 3-methyl, butanal 3-methyl, nonane. **Pentane** and **1-butanol 3-methyl** peak area concentration in **Pork+*S.* Typhimurium** are **higher** compared to **Pork+*C. jejuni*** and **Pork+*S. aureus***: the compounds could be considered **index of pork contamination due to *S.* Typhimurium**. Other compounds abundances in the combination do not describe significant differences and are not considered indicative of the contamination due to a specific microorganism, rather **index of common metabolic pathways**.

4. Volatiles that can be considered **potential marker of Pork contamination due to a single microorganism** are:
    - **Pork + *S.* Typhimurium**: 2-propanone, 1,1-dibutoxy-, dimethylsulfide, benzoic acid, hexanol, oxirane, (1-methylbutyl)-.
    - **Pork + *C. jejuni***: propanoic acid, pyridine 2-(1,3,4-oxadiazol-2-yl)-, 1,1-ethanediol diacetate, 1-hexanol 2-ethyl-, indolizine.
    - **Pork + *S.aureus***: cycloundecene, propane 2-ethoxy-2-methyl-, butanoic acid, 1,4-heptadiene, ethylhexanol, hydroxyurea, trimethylcyclohexane, ethosuximide.

4.5. Meat and bacterial mixes

Each combination was **individually analyzed** and **compared to the corresponding samples** for the research of similarities and different substances:

1. **Common substances** comparing the sample with the **meat matrix, the single and mixed bacterial inocula, and the combinations** between meat matrices and single pathogens.
2. **Correlation analysis between the available meat + bacterial mixes combinations**.
3. Potential **marker compounds** produced by each mix: substance not detected in other combinations within the study.

Whereas the same substance was produced, **decrease/increase/similarity was described**. To suggest a clear comparison, abbreviations were used for the combinations.

In addition, data were analyzed **without describing generic spoilage substances variations**, substances previously indicated: ethanol, 2-butanone 3-hydroxy, 1-butanol 3-methyl, hexane, ethyl acetate.

4.5.1. Beef and bacterial mixes

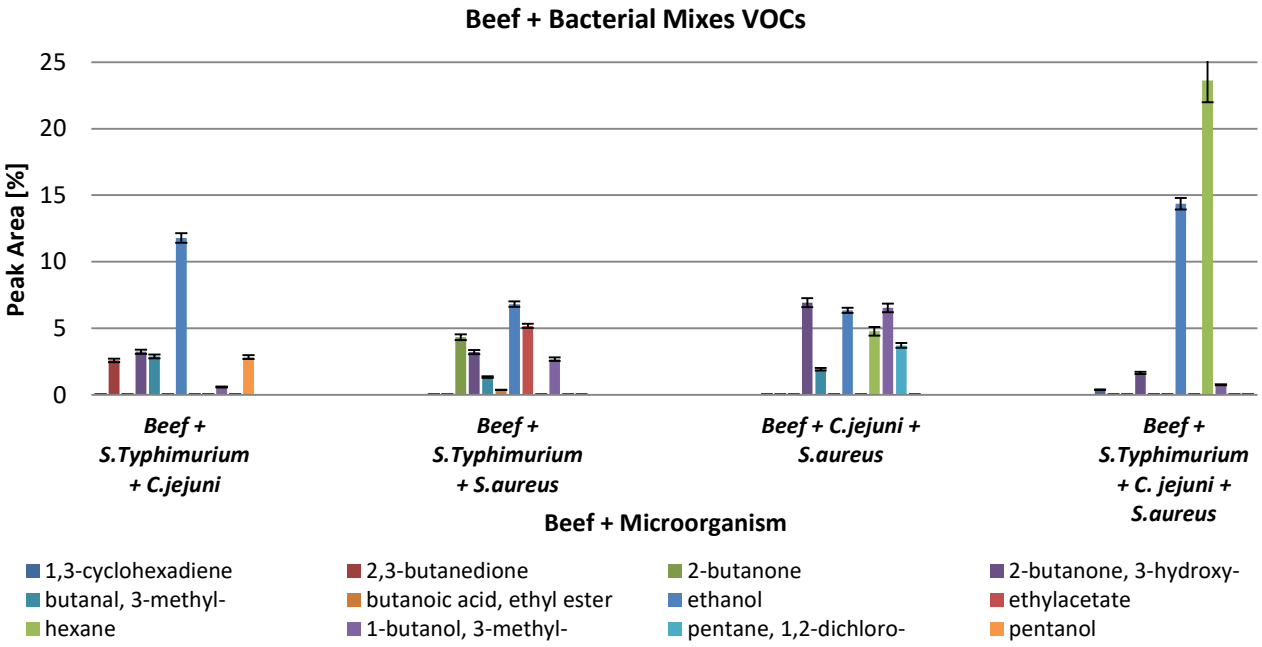


Figure 15 Analysis of substances produced by mixes of 2 or 3 target microorganisms inoculated on beef samples.

The association analysis comparing VOC profiles of beef spiked with the three bacteria available mixes (**B+S+C**, **B+S+A**, **B+C+A**, **B+S+C+A**) brought out the production of compounds which are common to either contaminations, single beef matrix (negative control) and single and mixed bacterial inocula. The production of potentially marker compounds has been additionally noted (**Figure 15**).

1. Research of **common compounds between single matrices, bacterial inocula, bacterial mixes, matrix with single bacteria** shows that:
   - Comparing **Beef + *S.* Typhimurium + *C. jejuni* (B+S+C)** to **Beef (B)**, it can be highlighted that these samples share the production of **pentanol**, with similar concentrations.
   - **Pentanol** was detected also in **Beef + *C. jejuni* + *S. aureus***, without significant concentrations variations.
   - Analyzing **Beef + *C. jejuni* + *S. aureus*** and ***C. jejuni* + *S. aureus*** profiles, it is interesting the common production of **pentane 1,2-dichloro** (similar abundance).
   - **Beef+ *S.* Typhimurium + *C. jejuni* + *S. aureus***, compared to the single inocula of **S**, **C**, and **A** and the combination **S+C+A**, describes a **sensitive increase of hexane**, registering a **33.63%** value.

2. The research of **common compounds within the combinations of beef spiked with the 4 bacterial mixes** evidenced that **ethanol**, **2-butanone 3-hydroxy**, and **isopenthyl alcohol are produced by all of the 4 combinations**: **differences** can be noted **in terms of concentrations**, which could result

useful to the generation of typical profiles. On the other hand, the presence of mostly common substances rather than unique compounds could not be convenient to the proper identification of the contamination due to bacterial combinations.

3. Volatiles that can be considered **potential marker of beef contamination due to a specific microorganisms mix** are:
   - **Beef + *S.* Typhimurium*+ C. jejuni***: 2,3-butanedione.
   - **Beef + *S.* Typhimurium*+ S. aureus***: 2-butanone, butanoic acid, ethyl ester (**also in Ch+C**).
   - **Beef + *C. jejuni+S. aureus***: pentane 1,2-dichloro.
   - **Beef + *S.* Typhimurium*+ C. jejuni S.aureus***: 1,3-cyclohexadiene.

Unique substances are less abundant in terms of number, compared to meat samples spiked with single bacteria. The generation of profiles, as suggested above, could more adequately support the identification of a specific multi-bacterial contamination on meat matrices.

4.5.2. Chicken and bacterial mixes

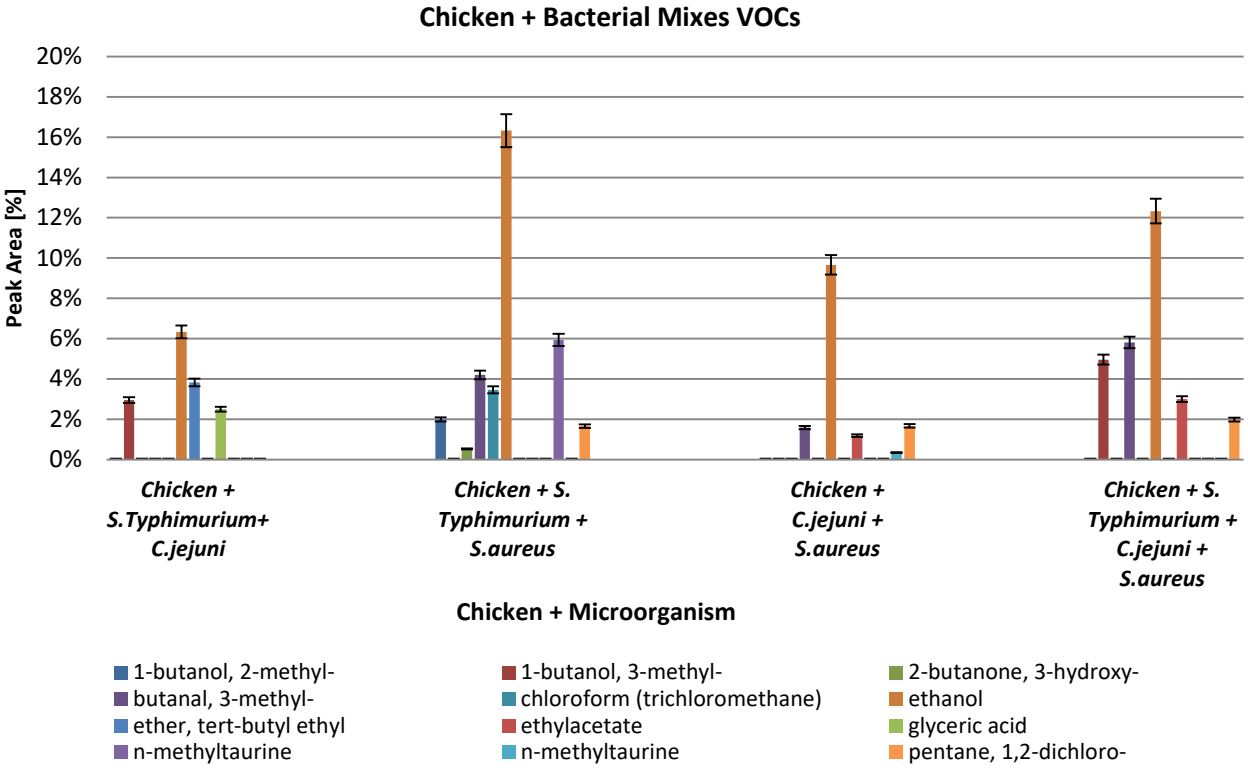


Figure 16 Analysis of substances produced by mixes of 2 or 3 target microorganisms inoculated on chicken samples.

1. **Common compounds between single matrices, bacterial inocula, bacterial mixes, matrix with single bacteria** are listed below:
   - **Chicken + *S.* Typhimurium + *C. jejuni* (Ch+S+C)** interesting common compound is **ether, tert-butyl ethyl**: produced by **S**, **C, S+C**, **Ch+S** (similar in S, S+C, Ch+S, lower in C).
   - **Chicken + *S.* Typhimurium + *S. aureus* (Ch+S+A)**, **Chicken+*C. jejuni*+*S. aureus* (Ch+C+A)**, and **Chicken+ *S.* Typhimurium + *C. jejuni*+*S. aureus* (Ch+S+C+A)** produced **pentane 1,2-dichloro**, detected even in **S** and **C** (similar in S, decreased in C).
   - **Chicken + *S.* Typhimurium + *C. jejuni*+*S. aureus* (Ch+S+C+A)** and **Ch+A** share the production of **isopenthyl alcohol**, which **highly decreased in the Ch+S+C+A** combination.

2. **Ethanol** was the **only common compound within the combinations of chicken spiked with the 4 bacterial mixes**: its concentrations vary but is not **useful to the generation of typical profiles**.
   The compound **n-methyltaurine is common to Ch+S+A and Ch+C+A:** it probably is **produced by *S. aureus* metabolism on chicken.**
   While **pentane 1,2-dichloro** is produced by **all combinations, excepting** for **Chicken + *S.* Typhimurium + *C. jejuni***: it is hypothesized but not confirmed that its production depends on *S. aureus*, considering that, the **single inoculum of *S. aureus*, despite Chicken+*C. jejuni*+*S. aureus* inoculum, does not produce pentane 1,2-dichloro.**

3. Volatiles that can be considered **potential marker of chicken contamination due to a specific microorganisms mix** are:
   - **Chicken + *S.* Typhimurium + *C. jejuni***: no unique compounds.
   - **Chicken + *S.* Typhimurium + *S. aureus***: 1-butanol 2-methyl.
   - **Chicken + *C. jejuni* + *S. aureus***: glyceric acid.
   - **Chicken + *S.* Typhimurium + *C. jejuni S.aureus***: no unique compounds.

   In the majority of cases, because of the **few peaks registered** by samples of chicken spiked with bacterial mixes**, combinations present only one unique substance**. The generation of profiles, in this case, could be the only chance to support the identification of a specific multi-bacterial contamination on chicken (**Figure 16**).

4.5.3. Pork and bacterial mixes

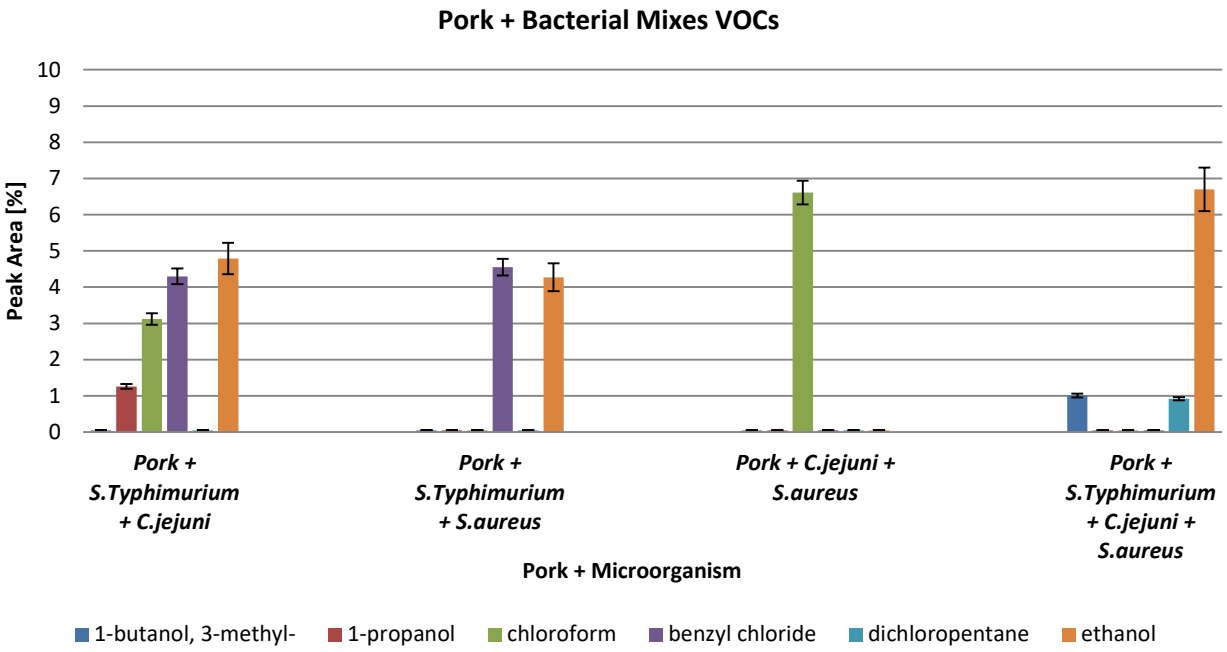


Figure 17 Analysis of substances produced by mixes of 2 or 3 target microorganisms inoculated on pork samples.

1. Research of **common compounds in single matrices, bacterial inocula, bacterial mixes, matrix with single bacteria,** brought out that **ethanol** was the **only common compound:** its concentration varies but is not **useful to the generation of typical profiles**.

2. **Chloroform is produced by both P+S+C and P+C+A:** it probably results from ***C.jejuni*** **metabolism on pork.** While **benzyl chloride** is produced by **Pork + *S.* Typhimurium + *C. jejuni*** and **Pork + *S.* Typhimurium + *S. aureus***: it is possible that **its production depends on *S.* Typhimurium**.

3. Volatiles that can be considered **potential marker of pork contamination due to a specific microorganisms mix** are:
   - **Pork + *S.* Typhimurium + *C. jejuni***: 1-propanol (**also in Ch+S**, **Ch+C**, and **P+S**).
   - **Pork + *S.* Typhimurium + *S. aureus***: no unique compounds.
   - **Pork+ *C. jejuni* + *S. aureus***: no unique compounds.
   - **Pork + *S.* Typhimurium + *C. jejuni S. aureus***: dichloropentane.

