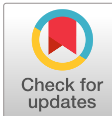
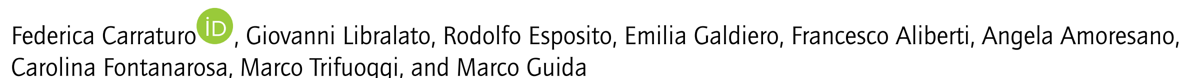


Metabolomic profiling of food matrices: Preliminary identification of potential markers of microbial contamination



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Abstract: The research aimed to generate an early warning system highlighting in real-time bacterial contamination of meat matrices and providing 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 purpose of this research was to evaluate the ability of the headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC/MS) methodologies to detect volatile organic carbons (VOCs), which may be associated to a peculiar microbiological contamination of food. The disposal of fast headspace gas chromatography-mass spectrometry (HS-SPME-GC/MS) able to accurately and rapidly (30 min per sample) detect pathogens in raw meat could replace the traditional and time-consuming (3 to 4 days) standardized microbiological analysis required by regulations. Experiments focused on qualitative and quantitative evaluations of VOCs produced by *Salmonella* Typhimurium, *Campylobacter jejuni*, and *Staphylococcus aureus* in different types of raw meat (beef, pork, chicken). HS-SPME-GC/MS allowed to use smaller sample volumes compared to traditional methods with no sample processing and the potentiality for its application on various food matrices for the detection of a wide variety of pathogens. Data analysis showed the identification of unique VOCs' profiles being possible markers of meat contamination due to their association to specific pathogens. The identification of VOCs markers in association to selected bacterial pathogens and their metabolites could support the rapid determination of specific meat samples contamination. Further research is required to outline-specific metabolic profiles for each microorganism responsible of meat contamination and prevent false positives.

Keywords: fingerprinting, GC/MS, meat, metabolomics, salmonella, VOC

1. INTRODUCTION

Detection of pathogens and their byproducts in food contaminated by harmful 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), *Salmonella* spp., *Campylobacter* spp., and bacterial toxins, including *Staphylococcus aureus* toxins, represent the main causative agents of foodborne outbreaks reported in Europe: 2017 report, over the 5.079 registered cases, indeed described *Salmonella* (27.4%) as the main responsible, followed by bacterial toxins, and *Campylobacter* spp. (European Food Safety Authority, 2018). 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. Current gold standard techniques for food pathogens identification consists of microbiological analysis, providing results in 3 to 4 days. Molecular methods (qPCR) have been considered useful approaches to rapidly identifying microorganisms (Yost & Nattress, 2002), but require complex and long sample preparations steps (e.g., extraction), and results are available within one day. Several studies have tested -omics based protocols to detect food contamination within a few minutes, using real-time meat monitorings (Ercolini et al., 2011; Jääskeläinen, Hultman, Parshintsev, Riekkola, & Björkroth, 2016; Mikš-Krajnik, Yoon, Ukuku, & Yuk, 2016): among these the headspace gas chromatography-mass spectrometry (HS-SPME-GC/MS), based on the analysis of volatile organic compounds (VOCs), produced as metabolites by bacteria exploiting food components. HS-SPME-GC/MS is rapid, costs are similar to microbiological evaluations, but do not require sample preparations, the contemporary analysis of huge amounts of samples can be performed, and the results are available in 20 to 30 min (Mayr et al., 2003). The aim of the present research was indeed to evaluate the ability of the HS-SPME-GC/MS methodologies to detect VOCs, which may be associated with a target microbiological contamination of food. The three microbiological descriptors of the study, based on EFSA data on foodborne pathogens health risks, were *Salmonella* Typhimurium, *Campylobacter jejuni*, and *S. aureus* (European Food Safety Authority 2018). Considering that pathogenic microorganisms' contamination potential is highly related to their proteolytic activity on food, the choice of meat matrices (beef, chicken, pork), additionally holding a low shelf-life, resulted functional to metabolomics analysis. Hence, the suggested scientific approach aims to the rapid determination and association of selected bacterial pathogens and their metabolites on specific meat samples. Using multivariate

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analysis, the study pointed at discriminating the contamination by the three pathogens, analyzing the specific volatilomes on three meat matrices (beef, pork, chicken). The employment of the suggested technology, followed by data association analysis, to generate typical profiles, and identify potential unique biomarkers of contamination, aimed to shorten analytical time required to detect the presence of specific pathogens contaminations of meat samples.

2. MATERIALS AND METHODS

2.1 Study design and sampling

The research was conducted from 2015 to 2017 and was performed on 126 raw meat (beef, pork, and chicken) 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. The samples were kept cold during transport to the laboratory. Samples (kept at 4 °C for 1 to 2 days) were prepared starting from three 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 VOCs. Contemporary microbiological analyses were conducted using standardized procedures.

2.2 Samples preparation

Selected food matrices, when microbiological evaluations proved the absence of specific pathogens, were analyzed by HS-GC/MS. The evaluations workflow consisted on several stages, based on the analysis of profiles produced by: inoculums of ATCC® strains of *S.* Typhimurium, *C. jejuni*, *S. aureus* (ATCC® 14028, 33291, and 26923); mixed inoculums of the three ATCC strains; not spiked and not sterile meat matrices (beef, chicken, pork); meat matrices spiked with a 10^6 UFC/mL concentration of the three microorganisms; meat spiked with the microorganisms combinations; meat spiked with a 10^3 and 10^2 CFU/mL concentration of the three bacteria (Table S1). A replicate of each sample was evaluated with standard cultural methods for *Salmonella* spp., *Campylobacter* spp., and *S. aureus* identification, to confirm the proper contamination procedure and the adequate inoculum concentration. The approach aimed to the identification of typical compounds for each contamination. Applying SPME to mass spectral GC/MS technique, the mass spectra of the headspace volatiles produced by *S.* Typhimurium, *C. jejuni*, and *S. aureus*, and blank samples 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. 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.

