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NEW TOOLS FOR AUTHENTICATION AND TRACEABILITY TO ASSURE THE INTEGRITY OF PIG CHAIN

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Ph.D. THESIS

New tools for authentication and traceability to assure the integrity of pig chain

by

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To my beloved Mother

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It is our choices that show what

we truly are, far more than our abilities.

J.K. Rowling, Harry Potter and the Chamber of Secrets

Preface

Several voluntary label claims are declared on the label of meat and meat-based products assuring high-quality food with peculiar properties - *Raised without antibiotics, No antibiotics ever, Antibiotic-Free* - as a consequence of the green transition that meat production systems have undergone. This trend may hide novel threats to the integrity of food systems since new kinds of fraudulent practices may emerge. In this regard, approaching the issue with the classic tools may be not successful in the modern meat market since new assurances may require innovative approaches. The omics sciences represent an emerging field spread on distinct levels - Genomic, Transcriptomic, Proteomic, Metabolomic, and Lipidomic - widely explored in different scientific fields such as food authenticity, and precision medicine. Among the omics hierarchy, the metabolome represents the most sensitive to internal factors like the health *status* of the organism and the genetic background, and to external factors like environmental inputs, nutrition, and pollutants. All these factors can affect the phenotypic outcome of an organism which can be investigated on metabolite levels through metabolomics.

In this Ph.D. thesis, the definition of animal welfare was considered as a starting point to gain a comprehensive overview of the internal and external factors affecting animals' phenotypic outcomes. Finally, the theoretical framework allowed to set up the experimental framework by employing the untargeted metabolomic approach to compare two different phenotypes of pigs, antibiotic-free and antibiotic-treated, respectively.

Therefore, the current Ph.D. thesis was divided into five parts, as follows.

In the first chapter, Introduction, a brief contextualization of the case of the antibiotic-free claim as a vulnerable factor for the integrity of the meat chain was introduced considering the scientific and legislative background and the future projections.

In the second chapter, Aims of the Ph.D. thesis, both the overall scope of the present thesis and the aim of each study were described.

The third chapter was organized into two main sections dealing with the feasibility of metabolomics as a new tool to explore animal welfare and the case of antibiotic-free label claims. In the first section, Metabolomic Insights into Animal Welfare, both the Animal Welfare definition and the application of omics approaches in the field of animal sciences were reviewed. All domains - nutrition (I), environment (II), health (III), behaviour (IV), and mental state (V)- were discussed considering the omics application within each domain. Additionally, the workflow of metabolomics was described to discuss the feasibility of this approach as new a tool for investigating new objects in the field of animal welfare. In the second section, Metabolomic insights into Antibiotic-Free label claims, three case studies exploring both an NMR- and HRMS-based untargeted metabolomics for the comparison between antibiotic-free *versus* antibiotic-treated pigs were illustrated.

In the fourth chapter, Discussion, a general discussion was performed to highlight critical points both on experimental and theoretical levels.

The fifth chapter, Conclusion, overall conclusion, and outlooks were outlined.

Chapter 1

Introduction

Projections regarding population growth, climate change, and new consumer needs introduced multiple challenges in food systems, especially in livestock meat production (Parlasca & Qaim, 2022). Livestock systems are a source of great debate when dealing with environmental footprint and ethical concerns as regards the protection of animal welfare (Dumont et al., 2019). The Sustainable Development Goals (SDGs), a global strategic plan that the FAO endorsed, required more efforts to ensure the sustainable transition of the meat production systems (FAO, FAO and Sustainable Development Goals).

In response to these urgent needs, the meat production systems implemented significant enhancement actions within the meat chain, from the raising of livestock to the distribution of the meat products. Many events can be stressful for animals, and these represent a threat to be monitored when dealing with animal welfare. For example, the weaning phase is widely recognized for negatively affecting the welfare of the calves, sheep, and pigs, also the choice of feed, and the type of housing systems (Orihuela, 2021). All these aspects highlighted that animal welfare is a multidimensional reality, where the interaction between the producer and the animal with a One Health approach covers a key role (FAO, One Health).

The introduction of good practices of management in livestock improved the safety and security of the meat and meat-based products together with the protection of animal welfare. In this context, the consumers highly promoted the efforts toward the protection of animal welfare, given the cheerful outlook to consider animal welfare as a relevant criterion for meat purchasing choice (Denver et al., 2017; Miranda-de la Lama et al., 2017).

Given these changes, the meat production systems faced new virtuous objectives to satisfy the meat market needs. The introduction of new labels claim (e.g., *Raised without Antibiotics* or *Antibiotic free*) defining high value-added meat and meat-based products, after the adoption of voluntary certification schemes (Main et al., 2014) positively affect brand and the market positioning parameters. In this framework, the voluntary nature of certifications suggest that all producers can adopt different certification schemes according to the level of assurance that they want to guarantee.

Among all animal-based topics to which consumers may be sensitive, the usage of antibiotics in livestock and the antimicrobial resistance represent a source of concern (Meerza et al., 2022).

As a general remark, the spread of new voluntary label claims for all those untouchable and unobservable attributes (Kaczorowska et al., 2021) resulted in a niche meat market with premium prices highly vulnerable to food fraud (Manning et al., 2021). Therefore, the spread of new voluntary label claims may affect the integrity of meat production systems with the introduction of new grey areas in which meat frauds can take place. As a result, several concerns emerged related to the harmonization of requirements for the voluntary certification schemes and the need for new tools for fraud investigation. Up to 2013, the number of certification schemes for label claims was 901 within the European Scenario, with an internal repartition between certifications and self-declarations, covering Meat, and Fruit and Vegetables, as predominant food commodities (European Commission, Voluntary Food Labelling Schemes Study). The magnitude of the number of voluntary certification schemes covering various food products led to suppose that different certification schemes exist under the same umbrella topic (i.e., Animal Welfare). This aspect suggested that the lack of harmonization of requirements behind the scheme can populate the field of voluntary standards without increasing the level of assurance related to food products. In other terms, the availability of different label claims covering the same aspect can be considered a confounding factor in the purchasing choice of consumers given that they can find the same type of food product, such as meat, labelled with different types of label claims referring to the same aspect of production and/or processing (i.e. Antibiotic Free).

Additionally, the plethora of voluntary and tailor-made schemes intended for food labelling requires sensitives approaches to detect the truthfulness of the label claim. For the current manuscript, all those label claims related to the assurance of “Antibiotic Free” will be considered. The current state-of-art highlighted that the combo antibiotic-free and meat is investigated for the detection of antibiotic residues with screening and confirmatory methods (Ahmed et al., 2017). Broadly speaking, the association between meat and

antibiotics has a long scientific literature tradition mainly dealing with immunological and hyphenated chromatographic methods (Ahmed et al., 2020; Barros et al., 2023) for antibiotic residues detection.

In the field of Antibiotic-Free claims for authentication purposes, these methodologies may not be effective in clarifying the truthfulness of label claim since the hypothesis to be tested is not aimed at identifying and quantifying the antibiotic residues, but it is aimed at revealing if antibiotic treatment occurred during the rearing periods of the animal. Overall, producers must respect the withdrawal period for the administration of veterinary medicine considering both antibiotic-free and conventional meat chains to avoid levels of residues above the maximum residue limit in food (EMA, Withdrawal Period; Regulation (EU) n. 37/2010).

Hence, the legal administration of antibiotics allows the producer to comply with the mandatory legislation; however, compliance with mandatory legislation does not mean that the Antibiotic-Free condition is also satisfied, as a general rule. This assumption led to the consideration of metabolomics as a new tool for the authentication of Antibiotic-Free label claims in meat chain.

Metabolomics is the study of low molecular weight molecules (< 1000-2000 Da) of tissues and biofluids in living organisms (Pedrosa et al., 2021) defining the phenotypic outcome (Zhang et al., 2021). In this regard, the authentication of the Antibiotic-Free claim may be performed considering that with an untargeted metabolomic approach, no priori hypotheses drive the experimental design (i.e., research of targeted veterinary medicine), resulting in a comprehensive exploration of metabolic changes due to a defined condition such as putative exposure to antibiotics administration during the rearing period of the animal. Currently, the two main analytical platforms employed are Nuclear Magnetic Resonance (NMR) and High-Resolution Mass Spectrometry (HRMS) which allows to perform the detection of metabolites with different sensitivities in the recovery (Zhang et al., 2021). Therefore, testing Antibiotic-Free meat with an untargeted metabolomics approach may allow the identification of novel biomarkers able to identify in a univocal way that animals never experienced the antibiotic administration during the entire lifecycle or

prove the opposite hypothesis. For the current manuscript, both NMR- and HRMS-based metabolomics were exploited to highlight if metabolomics can successfully prove differences in pigs reared according to the Antibiotic-Free chain requirements from antibiotic-treated pigs. In this sense, the literature search displayed positive outcomes for discriminating both treated from untreated animals without prior knowledge of the type of veterinary treatment in a fully untargeted approach (Liesenfeld et al.,2020) and detecting changes in metabolome with a more informative experimental design (Kieken et al., 2009).

Although in this section a clear distinction was made between all methods adopted to detect antibiotic residues in meat from methods for the authentication of the antibiotic-free label claims, the current state-of-art highlights the overlapping of two topics. The great advantages of high-resolution mass analysers for performing a risk-based workflow in the field of veterinary drugs for compliance with legislation were reviewed with the conclusion that this strategy may become the workhorse of residue analysis in a future perspective (Jongedijk et al., 2023).

Hence, all these premises are prominent for a future in which it may be possible that the mandatory and voluntary fields will cross to simultaneously assure food safety and the integrity of supply chain.

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Chapter 2

Aims of Ph.D. Thesis

The overall aim of the current Ph.D. thesis was focused on the research of new effective analytical tools that in a univocal way can authenticate meat and meat-based products to improve the traceability of the pig supply chain. In other terms, this project aimed to investigate the feasibility of the untargeted metabolomics approach to detect potential fraud along the meat supply chain. The biological question first formulated was related to understanding the potential cause-effects relationship between the administration of antibiotics and the metabolome in pigs.

In premise, for all the investigations described in the following section, an untargeted metabolomics approach was carried out with a bottom-up strategy. This approach can be described as hypothesis-generating and, therefore, we started from the formulation of wide biological questions and assumptions. Also, the metabolomic workflow adopted was designed to collect as much biological information as possible to conduct a retrospective analysis with the help of metadata. According to the overall objective, different case studies were conceptualized and conducted to face the challenge from different points of view, as displayed in **Figure 1**.

Therefore, in the *Case Study 1* - ^{1}H NMR metabolomics on pigs' liver exposed to antibiotics administration: an explorative study- the metabolome of liver of pigs was explored to capture and compare the metabolomic fingerprinting. The study was designed without *a priori* information about the potential role that antibiotics could exert on metabolome. However, supposed that the metabolomics approach represents a molecular snapshot of tissue and biofluids, the liver was investigated to explain different biochemical phenotypes according to the antibiotic administration. The choice of the liver, as the first organ to be investigated, was mainly due to the biological role that this organ covers as the chemical factory of all organisms. Also, Nuclear Magnetic Resonance (NMR) was used to carry out this study to evaluate the results and the feasibility of this analytical platform within the metabolomics approach.

Once the role of the liver was clarified in discriminating samples of pigs treated with antibiotics from samples never treated with antibiotics, in the subsequent experimental application a second concern emerged related to the

biological role of other types of samples in discriminating antibiotic-free from antibiotic-treated animals.

Therefore, in *Case Study 2*- NMR-based untargeted metabolomic to explore the role of kidney and muscle for Antibiotic Free pig meat authentication- the metabolome of the kidney and muscle of pigs was investigated by an untargeted NMR metabolomics approach. This study was formulated to confirm the effectiveness of the ^{1}H NMR-based metabolomics strategy coupled with the multivariate statistics to discriminate Antibiotic-Free pigs versus antibiotic-treated pigs. Also, it was possible to understand which metabolites, according to the chemical properties, returned better results in terms of discriminating animals according to the antibiotics exposure.