Pork spiked by the 4 mixed bacterial inocula was the meat matrix **producing the lowest number of volatiles**. The generation of profiles, could be the only chance, although not adequate, to support the identification of a specific multi-bacterial contamination on pork (**Figure 17**).

4.6. Meat and bacteria in varying concentrations

4.6.1. Beef and bacteria in varying concentrations

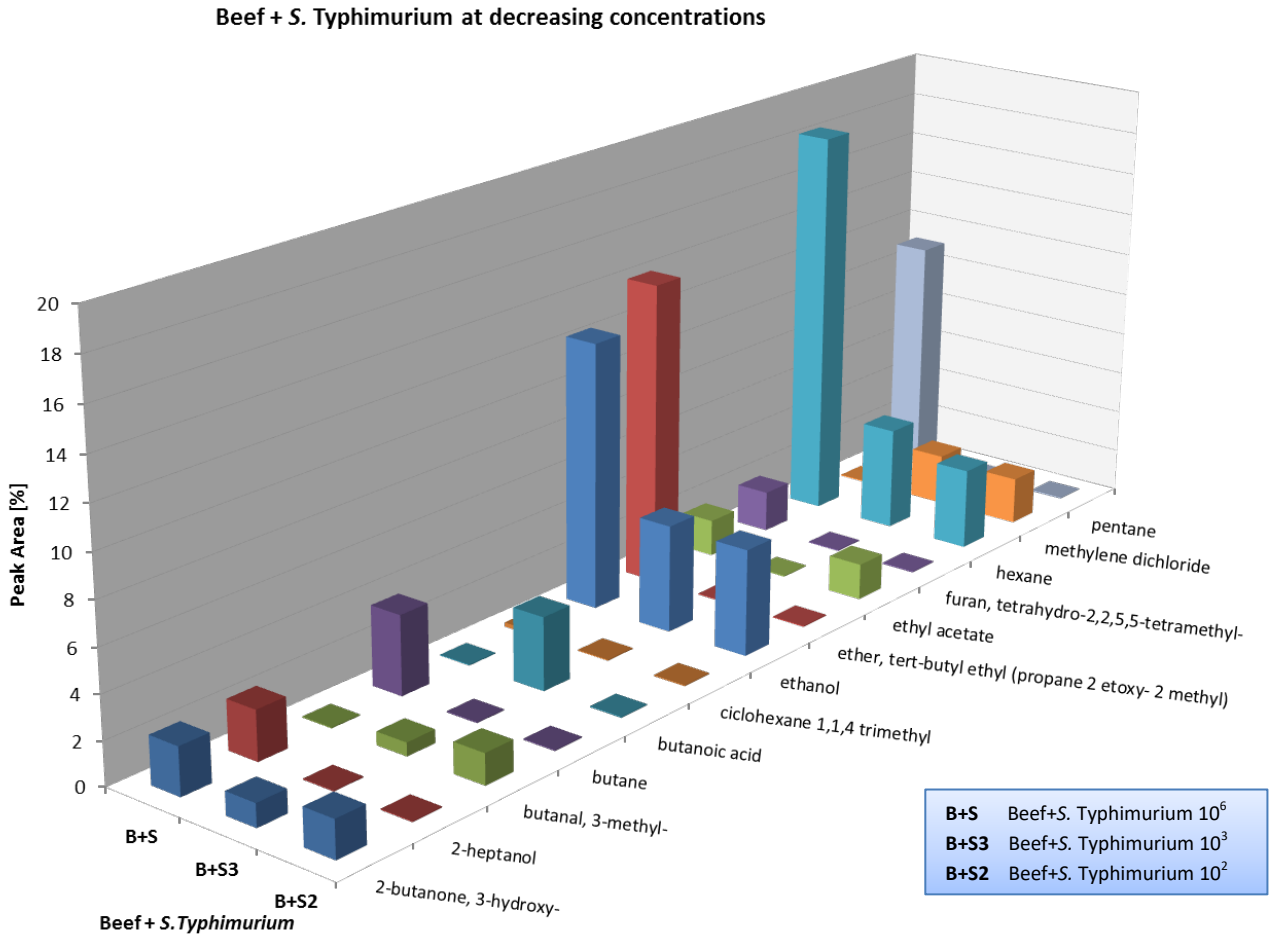


Figure 18 VOCs profiles and comparison of beef spiked with decreasing concentrations of *S.* Typhimurium (10^6, 10^3, 10^2).

The comparison between samples of beef inoculated with different concentrations of *S.* Typhimurium showed that the compounds produced by the three concentrations are **2-butanone 3-hydroxy**, **ethanol** and **hexane**, whose concentrations vary with the reduction of the bacterial load.

On the other hand, the **decrease of these substances, does not result in the increase or decrease of substances produced by the samples inoculated with 10^6 UFC/mL of *S.* Typhimurium**. Metabolism changes and when some substances from the 10^6 contamination are not detected (e.g. 2-heptanol, pentane, butane), the production of new substances is evident: **butanal 3-methyl** and **methylene chloride** abundances **grow proportionally to the decrease of bacterial load**, indicating maybe the prevalence of the resident microbial flora exploiting meat matrix nutrients (**Figure 18**).

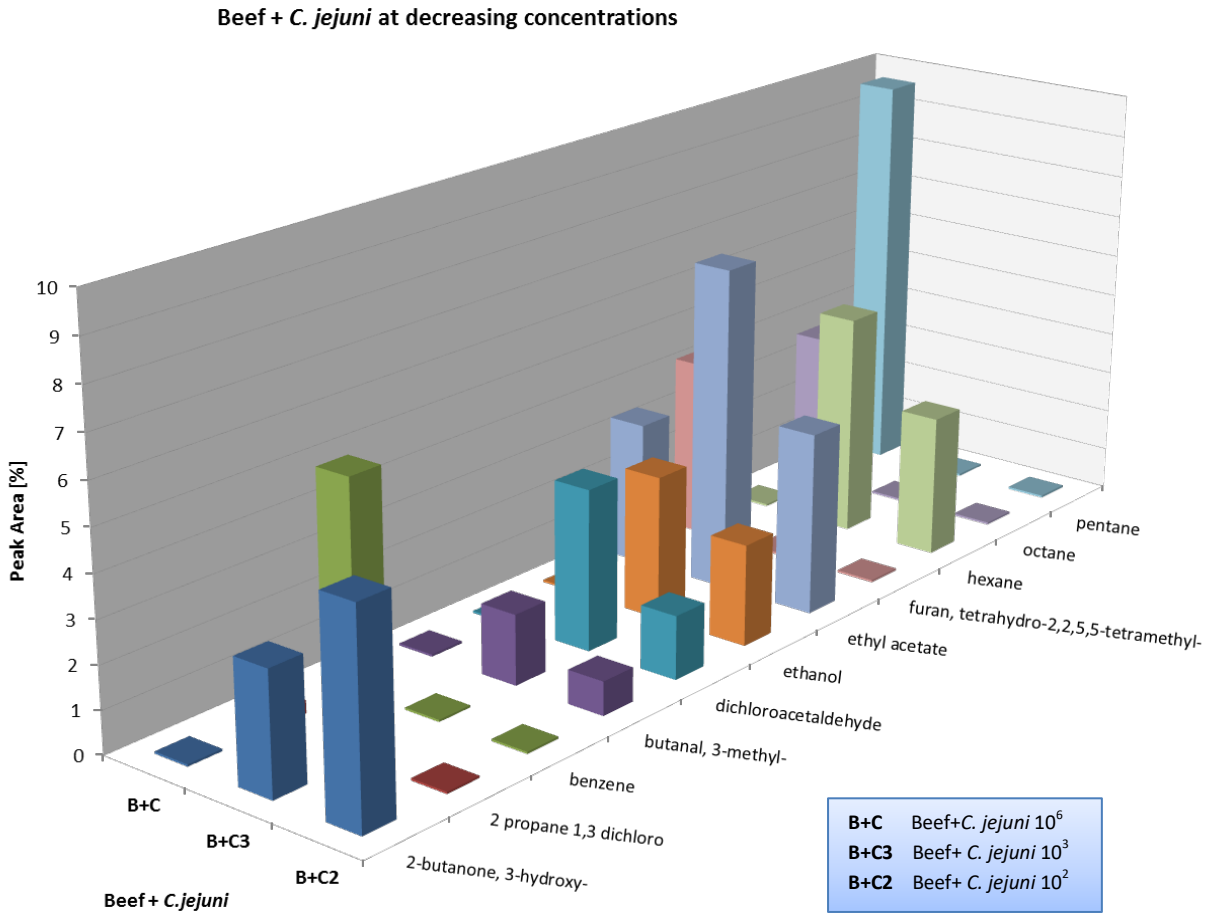


Figure 19 VOCs profiles and comparison of beef spiked with decreasing concentrations of *C. jejuni* (10^6, 10^3, 10^2).

Profiles of beef spiked with the three concentrations of ***C. jejuni*** **(10^6, 10^3, 10^2 UFC/mL)** are very **different** and the production of totally different pools of substances in the two combinations with lower bacterial loads describes changes in metabolism: **2-butanone 3-hydroxy**, **butanal 3-methyl**, **dichloroacetaldehyde**, and **hexane**, not detected in the 10^6 sample, were produced (**Figure 19**).

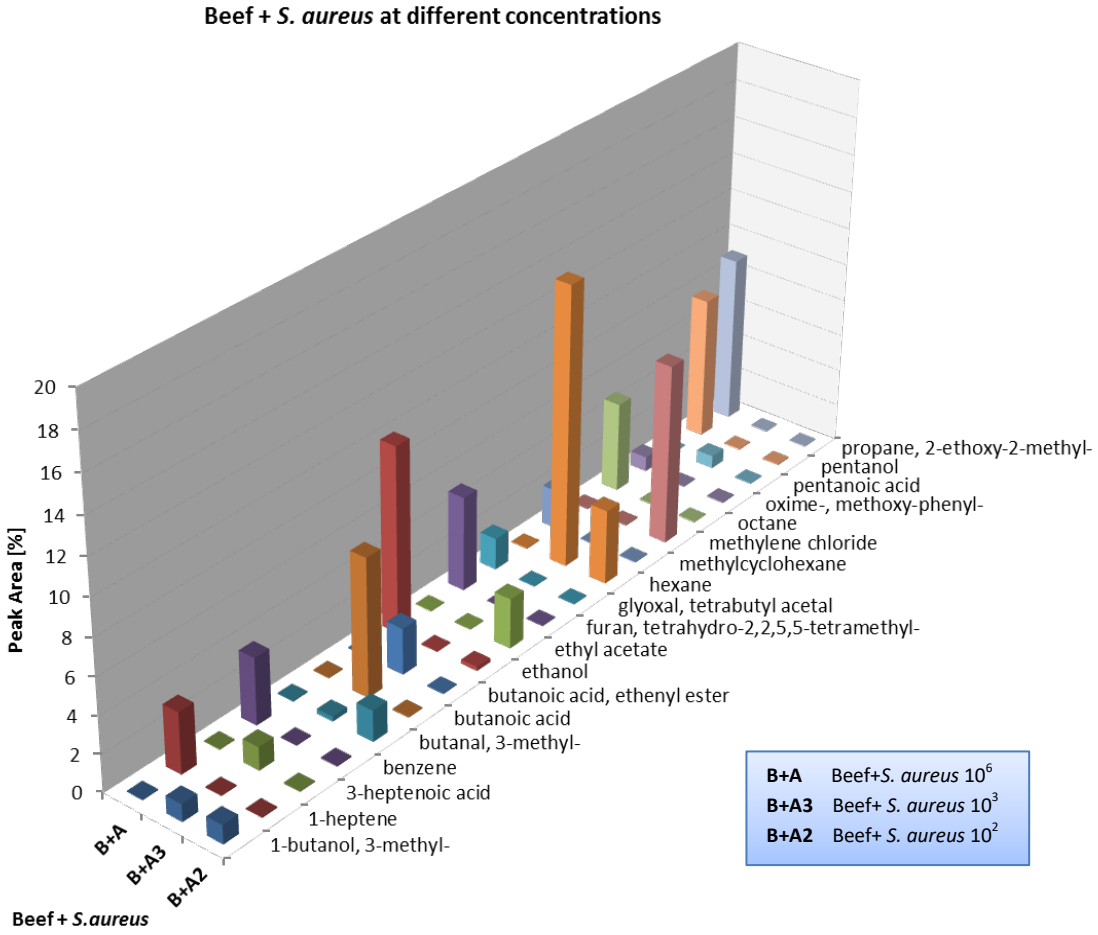


Figure 20 VOCs profiles and comparison of beef spiked with decreasing concentrations of *S. aureus* (10^6, 10^3, 10^2).

Beef contaminated with the **three concentrations of *S. aureus*** describes really **different profiles**, especially compared to the most concentrated combination: **no common substances** were produced crossing the data of the 10^6 to the lower loads. Besides, different substances are produced by the less *S. aureus* contaminated inocula: while **3-heptenoic acid**, **butanoic acid ethenyl ester,** and **pentanoic acid** were detected exclusively in the **10^3 sample**, compounds like **benzene**, **ethyl acetate**, and **methylene chloride** were produced by the 10^2 sample.

This outcome evidences the need of more accurate analysis on *S. aureus* metabolic pathways and the **competition with other species colonizing the meat** (**Figure 20**).

4.6.2. Chicken and bacteria in varying concentrations

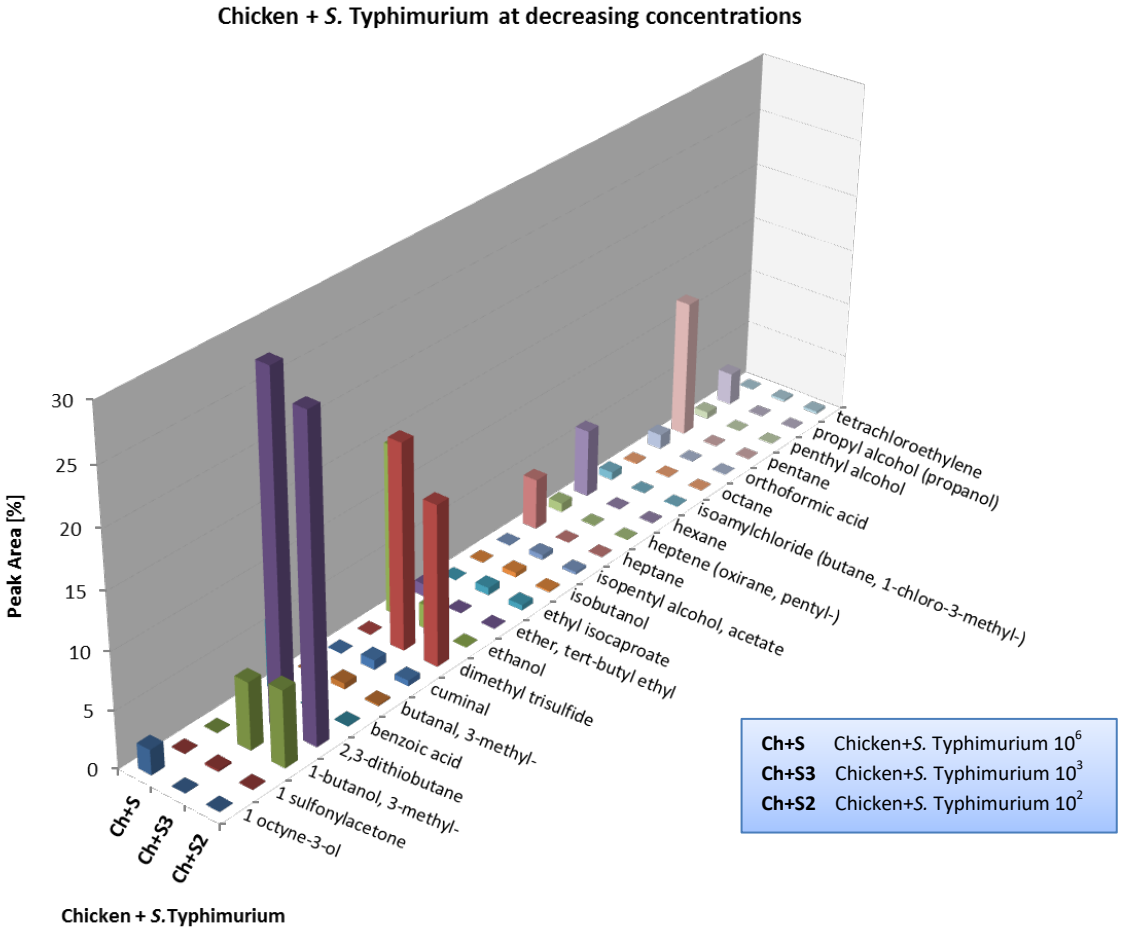


Figure 21 VOCs profiles and comparison of chicken spiked with decreasing loads of *S.* Typhimurium (10^6, 10^3, 10^2).