2.3 Microbiological analysis

2.3.1 *Salmonella* spp. analysis. According to UNI EN ISO 6579:2017, an aliquot of 25 g meat sample and 225 mL buffered peptone water (BPW – OXOID) were added to a Stomacher bag. Bags were homogenized and incubated at 37 ± 1 °C for 24 hr. Homogenate underwent a subsequent enrichment step: 100 µL from the sample were added to 10 mL Rappavort Vassilliadis broth (RPV – OXOID). Samples were incubated at 41 ± 1 °C for further 24 hr and then surface plated on selected media, XLD, and Salmonella chromogenic agar base (OXOID), incubated at 37 ± 1 °C for 24 hr (International Organization for Standardization 2017). *Salmonella* spp. evaluation is qualitative (presence/absence). 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.

2.3.2 *Campylobacter* spp. analysis. An aliquot of 10 g meat sample and 90 mL Bolton Broth Selective Enrichment Broth (OXOID) were added to a Stomacher bag. Stomacher bag was first homogenized, and then incubated at 37 ± 1 °C for 4 hr and 41 ± 1 °C for 48 hr, under microaerophilic conditions (5% O_2). 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 hr, under microaerophilic conditions (5% O_2) (ISO 10272:2017; International Organization for Standardization 2017). *Campylobacter* spp. evaluation is qualitative (presence/absence): colonies result grey, flat, and swarming.

2.3.3 *S. aureus* analysis. An aliquot of 10 g meat sample and 90 mL BPW (BPW – OXOID) were homogenized into a Stomacher bag and an 100 µL aliquot is spread plated on Baird Parker selective agar (OXOID); agar plates are then incubated at 37 ± 1 °C for 24 to 48 hr. Plates are incubated for 48 hr, and the presence of typical colonies is verified after 24 and 48 hr. *S. 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. (International Organization for Standardization 1999).

2.4 GC/MS analysis

2.4.1 Bacterial strains, medium, growth conditions, and sample preparation. The strains employed in the current research were *S.* Typhimurium ATCC® 14028, *C. jejuni* ATCC® 33291, and *S. aureus* ATCC® 26923. Monocultures of all strains were cultured according to each microorganism's growth protocols for 24 hr at 37 °C with constant shaking at 150 to 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 cells/mL). Mixed cultures of the three pathogens were prepared by individually culturing each microorganism in 30 mL TSB for 23 hr at 37 °C, shaking at 150 to 200 rpm. The monocultures were then mixed in same proportions of *S.* Typhimurium, *C. jejuni*, and *S. aureus*, to obtain a final volume of 30 mL and then incubated for an additional hour at 37 °C and 150 to 200 rpm, followed by GC/MS spectrum acquisition.

In order to perform analysis for the characterization of VOCs, samples were prepared as described in Table S1. All samples were prepared in triplicate and analyzed via HS-GC/MS. A replicate from each sample underwent microbiological analysis, to confirm the presence of pathogens

2.4.2 SPME-GC/MS settings. 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.

The GC 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, 0.25 µm 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. Solid-phase microextractions 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. 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 to 450 mass m/z. For the evaluation of volatile components, the National Institute of Standards and Technology Mass Spectral library and comparison with the electron impact mass spectra and retention times of standards were used. The volatile compounds were analyzed 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 detected. The peak area values described in the Tables and Figures indicate the mean between five different samples each considered evaluation, analyzed in triplicate by SPME-GC/MS.

2.4.3 Statistical analysis. The correlation between key metabolites determined by SPME-GC/MS and single microorganisms or group of microorganisms was determined by employing principal component analysis (PCA) model, part of multivariate analysis. GC/MS data were organized in a matrix (Y), which was then compared with another matrix (X) containing data related to microbial counts. Statistical analysis was carried out employing XLStat Version 2019.

3. RESULTS AND DISCUSSION

The analysis of individual volatile species produced by pathogenic bacteria inoculation in different meat samples was pointed at investigating the possibility to discriminate the microorganisms using HS-SPME-GC/MS. The metabolomics approach to food analysis allowed the identification of typical VOCs in contaminated meat samples. It was also found that, not only some volatiles appeared as a result of their release because of the growth of a peculiar bacterium, but also that the volatile compounds profile changed significantly between the contaminated and control meat samples (Ercolini, Russo, Nasi, Ferranti, & Villani, 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. Qualitatively, the resulting 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 indeed captures more information about the bacterial volatiles than just the smell of the culture (Zhu, Bean, Kuo, & Hill, 2010). 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. In addition, no naturally occurring *Salmonella* spp., *Campylobacter* spp., and/or *Staphylococcus* spp. were present in the control samples. It is necessary to specify that sample preparation stage described in the suggested protocol included the artificial spiking of the meat samples, allowing the colonization of the bacteria to the matrix. The step would not be necessary in real monitoring, considering that even low concentrations of the germs could be detected, either employing either molecular, or analytical chemistry analyses. Therefore, the lower sample pre-processing times, in the system implementation in the development phase, would consist in an enrichment step of maximum 3 to 4 hr, consenting the production of VOCs in the autosampler vials, followed by the analysis via SPME-GC/MS, providing the results in 30 to 40 min. According to the workflow scheme planned, VOCs 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. In order to ensure a better data interpretation, abbreviations have been employed to describe the samples. Table S2 summarizes the list of abbreviations referred to each sample evaluated.