Considering the optimistic results obtained from all samples investigated, we decided to change the analytical platform for varied reasons. Firstly, the lipidome of all investigated samples (liver, kidney, and muscle) returned discrete results; this output was discussed taking into consideration the sensitivity of the analytical platform. For this reason, the same biological question was evaluated in the same type of samples with a different analytical platform, using high-resolution mass spectrometry.

Therefore, in *Case Study 3*- HRMS-based untargeted metabolomic coupled to chemometrics to discriminate Antibiotic Free meat from Conventional meat: a study on the liver, kidney an muscle of pig- the investigation of metabolome and lipidome on liver, kidney, and muscle of pigs aimed at comprehensively highlight the impact of antibiotic treatment (Presence *vs* Absence) on metabolite levels. Therefore, UHPLC-HRMS coupled with multivariate statistics approaches was conducted to reach this aim.

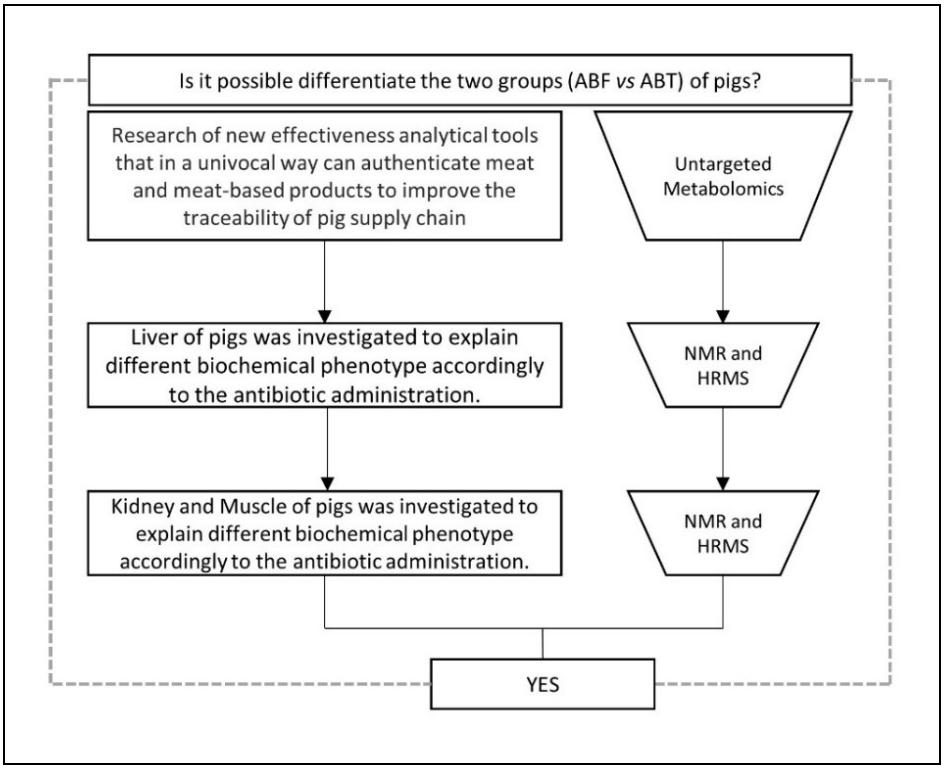

Figure 1. Graphical representation of the work breakdown structure according to the aims.

Chapter 3

Theoretical and Experimental Framework

Summary

In this chapter, the theoretical and experimental framework of the feasibility of metabolomic approach as new tool for the integrity of meat chain will be presented. This chapter is organized into two sections titled “Metabolomic insights into Animal Welfare” and “Metabolomic insights into Antibiotic Free label claims”, according to the following structure:

Section I – Metabolomic insights into Animal Welfare

Literature Review: Filling gaps in animal welfare assessment through metabolomics.

Section II - Metabolomic insights into Antibiotic Free label claims

Case Study 1: ^{1}H NMR metabolomics on pigs’ liver exposed to antibiotics administration: an explorative study.

Case Study 2: NMR-based untargeted metabolomic to explore the role of kidney and muscle for Antibiotic Free pig meat authentication.

Case Study 3: UHPLC-HRMS untargeted metabolomic and lipidomic coupled to chemometric techniques to discriminate Antibiotic Free from Antibiotic treated pig: a study on liver, kidney, and muscle of pigs.

Section I

Metabolomic insights into Animal Welfare

Review

Filling gaps in animal welfare assessment through metabolomics

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Keywords: Five Domains model, biomarkers, metabolomics workflow, NMR-based metabolomics, HRMS-based metabolomics, animal-based measures

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Abbreviations

AA, amino acids; **AW**, animal welfare; **DoE**, design of experiments; **DON**, deoxynivalenol; **EU**, European Union; **FA**, Fatty acid, **GC-MS**, gas chromatography-mass spectrometry; **HRMS**, high resolution mass spectrometry; **HCA**, hierarchical cluster analysis; **LC-MS**, liquid chromatography-mass spectrometry; **MVDA**, multivariate data analysis; **NEB**, negative energy balance; **NMR**, nuclear magnetic resonance spectroscopy; **NOESY**, nuclear overhauser enhancement spectroscopy; **OPLS-DA**, orthogonal partial least squares-discriminant analysis; **PLS-DA**, partial least squares-discriminant analysis; **PCA**, principal component analysis; **RH**, relative humidity; ToF, time-of-flight; **VIP**, variable importance in projection; **WOAH**, World Organisation for Animal Health.

3.1.1 Abstract

Sustainability has become a central issue in Italian livestock systems driving food business operators to adopt ambitious standards of production concerning animal husbandry conditions. Meat sector is largely involved in this ecological transition with the introduction of new label claims concerning the defence of animal welfare (AW). These new guarantees referred to AW provision require new tools for the purpose of authenticity and traceability to assure meat supply chain integrity. Over the years, European Union (EU) Regulations, national, and international initiatives proposed provisions and guidelines for assuring AW introducing requirements to be complied with and providing tools based on scoring systems for a proper animal status assessment. However, the comprehensive and objective assessment of the AW status remains challenging. In this regard, phenotypic insights at molecular level may be investigated by metabolomics, one of the most recent high-throughput omics techniques. Recent advances in analytical and bioinformatic technologies have led to the identification of relevant biomarkers involved in complex clinical phenotypes of diverse biological systems suggesting that metabolomics is a key tool for biomarker discovery.

In the present review, the Five Domains model has been employed as a *vademecum* describing AW. Starting from the individual Domains - nutrition (I), environment (II), health (III), behaviour (IV), and mental state (V) - applications and advances of metabolomics related to AW setting aimed at investigating phenotypic outcomes on molecular scale and elucidating the biological routes most perturbed from external solicitations, are reviewed. Strengths and weaknesses of the current state-of-art are highlighted, and new frontiers to be explored for AW assessment throughout the metabolomics approach are argued. Moreover, a detailed description of metabolomics workflow is provided to understand dos and don'ts at experimental level to pursue effective results. Combining the demand for new assessment tools and meat market trends, a new cross-strategy is proposed as the promising combo for the future of AW assessment.

3.1.2 Introduction

In recent years, animal welfare (AW), primarily related to food-producing animals, became a relevant topic of public health due to the overall impact on the condition of the animals, with consequences for disease, productivity, and food safety [1]. The 13th article of the Treaty on the Functioning of the European Union (EU) marked a new beginning in the EU policy concerning AW, recognizing animals as sentient beings [2]. Over the years, the debate on this issue has involved several international organizations that have focused their attention on awareness themes aiming to promote a state of wellbeing affecting human, animals, and environment. In this context, it is worth mentioning 2030 Agenda for Sustainable Development adopted in 2015 by the members of the United Nations, a 15-years plan of action for people, planet, and prosperity expected to guide the actions of the international community. Among its seventeen sustainable development goals, some are related to AW and food consumption, such as goal #3 "Good health and well-being," goal #12 "Responsible consumption and production," and goal #15 "Life on land" [3]. This framework fits into the concept of One Health, the multisectoral and multidisciplinary approach adopted by the tripartite collaboration between the Food and Agriculture Organization of the United Nations, the World Organisation for Animal Health (WOAH), and the World Health Organization to address health threats at the human animal-environment interface facing the world today [4]. In this context, a central role is played by the international standards set by WOAH related to different aspects of AW for disease control purposes [5]. The WOAH science-based standards embrace manifold issues of AW that at European level have been regulated to encourage measures for the protection of food producing animals kept for farming purposes [6], during transport and related operations [7], and at the time of killing [8]. However, despite numerous international initiatives aiming to promote and achieve a welfare status, one of the main challenges remains uniquely identifying a welfare's condition; this limitation is due to the multidisciplinary nature of AW assessment, given that it concerns aspects related to behavioral and cognitive sciences, animal husbandry, veterinary pathology, biochemistry, physiology, and nutrition [9].

In the EU, claims related to animal husbandry conditions included those related to AW, are voluntary indications more and more often indicated on meat labels to meet consumer's demand for a transition toward more ethical and sustainable farming systems. European consumers' initiatives, such as that entitled "End of the Cage Age" [10] evidence the importance of ethical values related to AW issue defining the so-called untouchable quality to be complied with for the food market. Communication about AW is a current requirement for food business operators encouraged by this increased awareness. A study on AW labeling for the European Commission showed that at least half of the European consumer population would like to receive information on the conditions under which farmed animals are kept and treated. Currently, fifty-one labels with AW claims present in the EU Member States and the UK were monitored [11]. In the case of beef, for labels containing indications other than mandatory, each operator or organization shall send a specification for approval to the competent authority of the Member State in which production or sale take place [12]. In the case of other farm animal species, some national and international transversal standards encompass only a few aspects of AW marking the unavailability of harmonized approaches. All the standards, which may be voluntarily adopted by the operators, are usually represented by requirements to be met in order to pursue AW [13]. Compliance with the management-based requirements (e.g., stock density, bedding type, water facilities) is evaluated using outcomes of scoring systems during periodic control visits. Moreover, animal-based measures are often included in the protocols and guidelines proposed to be implemented on farm and at slaughter to assess and improve welfare [14]. Protocols are constantly evolving and improving; recently, the panel on Animal Health and Welfare of the European Food Safety Authority has suggested a set of animal-based measures to use at slaughter for monitoring on-farm welfare of cull sows and rearing pigs [15]. In Italy, some protocols for AW assessment of various animal species—based on scoring grids of general farm management, structures, equipment, major risks, and animal-based measures—have been developed within ClassyFarm, an integrated system aimed at categorizing livestock farms [16]. Moreover, an initiative called Il Sistema di Qualità Nazionale per il Benessere Animale has been recently drawn up throughout a concerted action between the Ministry

of Health and the Ministry of Agriculture Food and Forestry Politics with the aim to achieve production standards beyond the legal limits and not just for AW [17].

The development of tools for an objective assessment of the AW is a topic of great interest to the food sector for the purposes of authentication and traceability of food of animal origin, and to implement efficient control systems by food business operators, competent authorities, and certification bodies. To date, stress blood levels of cortisol, creatine kinase, and lactate are used as common biomarkers of animal stress; a recent proteomic study enabled to demonstrate the relationship between some meat quality defects associated with AW, and some structural-contractile skeletal proteins have been proposed as biomarkers of heat stress during the rearing and pre-slaughter management for beef meat, pork, and poultry [18].

However, as reported by Keeling et al., the "one perfect indicator", reflecting an integrative measure of both negative and positive welfare and placing an individual on a scale from having very poor to very good welfare, is still the holy grail of welfare assessment [19].

Because AW phenotypic understanding may be based upon what it can be observed about both the temperament of the animal and the metabolic changes, new phenotypic insights at molecular level may be investigated by metabolomics, one of the most recent high-throughput omics techniques. Metabolomics is a comprehensive analysis of endogenous and exogenous low molecular-weight (typically <1,500 Da) metabolites in biological systems. Metabolites intended as precursors, intermediates, and products of biochemical processes, provide a molecular snapshot of the complex interplay between genome and environment; for this reason, metabolomics has been defined as the link between genotypes and phenotypes [20, 21].