***S.* Typhimurium** different microbial loads on chicken **produced different profiles**, comparing the higher load, to the 10^3 and 10^2 samples: the **profile changes when *S.* Typhimurium concentration is less concentrated**. In contrast, the **10^3 and 10^2** samples produced three highly concentrated compounds (**1-butanol 3-methyl**, **2-3 dithiobutane**, and **dimethyltrisulfide**) and two less concentrated substances (**cuminal** and **ethyl isocaproate**), whose abundances decrease proportionally to the pathogen load (**Figure 21**).

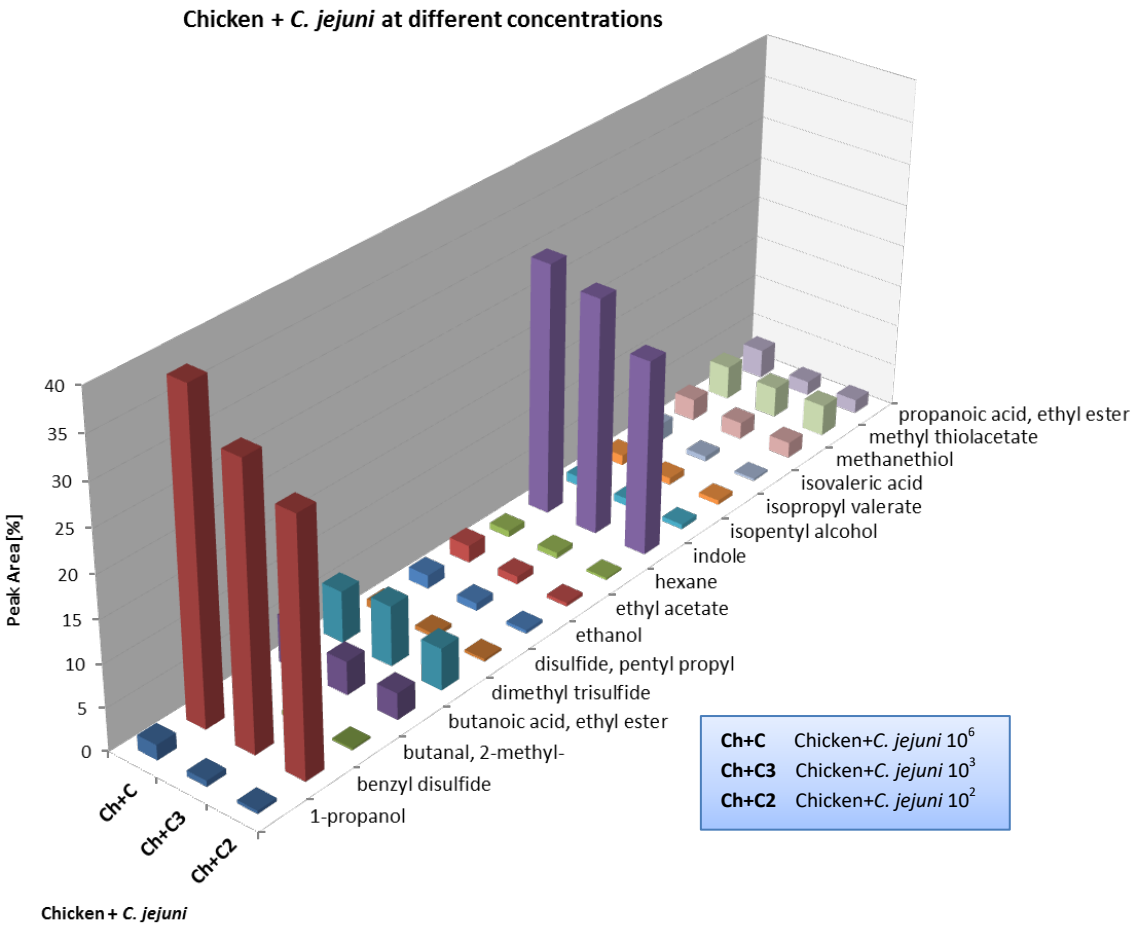


Figure 22 VOCs profiles and comparison of chicken spiked with decreasing concentrations of *C. jejuni* (10^6, 10^3, 10^2).

C. jejuni exploitation of chicken resulted in **profiles that can be highly comparable**: with the decrease of microbial loads, the **pathogens produce mostly the same substances** with lower abundances: the most concentrated substances detected are **benzyl disulfide**, **butanoic acid ehtyl ester**, **dimethyl trisulfide**, **indole**, and **methyl thioacetate** (**Figure 22**).

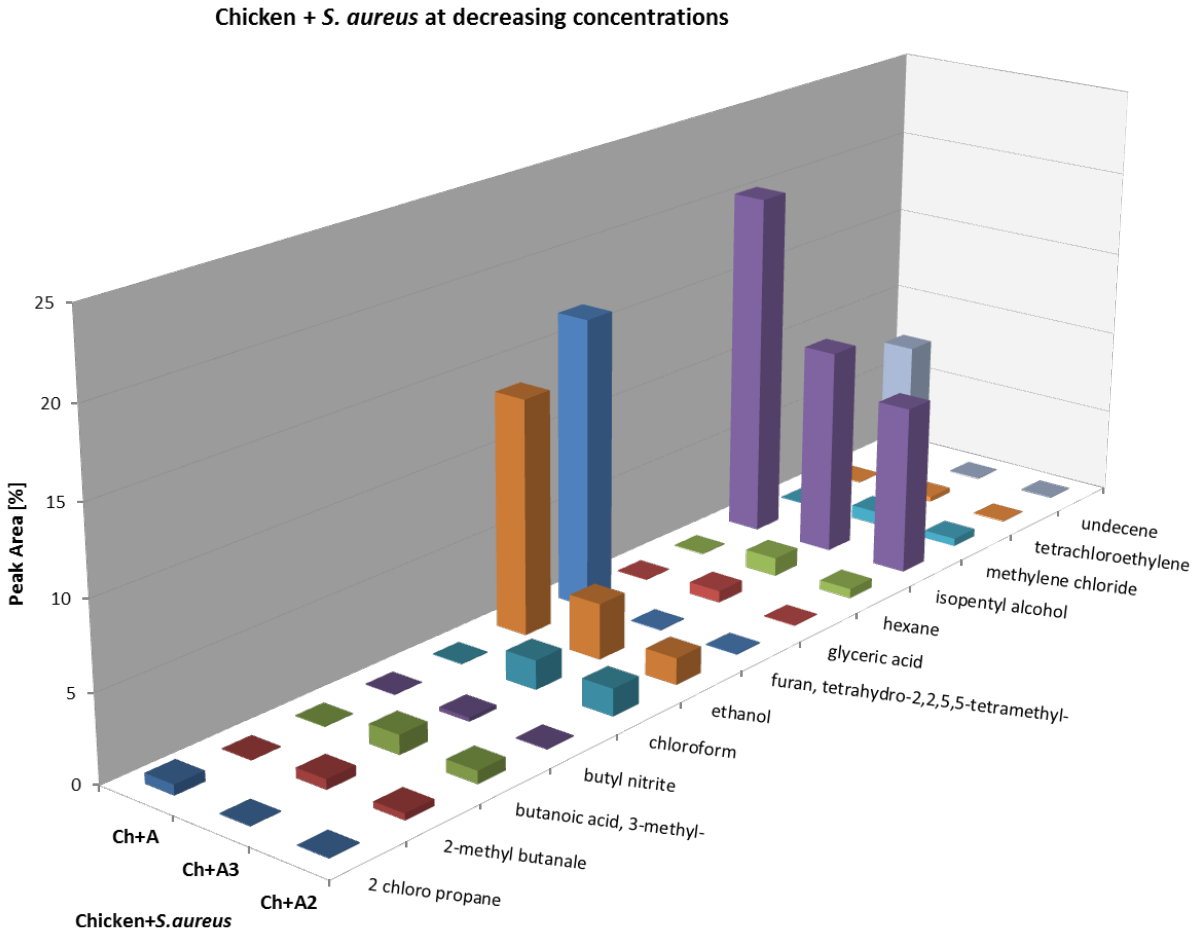


Figure 23 VOCs profiles and comparison of chicken spiked with decreasing concentrations of *S. aureus* (10^6, 10^3, 10^2).

VOC profiles of different concentrations of ***S. aureus*** spiked on chicken highlighted the **common production** of **ethanol** and **isopenthyl alcohol**, with gradually decreasing peak area abundances. **Butanoic acid 3-methyl**, **chloroform**, and **hexane** were produced only by **10^3 and 10^2** samples, while **furan tetrahydro-2,2,5,5-tetramethyl-** and **undecene**, detected with high concentrations in the **10^6** sample, were not produced by the less concentrated combinations: the results may describe **modifications in the metabolic pathways of *S.* Typhimurium**, due to the reduction of its microbial load on chicken (**Figure 23**).

4.6.3. Pork and bacteria in varying concentrations

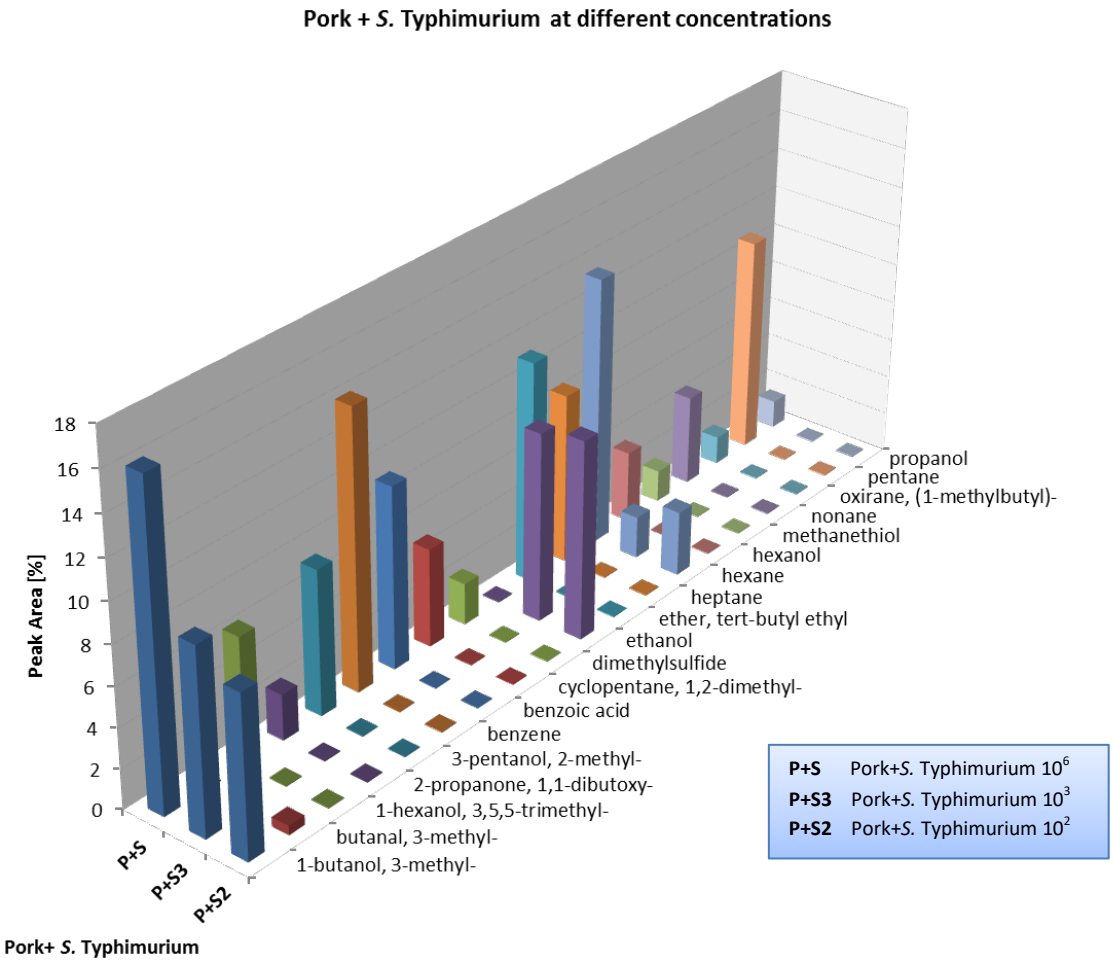


Figure 24 VOCs profiles and comparison of pork spiked with decreasing concentrations of *S.* Typhimurium (10^6, 10^3, 10^2).

From the evaluation of the reduced ***S.* Typhimurium** loads on **pork**, the common production of only one substance (1-butanol 3-methyl) is underlined: VOCs profiles are different. Indeed, while the 10^6 sample produce a large variety of compounds (16 volatiles), the **metabolism of the pathogen on pork** appears **sensibly reduced**. Three substances were detected in the **10^3** and **10^2** samples: **1-butanol 3-methyl**, **ethanol**, and **hexane**, which have been widely described as common to several combinations (**Figure 24**).

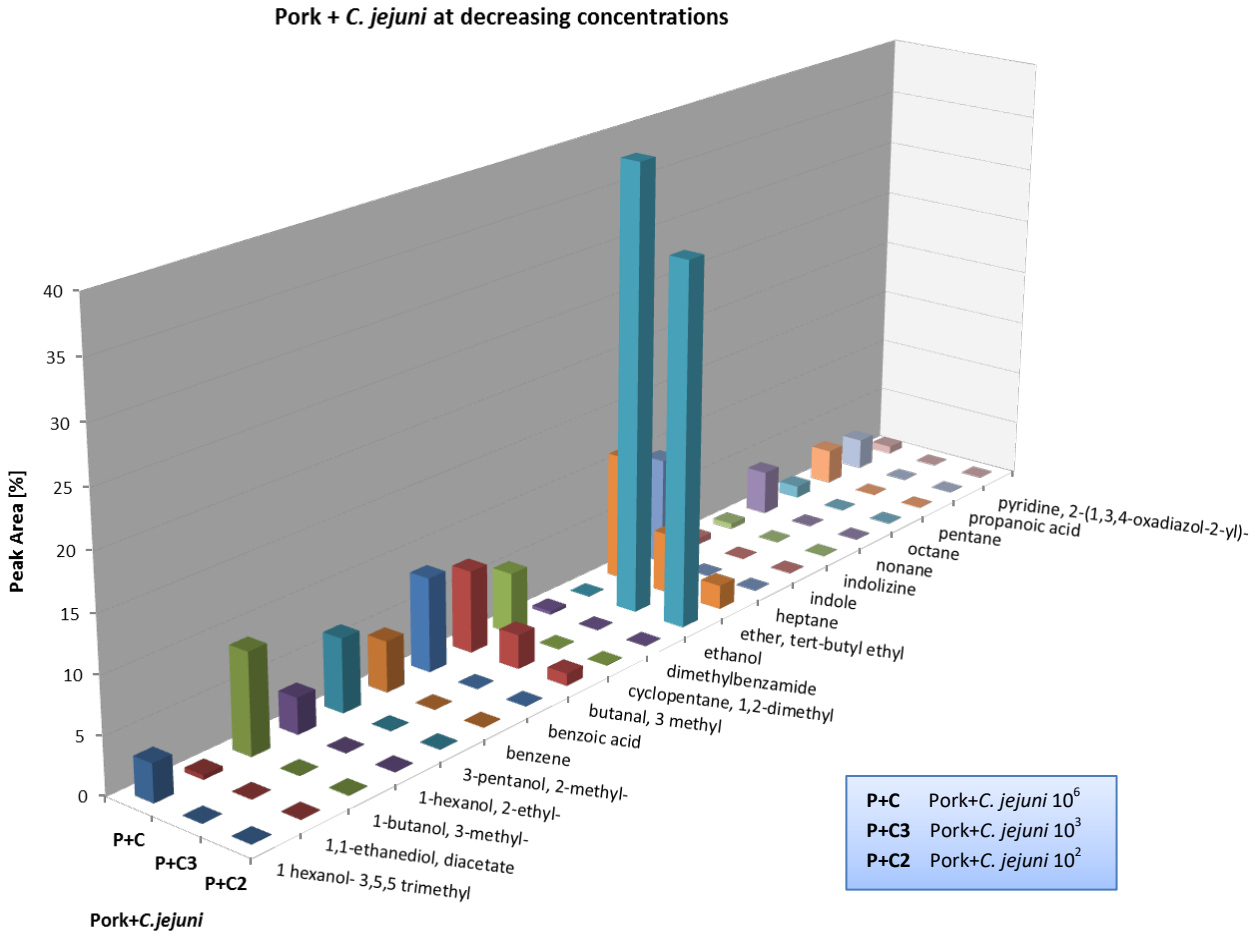


Figure 25 VOCs profiles and comparison of pork spiked with decreasing concentrations of *C. jejuni* (10^6, 10^3, and 10^2).