3.1 Profiles generation of meat spiked with each bacterium

The generation of typical profiles to describe the contaminations resulted essential, especially when the number of the produced volatiles is low in specific combinations. Meat samples contaminated with single bacteria (Tables S7–S9) were compared to the relative negative controls (Tables S4–S6) and to the combinations with the other two bacteria contaminations on the same meat type (Tables S10–S12). VOCs profiles changed significantly between the contaminated and control meat samples. Thus, 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, although a meat and 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. For example, 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. Table 1 to 7 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 an asterisk (*) in the "marker compounds" column.

3.2 Meat and bacteria in varying concentrations

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 CFU/mL, 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. Hence, it may be exclusively possible to discriminate the contaminations of the three descriptors by considering both profiles and marker substances.

3.2.1 Beef and bacteria in varying concentrations. The comparison between samples of beef inoculated with different concentrations of *S.* Typhimurium showed that the compounds produced by the three bacterial loads (2-butanone

Table 1–Beef spiked with each bacterium: VOCs profiles generated through the comparison of each beef sample contaminated by the microorganisms with relative negative controls (not contaminated beef and single bacterial inoculum) and respective contaminations.

Combination Beef + *Salmonella* Typhimurium	Marker compounds	Negative controls		Contaminations		
(B+S)	B+S	B	S	B+C	B+C	Notes
2-Butanone, 3-hydroxy-		+	-	-	-	
2-Heptanol	*	-	-	-	-	
Butane	*	-	-	-	-	
Ciclohexane 1,1,4 trimethyl	*	-	-	-	-	
Ethanol		+	-	-	+	
Ether, tert-butyl ethyl		-	+	-	-	
Ethyl acetate		-	-	+	-	
Furan, tetrahydro-2,2,5,5-tetramethyl-		-	-	+	+	
Hexane		+	+	-	-	3* B; 10* S
Pentane		-	-	+	-	

Combination Beef + *Campylobacter jejuni*)	Marker compounds	Negative controls		Contaminations		
(B+C)	B+C	B	C	B+S	B+A	Notes
2 Propane 1,3 dichloro	*	-	-	-	-	
Benzene		+	-	-	+	
Ethyl acetate		-	-	+	-	
Furan, tetrahydro-2,2,5,5-tetramethyl-		-	-	+	+	3* B; 10* S
Octane		+	-	-	+	
Pentane		-	-	+	-	

Combination Beef + *Staphylococcus aureus*	Marker compounds	Negative controls		Contaminations		
(B+A)	B+A	B	A	B+S	B+C	Notes
Benzene		+	-	-	+	
Ethanol		+	-	+	-	
Furan, tetrahydro-2,2,5,5-tetramethyl-		-	-	+	+	3* B; 10* S
Glyoxal, tetrabutyl acetal	*	-	-	-	-	
Methylcyclohexane	*	-	-	-	-	
Octane		+	-	-	+	
Oxime-, methoxy-phenyl-	*	-	-	-	-	
Pentanol		+	-	-	-	
Propane, 2-ethoxy-2-methyl-	*	-	-	-	-	

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. Typhimurium*			

3-hydroxy, ethanol, and hexane) registered decreased values with the reduction of the bacterial load: such decrease does not result in the increase or decrease of substances produced by the 10^6 CFU/mL sample. Metabolism changes and, when some substances from the 10^6 CFU/mL contamination are not detected (e.g., 2-heptanol, pentane, butane), the production of new substances is evident (butanal 3-methyl and methylene chloride), indicating the potential prevalence of the resident microbial flora exploiting meat matrix nutrients. Profiles of beef spiked with the three concentrations of *C. jejuni* are very different and the production of totally different pools of substances in the two combinations with lower bacterial loads describes changes in metabolism. Also, 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 CFU/mL sample to the lower loads. Besides, different substances are produced by the less *S. aureus* contaminated inocula. This outcome evidences the need of more accurate analysis on *S. aureus* metabolic pathways and the competition with other species colonizing the meat (Table S12).

3.2.2 Chicken and bacteria in varying concentrations. *S.* Typhimurium different microbial loads on chicken produced different profiles, comparing the higher load, to the 10^3 and 10^2 CFU/mL samples: the profile changes when *S.* Typhimurium concentration is less concentrated. In contrast, the 10^3

Table 2–Chicken spiked with each bacterium: VOCs profiles generated through the comparison of chicken contamination due to *Salmonella* Typhimurium with relative negative controls (not contaminated beef and single bacterial inoculum) and respective contaminations.

Combination Chicken + *Salmonella* Typhimurium	Marker compounds	Negative controls		Contaminations		
(Ch+S)	**Ch+S**	**Ch**	**S**	**Ch+C**	**Ch+A**	**Notes**
1 Octyne-3-ol	*	-	-	-	-	
Benzoic acid	*	-	-	-	-	
Ethanol		+	-	+	+	
Ether, tert-butyl ethyl		-	+	-	-	
Heptane	*	-	-	-	-	
Heptene (oxirane, pentyl-)	*	-	-	-	-	
Hexane		-	+	+	-	6* S
Isoamylchloride	*	-	-	-	-	
Octane		+	-	-	-	1/3* C
Orthoformic acid	*	-	-	-	-	
Pentane	*	-	-	-	-	
Penthyl alcohol		-	-	-	-	
Propyl alcohol (propanol)		-	-	+	-	
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*			

Table 3–Chicken spiked with each bacterium: VOCs profiles generated through the comparison of chicken contamination due to *Campylobacter jejuni* with relative negative controls (not contaminated beef and single bacterial inoculum) and respective contaminations.