Over the last decade advances in analytical and bioinformatic technologies have led to the identification of relevant biomarkers involved in complex clinical phenotypes of diverse biological systems suggesting that metabolomics is a key tool for biomarker discovery. In this respect, being welfare status the result of internal cellular activities that may be triggered by external stimuli, the

metabolomics approach may help to gain molecular fingerprints related to AW by revealing metabolites and in which way their levels may change as a result of external conditions [22].

Therefore, the present review is aimed at highlighting the link between AW and metabolomics beyond the state-of-the-art and exploring new frontiers for AW assessment. In particular, the recent applications and advances of metabolomics in the AW context are argued. The methodological aspects of the metabolomic workflow, as well as strengths and weaknesses, are presented. Challenges and outlooks of metabolomics as a new tool for a comprehensive AW assessment are also discussed in the final part of the review.

1.2 Animal welfare from Five Freedoms paradigm to Five Domains model

A milestone in the description of AW dates back to 1965 when the Brambell Report introduced for the first time the Five Freedoms paradigm, later considered also by the British farm animal welfare Council in 1979. That paradigm established five macro-areas of freedom to satisfy aiming to guarantee the animal a life worth living and free animals from discomforts [23]. The Five Freedoms paradigm embodies several animal needs and establishes a view considering welfare just as a condition devoid of negative experiences without promoting positive experiences. This paradigm is based upon avoiding unfavourable conditions able to induce welfare-compromising negative effects. However, a good welfare status stands for reducing negative experiences and encouraging positive experiences along the entire life of the animal including, for example, feeds having attractive smells, access to preferred sites for resting, and protection during transport and at the slaughterhouse [24]. Therefore, despite its acclaim, the Five Freedoms model carried a great limitation concerning a vision just limited on “what to avoid” and not “what to pursue” to guarantee AW; in keeping with this, the logic behind the paradigm was “animal should be free from thirsty” and not “provide the animal with adequate water supply”. Through the years, more and more attention has been paid to the provisions ensuring AW by leading to the development of the Five Domains

model intended not as freedoms but in terms which highlighted welfare positive connotation [25]. Through this latter model the description of AW passed from "animals free from thirst" to "good feedings" by introducing new ways and perspectives of approaching the issue [23, 26]. The Five Domains model is accurately defined by "five Domains" that are, respectively, (I) nutrition, (II) environment, (III) health, (IV) behaviour, and (V) mental state, seen as gear wheels which worked if wedged. The first three Domains mainly referred to physiological needs to be met from a nutritional, environmental, and health point of view. The vision changes with the fourth Domain—behaviour—characterized by a faceted standpoint; in fact, if on the one hand, the behaviour may be the answer to objective conditions, such as breeding type or environmental conditions, on the other hand, it could have a strong subjective connotation. The shift from objective to subjective connotation led to the fifth Domain mental state—highly influenced both by the first four ones [24].

3.1.3 Metabolomics applications within animal welfare: Domain-by Domain

Metabolomics is a young discipline grown in the last years within the omics techniques. Although many applications are already available in various scientific fields, this science has a great potential expected to be increasingly exploited for new scopes of research. Elucidating the structure of metabolites and relative interactions, metabolomics provides a global vision of a living organism strongly related to the phenotypic outcome [27]. The phenotype understanding on molecular scale occurs by monitoring the changes in composition and levels of metabolites providing information on the cellular regulatory processes involved in the latest biological response of systems to genetic and environmental solicitation [28]. Within the hierarchy of -omics approaches focused on the study of the molecular expressions of the biological systems, metabolomics is the most sensitive to variations, hence the best option to provide direct information about the physiological state of a living being. This high responsiveness allows to capture a snapshot of what happens at the metabolite level to discover biological pathways and related specific biomarkers, evidence of a well-defined condition [29, 30]. For the scope of

application, metabolomics in plant systems results widely explored demonstrating to be a valid tool when investigating the cause of biological effects, such as plant-pathogen interactions [20]. In the same way, the effectiveness may suggest that metabolomics can be exploited to decipher the combination of "living organism-external stimuli" aiming to highlight the biological response to different conditions (**Figure 2**).

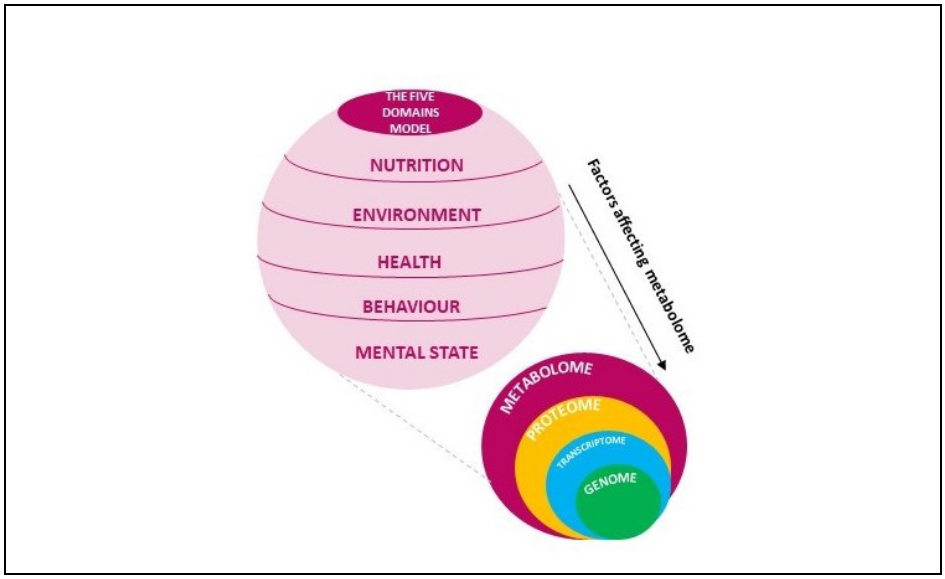

Figure 2. The Five Domains model embodies all factors affecting changes in metabolome that may be explored with -omics sciences.

AW evaluation is based on a metrics considering a scale ranging from poor to good welfare, where the lower extreme is a condition in which the animal is suffering or stressed. With this in mind, AW is often investigated at the lower extreme, to clarify the biological mechanisms elicited when the animal perceives a threat to its homeostasis. In this regard, the time of exposure to the threat (short-term and long-term) is an important issue since living organisms cope with stress in different ways. Generally, the activity of sympathetic-adrenal medullary system, especially in the hypothalamic-pituitary-adrenal cortex system, is the focus for short-term stress studies, while enzyme activity and metabolites measurement for long-term stress [31]. Usually, threats to the

animal fall into one of the areas building the Five Domains model. However, free an animal from stress cannot be reduced to one Domain due to the very high interactivity of biological functions within the organism. Welfare Domains are strongly interrelated and therefore any form of disruptive instability concerning welfare has distinctive detectable using physiologically, pathologically, and clinically measurable outcomes [24]. For instance, in an overcrowd pens, pigs will be most probably affected by heat stress (temperature, environment, Domain II), will struggle to grab food (nutrition, Domain I; agitation, mental state, Domain V), and cumulative stress will cause internal quarrels (behavior, Domain IV). Similarly, a loud noise in pen (auditory discomfort, environment, Domain II) will incite considerable fear in the animal (negative emotion, mental state, Domain V) that will assume an attitude of defense (behavior, Domain IV) since the noise is recognized by the animal as a threat [32]. The last example clarifies the assumption of interactive Domains since an environmental stress (Domain II) induces a particular behavior (Domain IV) stimulated by different feelings (Domain V).

In line with this, some relevant examples from the scientific literature of metabolomics studies aimed at elucidating individually the role of factors belonging to defined Domains of the Five Domains model are listed in **Table 1** and discussed in the following section. Although confined to single Domains, the study of metabolome allows to broaden the perspective, decipher comprehensive metabolic perturbations, leading ultimately to the identification of a specific biomarkers evidence of a general animal's status.

Table 1. Overview of metabolomics approach explored within the Five Domains model.					
Focus	**Purpose of Analysis**	**AS**	**Sample**	**AP**	
Domain: Nutrition (I)					
Feed supplementation	Study on changes in metabolites after dietary supplementation of arginine to basal diet in pigs reared in controlled facilities. Hypothesis to be tested: arginine provision may affect metabolites of AA, glucose and FA.	Pig	Serum	^{1}H NMR	*36*
Feed supplementation	Explore change in metabolic profile caused by the addition of sesamin in vegetable oils-based diets with varying n-6/n-3 FA ratio. Six different diets were compared for a period of 4-month feeding trial.	Salmon	Liver Muscle	^{1}H NMR	*35*
Feed regimens	Characterize metabolome to ascertain pathways differences originating from grass-fed and grain-fed steers to understand possible implications in meat quality for public health and animal welfare.	Beef	Blood Muscle	LC-MS GC-MS	*34*
Feed deprivation	Assessment of metabolomic fingerprinting for malnutrition biomarkers identification in short-term fasted fish.	Sea bream	Serum	LC-MS	*39*
	Obtain a perspective of metabolites regulation during feed deprivation and understand metabolic fluxes characterizing fed and fasted fish, also monitoring growth and energy stores.	Trout	Plasma Liver Muscle	^{1}H NMR	*40*

Domain: Environment (II)					
Heat stress and Relative Humidity	Determine a comprehensive impact of heat stress on lipid and nonlipid metabolites to identify signalling molecules of adaptation to heat stress.	Pig	Adipose tissue	LC-MS	*47*
	Study to identify metabolic pathways associated with the hot-humid or dry climate.	Chick	Faecal	LC-MS	*48*
Heat stress	Investigate the effects of chronic heat stress on serum metabolome in finishing pigs reared in monitored facilities for area, luminance, light and feeding *ad libitum*.	Pig	Serum	GC-MS	*46*
Heat stress	Understanding gender specific heat stress-mediated metabolic changes and investigating metabolites potential biomarkers.	Pig	Saliva	^{1}H NMR	*76*
Preslaughter stress	Understand molecular mechanism in response to stress occurring during preslaughter period.	Chick	Muscle	LC-MS	*109*
Domain: Health (III)					
Tumor	Identify metabolic biomarkers evidence of tumor presence and disease characterization with metabolic networks.	Dab	Liver	^{1}H NMR	*51*
Clinical mastitis	Discover serum metabolites and lipids that precede clinical mastitis during the imminent period in dairy cows.	Cow	Serum	LC-MS	*110*

Periparturient period	Assess individual metabolic patterns at different stages of the periparturient period and following the changes of these patterns during both pregnancy and lactation period.	Cow	Serum	LC-MS	*111*
Vaccine	Identify metabolomic markers of the primary and secondary immune response to administration of an intranasal vaccine.	Calves	Plasma	LC-MS	*112*
Domain: Nutrition (I), Environment (II) Behaviour (IV)					
Feed High temperature space, gender	Evaluate the potential of volatile organic compound and metabolites in animals at 12th day from birth to identify biomarkers evidence of a negative postnatal experience. Animals were deprived of feed and water and moved in bad conditions both in terms of movement and temperature.	Chick	Faecal	LC-MS GC-MS	*113*
Domain: Environment (II), behaviour (IV), mental state (V)					
Toys, socialization	Determine the combined effects of early socialization (opened pens) and neonatal enriched environment (hearty chew dog toys, squid-shaped toys, and natural ropes) during lactation (critical life phase) on the metabolome of piglets fed *ad libitum*.	Pig	Serum	^{1}H NMR	*59*
Domain: Nutrition (I), Health (III)					
Diet disease	Identify compounds and metabolic perturbation involved in periparturient aetiology and unravel the effect of diet on health disease.	Cow	Rumen fluid	^{1}H NMR GC-MS	*69*
Met aboli	Changes in metabolomic status of animal during hyperketonemia.	Cow	Serum	^{1}H NMR	114

	Study on changes of metabolites due to the development of hyperketonemia to understand functional mechanism of ketosis.	Sheep	Serum	^{1}H NMR	115
	Analysis of lipid fraction to highlight metabolic changes during the foaling period (pre- and post-).	Donkey	Plasma	GC-MS	116

For each Domain, focus, purpose of analysis, animal species (AS), type of sample, adopted analytical platform (AP), and reference number (References) have been highlighted.
AA, amino acids; FA, fatty acids; GC-MS, gas-chromatography-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; NMR, nuclear magnetic resonance; RH, relative humidity.