While pork has been proved to be a good nutrients source for the three pathogens inoculated with a 10^6 UFC/mL concentration, it is evident how, **by decreasing the microbial loads**, the **number of produced volatiles drops**: ***C. jejuni* profiles** show the absence of common compounds, the production of few volatiles in the less spiked samples and the significant abundance – decreasing proportionally to pathogen load – of two substances: **butanal 3-methyl** and **ethanol** (**Figure 25**).

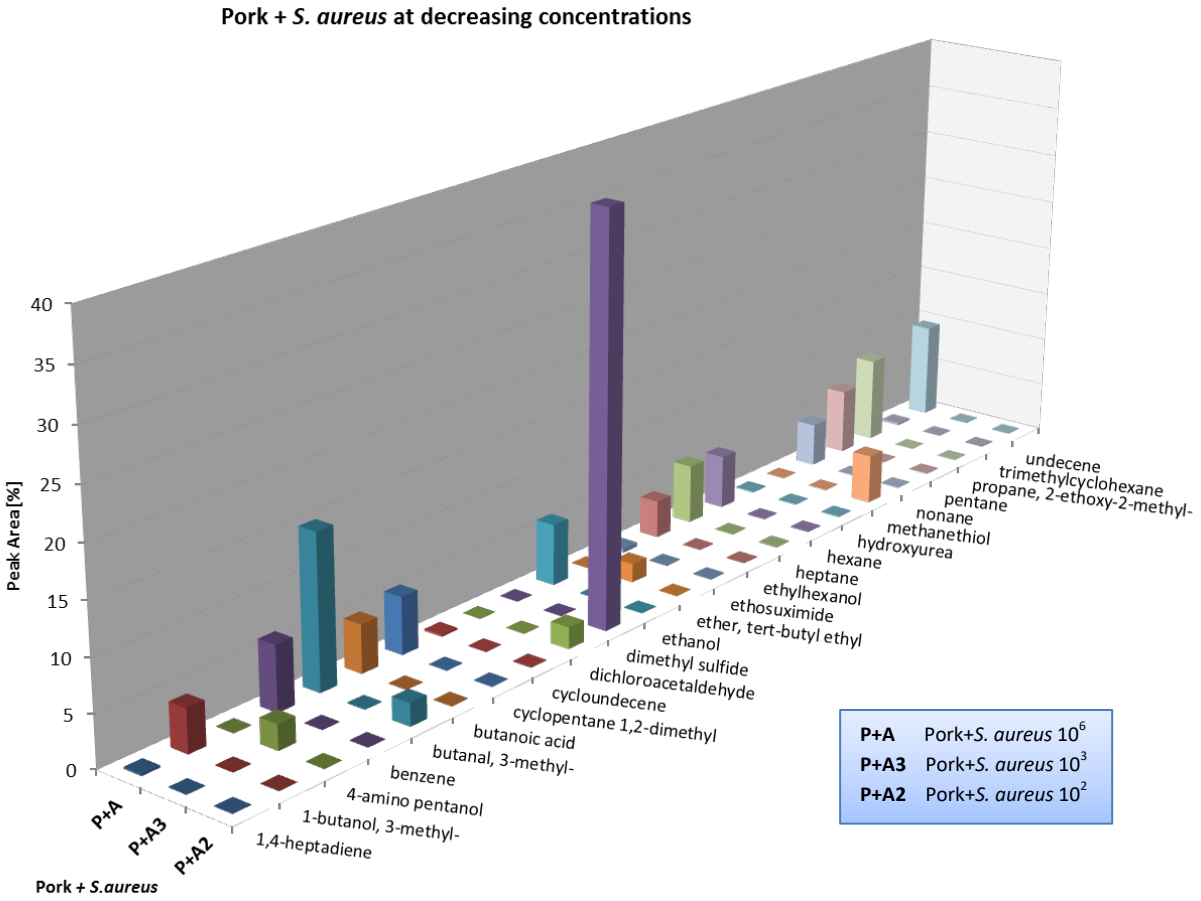


Figure 26 VOCs profiles and comparison of pork spiked with decreasing concentrations of *S. aureus* (10^6, 10^3, and 10^2).

***S. aureus* decreased microbial charges on pork** follow *S.* Typhimurium and *C. jejuni* trends: over the 13 compounds detected from 10^6 UFC/mL contaminated samples, **the only common substance** produced, by the **10^2 sample**, but not by the 10^3, is **butanal 3-methyl**. Profile of **pork** spiked with a **10^3 pathogen load** produced **2 substances** (**4-amino pentanol** and **ether tert-butyl ethyl**), which are different from the 10^2 sample volatiles. Noteworthy the profile of **pork** spiked with a **10^2 *S. aureus* load**, producing **dimethylsulfide**, with a high abundance (39.76%) (**Figure 26**).

4.7. Comparison with available bibliography

The analysis of volatile compounds produced from the metabolisms of bacteria on meat samples is not available for the majority of considered descriptors. **Volatiles profiling has been mostly focused on the detection of the generic spoilage evaluation, based on the molecular characterization of the endogenous flora**, associated with the related volatiles produced along the food matrices deterioration (Ercolini and others 2011; Flores and others 2013; Hernandez-Macedo and others 2014; Jääskeläinen and others 2016; Mikš-Krajnik and others 2014; Mikš-Krajnik and others 2015; Gianelli and others 2012).

Furthermore, clinical and environmental **bacterial isolates** have been submitted for GC/MS analysis in order to define typical bacterial profiles (O' Hara and others 2008; Tait and others 2013; Arnold and others 1998; Bunge and others 2008; Zhu and others 2010; Sohrabi and others 2014). Few studies have been conducted on *Campylobacter* genus volatiles production (Sohrabi and others 2014).
Furthermore, researches focusing on the **detection of markers of meat spoilage due specific pathogens** have been **rarely described** (Bhattacharjee and others 2011).

Volatiles organic compounds produced by negative controls and spiked meat samples have been **compared with available literature**. **Table 20**, **Table 21**, and **Table 22** summarize the compounds detected in both references and the present research. Whereas **interesting comparisons** were observed, the **compound was evidenced in bold**: for example, **1-pentanol, 2,3-butanedione, 2-butanone, 3-hydroxy-, and ethanol**, emitted from different beef samples analyzed in previous researches, were **also produced by spiked and not spiked beef samples under analysis**.

As previously stated, it is possible to identify the production of compounds which can be considered **generic meat spoilage indicators**: **ethanol, ethyl acetate, 1-butanol, 3 methyl, 2-butanone, 3-hydroxy-**.

In general, especially regarding bacterial inocula, data do not reflect the results obtained: this can depend the non-correspondence of the microbial species with the strains selected for this study. The disposal of additional analysis, **testing a higher number of species for each genus**, will allow sharpening **the markers identification process**.

Moreover, the **extreme variability of meat cuts**, **proteins and fatty acids compositions** modifications across countries needs to be taken into account: the GC/MS methodology will be indeed optimized in order to **reduce interferences depending on the matrix**.

Compounds BEEF	References					Samples from the research
	Bhattacharjee and others 2011	Ercolini and others 2011	Flores and others 2013	Hernandez-Macedo and others 2014	Jääskeläinen and others 2016	
1-butanol, 3 methyl	**x**			**x**		**In several samples**
1-hexanol				x		Pork+ *S.*Typhimurium
1-octyn, 3-ol		x			x	Pork+ *S.aureus*
1-pentanol				**x**		**Beef; Beef+*S.aureus***
1-propanol				x		Pork+*S.*Typhimurium
2,3-butanedione	**x**					**Beef+*S.*Typhimurium+*C.jejuni***
2-butanone	**x**			**x**		**Beef+S.Typhimurium+*S.aureus***
2-butanone, 3-hydroxy-	**x**					**In several samples**
2-heptanol			**x**	**x**		**Beef+ *S.*Typhimurium**
butanal, 3-methyl			x			Pork+ *S.aureus*
butanoic acid		x		x	x	Pork+ *S.aureus*
dimethylsulfide		x		x		Pork+ *S.*Typhimurium
ethanol			**x**	**x**	**x**	**In several samples**

Compounds CHICKEN	References		Samples from the research
	Mikš-Krajnik and others 2014	Mikš-Krajnik and others 2015	
1-butanol, 3 methyl	**x**	**x**	**In several samples**
2-butanone, 3-hydroxy-		**x**	**In several samples**
butanoic acid		x	Pork+ *S.aureus*
dimethyltrisulfide	**x**	**x**	**Chicken+*C.jejuni***
ethanol	**x**	**x**	**In several samples**
methanethiol		**x**	**Chicken/Pork+*S.*Typhimurium**

Compounds PORK	References		Samples from the research
	Gianelli and others 2012	Flores and others 2013	
1-butanol, 3 methyl	**x**		**In several samples**
1-octyn, 3-ol	**x**		**Pork+ *S.aureus***
2-butanone	x	x	Beef+*S.*Typhimurium+*S.aureus*
2-heptanol	x		Beef+ *S.*Typhimurium
butanal, 3-methyl	**x**		**Pork+ *S.aureus***
butanoic acid	**x**		**Pork+ *S.aureus***
ethanol	x	x	In several samples
undecene	**x**		**Pork+ *S.aureus***

Table 20 Comparison of the results with the available bibliography reporting VOCs analysis on basic metabolisms of endogenous microbial flora products on beef, chicken and pork samples (negative controls, i.e. not contaminated samples). Compounds in bold represent the evidence of similarities with the present research outcomes.

Compounds *Staphylococcus aureus*	References		Samples from the research
	O' Hara and others 2008	Tait and others 2013	
1-butanol, 3 methyl		**x**	**In several samples**
1-propanol		x	Pork+*S.*Typhimurium
2-butanone	**x**	**x**	**Beef+ *S.*Typhimurium+*S.aureus***
butanoic acid		**x**	**Pork+ *S.aureus***
butanoic acid, ethyl ester		**x**	**Beef/Pork+*S.aureus***
ethanol	**x**	**x**	**In several samples**
ethyl acetate		**x**	**In several samples**

Compounds *Salmonella* spp.	References			Samples from the research
	Arnold and others 1998	Bunge and others 2008	Zhu and others 2010	
1-butanol, 2 methyl		**x**		**Chicken+*S.*Typhimurium**
1-butanol, 3 methyl	**x**			**In several samples**
1-pentanol			**x**	Beef; Beef+*S.aureus*
1-propanol	**x**			**Pork+*S.*Typhimurium**
2-butanone		**x**		**Beef+ *S.*Typhimurium+*S.aureus***
ethanol	**x**	**x**	**x**	**In several samples**
methanethiol		**x**		**Chicken/Pork+*S.*Typhimurium**
indole			x	Chicken+*C.jejuni*

Table 21 Comparison of the results with previous studies reporting volatile organic compounds produced by *S.* Typhimurium and *S. aureus* (single bacterial inocula). Compounds in bold represent the evidence of similarities with the present research outcomes.

Compounds Beef + *Salmonella* spp.	References		Samples from the research
	Bhattacharjee and others 2011 *S.* Typhimurium (fresh meat)	Bhattacharjee and others 2011 *S.* Typhimurium (aged meat)	
1-butanol, 2 methyl		**x**	**Chicken+*S.*Typhimurium**
1-butanol, 3 methyl		**x**	**In several samples**
2,3-butanedione	**x**	**x**	**Beef+ *S.*Typhimurium+*C.jejuni***
2-butanone	**x**	**x**	**Beef+ *S.*Typhimurium+*S.aureus***
2-butanone, 3-hydroxy-	**x**	**x**	**In several samples**

Table 22 Comparison of the results with previous studies reporting volatile organic compounds produced by *Salmonella* Typhimurium on beef samples. Compounds in bold highlight similarities with the present research outcomes.

4.8. Molecular biology results analysis

Molecular biology approach, to **directly detect microbial contaminations on food**, resulted accurate and reliable. Extraction protocol, employing a **modified CTAB-chloroform-isoamylic alcohol method**, allowed the extraction of bacterial DNA in **3 hours**. Amplification using **specific primers** for the 3 pathogens, and **agarose gel electrophoresis are performed in 2 to 3 hours**, consenting to detect contaminations **within 1 day**. Resulting contamination can be evidenced by the visualization, on the agarose gel, of a **band, indicating the amplification (i.e. the presence) of the DNA of each specific pathogen** (Figure 27). Aiming to simplifying sample preparation protocols and sensibly reducing analysis times, although this methodology is highly reliable and describes a high reproducibility, it is **more expensive (3-4 times more) than a VOCs profiling**, therefore **less applicable for routine analysis**, requiring lower costs and sample processing.

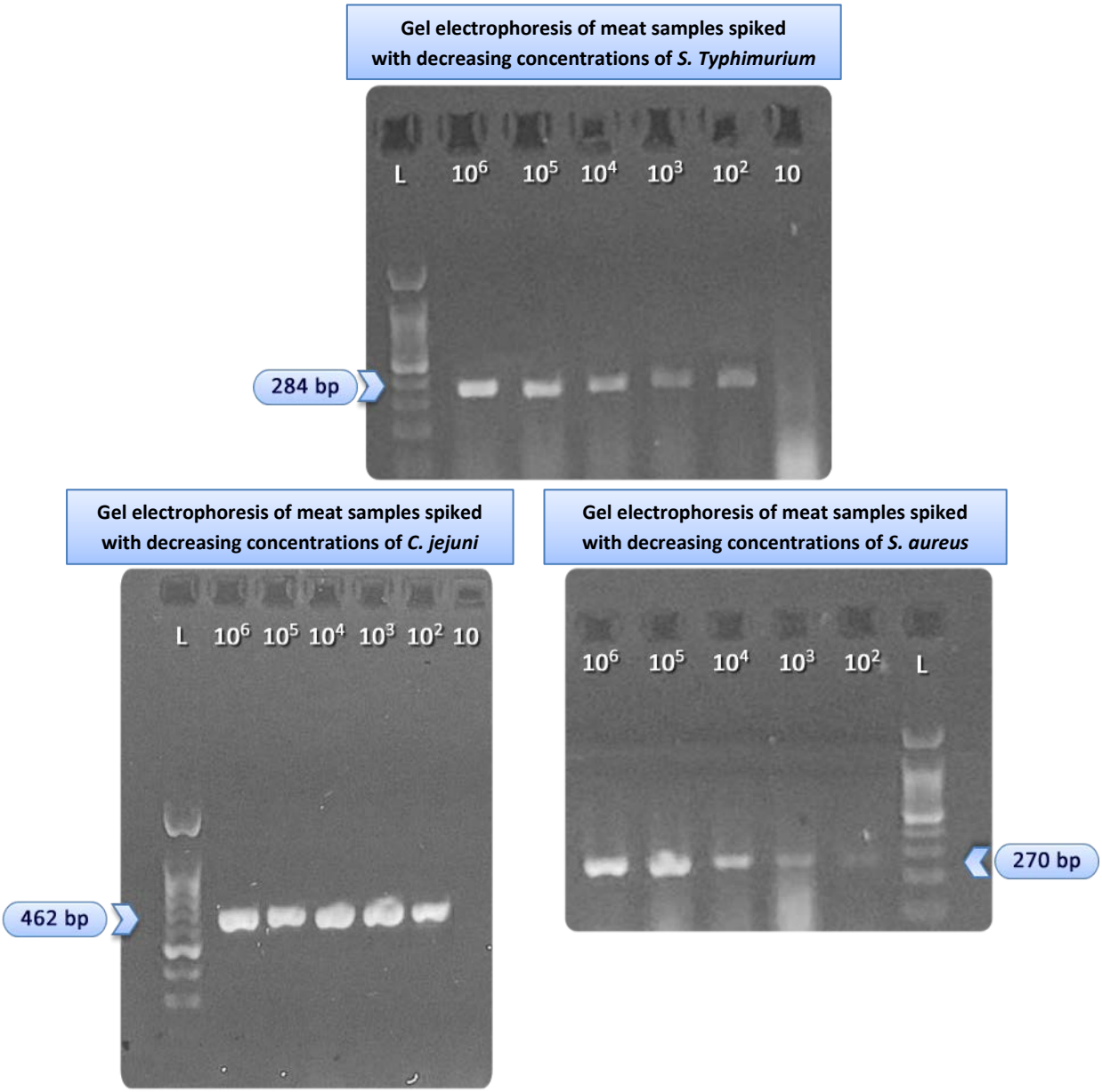


Figure 27 1.5% agarose gels of *S.Typhimurium*, *C.jejuni*, and *S. aureus* DNA extracted from meat samples spiked with the 3 pathogens at varying microbial loads (10^6, 10^5, 10^4, 10^3, 10^2, and 10 UFC/mL) [L: 100-bp DNA ladder].