Combination Chicken + *Campylobacter jejuni*	Marker compounds	Negative controls		Contaminations		
(Ch+C)	**Ch +C**	**Ch**	**C**	**Ch+S**	**Ch+A**	**Notes**
1-Propanol		-	-	-	-	
Benzyl disulfide	*	-	-	-	-	
Butanal, 2-methyl-	*	-	-	-	-	
Butanoic acid, ethyl ester	*	-	-	-	-	
Dimethyl trisulfide	*	-	-	-	-	
Disulfide, pentyl propyl	*	-	-	-	-	
Ethanol		+	-	+	+	1/3* C
Ethyl acetate		-	-	-	-	
Hexane		-	+	+	-	1/5* Ch
Indole		-	-	-	-	
Isopentyl alcohol		-	-	-	+	
Isopropyl valerate	*	-	-	-	-	
Isovaleric acid	*	-	-	-	-	
Methanethiol		-	-	-	-	
Methyl thiolacetate	*	-	-	-	-	
Propanoic acid, ethyl ester	*	-	-	-	-	
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*	
1/3* C	3 times less concentrated than *C. jejuni*	**1/5* Ch**	5 times less concentrated than chicken			

and 10^2 CFU/mL samples produced three highly concentrated compounds (1-butanol 3-methyl, 2–3 dithiobutane, and dimethyl trisulfide) and two less concentrated substances (cuminal and ethyl isocaproate), whose abundances decrease proportionally to the pathogen load. *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 (e.g., benzyl disulfide, butanoic acid ethyl ester, and dimethyl trisulfide). 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, whereas substances detected with high concentrations in the 10^6 CFU/mL sample, were not produced by the less concentrated combinations: the results may describe modifications in the metabolic pathways of *S.*

Table 4–Chicken spiked with each bacterium: VOCs profiles generated through the comparison of chicken contamination due to *Staphylococcus aureus* with relative negative controls (not contaminated beef and single bacterial inoculum) and respective contaminations.

Combination Chicken + *Staphylococcus aureus*	Marker compounds	Negative controls		Contaminations		
(Ch+A)	Ch +A	Ch	A	Ch+S	Ch+C	Notes
2 chloro propane	*	-	-	-	-	
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*	

Table 5–Pork spiked with each bacterium: VOCs profiles generated through the comparison of pork contamination due to *Salmonella* Typhimurium with corresponding negative controls (not contaminated beef and single bacterial inoculum) and respective contaminations.

Combination Pork + *Salmonella* Typhimurium	Marker compounds	Negative controls		Contaminations		
(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-	*	-	-	-	-	
3-Pentanol, 2-methyl-		-	-	+	-	
Benzene		-	-	+	+	
Benzoic acid		-	-	+	-	
Cyclopentane, 1,2-dimethyl-		-	-	+	+	
Dimethylsulfide	*	-	-	-	-	
Ether, tert-butyl ethyl		-	+	+	-	8* S
Heptane		+	-	+	+	
Hexane		-	+	-	+	13* S
Hexanol	*	-	-	-	-	
Methanethiol		-	-	-	-	
Nonane		-	-	+	+	
Oxirane, (1-methylbutyl)-	*	-	-	-	-	
Pentane		-	-	+	+	
Propanol		-	-	-	-	
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*	
3* P	3 times more concentrated than pork	**8* S**	8 times more concentrated than *S. Typhimurium*	**13* S**	13 times more concentrated than *S. Typhimurium*	

aureus, due to the reduction of its microbial load on chicken (Table S13).

3.2.3 Pork and bacteria in varying concentrations. Analyzing decreasing *S.* Typhimurium loads on pork, the common production of only one substance (1-butanol 3-methyl) is underlined: VOCs profiles are different. Indeed, although the 10^6 CFU/mL sample produces a large variety of compounds (16 volatiles), the metabolism of the pathogen on pork appears sensibly reduced. *C. jejuni* decreasing loads 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. *S. aureus* decreased microbial charges on pork follow *S.* Typhimurium and *C. jejuni* trends: over the 13 compounds detected from 10^6 CFU/mL contaminated samples, the only common substance produced, by the 10^2 sample, but not by the 10^3, is butanal 3-methyl (Table S14). The outcomes do not allow to adequately compare the decreasing concentrations, detecting common compounds.

3.3 Identification of marker compounds of microbial contamination

VOC profiles on meat samples spiked by *S.* Typhimurium*, C. jejuni*, and *S. aureus*, and useful biomarkers of the respective contaminations are not available in literature. 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. 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

Table 6–Pork spiked with each bacterium: VOCs profiles generated through the comparison of pork contamination due to *Campylobacter jejuni* with corresponding negative controls (not contaminated beef and single bacterial inoculum) and respective contaminations.

Combination Pork + *Salmonella* Typhimurium	Marker compounds	Negative controls		Contaminations		
(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-	*	-	-	-	-	
3-Pentanol, 2-methyl-		-	-	+	-	
Benzene		-	-	+	+	
Benzoic acid		-	-	+	-	
Cyclopentane, 1,2-dimethyl-		-	-	+	+	
Dimethylsulfide	*	-	-	-	-	
Ether, tert-butyl ethyl		-	+	+	-	8* S
Heptane		+	-	+	+	
Hexane		-	+	-	+	13* S
Hexanol	*	-	-	-	-	
Methanethiol		-	-	-	-	
Nonane		-	-	+	+	
Oxirane, (1-methylbutyl)-	*	-	-	-	-	
Pentane		-	-	+	+	
Propanol		-	-	-	-	
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*	
3* P	3 times more concentrated than pork	**8* S**	8 times more concentrated than *S.* Typhimurium	**13* S**	13 times more concentrated than *S.* Typhimurium	

Table 7–Pork spiked with each bacterium: VOCs profiles generated through the comparison of pork contamination due to *Staphylococcus aureus* with corresponding negative controls (not contaminated beef and single bacterial inoculum) and respective contaminations.