Nutrition (Domain I)

It is widely accepted by scientists that diet influences the animal as a whole entity, affecting nutrient assimilation, disease resistance, and behaviour. The scientific interest in nutritional aspects in relation to AW is particularly relevant for exploring the impact of diet on AW, as well as many other features such as meat quality and sustainability in case of food producing animals. Metabolomics has been used to characterize the effects of both a deficiency or a supplementation of different nutrients in order to compare and explore the metabolic effects on opposite levels, also distinguishing all those variable factors such as age, gender, and lifestyle from diet [53]. Overall, metabolomics approach proved the effects of nutritional intervention, but at the same time offers the advantage of shedding light on metabolic perturbations related to the general context in which the animal is located, allowing to acquire a more holistic view of the animal's status.

An interesting example showing the involvement of different Domains is the observational case-control study carried out by Carrillo et al. [35] focusing on two feeding systems. In particular, these authors applied—omics techniques, metabolomic and transcriptomic, to compare grain-fed and grass-fed beef. A clear difference between the two feed regimens was shown by changes in metabolites levels from blood and muscle tissue, mainly attributable

to glucose metabolism and lipid oxidation. Also, grassfed animals reared in their natural habits showed lower levels of circulating cortisol than the grain-fed group reared in limited spaces and fed ad libitum, source of competition among animals; the authors attributed this finding to the less stressful condition experienced by grass-fed animals, free to move and express their behaviour [35]. In this view, the integrated view of the Five Domains model for AW assessment is supported. Therefore, despite the high energy diet ensured to grain-fed animals, the environmental condition and agonistic behaviour played an important role in defining the overall welfare condition, confirming the need to take into consideration more than one Domain and balance them to correctly estimate the contribute of the experiences played by nutrition (I), environment (II), and behaviour (IV) Domains.

Feed supplementation with bioactive compounds of both water- and land-species animals may have an impact on AW. Metabolomics was employed by Wagner et al. [34] to investigate the metabolic profile of liver and white muscle of Atlantic salmon (*Salmo salar*) fed with vegetable oils-based diets enriched with sesamin, a lipid modulator able to convert short 18-carbon fatty acids (FA) to long chain polyunsaturated FA, to ensure their content in farmed fish tissues. Most perturbed metabolisms for liver were amino acids (AA), carbohydrate, and lipid, while for the muscle lactate, creatine/phosphocreatine, and nucleosides. According to their findings, the up-regulation of a metabolites marker of xenobiotic exposure was observed in the fish fed with high level of sesamin, since fish recognized sesamin as xenobiotic compound; moreover, high lactate levels measured in the treated salmons, were indicative of response to a wide variety of stress. Furthermore, salmon fed with high sesamin levels showed lower final body weight, especially relevant for producers [34]. Turning to land animals, the effect of dietary arginine supplementation on the metabolome of growing pigs was evaluated by He et al. [33] exploiting 1H Nuclear Magnetic Resonance (NMR)-based metabolomics. According to their findings, L-arginine supplementation greatly affected serum concentrations of nitrogenous and lipid signalling molecules and intestinal bacterial metabolites, also suggesting a great potential for the enhancement of protein synthesis in skeletal muscle and the modulation of the gut microbiota [33]. These results prompted the same authors to investigate the dietary intervention on weaning

piglets to explore how arginine may relieve early life stress [54]. Weaning is a piglets stressful condition that deals with *(i)* psychological stress, early separation from mother; *(ii)* environmental stress, moving to new facilities; *(iii)* health stress, gastrointestinal dysfunction and metabolic disorders; *(iv)* nutritional stress, dietary change, and *(v)* piglets sometimes behave more aggressively, as observed in the common increase in fighting in the new pens [31, 54, 55]. He et al. observed that the arginine supplementation was more effective for weaned piglets than the growing ones in terms of growth performance, but less successful to restore the most perturbed ecology of the gut microbiota [54].

Nutritionally regulated biomarkers capable of assessing the AW status have been identified in farmed animals in malnutrition conditions. Nutritional deficiency is a negative experience within the first Domain explored by several authors by metabolomics. Gil-Solsona et al. [36] used gilthead sea bream (*Sparus aurata*) to compare metabolic fingerprinting of serum from fed and 10-days fasted fish by an untargeted metabolomics approach. The main perturbed biological processes were seven: FA oxidation, AA catabolism, lipolysis, gluconeogenesis, Maister's cycle, FA/phospholipid metabolism, and biotin metabolism. Higher circulating levels of fatty acyl carnitines, known as carrier of FA, were found in fasted fish suggesting that organism mobilizes all energy stores to cope with the stress condition. Moreover, elevated levels of urea cycle metabolites confirmed that both FA metabolism and AA catabolism are highly involved in negative energy balance (NEB) assuming a key role in the specific response to the condition of feed deprivation [36]. On the other hand, this study highlighted the possibility to identify biomarkers concerning fish general welfare status since some identified metabolites were relevant for both malnutrition and pollutants toxicity [34, 36]. In the context of feed deprivation of fish, Kullgren et al. [37] have obtained a general picture of which metabolites are up- or down-regulated during feed deprivation in salmonids considering juvenile rainbow trout (Oncorhynchus mykiss) vs. a prolonged stress condition related to 28-day fasting period. Both polar and non-polar extracts from muscle, liver, and plasma were investigated by 1H NMR spectroscopy analysis. A remarkable discrimination between two groups was observed, and the fasting-induced changes detected in tissues and plasma contributed to elucidate the

effect of nutritional long-term stress on metabolic response. Higher levels of muscle phosphocreatine detected in fasted fish is considered the mechanism that allows fish to keep readily available energy for burst swimming and important behavior responses -such as foraging activities and predator avoidance—during fasting, contributing not only to the survival, but also to the general fish welfare status [37].

Environment (Domain II)

Environmental Domain opens the window to a wide range of variables to be considered. For food producing animals, depending on the outdoor or indoor type of rearing, animals may be exposed to uncontrollable extreme weather conditions or, on the contrary in confined spaces to an overcrowding condition; all these discomforts may cause severe physiological stress and debilitating state, even pathologies, for animals [9]. One of the most widely explored environmental stressor is heat stress that, in energy terms, refers to a particular NEB since the amount of heat produced by the animal may exceed the capacity to dissipate the heat in environment, creating an imbalance. This state can be defined by external variables, e.g., air temperature and humidity, and internal variables specific of the animal, e.g., species [56]. Temperament, genetics and the early life experience (i.e., maternal contact during development stage) play a pivotal role in determining how animals, both during childhood and adulthood, experience the environment. In fact, the neural function of animals is strictly related to epigenetic factor: new sensory experiences may alter genes expression in discrete regions thanks to the plasticity of central nervous system. In this sense, a key physiological role is played by hippocampus. Therefore, animals may experience the same environment in different ways [14, 57–59]. A common response of animals to heat stress is the decreasing in feed intake to silence all those processes generating heat—such as digestion—with the aim to survive [60]. In case of investigations dealing with thermal stress, environment is the main Domain of interest, but the repercussion on other Domains, i.e., nutrition, is tangible. This involvement needs to be taken into consideration for an integrated assessment of AW enabling to develop adequate management strategies able to minimize or reduce the negative consequences of heat stress.

In the swine sector, Cui et al. [40] investigated metabolome changes in serum of finishing pigs exposed for 3 weeks to 30°C, compared to a thermal neutral condition of 22°C, aiming to discover potential biomarkers related to heat stress. Five metabolic pathways were considered relevant for the description of chronic heat stress exposure, as follows: carbohydrates, AA, amines metabolism and gut microbiome-derived metabolism. Pigs showed decreasing in feed intake and glucose levels leading to NEB, partly attributable to the decrease in concentration of 6-phosphogluconic acid; as a short-term compensatory mechanism, the finishing pigs used glucose precursors, protein, and AA, through the deamination process and the gluconeogenesis, to overcome the lowering levels of glucose in the serum. A role in NEB adaptation has been shown by ketone bodies and non-esterified FA, whose levels were elevated in the stressed group. These findings confirmed that heat stress condition urges the mobilization of all energy stores to cope with the stress; however, particular attention has been paid to the higher levels of gut-microbiome metabolites observed in pigs reared at high temperature whose induced an increased permeability of intestine conducting to systemic inflammation. This last remark is fundamental in view of a general AW assessment because the symptoms related to the disease (health, Domain III) are the outcome of the activation of a series of biological pathways triggered by multiple stress originating from different Domains (environment, Domain II; nutrition, Domain I). Metabolomics also revealed alteration of phospholipid and FA composition during heat stress [40]. The involvement of lipid metabolism in thermal stress response was observed by Qu and Ajuwon [38] in adipocytes differentiated in culture and mesenteric adipose tissue of pigs exposed to extreme temperature conditions. In vitro and in vivo settings showed distinct metabolite profile, reflecting the in vivo complexity of heat stress adaptation. Moreover, a gender effect was observed by the Principal Component Analysis (PCA) of mesenteric fat that showed a better separation of metabolites in the boars than gilts, suggesting a further variable (gender) of the adaptive response to heat stress in pigs [38]. Untargeted metabolomics analysis was performed by Zhou et al. [39] to explore the combined effects of air temperature and relative humidity on metabolic pathways in broilers. Fecal samples of broilers assigned to three different treatments—reared, respectively, at 35, 60, or 85% relative

humidity (RH) with a gradually increased temperatures to 32°C, over the course of 15 days—were investigated. Based on the results, the authors made evidence that RH affects several metabolic pathways (in particular glucose, adenosine triphosphate– binding cassette transporter metabolisms, and the aminoacyl-tRNA anabolic pathway, metabolic pathway of taurine and hypotaurine) associated to heat dissipation and growth decline, confirming that environmental stress may induce perturbations on different Domains contributing to the definition of the overall wellbeing of the animal [39].

Health (Domain III)

Animal health is defined as the ability of the animal to adapt to the pathology [31]. It is widely accepted that health is a key part of welfare representing the condition of existence for the AW definition. Sources of threat to health domain can be metabolic disorder (i.e., ketosis for dairy cows and ewes), mycotoxin exposure (i.e., fumonisin causing leukoencephalomacia in horses and pulmonary edema in pigs), skin lesions (i.e., aggression in pens) and other unpleasant events (i.e., tail biting with evidence of chewing and possible infection) [43, 61–63]. Metabolomics has been used as a tool to explore the health (Domain III) in relation with other aspects, in particular environment (Domain II) and nutrition (Domain I). As an example, Southam et al. [64] applied the NMR-based metabolomic profiling and correlation networks alongside metabolite fingerprinting, to explore the metabolic network involved in hepatic tumors of flatfish, species used for environmental monitoring as they live close the marine floor where toxicants and carcinogen agents can accumulate in the sediment. Therefore, the dab (*Limanda limanda*), a disease sentinel used in European marine monitoring programs, was chosen for this study. Metabolic differences between two phenotypes (health vs. diseased) were small, effect probably masked by the high intragroup variability; for this reason, after histological evaluation of neoplastic lesions, the comparison between metabolome of healthy and tumor tissues sampled from the same liver was considered. Changes in metabolites abundance were clearly demonstrated, perhaps accountable to tumor development stage. Also, an increased anaerobic respiration—supported by the higher levels of lactate, succinate, and acetate—

and the depletion of choline were evidenced in tumor tissue, leading the authors to hypothesize that disruption of the choline oxidation pathway may be the key metabolic change in flatfish liver tumor enabling DNA hypomethylation and oncogene activation [64].