4.9. Profiles generation of meat spiked with each bacterium

Besides the identification of unique markers of bacterial contaminations of food, it is **useful to generate typical profiles to describe the contaminations**, especially when the number of the produced volatiles is low in specific combinations (e.g. the combination between meat and multiple bacteria mixes).

Meat samples contaminated with single bacteria were compared to the relative negative controls and to the combinations with the other two bacteria contaminations on the same meat type. The data analysis consented to generate **typical profiles comprising common substances**, whose concentrations varied significantly in one combination, rather than others, describing peculiar changes, attributable to a specific contamination (e.g. hexane highly increased abundance when meat is spiked with *S.* Typhimurium).

Hence, whereas a **meat + microorganism peculiar combination evidenced strong increases or high decreases of the relative abundances of a substance produced by further combinations**, the **substance was retained significant in the creation of that typical profile**. Some examples are indicated below:

- **Hexane** in produced by several negative controls and by some contaminations: its concentration is highly increased only when the contamination is due to ***S.* Typhimurium** (beef: 18.33%, chicken: 6.03%, pork: 13.50%).
- **Isopentyl alcohol** is produced on **chicken** by the three pathogens, but its abundance in the matrix contaminated by ***S. aureus* is higher** (20% abundance in *S. aureus* compared to the 0.6% and 1.09% of *S.* Typhimurium and *C. jejuni*).
- **Pentane** results from several combinations but its production in meat spiked with ***S.* Typhimurium is on average higher** (beef: 11.06%, chicken: 12.28%, pork: 10.88%) than the contaminations due to the other two bacteria.
- **Butanal 3-methyl** is a product of both *S. aureus* (15.02%) and *C. jejuni* (7.29%) on **pork**: its relative abundance is strongly **increased in the contamination due to *S. aureus***.
- **1-Butanol 3-methyl** is produced on **pork** by the three pathogens, but its abundance in the matrix contaminated by ***S.* Typhimurium** is higher (16.11% abundance in *S.* Typhimurium compared to the 8.92% and 4.2% of *S. aureus* and *C. jejuni*).

Table 23, **Table 24**, and **Table 25** show the profiles generation approach and describe the **profiles of the contaminations due to the three bacteria on the three different meat matrices**. Potential **marker compounds** of a specific contamination are indicated with a red "X".

4.9.1. Profiles of beef spiked with each bacterium

Combination	Marker compounds	Negative controls		Contaminations		
Beef + *Salmonella* Typhimurium (B+S)	**B+S**	**B**	**S**	**B+C**	**B+A**	**Notes**
2-butanone, 3-hydroxy-		+	-	-	-	
2-heptanol	x	-	-	-	-	
butane	x	-	-	-	-	
ciclohexane 1,1,4 trimethyl	x	-	-	-	-	
ethanol		+	-	-	+	
ether, tert-butyl ethyl		-	+	-	-	
ethyl acetate		-	-	+	-	
furan, tetrahydro-2,2,5,5-tetramethyl-		-	-	+	+	
hexane		+	+	-	-	3* B; 10* S
pentane		-	-	+	-	

Combination	Marker compounds	Negative controls		Contaminations		
Beef + *Campylobacter jejuni* (B+C)	**B+C**	**B**	**C**	**B+S**	**B+A**	**Notes**
2 propane 1,3 dichloro	x	-	-	-	-	
benzene		+	-	-	+	
ethyl acetate		-	-	+	-	
furan, tetrahydro-2,2,5,5-tetramethyl-		-	-	+	+	3* B; 10* S
octane		+	-	-	+	
pentane		-	-	+	-	

Combination	Marker compounds	Negative controls		Contaminations		
Beef + *Staphylococcus aureus* (B+A)	**B+A**	**B**	**A**	**B+S**	**B+C**	**Notes**
1-heptene		+	-	-	-	
benzene		+	-	-	+	
ethanol		+	-	+	-	
furan, tetrahydro-2,2,5,5-tetramethyl-		-	-	+	+	3* B; 10* S
glyoxal, tetrabutyl acetal	x	-	-	-	-	
methylcyclohexane	x	-	-	-	-	
octane		+	-	-	+	
oxime-, methoxy-phenyl-	x	-	-	-	-	
pentanol		+	-	-	-	
propane, 2-ethoxy-2-methyl-	x	-	-	-	-	

B	Beef	**S**	*S.* Typhimurium	**C**	*C. jejuni*	**A**	*S. aureus*
B+S	Beef + *S.*Typhimurium	**B+C**	Beef + *C.jejuni*	**B+A**	Beef + *S. aureus*		
3* B	3 times more concentrated than beef	**10* S**	10 times more concentrated than *S.*				

Table 23 VOCs profiles generated through the comparison of each beef + microorganism contamination with relative negative controls (not contaminated beef and single bacterial inoculum) and respective contaminations.

4.9.2. Profiles of chicken spiked with each bacterium

Combination	Marker compounds	Negative controls		Contaminations		
Chicken + *Salmonella* Typhimurium (Ch+S)	Ch+S	Ch	S	Ch+C	Ch+A	Notes
1 octyne-3-ol	x	-	-	-	-	
benzoic acid	x	-	-	-	-	
ethanol		+	-	+	+	
ether, tert-butyl ethyl		-	+	-	-	
heptane	x	-	-	-	-	
heptene (oxirane, pentyl-)	x	-	-	-	-	
hexane		-	+	+	-	6* S
isoamylchloride	x	-	-	-	-	
octane		+	-	-	-	1/3* C
orthoformic acid	x	-	-	-	-	
pentane	x	-	-	-	-	
penthyl alcohol		-	-	-	-	
propyl alcohol (propanol)		-	-	+	-	

Combination	Marker compounds	Negative controls		Contaminations		
Chicken + *Campylobacter jejuni* (Ch+C)	Ch +C	Ch	C	Ch +S	Ch +A	Notes
1-propanol		-	-	-	-	
benzyl disulfide	x	-	-	-	-	
butanal, 2-methyl-	x	-	-	-	-	
butanoic acid, ethyl ester	x	-	-	-	-	
dimethyl trisulfide	x	-	-	-	-	
disulfide, pentyl propyl	x	-	-	-	-	
ethanol		+	-	+	+	1/3* C
ethyl acetate		-	-	-	-	
hexane		-	+	+	-	1/5* Ch
indole		-	-	-	-	
isopentyl alcohol		-	-	-	+	
isopropyl valerate	x	-	-	-	-	
isovaleric acid	x	-	-	-	-	
methanethiol		-	-	-	-	
methyl thiolacetate	x	-	-	-	-	
propanoic acid, ethyl ester	x	-	-	-	-	

Combination	Marker compounds	Negative controls		Contaminations		
Chicken + *Staphylococcus aureus* (Ch+A)	Ch +A	Ch	A	Ch +S	Ch +C	Notes
2 chloro propane	x	-	-	-	-	
ethanol		+	-	+	+	
furan, tetrahydro-2,2,5,5-tetramethyl-		-	-	-	-	
isopentyl alcohol		-	-	-	+	
undecene		-	-	-	-	

Ch	Chicken	**S**	*S.* Typhimurium	**C**	*C. jejuni*	**A**	*S. aureus*
Ch+S	Chicken + *S.*Typhimurium	**Ch+C**	Chicken + *C.jejuni*	**Ch+A**	Chicken + *S. aureus*		
6* S	6 times more concentrated than *S.* Typhimurium	**1/3* C**	3 times less concentrated than *C. jejuni*	**1/5* Ch**	5 times less concentrated than chicken		

Table 24 VOCs profiles generated through the comparison of each chicken + microorganism contamination with relative negative controls (not contaminated beef and single bacterial inoculum) and respective contaminations.

4.9.3. Profiles of pork spiked with each bacterium

Combination	Marker compounds	Negative controls		Contaminations		
Pork + *Salmonella* Typhimurium (P+S)	P+S	P	S	P+C	P+A	Notes
1-butanol, 3-methyl-		+	-	+	+	3* P
1-hexanol, 3,5,5-trimethyl-		-	-	+	-	
2-propanone, 1,1-dibutoxy-	x	-	-	-	-	
3-pentanol, 2-methyl-		-	-	+	-	
benzene		-	-	+	+	
benzoic acid		-	-	+	-	
cyclopentane, 1,2-dimethyl-		-	-	+	+	
dimethylsulfide	x	-	-	-	-	
ether, tert-butyl ethyl		-	+	+	-	8* S
heptane		+	-	+	+	
hexane		-	+	-	+	13* S
hexanol	x	-	-	-	-	
methanethiol		-	-	-	-	
nonane		-	-	+	+	
oxirane, (1-methylbutyl)-	x	-	-	-	-	
pentane		-	-	+	+	
propanol		-	-	-	-	

Combination	Marker compounds	Negative controls		Contaminations		
Pork + *Campylobacter jejuni* (P+C)	P +C	P	C	P +S	P +A	Notes
1 hexanol- 3,5,5 trimethyl		-	-	-	+	
1,1-ethanediol, diacetate	x	-	-	-	-	
1-butanol, 3-methyl-		+	-	+	+	
1-hexanol, 2-ethyl-	x	-	-	-	-	
3-pentanol, 2-methyl-		-	-	-	+	
benzene		-	-	+	+	
benzoic acid		-	-	+	+	
butanal, 3 methyl		-	-	+	-	
cyclopentane, 1,2-dimethyl		-	-	+	+	
ether, tert-butyl ethyl		-	+	-	+	1/2* C
heptane		+	-	+	+	
indole	x	-	-	-	-	
indolizine	x	-	-	-	-	
nonane		-	-	+	+	
octane		+	-	-	-	
pentane		-	-	+	+	
propanoic acid		-	-	-	+	
pyridine, 2-(1,3,4-oxadiazol-2-yl)-	x	-	-	-	-	

Combination Pork + *Staphylococcus aureus* (P+A)	Marker compounds P +A	Negative controls P	 A	Contaminations P +S	 P +C	Notes
1,4-heptadiene	x	-	-	-	-	
1-butanol, 3-methyl-		+	-	+	+	
benzene		-	-	+	+	
butanal, 3-methyl-		-	-	-	+	
butanoic acid	x	-	-	-	-	
cyclopentane 1,2-dimethyl		-	-	+	+	
cycloundecene	x	-	-	-	-	
ethanol		+	-	-	-	1/3* P
ethosuximide	x	-	-	-	-	
ethylhexanol	x	-	-	-	-	
heptane		+	-	+	+	
hexane		-	+	+	-	5* A
hydroxyurea	x	-	-	-	-	
nonane		-	-	+	+	
pentane		-	-	+	+	
propane, 2-ethoxy-2-methyl-	x	-	-	-	-	
trimethylcyclohexane	x	-	-	-	-	
undecene		-	-	-	-	

P	Pork	**S**	*S.* Typhimurium	**C**	*C. jejuni*	**A**	*S. aureus*
P+S	Pork + *S.*Typhimurium	**P+C**	Pork + *C.jejuni*	**P+A**	Pork + *S. aureus*		
13* B	13 times more concentrated than *S.* Typhimurium	**8* S**	8 times more concentrated than *S.*Typhimurium	**3* P**	3 times more concentrated than pork		
1/2* C	2 times less concentrated than *C.jejuni*	**1/3* P**	3 times less concentrated than pork	**5* A**	5 times more concentrated than *S. aureus*		

Table 25 VOCs profiles generated through the comparison of each chicken + microorganism contamination with corresponding negative controls (not contaminated beef and single bacterial inoculum) and respective contaminations.

4.10. Identification of marker compounds of microbial contamination

VOC profiles on meat samples spiked by ***S. Typhimurium***, ***C. jejuni*** and ***S. aureus*** are not available in literature: the **characterization of marker compounds and characteristic profiles** able to indicate the presence of a microorganism in raw meat is the purpose of the study. Data interpolation of **examined combinations** for meat samples spiked with bacterial strains has been pointed to the generation of **unique correlations between VOC profiles and pathogens** spiked on the three meat types, based on evaluated samples.

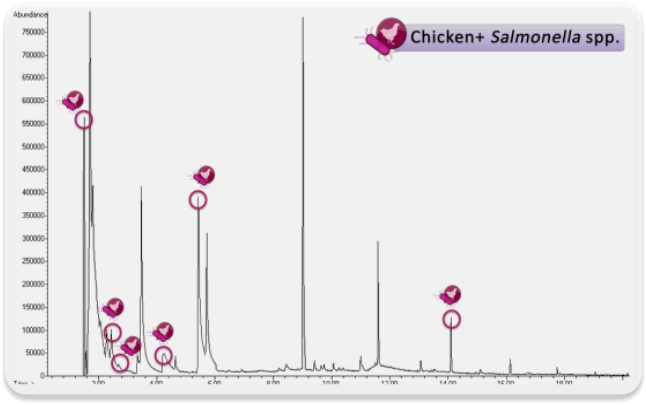


Figure 28 Chromatogram of VOCs produced by *S.*Typhimurium on chicken. Colored circles identify compounds produced exclusively by this contamination.

Table 26 shows **typical and unique compounds** associated to the contamination of meat samples due to a specific microorganism.

+	Beef
***S.* Typhimurium**	butane 2-heptanol ciclohexane 1,1,4 trimethyl
C.jejuni	2 propane 1,3 dichloro
S.aureus	propane, 2-ethoxy-2-methyl- methylcyclohexane oxime-, methoxy-phenyl- glyoxal, tetrabutyl acetal

+	Chicken
S.Typhimurium	pentane orthoformic acid heptane heptene (oxirane, pentyl-) benzoic acid 1 octyne-3-ol isoamylchloride
C.jejuni	isovaleric acid methyl thiolacetate butanal 2-methyl propanoic acid, ethyl ester benzyl disulfide butanoic acid, ethyl ester disulfide, pentyl propyl isopropyl valerate dimethyl trisulfide
S.Aureus	2 chloro propane

+	Pork
S.Typhimurium	2-propanone, 1,1-dibutoxy- dimethylsulfide hexanol oxirane, (1-methylbutyl)-
C.jejuni	propanoic acid pyridine, 2-(1,3,4-oxadiazol-2-yl)- 1,1-ethanediol, diacetate 1-hexanol, 2-ethyl- indolizine
S.aureus	cycloundecene propane, 2-ethoxy-2-methyl- butanoic acid 1,4-heptadiene ethylhexanol hydroxyurea trimethylcyclohexane ethosuximide

Table 26 Results of GC-MS data interpolation: unique volatile organic compounds (VOCs) detected as markers of each bacterial contamination.

The analysis of **chromatograms** lead to the identification of a **higher number of VOCs**, with relatively high peak areas (e.g. pentane 12.28%, butane 3.7%, acetoin 2.2%), in samples spiked with ***S.* Typhimurium** and ***C. jejuni*** (apart from *C.jejuni* products on beef) compared to meat spiked with *S. aureus*: this may depend on *S.* Typhimurium and *C. jejuni* pathogenicity and their ability to better exploit meat nutrients. **Pathogens spiked on pork meat** produced **broader lists of marker compounds for the three pathogens,** than the other combinations.