Combination Pork + *Staphylococcus aureus*	Marker compounds	Negative controls		Contaminations		
(P+A)	P+A	P	A	P+S	P+C	Notes
1,4-Heptadiene	*	-	-	-	-	
1-Butanol, 3-methyl-		+	-	+	+	
Benzene		-	-	+	+	
Butanal, 3-methyl-		-	-	-	+	
Butanoic acid	*	-	-	-	-	
Cyclopentane 1,2-dimethyl		-	-	+	+	
Cycloundecene	*	-	-	-	-	
Ethanol		+	-	-	-	1/3* P
Ethosuximide	*	-	-	-	-	
Ethylhexanol	*	-	-	-	-	
Heptane		+	-	+	+	
Hexane		-	+	+	-	5* A
Hydroxyurea	*	-	-	-	-	
Nonane		-	-	+	+	
Pentane		-	-	+	+	
Propane, 2-ethoxy-2-methyl-	*	-	-	-	-	
Trimethylcyclohexane	*	-	-	-	-	
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*	
1/3* P	3 times less concentrated than pork	**5* A**	5 times more concentrated than *S. aureus*			

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%; Table 8). A higher number of samples would consent a better results evaluation and reliability.

Table 8–Results of GC-MS data interpolation: unique volatile organic compounds (VOCs) detected as markers of each bacterial contamination [S: *Salmonella* Typhimurium; C: *Campylobacter jejuni*; A: *Staphylococcus aureus*].

+ Beef	+ Chicken	+ Pork
S 2-heptanol	Orthoformic acid	Dimethylsulfide
Ciclohexane 1,1,4 trimethyl	Heptane	Hexanol
C 2 propane 1,3 dichloro	S heptene (oxirane, pentyl-)	Oxirane, (1-methylbutyl)-
propane, 2-ethoxy-2-methyl-	Benzoic acid	Propanoic acid
A methylcyclohexane	1 Octyne-3-ol	***C*** pyridine, 2-(1,3,4-oxadiazol-2-yl)-
Oxime-, methoxy-phenyl-	Isoamylchloride	1,1-Ethanediol, diacetate
Glyoxal, tetrabutyl acetal	Isovaleric acid	1-Hexanol, 2-ethyl-
	Methyl thiolacetate	Indolizine
	Butanal 2-methyl	Cycloundecene
	Propanoic acid, ethyl ester	Propane, 2-ethoxy-2-methyl-
	C benzyl disulfide	Butanoic acid
	Butanoic acid, ethyl ester	***A*** 1,4-heptadiene
	Disulfide, pentyl propyl	Ethylhexanol
	Isopropyl valerate	Hydroxyurea
	Dimethyl trisulfide	
	A 2 chloro propane	

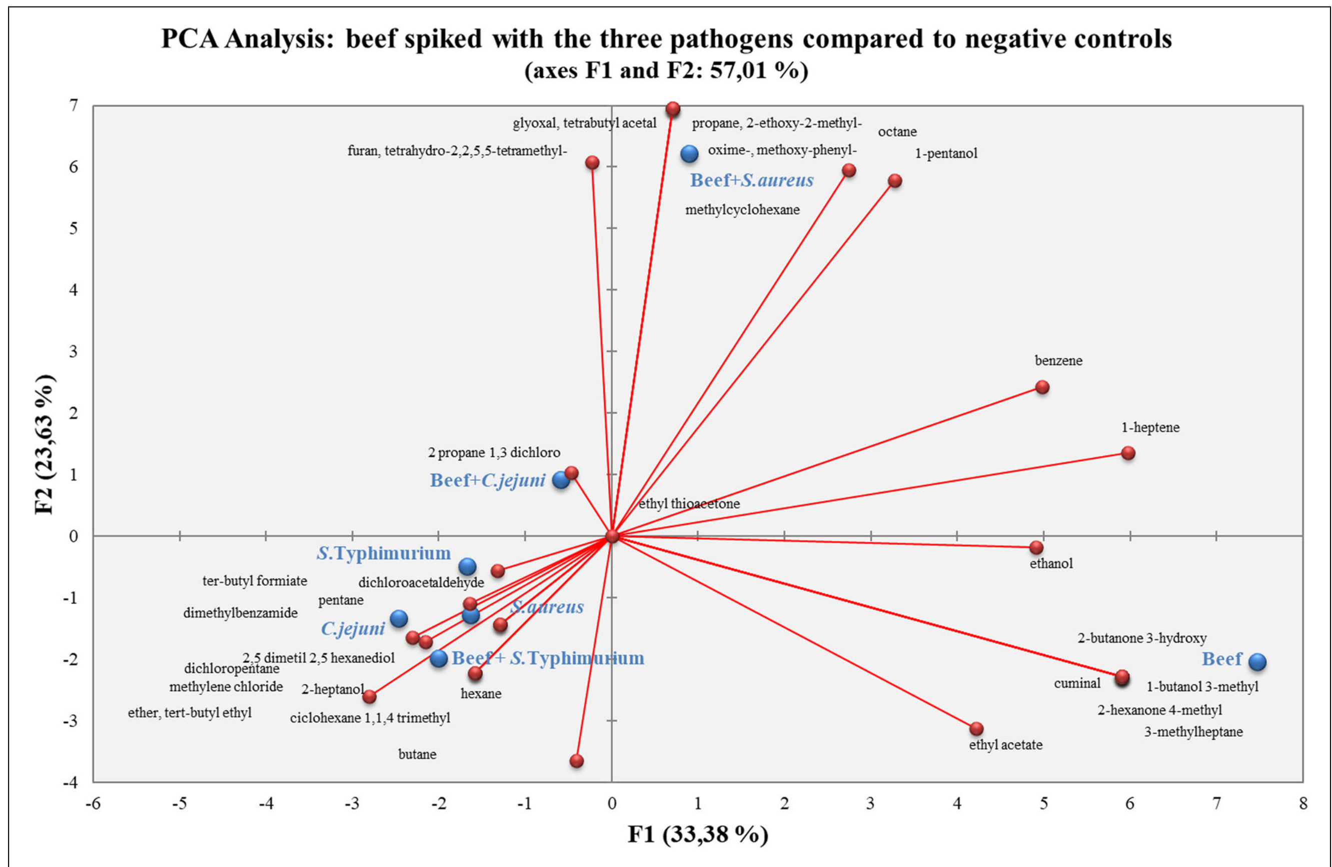

Figure 1–Principal component analysis of beef spiked with the three pathogens compared to all the negative controls. The contaminated beef profiles are different from the controls, allowing to identify each specific contamination.