As mentioned above, feed represents not only an important factor defining AW in toto but also a source of threat for animal health, in the case of mycotoxin-contaminated feed. In that context metabolomics proved to be a powerful technique to investigate the relationship between health and nutrition Domains. In particular, Wu et al. [65] applied metabolomics to study the relationship among metabolic changes mycotoxin-induced, mycotoxin contaminated-feed (treat), and feed supplementation. In detail, both the toxic effects of deoxynivalenol (DON) on pigs and the effects of supplemental glutamic acid on DON-induced toxic damage in piglets were investigated throughout an NMR-based metabolomic approach. The findings indicated that glutamic acid may be a useful nutritional supplement for regulating DON induced injury, being able to decrease oxidative stress, promote intestinal epithelial cell proliferation, and regulate energy, lipid, and AA metabolism disorders induced by the dietary exposure of trichothecene mycotoxin of pigs [65].

Behaviour (Domain IV) and mental state (Domain V)

Currently, very limited scientific evidence is available concerning metabolomics studies addressed to animals' emotional experiences within the welfare context. Despite this, the theoretical framework supports the assumption that the welfare of an individual is the totality of its efforts to live positive experiences in each of the Five Domains, suggesting that both mental state and behavior are very impactful topic to explore [66]. A strong correlation between behavior and mental state may be assumed because the animals manifest feelings through specific behaviors [67] following stimuli such as social interaction (e.g., separation or socialization), mental stimulation, stress (e.g., weaning for piglets) as well as nutrition, environment, and health [68]. There is a wide consensus that cognitive development may arise from environmental inputs such as the interaction with new material or sensory stimuli. In the pig

sector, behavioral development and stress adaptation are positively affected by an enriched environment during the early life of piglets and social interaction between litters during lactation [69]. The mechanisms underlying the decrease in agonistic behavior and reduced stress response in relation to the combined early socialization and environmental enrichment were successfully investigated by a recent study of Saladrigas-García et al. [48] throughout an untargeted metabolomics approach. The authors performed the study comparing pigs reared with enrichment objects (i.e., hearty chew dog toys, squid-shaped toys, and natural ropes per pen) vs. control group reared with standard management system. As a proof of differences, molecules related to energy metabolism (triglycerides, FA, very low- and low-density lipoproteins, and creatine) were significantly lower in the piglets of enriched environment probably due to the fact that pigs are engaged in playing with enriched material by reducing pen interaction, thus minimizing energy requiring. As a consequence, a reduction of aggression in these animals was observed suggesting that physically and socially enriched environment in early life can modify animal response after weaning, probably by means of diminishing social stress response. In fact, control group showed an increase in body lesions supported by the higher levels of stress markers, such as salivary cortisol and chromogranin [48]. However, to the best of the author's knowledge, the application of metabolomics approach to Domains IV and V of the Five Domains model is lacking, and further work on these issues is encouraged. For example, an abnormal behaviour, which is detrimental to both welfare and economy and for which metabolomics might provide adequate insights, is tail biting in pigs, for which a pig bites the tail of pen-mates [70]. This behavioural tendency is the manifestation of a set of several sub-optimal living conditions regarding nutrition, environment, health, and other triggering cause [71]. So far, only targeted approaches have been employed to explore the issue. Assuming that the tail biters may suffer from the inability to absorb nutrients, Palander et al. [72] focused their study on the determination of blood minerals and AA and gut cell wall structure. According to their findings, no signs of nutritional deficiency in tail biters on the basis of their intestinal morphology or blood metabolites were observed. On the other hand, data suggested differences in relation to the tail-biting environment and being bitten. Free access feeding (nutrition, Domain

I) with restricted feeding space (environment, Domain II), compared with feeding twice a day with unrestricted feeding space, was associated with an overall reduction in AA levels in plasma and deepened crypts in the jejunum. In this context, metabolomics could be exploited to identify metabolic changes enabling to recognize early signs leading to tail-biting behaviour and formulate appropriate remedial strategies [72].

Metabolome analysis

The study of the metabolome is considerably complicated being highly susceptible to external variations. Depending on the aim to pursue, metabolomics research may be performed following a targeted or untargeted strategy, mainly different in terms of metabolites coverage, being the first pointed to a selective recovery of groups of metabolites, whereas the second one aimed at maximizing the number of metabolites. Targeted strategy is a hypothesis testing approach and generally, throughout known chemical compounds used as internal standards, provides the quantification of targeted molecules [73–75]. The untargeted strategy is aimed at characterizing the whole metabolome and designed for the identification of as many metabolites as possible, up to thousands of molecules, both known and unknown (or novel) providing information about changes in metabolites [27, 75]. Combining targeted and untargeted strategies can be considered a potential choice for metabolomics studies since this integration may help to pinpoint and quantify metabolites [53, 76, 77]. The biological understanding from metabolomics experiments relies on a good experimental design, after the formulation of a proper biological question [78].

Most of AW metabolomics studies are observational case– control studies and focused to grab the molecular response generated from the exposure to different factors belonging to individual Domains. Generally, studies consist of two or more groups of animals, respectively, identified as control and treatment/s [35, 39], but longitudinal studies are also considered to investigate AW aspects mainly related to Health (IV) and Nutrition (I) Domains [49, 79]. A

critical aspect concerning the study design is being able to consider all the conditions affecting AW simultaneously, because also ensuring the best living conditions, the interaction between the animal's previous experiences and temperament may completely reverse the situation. Animals' feelings eliciting certain behaviours are still a challenge that could find answer by the metabolome studies. Comprehensive planning of complex experiments may take advantage from the Design of Experiments (DoE), a statistical methodology to plan experiments efficiently [80], although no evidence is available for metabolomics studies.

Sample size is a pivotal issue to enable detection of statistical significance, but there are not standard methods for its estimation in metabolomics. And so, as often happens, ethical and economical restrictions mainly determine the number of samples (e.g., animals to be sacrifice) for a study [81]. Classical univariate sample size determination is usually not straight forward for highly dimensional and multi-correlated metabolomics data [78]. The minimum sample size may be estimated by a power analysis method described for high-dimensional metabolomics data [82]. Alternatively, an analysis-based approach by publicly available software package for selecting the optimal sample size has been proposed by some authors [83].

The choice of sample type, biofluids and/or tissues, relies on the objective of the investigation, although in longitudinal studies biofluids are the preferred choice for repeated measurements. In terms of feasibility of sampling, biofluids such as urine, stool, and saliva can be collected non-invasively, whereas blood and tissues' collection are much more complicated and may be arduous, therefore qualified personnel is required [84]. Changes in metabolome of saliva, serum, and adipose tissue were used to study the effect of heat stress in pigs [38, 40, 41]. Both biofluids and tissues were considered to gain a global view on metabolic processes and elucidate the effects of feed deprivation in salmonid fish [37] and in an integrated multi-omics study aimed at ascertaining the effects of the finishing forage on metabolic pathways related to AW and meat quality in bovine [35].

3.1.4 Metabolomics workflow

Metabolomics is one of the -omics techniques requiring wide and robust analytical strategies. By and large, scientists agree that metabolomics workflow consist of sample preparation, sample analysis, data processing, data analysis, and biological interpretation [85, 86].

Sample preparation

The ideal sample preparation for metabolomics analysis should guarantee high recovery of metabolites and be as rapid and simple as possible—to minimize degradation reactions and exogenous interferences—as well as reproducible. As a matter of fact, even minor variations in the centrifugation of plasma can affect the recovery of some metabolites, which can be degraded or created after the sample's extraction procedure [87, 88]. The broad number of metabolites different for chemical structure, reactivity, and concentration make the simultaneous extraction of all molecules challenging and no single method can cover the entire metabolome.

Sample preparation heavily depends on the approach, targeted or untargeted. In the first case, conditions are optimized according to the physicochemical properties of metabolites, in particular polarity, to maximize their recovery. In the second case, sample treatment aims for increased coverage of metabolites, often leading to suboptimal recoveries for specific compounds: this justifies the introduction of lipidomics to refine sample preparation enhancing the recovery and study of lipids [73, 76]. For tissues, the sample representativeness may be an issue because organs may be described and respond regio-specifically; for example, the liver presents five different topographic lobes characterized by different levels of some enzyme systems expression, as well as the kidney consists of medulla, cortex, and many cell types with different structures and roles [89]. Generally, 1–100 mg of tissue and 10–100 µl of biofluids are sufficient [34, 37, 41, 90], although the amount relies on the type of the analytical platform used for the analysis.

Challenges, pitfalls, and best practices to follow in sample preparation for metabolomic studies of biofluids, tissues, and mammalian cells have been extensively described in literature [91, 92]. However, some trends and tissues in animal sample preparation are worth mentioning: such as for preliminary handling step, quenching may be necessary to switch off the enzyme activities of samples by extreme treatments based upon pH or temperature changes [92]. Dehydration of the sample may also be used for this aim, although water removal may cause metabolites' adsorption on cell walls and membranes leading to untrue results [20]. Frozen tissue should be thawed on ice to reduce changes in sample structure [89].

Literature often shows the use of biphasic extraction protocols to recovery simultaneously both polar and non-polar molecule classes. Among them, the Folch's and Bligh-Dyer methods, and their modifications, are those most widely employed [93, 94]. Both are two-step protocols based on the use of a mixture methanol/chloroform in different ratios and water. For environment and operator safety purposes, dichloromethane and methyl tert-butyl ether have been proposed as solvent alternatives to chloroform. The use of water helps to split the system in a polar upper layer and a non-polar lower one [95].

Sample analysis

Actually, none of the analytical platforms can detect the great heterogeneity of the metabolites. Nevertheless, NMR spectroscopy and Mass Spectrometry (MS) are the two major analytical platforms pillars adopted in metabolomics.

MS-based analysis provides a spectrum that sums up measurements of mass-to-charge ratio (m/z) and the relative abundance of ionized molecules [96]. Experiments MS-based may consider several options for separation and detection [53]. Both gas- and liquid-phase separation are employed, exploiting physicochemical properties (i.e., volatility of metabolites, interaction with phase in a column) of compounds [28, 97]. Application of capillary electrophoresis-MS are limited, despite this technique is particularly suitable for analysis of volume-limited biological samples [98, 99]. Various types of

detectors have been developed to improve the performance of analysis in terms of enhanced sensitivity and selectivity such as quadrupole, quadrupole ion trap, and time-of-flight (ToF) are the mass analyzers commonly used [84]. For targeted analysis purposes, triple quadrupole is the favourite choice because suitable for quantitative analysis by low resolution MS. On the contrary, for untargeted applications, also known as full scan approach, high resolution MS (HRMS) may be preferred [92]. Among the emerging MS techniques, no requiring prior sample preparation, MS imaging is very promising because it has the advantage of visualizing the distribution of metabolites in tissue or cells making this approach less time consuming and solvents employing [100, 101].

NMR spectroscopy proved to be a powerful tool for structural and quantitative information but also to identify unknown metabolites [102, 103]. Considerable progress has been made in NMR-based metabolomics considering that it is highly reproducible and allows the identification of compounds with identical masses; on the other hand, it provides the detection of most abundant metabolites being less sensitive than the hyphenated methods [97, 103]. Literature offers many examples of NMR-based metabolomics applications showing that the most commonly investigated nucleus is the proton ^{1}H, having a natural abundance of 99.98%, but also other nuclei such as ^{13}C, ^{15}N, and ^{31}P can be investigated [103, 104]. A critical aspect concerning the employment of NMR spectroscopy in metabolomics is that biological samples are complex matrixes characterized by the presence of proteins, lipoproteins, and water, whose signals in the spectrum may hide those metabolites at lower concentration. To overcome these issues, several pulse sequences have been developed to improve specific NMR signals. For water suppression, NOESY pulse sequence is the most popular applied for the acquisition of metabolomics profile [105], specifically, the ^{1}H NMR spectra of the polar extracts of both biofluids and tissues are usually recorded [34,106]. In the case of darkening-signals macromolecules, the Carr-Purcell-Meiboom-Gill (CPGM) pulse sequence may be employed to overcome the problem related to low concentration metabolites [105]. For example, the authors Le Roy et al. employed CPGM sequence for the acquisition of the ^{1}H NMR spectra of chicken's plasma and egg white to limit the contribution of albumin and ovoalbumin signals, respectively

[106]. Further, NMR spectrum may be severely affected by pH, osmolality, and concentrations of some ions creating a variable condition in ppm chemical shifts of proton signals [92, 96]. In this regard, it is recurrent the use of a buffer solution with pH = 7.0/7.4 during sample preparation to control ion concentration [34, 37, 106].