C. jejuni spiked on beef produced only one unique volatile per sample; the analysis of *S. aureus* contamination on raw chicken allowed detecting only one unique compound (2-chloropropane, 0,6%). A **higher number of samples** would consent a better results evaluation and reliability.

4.11. PCA analysis

In order to better visualize the differences between the profiles compared to negative control meat samples, **Principal Component Analysis (PCA)** has been employed. Considered the high number of data and analyzed variables, association analysis supported a better presentation of the results, confirming what described from the data analysis. Indeed, Principal component analysis (PCA) is a technique basically used to emphasize variation and bring out strong patterns in a dataset. It is indeed used to make data easy to explore and visualize.

Each combination is "isolated", compared to others, meaning that, considering the statistical significance approach, **the higher is the distance between the components, the major difference between the profiles can be described**.

PCA was used to show associations between:

1. The **three bacterial contaminations** on each meat matrix (e.g. Beef +*S* Typhimurium, Beef+*C.jejuni*, and Beef+*S.aureus*) (**Figure 29**, **Figure 33**, and **Figure 37**).

2. The **three bacterial contaminations** on each meat matrix compared to the **bacterial inocula** products (e.g. B+S, B+C, and B+A compared to *S.*Typhimurium, *C.jejuni*, and *S.aureus*) (**Figure 30**, **Figure 34**, and **Figure 38**).

3. The **three bacterial contaminations** on each meat matrix compared to the relative **negative control meat matrix**, whose products were ideally result of endogenous microbial flora metabolism (e.g. B+S, B+C, and B+A compared to Beef products) (**Figure 31**, **Figure 35**, and **Figure 39**).

4. The **three bacterial contaminations** on each meat matrix compared to **all the relative negative controls** (meat matrix and bacterial inocula) (e.g. B+S, B+C, and B+A compared to Beef products and Bacterial inocula) (**Figure 32**, **Figure 36**, and **Figure 40**).

4.11.1. PCA analysis of beef spiked with single bacteria

The association analysis comparing the samples of **beef spiked with the three pathogens** under analysis (*S.* Typhimurium, *C. jejuni*, and *S. aureus*) highlights the substantial differences (combinations are distant from each other in the figure) between the generated profiles, indicating the potential discrimination ability of the suggested analytical approach (**Figure 29**).

Compounds vectors directed to the combinations can be considered typical of an exact contamination, while **vectors of substances resulting far from the combinations**, produced by the three combinations (e.g. ethyl acetate and pentane) **cannot be considered significant for the profiles generation**.

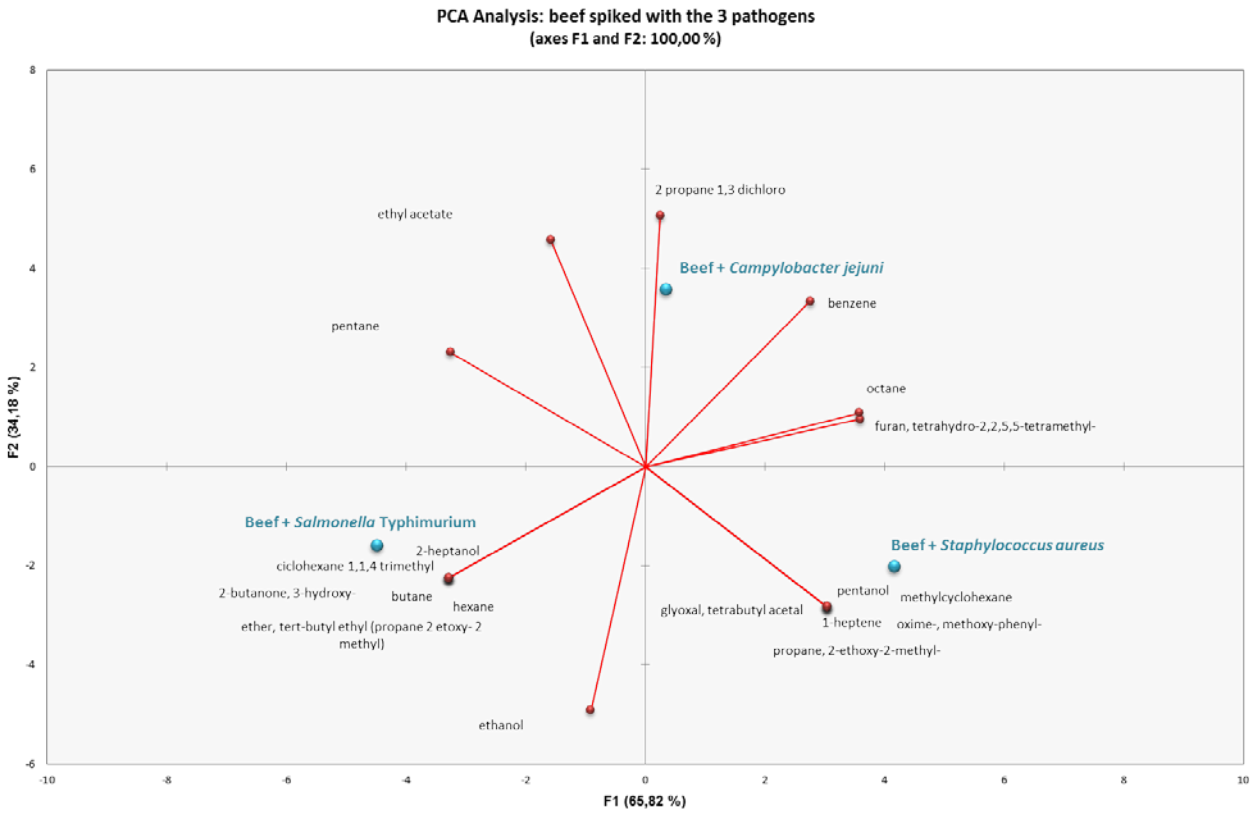


Figure 29 Principal component analysis of beef spiked with *S.*Typhimurium, *C. jejuni*, and *S.aureus*. Association analysis describe substantial differences between the generated profiles.

The picture changes when the **variable "bacterial inocula" is added** to the association analysis: the significant **differences are more evident comparing Beef + *S. aureus* to *S. aureus* inoculum**, less analyzing the contaminations due to *S.* Typhimurium and *C. jejuni*, although the two combination are distant from the respective inocula.

The three meat + bacterium combinations result however in different points of the chart, confirming the **differences in the profiles and therefore the potential of VOCs analysis to discriminate bacterial contaminations** (**Figure 30**).

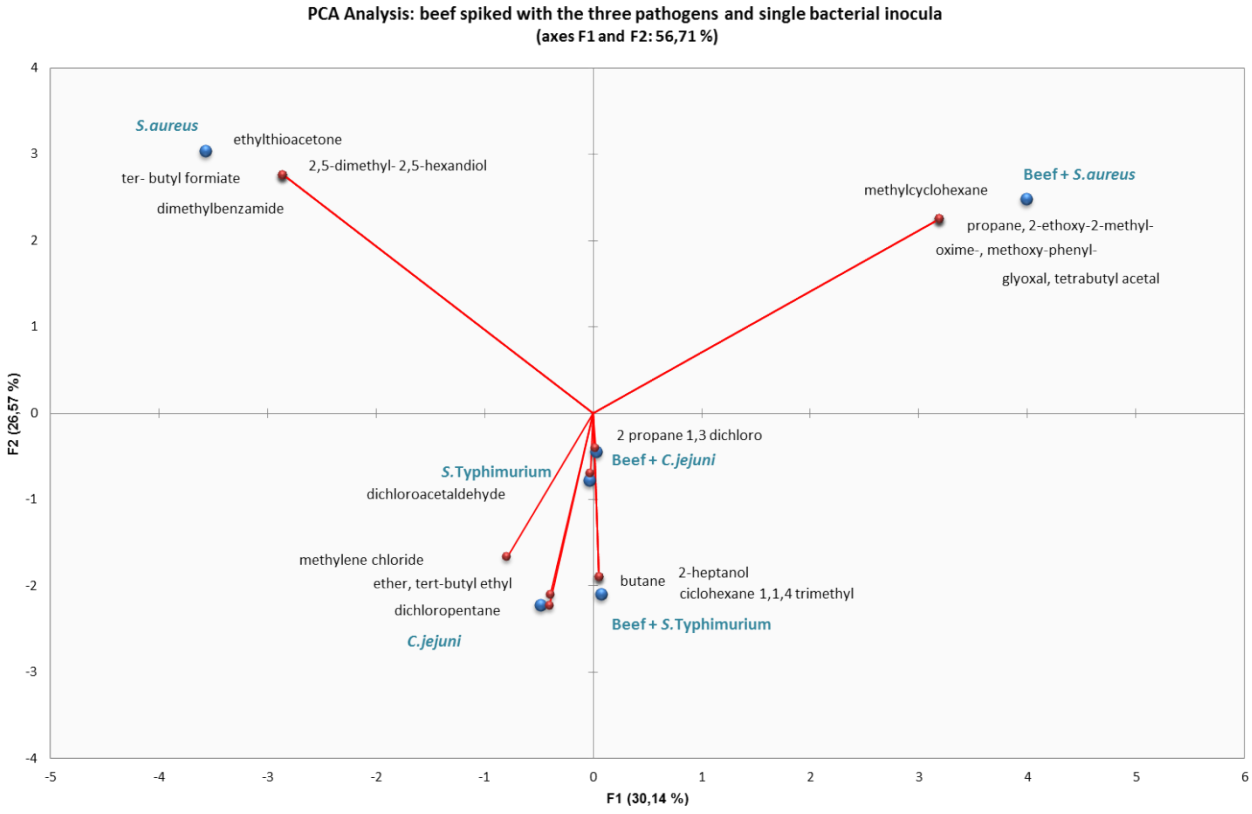


Figure 30 Principal component analysis of beef spiked with the 3 pathogens compared to the single bacterial inocula. Association analysis describe more evident differences comparing Beef+*S aureus* to *S. aureus* inoculum.

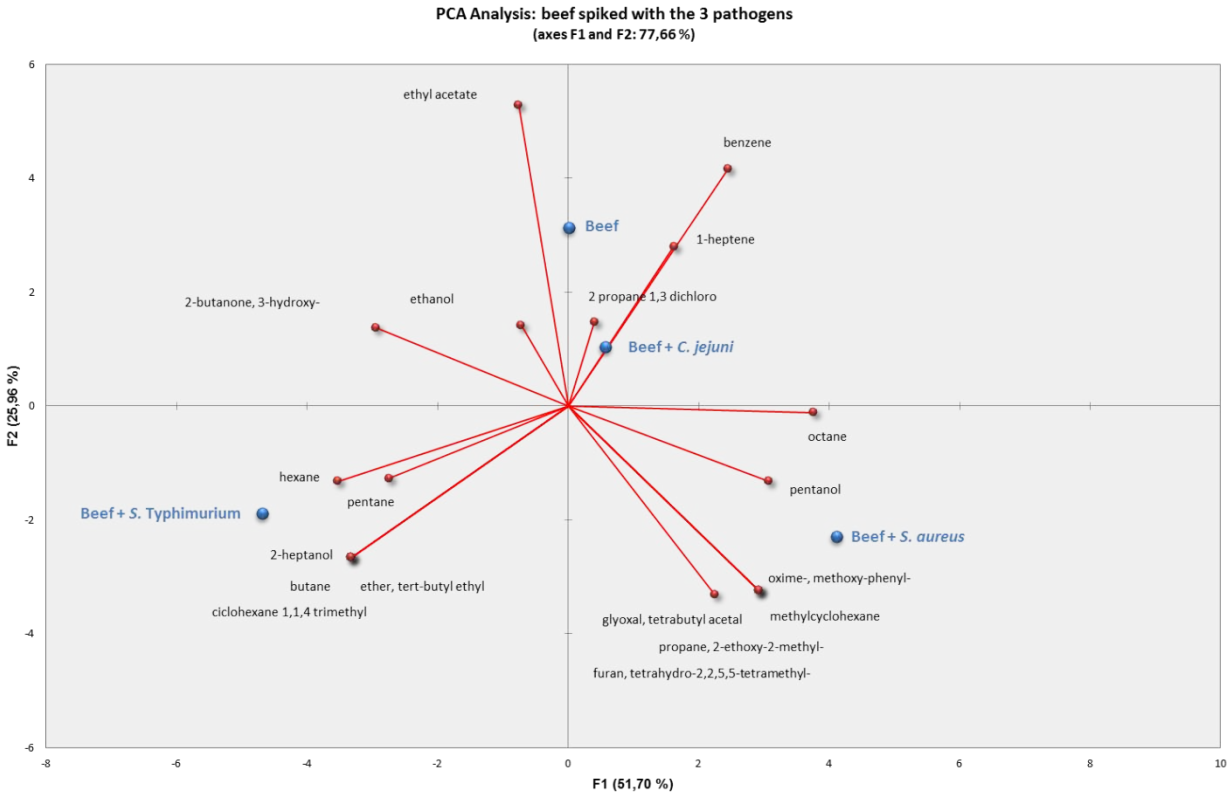


Figure 31 Principal component analysis of beef spiked with the 3 pathogens compared to the not spiked beef sample (negative control).

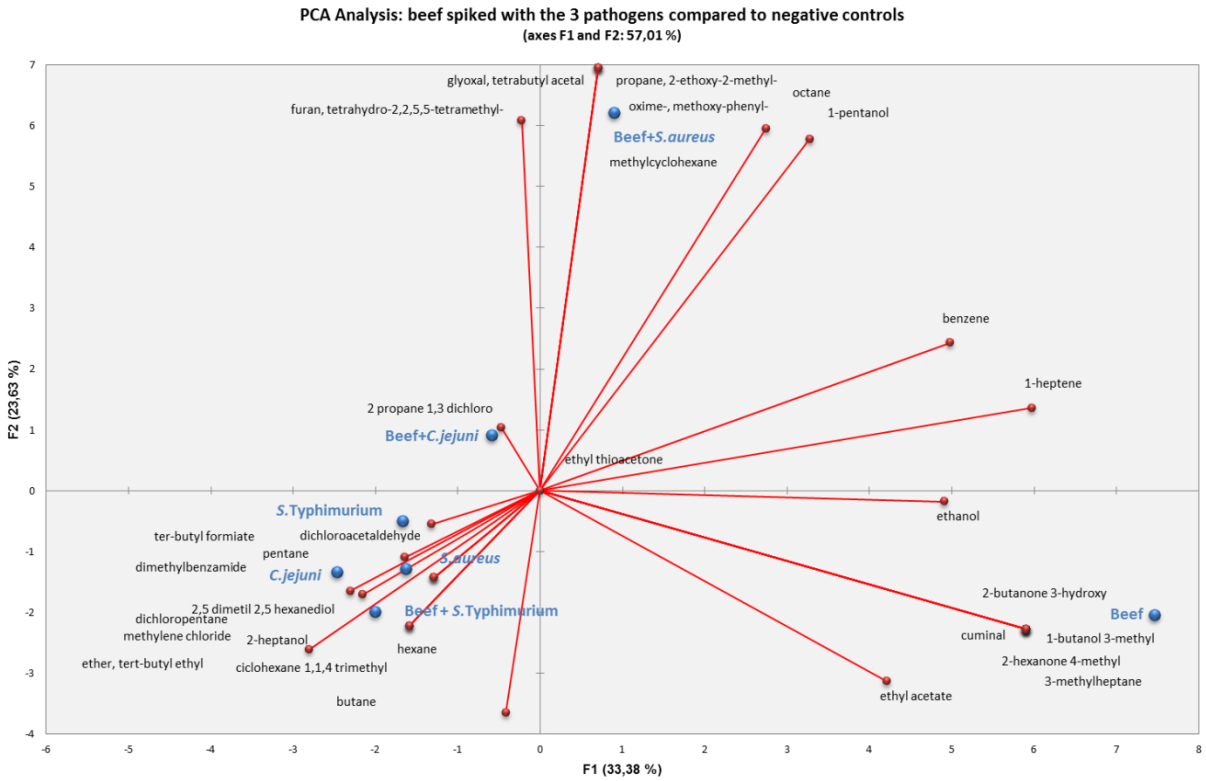


Figure 32 Principal component analysis of beef spiked with the 3 pathogens compared to all the negative controls. The contaminated beef profiles are different from the controls, allowing to identify each specific contamination.