3.4 Comparison with available studies

The analysis of volatile compounds produced from the metabolisms of specific bacteria on meat samples is not available for many descriptors considered in the present research. 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 or shelf-life (Ercolini et al., 2011; Flores, Olivares, Dryahina, & Spanel, 2013; Gianelli, Salazar, Mojica, & Friz, 2012; Hernandez-Macedo et al., 2012; Jääskeläinen et al., 2016; Mikš-Krajnik et al., 2016; Mikš-Krajnik, Yoon, & Yuk, 2015; Xinfu et al., 2018; You et al., 2018). Furthermore, clinical and environmental bacterial isolates have been submitted for GC/MS analysis in order to define typical bacterial profiles (Arnold & Senter, 1998; Bunge et al., 2008; Chen, Tang, Shi, Tang, & Zhang, 2017; O' Hara & Mayhew, 2009; Sohrabi, Zhang, Zhang, Ahmetagic, & Wei, 2014; Tait, Perry, Stanforth, & Dean, 2014; Zhu et al., 2010). Few studies have been conducted on *Campylobacter* genus volatiles production (Núñez-Carmona, Abbatangelo, & Sberveglieri, 2019; Sohrabi et al., 2014). Furthermore, researches

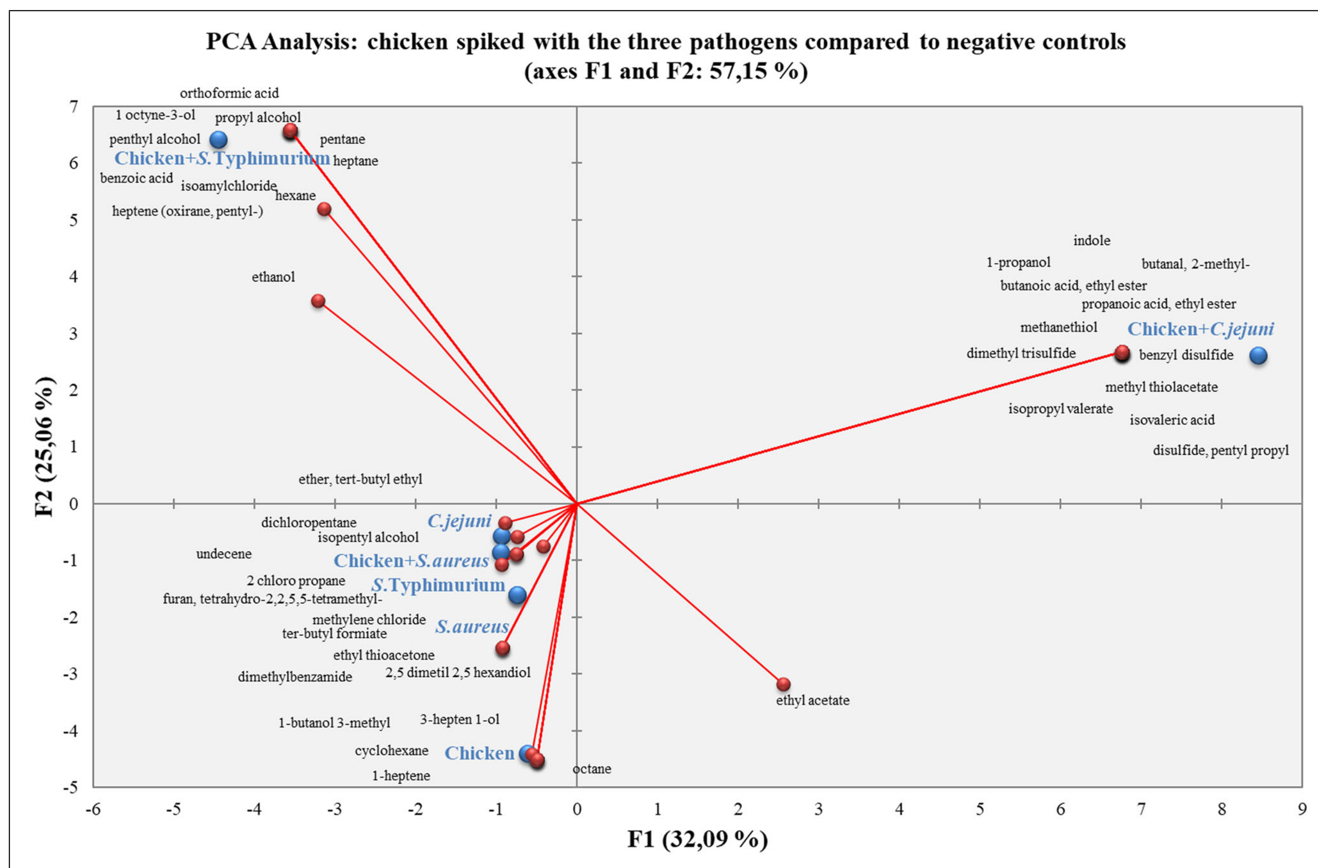

Figure 2–Spiked chicken compared to respective negative controls. The contaminated chicken profiles are different from the controls, although the *S. aureus* contaminated sample shares more similarities to negative control.

focusing on the detection of markers of meat contamination due specific pathogens have been rarely described (Bhattacharjee et al., 2011). Jadhav et al. published a similar study based on foodborne pathogens detection using -omics based approaches (GC/MS and MALDI-ToF MS), spiking minced beef meat with *Listeria monocytogenes*, *Salmonella enterica*, and *Escherichia coli* O157:H7: the research exploited multivariate analysis to identify useful biomarkers for detecting contamination due to the aforementioned pathogens (Jadhav et al., 2018). The development of studies based on the same methodological approach would surely support the implementation of databases to detect reliable markers.