Data processing

NMR and MS provide a considerable amount of data, of the order of thousands signals, which form the data sets of the putative metabolites. Initially, raw data sets contain undesirable information such as noise [107]. This unnecessary information might force some trends at a later stage and, at first, alleged interferences must be removed by adequate data pre-treatments [102]. Further data processing consists in setting the raw data into a format that can be used for the subsequent data analysis. To combine data from different samples, the alignment of signals is needed because often shifts in ppm and in retention time are observed in NMR and MS, respectively [102, 108]. To this aim, an internal standard may be added during sample preparation and used as a reference signal. Unwanted data variation related inevitably to both experimental and biological sources such as sample preparation, analysis of multiple batches, inter-instrument and inter-laboratory variation, constitution of the biological samples, may be removed by normalization. This step minimizes the risk of identifying false biomarkers, missing out true biomarkers, artificial classification or clustering of the samples or metabolites [109, 110]. Scaling is a step of data processing that places each metabolite in a comparable scale because metabolite levels may range over many orders of magnitude [111]. Autoscaling and Pareto scaling are the most commonly scaling methods used in metabolomics [97, 102].

Data analysis

Metabolomics data require reliable statistical methods, defined as chemometric tools, to efficiently extract the maximum useful information. For this purpose, the multivariate data analysis (MVDA) is generally employed to manage large and complex data sets, for data visualization and exploration, and

the identification of patterns [112, 113]. For explorative aim, PCA is often the first step applied to detect the presence of natural grouping trend or outliers by displaying with scores plot the relation among the observations or samples in the model plane. Hierarchical cluster analysis (HCA) is also used for preliminary evaluation of information contents in the data to study any differences or similarities, with a separative or agglomerative approaches, characterizing the investigated samples. The relationship between the samples is graphically summarized as a tree plot building the dendrogram, where the contribution of each variable is shown by an heatmap [114]. In the study of Carrillo et al. PCA and HCA revealed a perfect separation between grass- and grain-fed Angus steers based on the metabolite profiling from muscle tissue and blood, that may be indicative of the divergences in global metabolism [35]. According to Qu and Ajuwon metabolomics of heat stress response in pig adipose tissue revealed alteration of lipids during heat stress, and PCA provided a separation of metabolites by temperature [38].

Unlike PCA and HCA that do not take into account the information about the class membership (i.e., control vs. positive, exposed vs. non-exposed, feed supplementation vs. normal diet), Partial Least Squares-Discriminant Analysis (PLSDA) and Orthogonal Partial Least Squares- Discriminant Analysis (OPLS-DA) are discriminating analysis conducted for improving separation between groups by using class information [97]. In this framework, the contribution of metabolites in the discrimination is expressed by the Variable Importance in Projection (VIP) score, where the greater the VIP score, generally with a score value higher than unit, the greater the contribution to the separation between sample groups. For example, in a metabolomics study aimed at identifying biomarkers of malnutrition in farmed fish, using gilthead sea bream (*Sparus aurata*) as a model, PLS-DA clearly discriminated the fasted individuals from those of the fed group; moreover, by OPLS-DA around 850 features of the data set were highlighted as discriminatory between fed and fasted fish [36]. In the study performed by Zhou et al. [39] focused on metabolic profiling of chicken stools within environmental stress characterization, PLS-DA plots showed a clear separation between groups of broiler chickens reared at different relative humidity and temperatures. Moreover, 36 metabolites with VIP score >1 were

identified and further investigated as potential biomarkers related to hot humid or dry stress [39].

Biological interpretation

Statistical significance does not mean biological significance, and biological interpretation should not be derived on the change of biomolecules selected by statistical significance. Biological interpretation is the most challenging step and the real bottleneck of metabolomics workflow. Metabolism is a complex and dynamic network where known metabolites are connected by enzymatic and non-enzymatic reactions or structural similarities. Once a list of metabolites possible candidate biomarkers has been ranked by data analysis, bioinformatics is needed to support the metabolomics interpretation. Pathway analysis, metabolite set enrichment analysis, and metabolite correlation networks are the tools most frequently applied to gain biological insights [112]. For example, Zhou et al. [39] applied pathways analysis to identify pathways affected by hot-humid or dry climate in broiler chickens reared in different conditions of relative humidity. In particular, in this study the relevant metabolites were subjected to Metaboanalyst, a free online tool based on the KEGG metabolic pathways database; thirteen metabolic pathways were detected as enriched metabolic pathways for the hot-humid or dry stress, suggesting that those environmental conditions can affect all those metabolites involved in fat synthesis and associated with skin vasodilation and blood flow [39]. In the NMR-based metabolomics investigation of Southam et al. [64] addressed to hepatic tumors of flatfish, metabolic correlation networks analysis revealed increased anaerobic metabolism and reduced choline metabolism in diseased tissue compared to healthy phenotype. Moreover, significant negative correlations were observed between alanine-acetate and between proline-acetate in diseased tissues only, suggesting that alanine and proline are utilized as alternative energy sources in flatfish liver tumor tissue [64].

3.1.5 Metabolomics and animal welfare: A step forward

Metabolomics proved its effectiveness in clarifying biological cause-effects mechanisms of living organisms, confirming that the phenotypic understanding

can take place on molecular scale by investigating metabolite identity and abundance. Focusing on a set of metabolites may provide significant clarification on cellular regulatory processes involved in the latest response of the biological systems to genetic and environmental changes.

Therefore, this approach can metabolically answer to biological questions concerning AW issues even when applied to individual Domains, hence with a limited perspective and covering confined areas of welfare. As a matter of fact, none of the studies listed in **Table 1** answered the key question Did the animal live in a good welfare status? In fact, metabolomics applications show how changes in metabolome can be induced by different factors such as diet, environmental stress, health, and mental state, which are the same factors declared from the Five Domains model. Thus, successful results have been achieved considering the Five Domains individually; in other words, all described examples show that the condition under investigation is targeted toward a well-defined condition i.e., nutrition state. Although satisfactory, it may be a limiting strategy supported by the assumption that AW cannot be described on the basis of individual Domains considering, for example that a stressful condition can have consequences embracing all blocks of the Five Domains model, as in the case of mycotoxin-contaminated feed [65].

Overall, the Five Domains model has been poorly explored in its completeness with metabolomics. Thus, promoting linking and combining the theoretical and scientific frameworks—the Five Domains model and metabolomics—could be the turning point to make available objective tools enabling to fill the gaps in AW assessment. This cue is strongly supported by the holistic nature of metabolomics approach which provides a dynamic view of biological systems integrating the flow of information from DNA, RNA, and proteins, ensuring a comprehensive overview of living systems. In fact, in the hierarchy of omics methodologies that reflect the molecular expressions of living systems, metabolomics is the most sensitive to variations resulting from a complex interaction between genotype, lifestyle, nutrition, drug therapy, and environmental exposure [20, 115]; in this way it may capture and integrate all information relevant for the molecular definition of AW phenotypic outcome. Therefore, metabolomics might be exploited at the best for the evaluation of

the wellbeing status of the animal as a whole entity, and unravel biomarkers intended as a characteristic that is objectively measurable reflecting a given condition [29, 30].

In the opinion of the authors, there are some challenges to face for the successful application of metabolomics in the setting of AW mainly related to the following two questions.

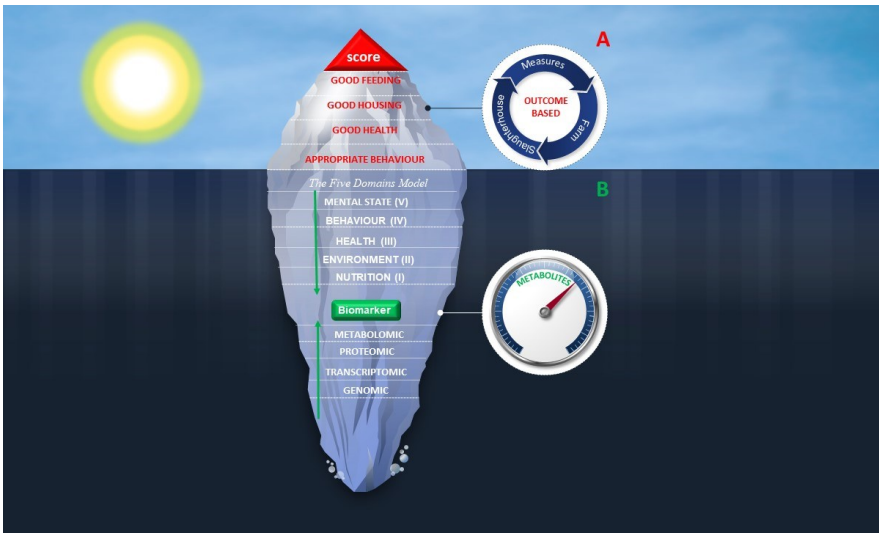

Figure 3. Iceberg represents the proposal of a model for the future AW assessment. Combining scoring systems **(A)** with metabolites identification and quantification **(B)** may greatly improve the understanding of welfare condition and assessment. Tip of the iceberg represents the current AW assessment protocols focused on management-based requirements and animal-based measures. Red items are welfare criteria on which are based measures on farm or at the slaughterhouse. These protocols provide a score indicating if welfare criteria are fulfilled. Underwater part of the iceberg represents a new path for AW assessment that includes an integrated view of the Five Domains model. All Domains can affect the phenotypic outcome of living organism which can be explored through the metabolomics approach. The Five Domains model meets untargeted metabolomics in the molecules measuring.

Might be the metabolomics approach a tool supporting the current AW assessment models?

Currently, AW assessment models are outcome-based systems obtained by adopting scoring systems as an evaluation metric. These models focus on the compliance with functional and management requirements as well as animal-based measures, all of them considered iceberg indicators of welfare (**Figure 3A**). In the opinion of the authors, coupling the Five Domains model with metabolomics might be the solution to provide the underwater part of the iceberg enabling for a whole picture of AW. By providing insights into metabolome, metabolomics could make available novel biomarkers spanning all Five Domains and that might be useful items for a comprehensive assessment of AW status (**Figure 3B**). Moreover, in this framework, the corrective actions will be targeted toward solving the problems due to the fact that metabolites are considered the "canaries" of genome and this nature as sensor might also be successfully employed as a preventive screening measure [116].

Which experimental workflow might be more powerful to give novel metabolic insights related to AW?

A well-conceived experimental design should be accomplished to gain a molecular snapshot as closer as possible to reality related to the condition of animal wellbeing. We have good reasons to think that further progress in analytical and computational technology will give even more performing tools able to increase metabolite coverage and biological interpretation, still the bottleneck of metabolomics workflow. It may be reasonable believe that carefully defining the study design might help in understanding the biological meaning of AW by remarking the idea that a condition in which animals live might be biologically demonstrable. In view of integrating metabolomics and Five Domains model, efforts should be addressed to include in the experimental studies animal behavior and mental state that, as far as the authors know, are the Domains least explored by metabolomics.