The most interesting comparisons regarded the **association analysis between the negative controls** (meat matrices) and the same **meat spiked with the single bacteria**. Analyzing **beef** compared to the contaminations, the clear distance between the components can be highlighted (**Figure 31**).

Considering the comparison with both not spiked beef and bacterial inocula, it is extremely evident **how different the contaminated profiles are with respect to the negative controls** (**Figure 32**). For this reason, basing on the results, it is possible **to distinguish beef contaminations due to *S.* Typhimurium, *C. jejuni*, and *S. aureus***.

4.11.2. PCA analysis of chicken spiked with single bacteria

PCA of the samples of **chicken spiked with *S.* Typhimurium, *C. jejuni*, and *S. aureus*** highlights the extreme differences between the generated profiles and underlines the **importance of detecting a higher number of marker compounds**, allowing distinguishing a bacterial contamination from the others (**Figure 33**).

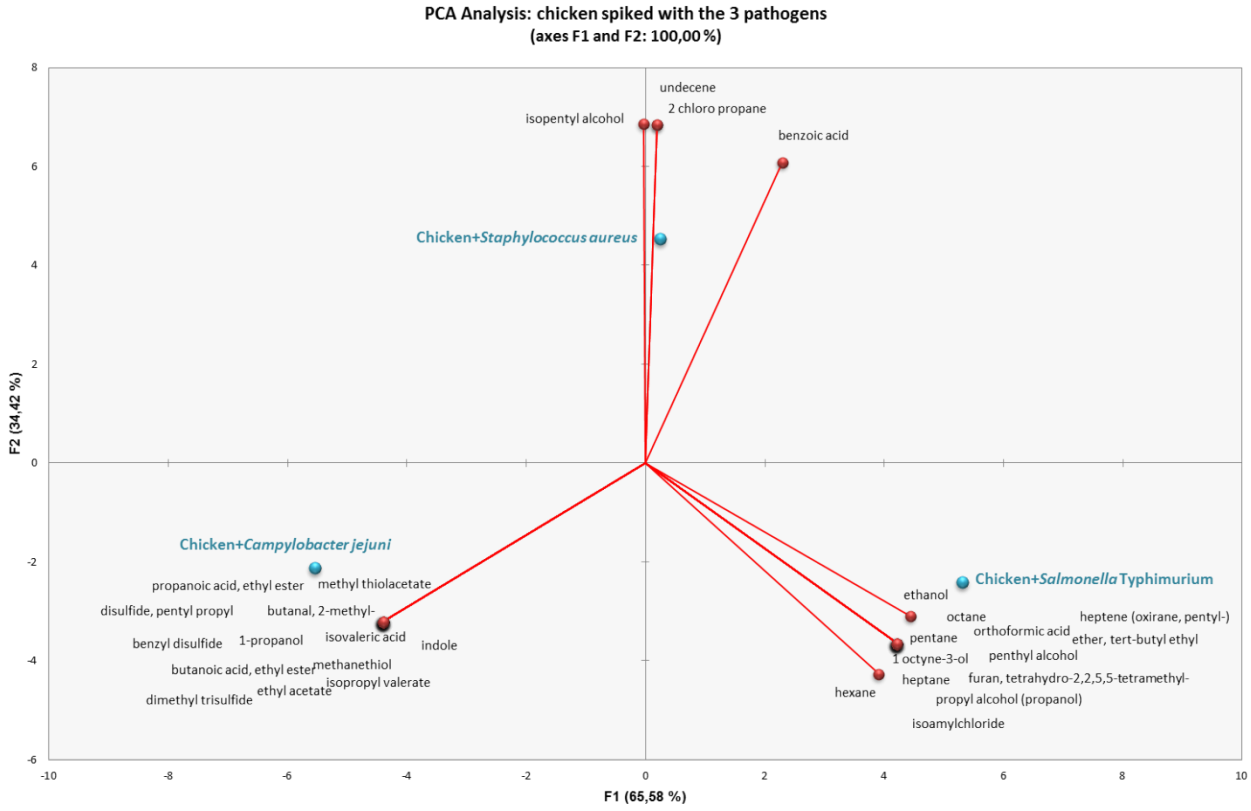


Figure 33 Principal component analysis of chicken spiked with *S.* Typhimurium, *C. jejuni*, and *S aureus*. Profiles are different, allowing to distinguish a contamination from the others.

Evaluating the comparison between the **three contaminations and the bacterial inocula**, the **differences between Chicken + *S.* Typhimurium** and **Chicken + *C. jejuni***, with respect to *S.* Typhimurium and *C. jejuni* inocula, **are evident**, allowing considering that the **metabolism of the two pathogens changes by the exploiting of chicken nutrients**. The **contamination of chicken due to *S. aureus* does not describe substantial differences**, compared to *S. aureus* inoculum: the result is strengthened by the detection, for **Chicken + *S.aureus*** of **only one marker compound** (2 chloro propane) (**Figure 34**).

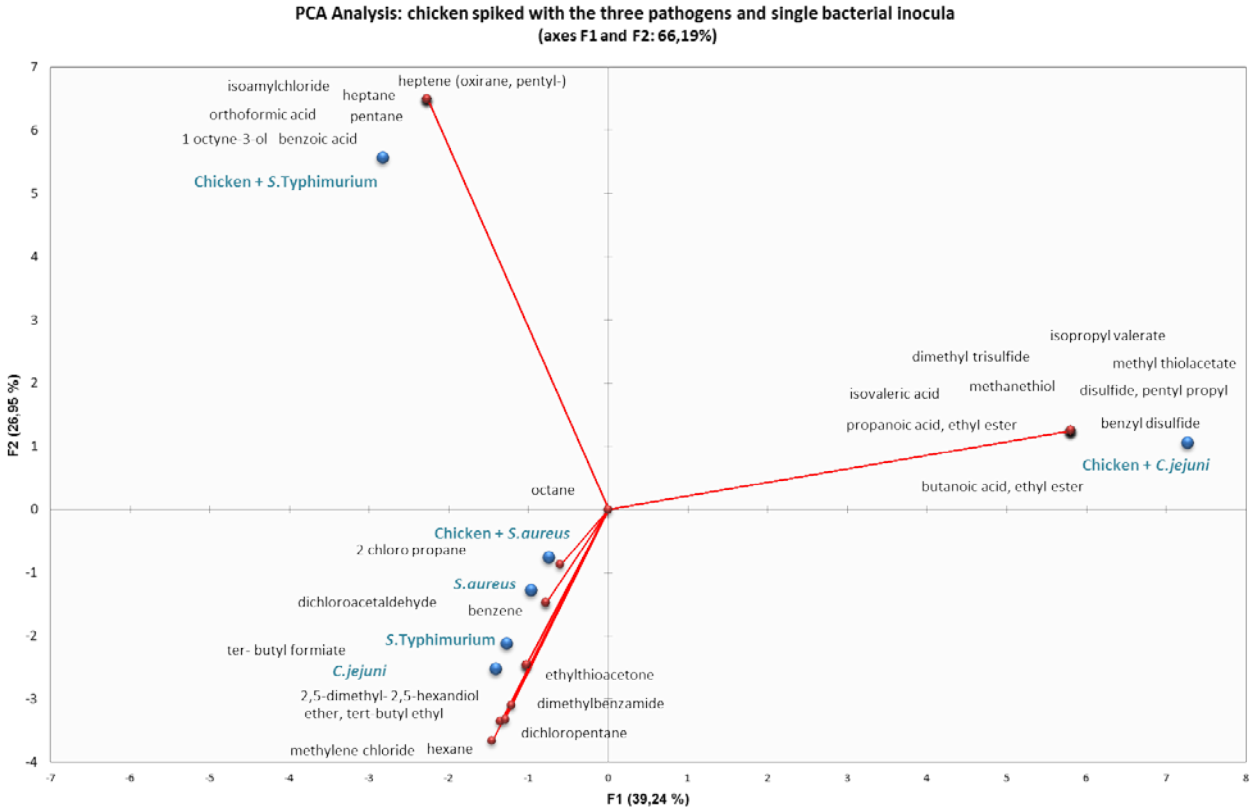


Figure 34 Principal component analysis of chicken spiked with the 3 pathogens compared to the single bacterial inocula. More significant differences are showed by differences in Chicken+*S.*Typhimurium and Chicken+*C. jejuni*.

The results are further confirmed by the association analysis comparing the contaminations with the **negative control chicken sample**: while **Chicken + *S.* Typhimurium** and **Chicken + *C. jejuni*** are largely different from the negative control, ***S. aureus* metabolism do not show significant differences** compared to not contaminated chicken, consenting to **hypothesize the low capability of the microorganism to employ chicken nutrients for its metabolic pathways** (**Figure 35**).

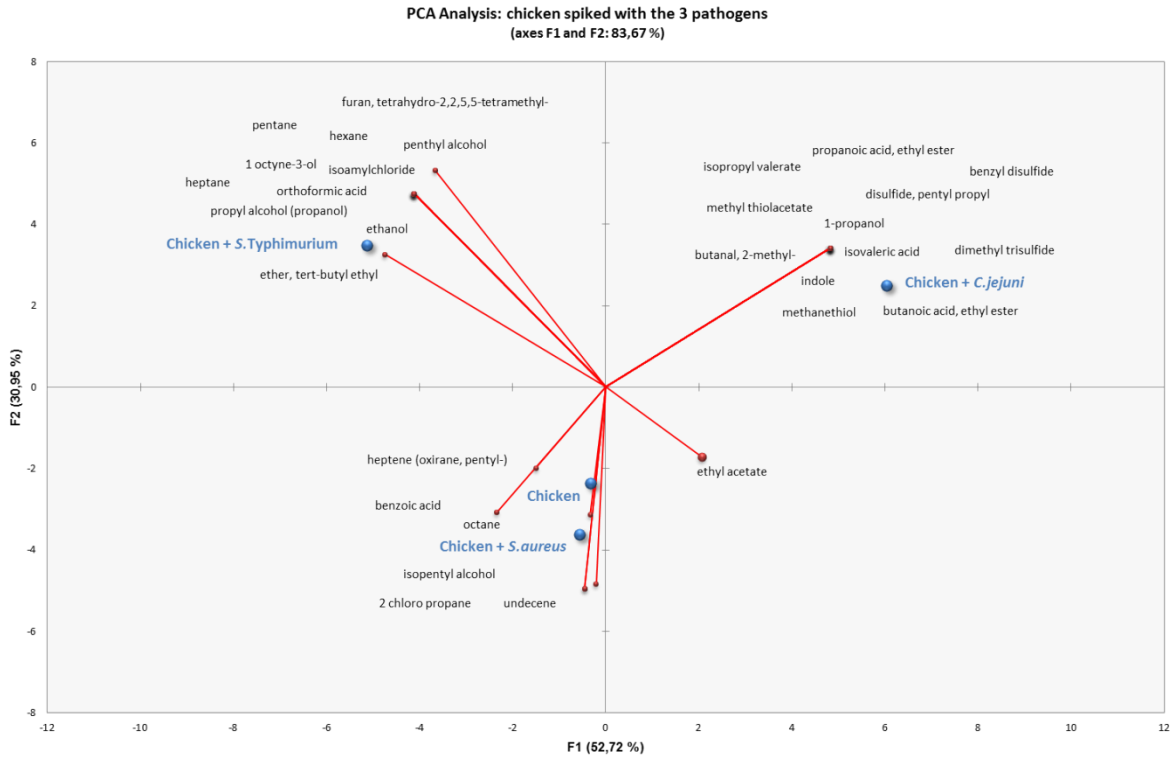


Figure 35 Principal component analysis of chicken spiked with the 3 pathogens compared to the not spiked chicken sample (negative control).

Figure 36 clearly summarizes the substantial differences between the spiked samples and the relative controls: the **discrimination of the three pathogens inoculated on chicken is therefore potentially possible**.

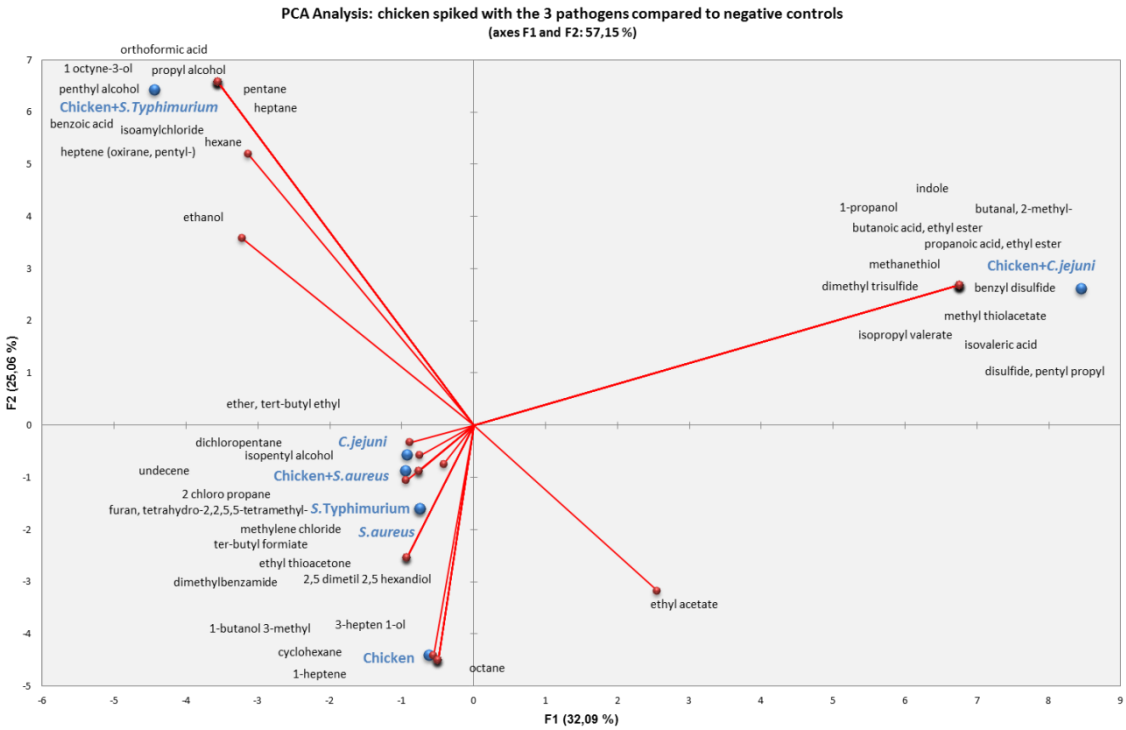


Figure 36 Spiked chicken compared to respective negative controls. The contaminated chicken profiles are different from the controls, although the *S.aureus* contaminated sample shares more similarities to negative control.

4.11.3. PCA analysis of pork spiked with single bacteria

In terms of metabolic products, **pork** resulted an **ideal matrix** for the three bacteria evaluated: the **highest number of volatiles was** indeed **identified**. The resulting PCA show **three different profiles**, evidencing a high number of **common substances and a relatively high amount of typical marker compounds identified** for each of the three contaminations (**Figure 37**).