3.5 Principal component analysis

In order to better visualize the differences between the profiles compared to negative control meat samples, PCA was 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. PCA was mainly used to show associations between the three bacterial contaminations on each meat matrix compared to all the relative negative controls (Figures 1 to 3). Compounds vectors directed to the combinations can be considered typical of an exact contamination, whereas vectors of substances resulting far from the combinations, produced by the three combinations cannot be considered significant for the profiles generation. For beef meat, considering the association analysis between the negative controls (not spiked meat and bacterial inocula) and the same meat spiked with the single bacteria, the contaminated profiles are highly different with respect to the negative controls (Figure 1). It is therefore possible to distinguish beef contaminations due to *S.* Typhimurium, *C. jejuni*, and *S. aureus*. Data analysis of the chicken samples spiked with the three pathogens highlights the extreme differences between the generated profiles and underlines the importance of detecting a higher number of marker compounds. The comparison evidences substantial differences between the spiked samples and the relative controls: the discrimination of the three pathogens inoculated on chicken is potentially possible (Figure 2). As for pork, 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 shows 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. The association analysis confirms the differences between the three profiles and the availability of marker volatile compounds to distinguish each contamination (Figure 3).

Based on the outcomes and on the data association analysis, it may be possible to identify a microbial contamination, using both fingerprints and marker compounds: fingerprints (integrating the preliminary database with additional data) would help the comparison between different kind of meat and different conditions; markers would support the identification of the contamination whereas differences in the profiles due to the microbial loads are evidenced, and therefore different fingerprints do not allow an accurate contamination detection. Future perspectives will focus on the upgrade of the database additional data, which will be implemented in a platform, generating an algorithm supporting the hypothesis, obtaining at least the qualitative (not quantitative) identification of specific pathogens.

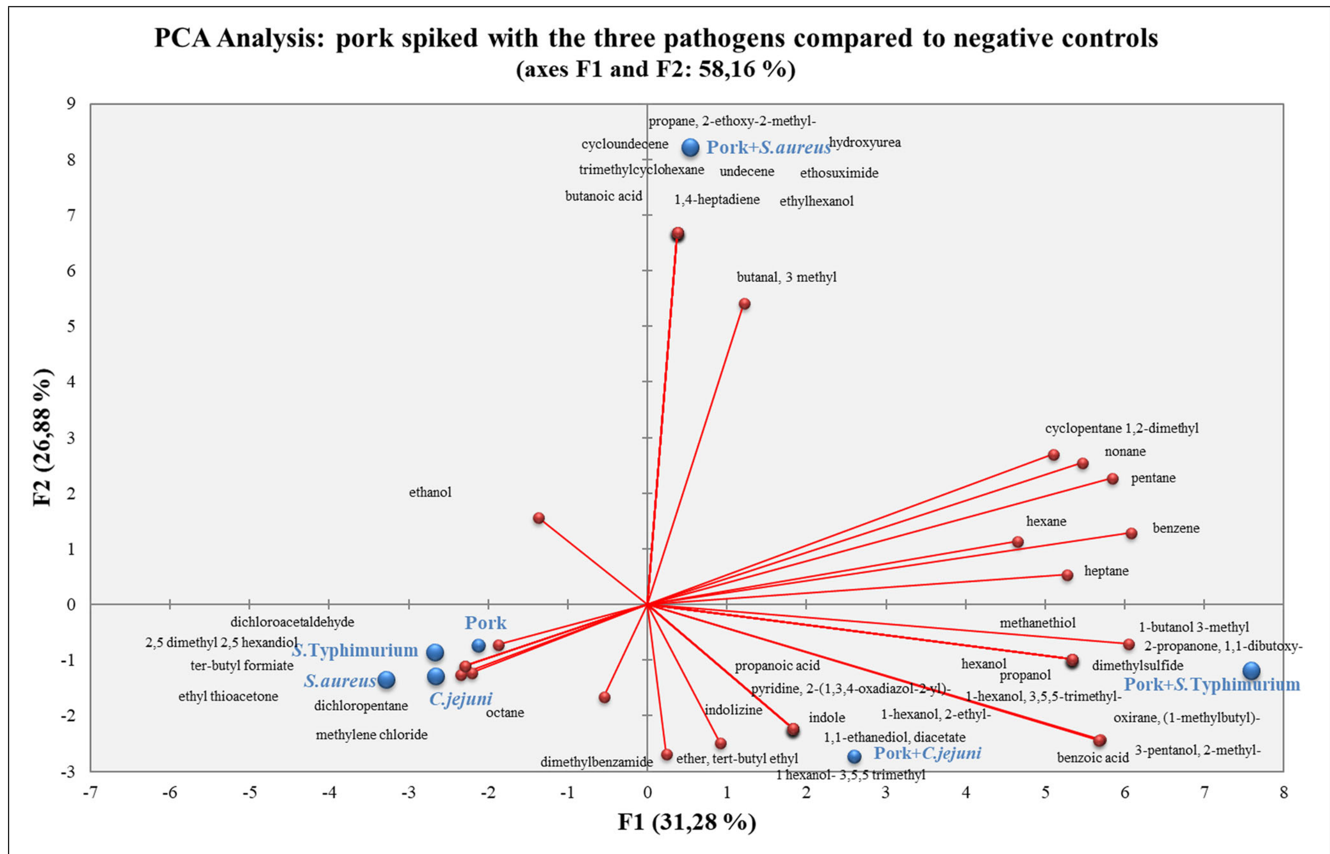

Figure 3–Principal component analysis of pork spiked with the three pathogens compared to all the relative negative controls. Contaminated pork profiles are highly different from the control, consenting the potential identification of each specific contamination.