3.1.6 Concluding remarks

The multidisciplinary nature of AW, well-represented by the different items according to the Five Domains model, makes the development of tools for the

objective assessment of the AW status challenging. Based on the literature, most of the metabolomics studies considering AW have approached the topic focusing on the study of one single Domain, with a limited perspective. Nevertheless, metabolomics has shown effectiveness in the discrimination between groups of animals subject to different conditions, the evaluation of the most perturbed metabolisms, and the detection of biomarkers. As a whole, even individually considered by metabolomics approach, the investigated conditions are able to induce tangible outcomes in the metabolic profile of the animal, by allowing for the correlation of biochemical changes with phenotype.

In this regard, in the last decades metabolomics has generated a remarkable amount of data through the improvement of high throughput technologies both as analytical platforms and data analysis strategies. However, specifically in the context of AW, metabolomics is still in its infancy, and the way to getting full coverage of all relevant metabolites is still long. Progress in metabolome coverage confidence and standardization of approaches across the entire workflow is needed for metabolomics to become a mature application to be widely adopted.

Within these limitations, efforts of the metabolomics scientific community have been made to gain meaningful biological and chemical insight from complex metabolomic data. Deciphering metabolites to be used as biomarkers of comprehensive AW status is a promising tool that may pave the way for new control strategies for both living animals and the food products thereof. Moreover, increased knowledge of metabolic pathways and biological interpretation will allow defining management strategies to improve the welfare in food-producing animals in livestock systems.

Looking forward, the inclusion in the experimental design of the last two Domains, behaviour, and mental state respectively, as well as nutrition, environment, and health, would represent a turning point for the integrated application of Five Domains model on molecular scale exploiting metabolomics. Regarding the biological interpretation, the final achievement to be reached, the combination of multi-omic data is a promising perspective but still a challenge to overcome. Further work on these issues is therefore encouraged.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Section II

Metabolomic insights into Antibiotic Free label claims

Case Study I

3.2.1 Case Study 1

Research Article

^{1}H NMR metabolomics on pigs' liver exposed to antibiotics administration: an explorative study.

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Keywords: antibiotic-free claim; untargeted metabolomics; NMR; pork; polar metabolites; non-polar metabolites

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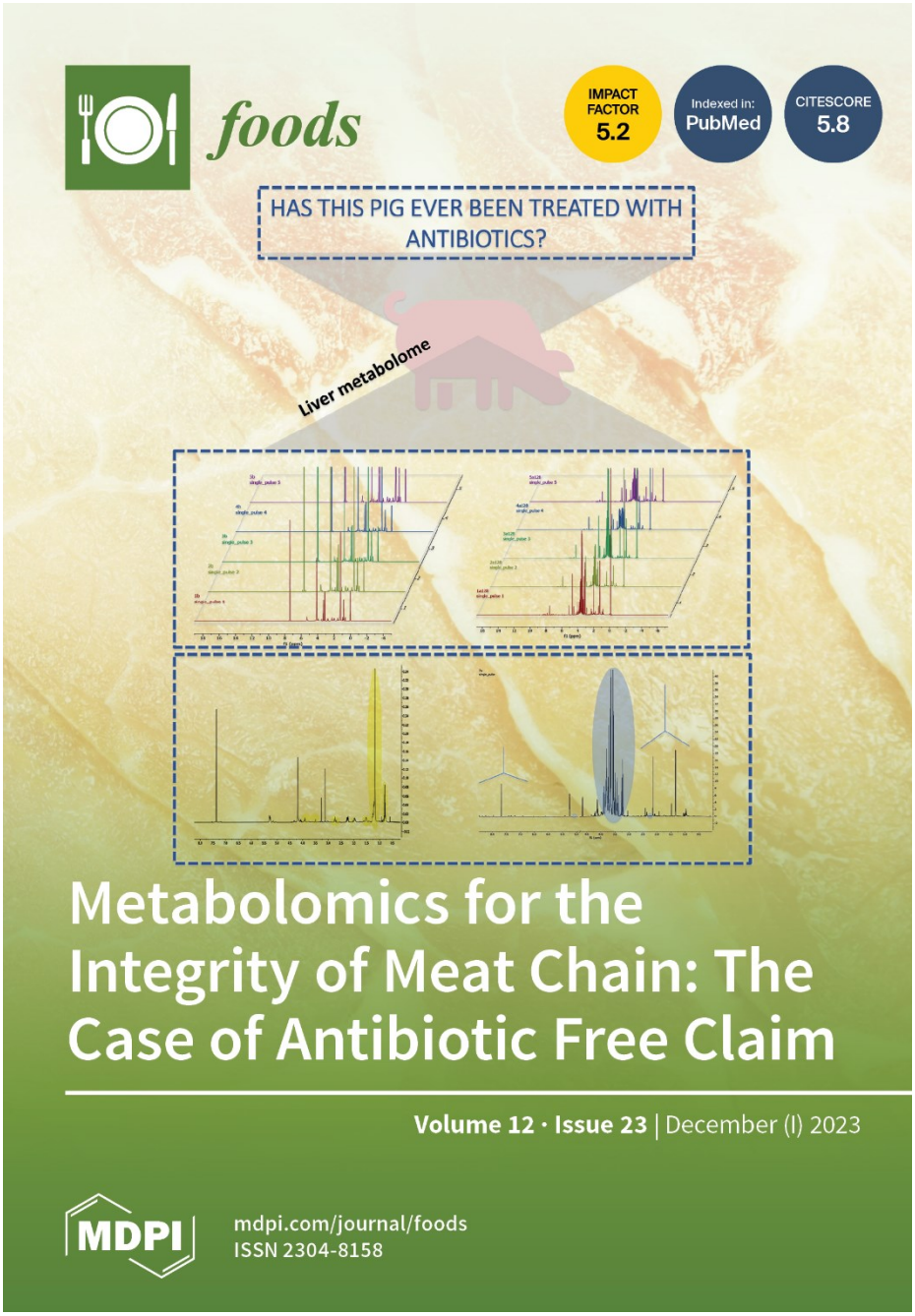


Figure 4. Cover of the Volume 12, Issue 2023 - Special Issue Food Fraud and Food Authenticity across the food supply chain (MDPI) FOODS.

3.2.1.1 Abstract

An untargeted Nuclear Magnetic Resonance (NMR) spectroscopy-based metabolomics approach was applied as a first attempt to explore the metabolome of pigs treated with antibiotics. The final goal was to investigate the possibility of discriminating between antibiotic-treated (TX group) and untreated pigs (CTRL group), with the further perspective of identifying the authentication tools for antibiotic-free pork supply chains. In particular, 41 samples of pig liver were subjected to a biphasic extraction to recover both the polar and the non-polar metabolites, and the ^{1}H NMR spectroscopy analysis was performed on the two separate extracts. Unsupervised (principal component analysis) and supervised (orthogonal partial least squares discriminant analysis) multivariate statistical analysis of ^{1}H NMR spectra data in the range 0–9 ppm provided metabolomic fingerprinting useful for the discrimination of pig livers based on the antibiotic treatment to which they were exposed. Moreover, within the signature patterns, significant discriminating metabolites were identified among carbohydrates, choline and derivatives, amino acids, and some lipid-class molecules. The encouraging findings of this exploratory study showed the feasibility of the untargeted metabolomic approach as a novel strategy in the authentication framework of pork supply chains and open a new horizon for a more in-depth investigation.

3.2.1.2 Introduction

Today, the meat sector faces many challenges in terms of sustainability, nutritional aspects and authenticity, leading meat operators to rethink the management of food producing husbandry systems. Additionally, as consumers incline toward making choices, which are seen as greener and more sustainable, greater attention is devoted to the positioning factors of meats in the market [1]. Currently, several certifications and labels guarantee various meat attributes, including antibiotic-free, halal, kosher, organic and GMO-free [2]. Certification marks concerning the usage of antimicrobials (AMU) (i.e., Antibiotic Free or Raised Without Antibiotics) fulfil consumer requests concerning value-added meat obtained from farm animal chains embracing animal health and welfare values [3]. On the other hand, responsible AMU in livestock farming responds to the global need to fight antimicrobial resistance (AMR)—a major threat to public health [4]. The definition of novel attributes in meat may provide opportunities for food fraud, and for this reason, fit-for-purpose tools are urgently needed for the protection of the integrity of the meat chain.

For public health protection, in the European Union (EU), the procedures establishing the maximum concentration of a residue of pharmacologically active substances, which may be permitted in food of animal origin, are laid down by Regulation (EC) n. 470/2009, and the maximum residue limits (MRLs) of those substances in foodstuffs of animal origin are set out in Commission Regulation (EU) n. 37/2010 [5,6]. The regulatory framework for the manufacturing, import, export, supply, distribution, pharmaco-surveillance, control and the use of veterinary medicinal products and medicated feed is represented by Regulation (EU) 2019/6 and Regulation (EU) 2019/4 [7,8]. Among the objectives, these provisions, together with further implementing and delegated regulations, aim at strengthening EU action against AMR through specific measures, such as prudent AMU and the reservation of certain antibiotics only for the treatment of infections in human medicine. For monitoring purposes, all member states must include official controls in the multiannual national control plans (MANCPs) with regard to antimicrobial agent

residues in animals and derived food; in particular, the specific requirements, criteria and uniform practical arrangements for the performance of official controls of residues are provided by Commission Delegated Regulation (EU) 2022/1644 and Commission Implementing Regulation (EU) 2022/1646 [9,10].

Concerning the analytical aspects in relation to residues of pharmacologically active substances in live food-producing animals and products of animal origin, the provisions applicable to the methods used for sampling, laboratory analyses and interpretation of analytical results are established by Commission Implementing Regulation (EU) 2021/808, which applies to official controls aimed at verifying compliance with MRLs [11]. In this framework, biological and biochemical methods are available mainly for screening purposes [12], while chromatographic analysis based on full-scan diode array detection spectrophotometry, fluorescence detection spectrophotometry or mass spectrometry detection is required as a confirmatory method, except for prohibited or unauthorized pharmacologically active substances, for which only mass spectrometry detection is suitable. By providing full or complementary information to unequivocally identify the substance, confirmatory methods for antibiotic residue monitoring are based on targeted multiresidue methods limited to a pre-defined number of known analytes [13,14]; the literature provides several examples of validated multiresidue multiclass methods, which cover more than one hundred targeted analytes in a single analysis [15,16]. However, the untargeted approach has emerged as an alternative and promising tool enabling the development of sensitive and wide-ranging screening of analytes, overcoming the limitations of targeted analysis. The untargeted workflow allows for the recovery of an enormous number of non-preselected compounds, even unknown ones, which may be simultaneously monitored and identified through a bottom-up strategy [17]; the screening of thousands of molecules may highlight new analytes as potential biomarkers of the physiological responses following the administration of antibiotics. In a long-term perspective, novel biomarkers might be a useful strategy for implementing more efficient control systems enabling the monitoring of a more representative number of samples within the residue control plans. As a matter of fact, the report of the European Food Safety Authority for 2021 on the results from the monitoring of veterinary

medicinal product residues in live animals and animal products reveals a continuous decline in the percentage of non-compliant samples over the last decade [18]. Despite the positive trend from a safety point of view, the very low non-compliance percentage (0.14% for antibacterials for all tested animal species, 0.09% for pigs in 2021) could be affected by the inadequacy of sampling and may highlight the need for new approaches.

Apart from compliance purposes for MRLs, the untargeted approach could play a role in assessing the specific quality certifications related to the use of antibiotics in animal husbandry beyond the presence or absence of residues in the samples under investigation. Changes in gut-microbiota-produced metabolites after antibiotic exposure in pigs are documented in the literature [19,20]. Moreover, there is evidence that antibiotic treatment elicits microbiota-independent changes in host metabolites [21], markedly affecting the metabolomic profiles of the gut and biofluids in antibiotic-treated piglets [22]. In this respect, it is conceivable that the metabolome—intended as a whole set of precursors, intermediates, and products of biochemical processes—may contain useful information for revealing the metabolites attributable to an antibiotic treatment.