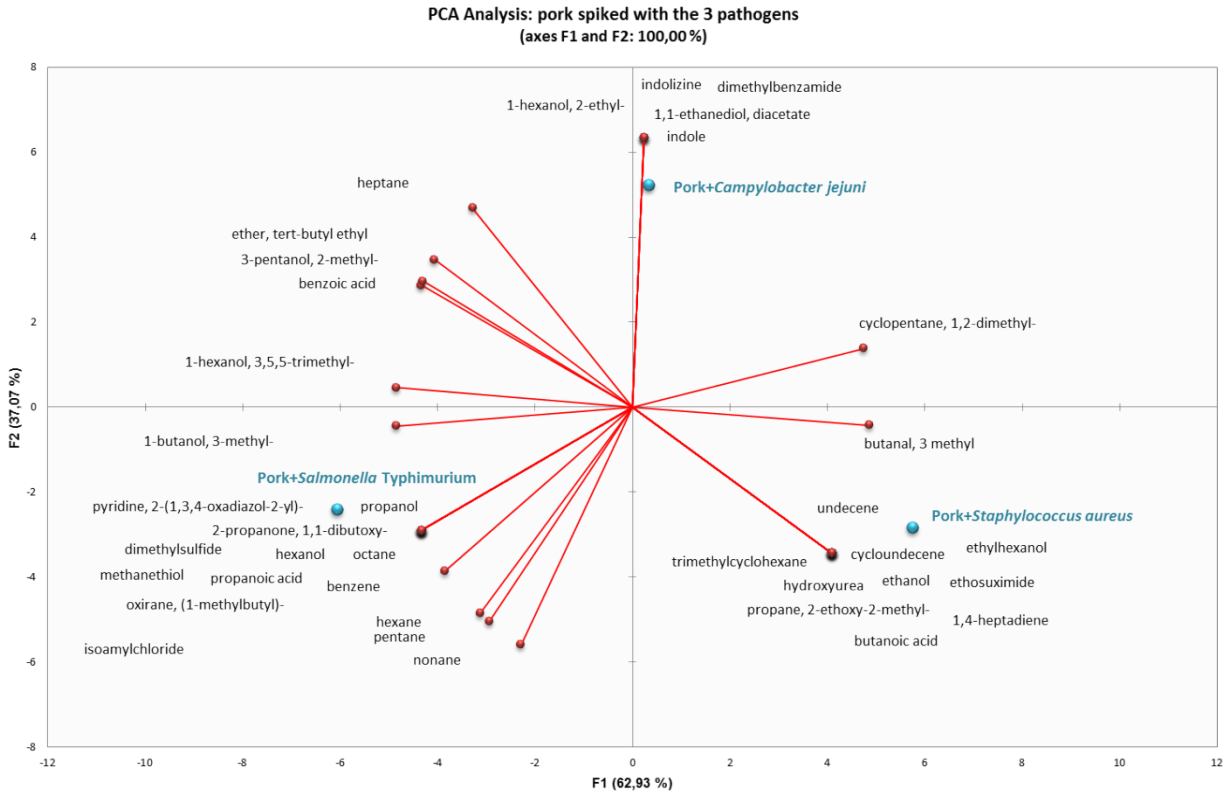


Figure 37 Principal component analysis of pork spiked with *S.*Typhimurium, *C.jejuni*, and *S.aureus*. Several substances are common to the three combinations: the detection of markers supported the discriminations between every different contamination.

As regards as the **comparison with the bacterial inocula**, **Pork + *C. jejuni*** and **Pork+*S. aureus***, with respect to *C. jejuni* and *S. aureus* inocula, are **highly different**. Chicken+*S.* Typhimurium metabolites are instead similar to *S.* Typhimurium profile (**Figure 38**).

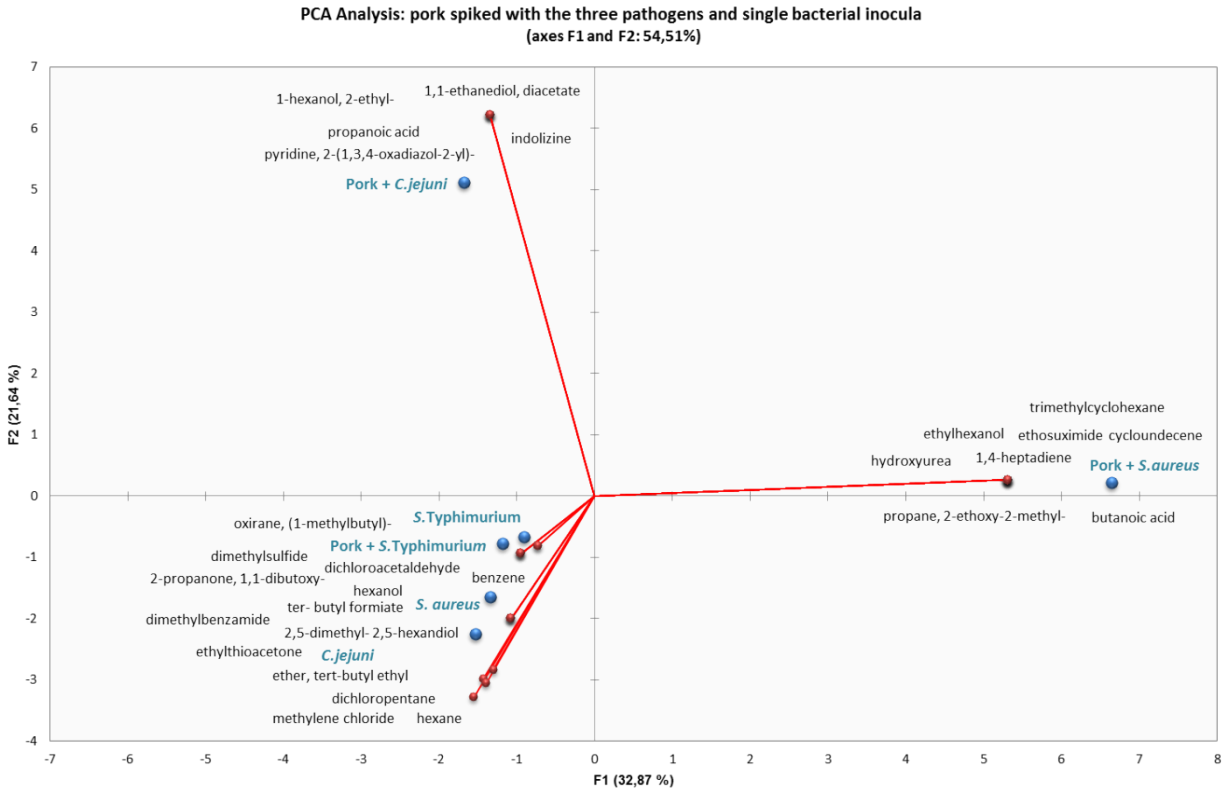


Figure 38 Principal component analysis of pork spiked with the 3 pathogens compared to the single bacterial inocula. Significant differences are showed by Pork+*C. jejuni* and Pork+*S.aureus*.

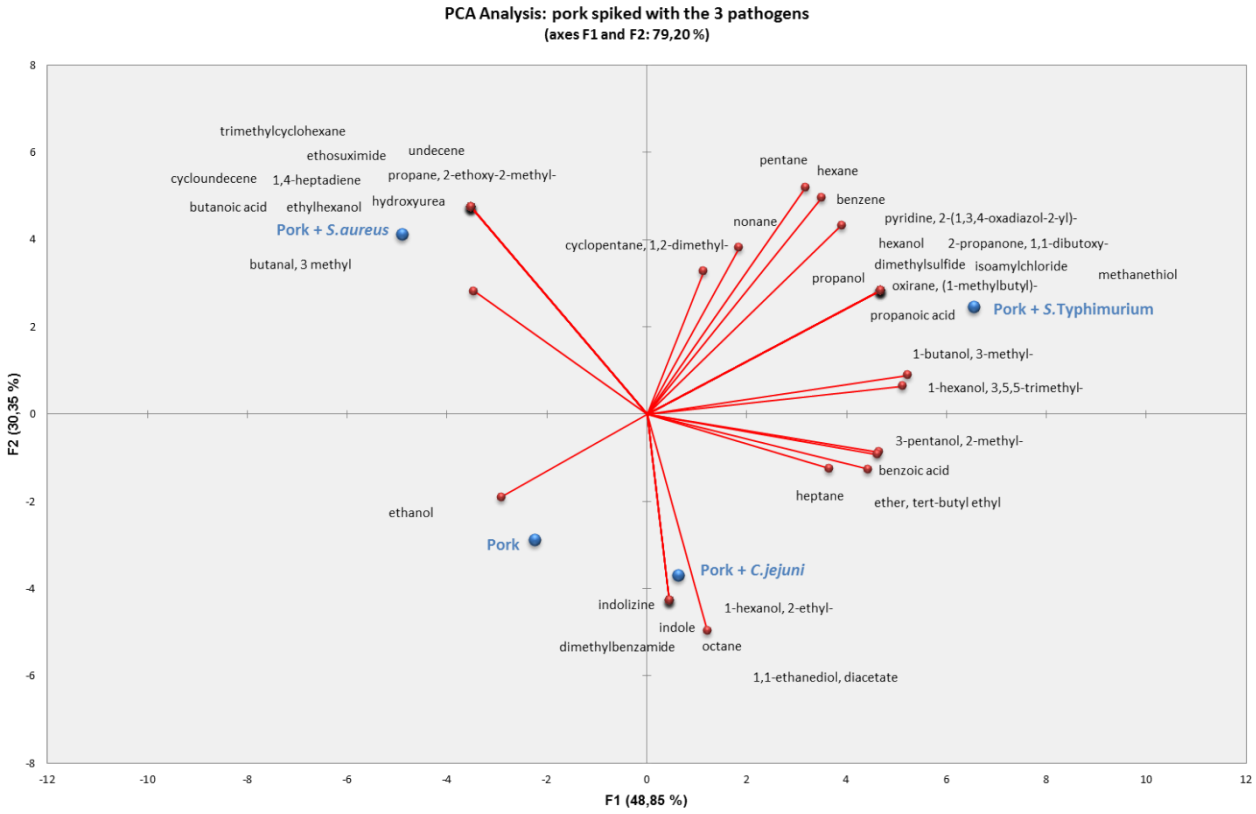


Figure 39 Principal component analysis of pork spiked with the 3 pathogens compared to the not spiked pork sample (negative control).

In contrast with the **not contaminated pork (negative control),** the **three contaminated samples**, basing on their positions on the graph, **underline significant differences**, almost being placed at the four corners of an ideal square (**Figure 39**).

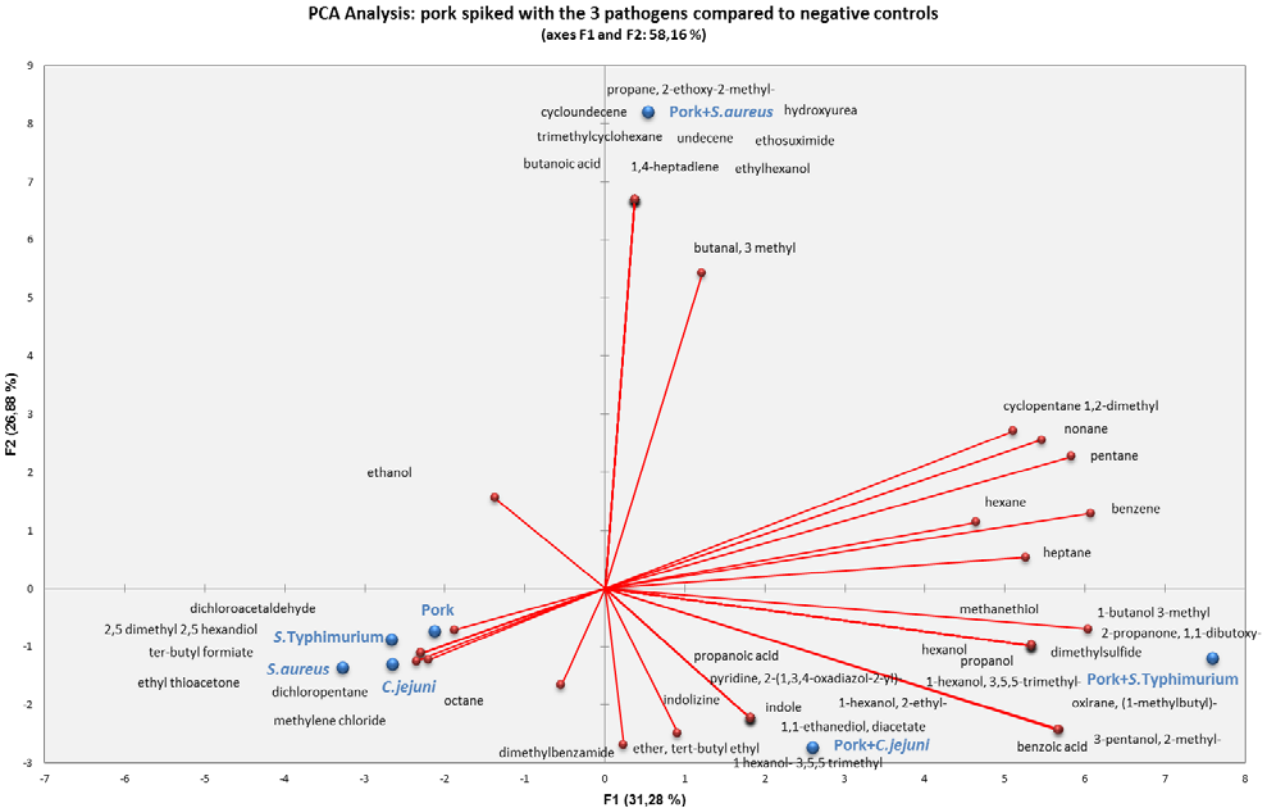


Figure 40 Principal component analysis of pork spiked with the 3 pathogens compared to all the relative negative controls. Contaminated pork profiles are highly different from the control, consenting the potential identification of each specific contamination.

The association analysis considering all the variables with **pork samples spiked with the three bacteria** further confirms **the differences between the three profiles** and the availability of marker volatile compounds useful to the characterization of each contamination (**Figure 40**).

5. CONCLUSIONS

HS-SPME-GC–MS analysis of VOC profiles appears to be a promising analytical approach for the identification of pathogenic bacteria. The identification of marker volatile compounds was crucial to delineating the metabolomics profiles of each microorganism responsible of meat spoilage, considering that **VOCs profiles changed significantly between the contaminated and control meat samples**. Data analysis allowed considering that:

1. The metabolism of **each single pathogen** (*S.* Typhimurium, *C. jejuni*, and *S. aureus*) **on meat** lead to the production of three different VOCs profiles each meat type. In fact, VOCs generated only when a specific pathogen was present: **spectra are unique**, useful to **distinguish the bacteria from others strictly by their volatile profiles**.

2. When **meat is contaminated with mixed bacterial inocula** (of 2 or 3 microorganisms) – although it has been rarely observable in real conditions – the resulting combinations produced **fewer substances**, compared to the single bacterial contaminations. In addition, some compounds are produced by more than one combination and only some combinations produced unique substances. Therefore, it is **not possible to suggest reliable typical profiles and marker compounds pools**.

3. **Changes in microbial loads influence the metabolism** of the pathogens on meat: **VOCs profiles sensibly change proportionally to microbial loads** drops and only some combinations produced unique compounds. In some cases lower loads share common substances to 10^6 and it often happens that concentrations decrease. These outcomes may depend on the **interference of endogenous microbial flora of meat**, whose competition could be stronger, when *S.* Typhimurium, *C. jejuni*, and *S. aureus* loads decrease.

4. It is **potentially possible to discriminate the contaminations of the three descriptors** by profiles and by marker substances.

5. The **detection**, for each contamination, **of VOCs marker compounds results essential**, although **profiles comprising common substances with substantial concentrations changes are useful**.

6. Multivariate analysis of data (i.e. **PCA**) better described the **associations** – in terms of similarities and differences **– between the combinations and the relative negative controls** (**Figures 29-40**).

PCA verified these findings and demonstrated that different VOC profiles could be generated for different bacterial species inoculated in the meat samples.

7. The study of **metabolomics descriptors** is **highly different from the molecular biology approach**: methodologies **employing DNA** are certainly valid and consolidated in terms of results, but rather **more expensive and not easily applicable in the routine analysis**.

The study aimed to the generation of a rapid system to establish, in short times, the presence and the abundance levels of specific pathogens contaminations of meat samples. The future perspective consists on supporting and subsequently completely replacing the traditional cultural methods, allowing faster intervention strategies to prevent public health risks.

The **hypothesis of employing metabolomics markers** detected using chromatographic methodologies and consisting of metabolic products released as a result of a particular pathogen contamination, was **confirmed by the research conducted**. It will be important to support the study with further statistical basis, which could allow the better characterization of the selected descriptors, in order to more clearly distinguish a pathogenic contamination from another.

Furthermore, given the **sensitivity of the analytical instrument** used and the relatively high concentrations of the VOCs emitted, it could be **possible to detect a contaminated food product from in vivo measurements**. This would be a tremendous advance in routine monitoring, since sampling and analysis could take place simultaneously and thereby provide major advantages for food industries, compared to the conventional time-consuming methods currently in use.

The suggested analytical approach was demonstrated to be **peculiar and essential in terms of health risks prevention**: it results innovative not only for the research of useful markers of microbial contaminations, but also to identify **alternative methods to detect microbial contaminations of food**, which could be **faster and simplified**. The reduction of analysis times is the key for the prompt application of preventive strategies aimed to the preservation of consumers health.

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