4. CONCLUSION

HS-SPME-GC/MS has been showed to be useful and promising to the detection of volatile compounds, produced by bacteria during meat spoilage, as markers of contamination due to harmful microorganisms, indeed fastening the identification of pathogens. Data allowed to demonstrate that the metabolism of each single pathogen on meat leads to the production different VOCs profiles for each combination. As regards to the challenging detection of either multiple germs, or pathogens in varying concentrations, the high variability in the available results makes not possible to suggest reliable typical profiles and marker compounds pools. Nevertheless, multivariate analysis of data better described the associations, in terms of similarities and differences, between the combinations and the relative negative controls, bringing out the possibility to generate metabolomic profiles for different bacterial species inoculated in the meat samples. This would be a tremendous advance in routine monitoring for food industries, because sampling and analysis could take place simultaneously. Based on the presented preliminary outcomes, it may be possible to discriminate particularly harmful pathogens like *Salmonella* spp. and *Campylobacter* spp., because any fingerprint or marker proving the presence of such pathogens indicates a risk for the consumers. In conclusion, the detection of foodborne pathogens in meats results embryonic: additional studies and the availability of higher number of data to support the study with further statistical basis are necessary, consenting to build up a reliable database and identify standardized biomarkers to be employed in rapid analysis. Moreover, the methodology will have to be further validated by testing naturally contaminated meat samples. The results provided may therefore represent a starting research targeted to the development of early detection on-field tools, saving times and costs, enhancing product assurance and foodborne outbreaks prevention, thus ensuring a better food safety management. The intent is to implement a methodology aimed to alarming producers in time along the production chains, avoiding that contaminated products reach the consumers. The objective may be reached realizing rapid test kits, featured with volatiles sensors (sensors arrays), detecting specific compounds, to identify target pathogens in food matrices.

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AUTHOR CONTRIBUTIONS

Federica Carraturo designed the study, with Marco Guida and Francesco Aliberti. Federica Carraturo organized microbiological experiments. Sample preparations and microbiological analysis were conducted by Rodolfo Esposito. Specimens for chemical evaluations were analyzed by Carolina Fontanarosa, which collected chemical data, subsequently reviewed and elaborated by Angela Amoresano and Marco Trifuoggi. Results interpretation and interpolation were made with the support of Giovanni Libralato. Federica Carraturo drafted the document, which was reviewed by Marco Guida, Giovanni Libralato, and Angela Amoresano.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

TABLE S1.Determination of VOCs produced by the sample prepared during the study.

TABLE S2.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.

TABLE S3.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].

TABLE S4.Results of GC/MS analysis: VOCs produced by bacterial inocula resulting from mixes of two or three pathogens (*S.* Typhimurium, *C. jejuni*, and *S. aureus*) [RT-1 (m), retention time; PA (%), peak area normalized].

TABLE S5.Volatile organic compounds produced by the meat matrices (beef, chicken, and pork) [RT-1 (m), retention time; PA (%), peak area normalized].

TABLE S6.Volatile organic compounds produced by beef spiked with *S.* Typhimurium, *C. jejuni*, and *S. aureus* (106 CFU/mL) [RT-1 (m), retention time; PA (%), peak area normalized].

TABLE S7.Volatile organic compounds produced by chicken spiked with *S.* Typhimurium, *C. jejuni*, and *S. aureus* (106 CFU/mL) [RT-1 (m), retention time; PA (%), peak area normalized].

TABLE S8.Volatile organic compounds produced by pork spiked with *S.* Typhimurium, *C. jejuni*, and *S. aureus* (106 CFU/mL). [RT-1 (m): Retention time; PA (%): Peak Area normalized].

TABLE S9.VOCs produced by beef spiked with mixed inocula of two or three bacteria (*S.* Typhimurium, *C. jejuni*, and *S. aureus* 106 CFU/mL) [RT-1 (m), retention time; PA (%), peak area normalized].

TABLE S10.VOCs produced by chicken spiked with mixed inocula of two or three bacteria (*S.* Typhimurium, *C. jejuni*, and *S. aureus* 106 CFU/mL) [RT-1 (m), retention time; PA (%), peak area normalized].

TABLE S11.VOCs produced by pork spiked with mixed inocula of two or three bacteria (*S.* Typhimurium, *C. jejuni*, and *S. aureus* 106 CFU/mL) [RT-1 (m), retention time; PA (%), peak area normalized].

TABLE S12.VOCs produced by beef spiked with decreasing microbial loads of the three microorganisms (103 and 102 CFU/mL) [RT-1 (m), retention time; PA (%), peak area normalized].

TABLE S13.VOCs produced by chicken spiked with decreasing loads of the three microorganisms (103 and 102 CFU/mL).

TABLE S14.VOCs produced by beef spiked with decreasing microbial loads of the three microorganisms (103 and 102 CFU/mL) [RT-1 (m), retention time; PA (%), peak area normalized].

Supplementary Results

Food Microbiology & Safety