Metabolomics is the comprehensive study of low-molecular-weight (typically <1500 Da) metabolites in biological systems. In recent years, this high-throughput molecular technology has been widely exploited to study several traits of interest in animal sciences. It has been explored as an efficient methodology for unravelling metabolic changes in food-producing animals, thus helping identify the candidate molecules representative of different physiological pathways and contributing to the development of diagnostic tools for better animal management [23]. In this regard, the advances in metabolomic profiling have led to an investigation of the effects of heat stress on the saliva and serum metabolome of pigs and identification of metabolites, which could be used as biomarkers of the stressful condition [24,25]. Metabolomics has been proposed as a promising tool for investigating novel biomarkers useful for a comprehensive assessment of animal welfare status; objective indicators of the overall status of the animal might be advantageous for defining adequate management strategies to improve the welfare of livestock animals and for the

purpose of authenticity and traceability of meat supply chains, which adopt high standards of production and label claims concerning animal husbandry conditions [26]. Two main analytical platforms—namely nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS)—are considered the workhorses for metabolomics analysis, leading to the generation of a massive amount of data due to the acquisition of thousands of metabolite signals [27,28].

To the best of the authors' knowledge, insights into the biochemical phenotype through the study of the metabolome of pigs treated with antibiotics have not been reported previously. Therefore, the aim of the present study was to explore the metabolome via an untargeted NMR-based approach in order to capture and compare the metabolomic fingerprinting of antibiotic-treated vs. untreated pigs. This study is intended as a first step of a wider investigation aimed at identifying and validating adequate biomarkers to be used as tools for assessing the pig chains' authenticity. In this regard, the pigs in this study were from commercial farms selected within the ClassyFarm system—the Italian national monitoring system aimed at categorizing livestock farms based on risk.

3.2.1.3 Materials and Methods

Chemicals and Reagents

HPLC grade methanol, chloroform and water were purchased from Labscan (Dublin, Ireland). Analytical grade sodium dihydrogen phosphate monohydrate and di-sodium hydrogen phosphate dihydrate were supplied by Merck (Darmstadt, Germany). Deuterium oxide (99.9% D), methanol-d4 (99.8% D) and chloroform-d4 (99.8% D) were obtained from VWR International BVBA (Geldenaakseban, Leuven, Belgium), and 3-(trimethylsilyl)-propionate-d4 (TSP) was obtained from Sigma-Aldrich (Milano, Italy).

Experimental Design

Forty-one heavy pigs (approximately 170 kg of live body weight) reared in 2020 on four fattening farms in northern Italy were selected for this study. Pigs were randomly allotted into two groups according to the object under

investigation: the control (group ID CTRL, *n* = 22) and treatment (group ID TX, *n* = 19) groups, respectively. Group classification was designed considering the AMU, which was estimated by calculating treatment incidence 100 (TI_{100}) using the Defined Daily Dose Animal for Italy (DDDAit) as a standard, as described in a previous study on Italian fattening farms [29]. The TI_{100} can be interpreted as the percentage of time a pig spent under treatment during its production cycle [30]. Data on AMU were extracted from the Italian Ministry of Health's surveillance system, ClassyFarm [31], which is available and mandatory for all Italian pig farms. In this study, for greater representativeness of the samples, pigs in the TX group were randomly chosen from two batches of about 100 pigs each from two farms, namely Farm 1, with a TI_{100} of 9.1, and Farm 2, with a TI_{100} of 20.8; similarly, pigs in the CTRL group were chosen from two batches from Farm 3 and 4, respectively, where no antibiotic treatments were recorded (**Figure 5**).

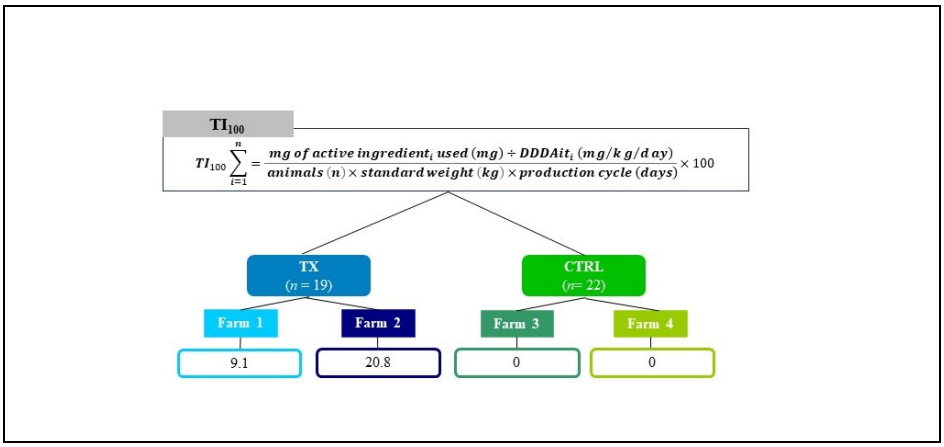


Figure 5. Graphical representation of the experimental design. The upper box sums up the mathematical formula for TI_{100} used as classification criterion for assigning group ID (CTRL and TX). (Active ingredient used (mg): amount of the active ingredient of the antibiotic used for treatment; DDDAit (mg/kg/day): Defined Daily Dose Animal for Italy expressed in mg of active substance per day per kg of body weight; Animals (n): number of animals considered for batch of livestock; Standard weight (kg): average animal weight during the treatment period, set up at 100 kg for heavy pigs; Production cycle (days): days of pig production cycle).

Except for antibiotic administration, other variables, such as feed and gender, were intentionally not considered to assure greater reliability of the study. However, all pigs were of the same age and were slaughtered on the same day at a commercial abattoir under the supervision of the veterinary team and were intended for human consumption. No ethical approval was required. The whole liver of all pigs was removed from the carcasses during slaughtering and immediately stored at 20°C until analysis. The liver was chosen as the matrix for the investigation since its role in drug metabolism is well established.

Sample Preparation

The biphasic extraction procedure, known as the Bligh and Dyer method [32], with slight modification was adopted for the recovery of polar and non-polar metabolites of the liver. Briefly, 100 mg of frozen liver manually grinded was mixed with 3 mL mixture of methanol/chloroform (2:1, *v/v*) in a 15 mL screw cap glass tube and vortexed for 30 s. Then, a sonication step in an ice-water bath for 30 min was performed. Further, 1 mL of water and 1 mL of chloroform were added and newly vortexed. The sample was then centrifuged for 35 min at 4°C, at a speed of 2220 *X g* (Beckman Coulter Life Sciences, Milano, Italy). Finally, phase separation was achieved, and the upper polar and lower non-polar fractions were separately transferred to new glass tubes, and each fraction was dried under nitrogen flow. Both fractions were stored at -20°C and reconstituted prior to ^{1}H NMR analyses. For the polar extracts, 700 µL of sodium phosphate buffer in D2O (0.25 M; pH = 7) and 100 µL of TSP as internal standard was added and transferred in 5 mm outer diameter NMR tubes. For the non-polar extracts, 600 µL of chloroform-d_4 and 200 µL of methanol-d_4 were employed for the reconstitution.

^{1}H NMR Spectroscopy Analysis

Polar and non-polar extracts were separately recorded using NMR spectrometer operating at a magnetic field of 600.17 MHz with a 5 mm ROYAL probe (JEOL ECZ 600, JEOL Ltd., Tokyo, Japan). For both extracts, each acquisition was preceded by a shimming phase along the *x*-, *x/z*-, *y*- and *y/z*-axes performed at 25°C (temperature delay, 120 s). Different setting conditions

were applied with the Delta software package (ver. 5.3) to optimize the quality of spectra acquisition, as follows. For the polar extracts, proton acquisition was performed at 298 K, with a field frequency lock on D_2O, 32,768 data points using a 30° pulse length and 5 s of relaxation delay. A total of 128 scans were collected over a spectral width of 24 ppm. The polar aqueous spectrum was collected by employing the basic shape pulse obs_DANTE_presaturation (JEOL ECZ600R database) for the suppression of water signal in the spectral region between 4.5 and 4.8 ppm (δ = 4.661 ppm). For the non-polar extracts, proton acquisition was conducted at 298 K, with a field frequency lock on methanol-d_4, 65,536 data points using 30° pulse length and 10 s of relaxation delay. A total of 32 scans were collected over a spectral width of 24 ppm.

Data Processing and Multivariate and Univariate Data Analysis

Raw spectra were transferred to the MestReNova 14.2.1 software (Escondido, CA, USA) to manually perform the correction for the phase and baseline. With regard to the polar fraction, all spectra were referenced to TSP (δ = 0 ppm). For non-polar fraction, δ = 0 ppm allowed a perfect alignment of the spectra; therefore, it was also used as the referencing signal. The assignment of ^{1}H NMR signals was supported by the literature [33–39] and online public databases for NMR, such as the Biological Magnetic Resonance Data Bank (BMRB) [40] and the Human Metabolome Data Base (HMDB) [41]. The integration step was manually performed on all fully overlapped spectra to identify and consider only the interesting region; thus, the integration pattern was built considering the region between 0 and 9 ppm for both polar and non-polar fractions. Spectral regions containing resonances only from noise, water and TSP were excluded prior to data analysis. To compensate for the concentration differences, each integral region was normalized to the TSP signal area for the polar fraction or to the total area of each spectrum in the case of non-polar fraction. Finally, the metabolomics dataset was built as a data matrix *N X R* [N = samples; R = ppm buckets], leading to the acquisition of a *41 X 84* (3 444) and a 41 *X* 76 (3 116) data matrix for polar and non-polar fractions, respectively. No missing data were detected in the two matrices. The two datasets were exported to the SIMCA 17 software package (v. 17.0.0, Sartorius Stedim Data Analytics AB, Umea, Sweden), and statistical analysis was

performed separately on the two different fractions. Unsupervised principal component analysis (PCA) and supervised orthogonal partial least squares discriminant analysis (OPLS-DA) were performed for the different objectives they pursue. PCA aims at exploring the natural distribution of the samples within a reduced dimensional space and looking for clustering trends among samples; the PCA score plots facilitate data projection from a higher dimensional space to a lower dimensional one and enable reconstruction of them without prior assumptions regarding their distribution. On the contrary, the OPLS-DA is a discriminant and classification method; it usually provides superior classification results to the PCA (which is not a classification method) because it focuses on the boundaries separating the pre-defined groups in the multidimensional space. Moreover, taking advantage of variable selection methods, OPLS-DA supports the identification of possible biomarkers, thus enhancing the interpretability of the datasets. In the present study, PCA was preliminarily performed on the auto-scaled datasets to explore their characteristics and detect clustering or trends among the samples. The presence of outliers was also checked by evaluating Hotelling's T2 range values (5% level of significance). The PCA models were internally validated with a 7-fold cross-validation (CV), and their quality was assessed by the performance indicators goodness-of-fit (R^2X) and predictive ability (Q^2). To further explore the datasets, the OPLS-DA was performed on the two matrices after Pareto scaling of the data; internal validation of the computed models was carried out with a 7-fold CV, and the R^2X, Q^2 and R^2Y (the fraction of Y variation modelled in that component) values were considered as useful performance indicators for evaluating the global quality of the models. Finally, the variable importance in projection (VIP) scores for OPLS-DA components were designed to find the strongest influence exerted by NMR signals over sample discrimination: as a rule, VIP values > 1 were used to identify the most relevant buckets (selected features), i.e., those with the largest discriminatory power, since scores smaller than one indicate a non-important variable for discrimination between groups [42]. The OriginPro 2019 software (OriginLab Corporation, Northhampton, MA, USA) was used for univariate data analysis. To check the normality of the selected features, the Shapiro–Wilk test was conducted. The non-parametric Mann–Whitney U-test ($p < 0.05$) and the two-sample t-test ($p < 0.05$) were

applied to the selected features not fitting and fitting the normal distribution, respectively, to investigate the differences between the two groups—TX vs. CTRL. The fold change (FC) ratio was calculated by taking the median value of selected features in the TX group over that of the CTRL group to highlight the metabolites' accumulation, where <1 = down accumulated and <1 = up-accumulated [43].

3.2.1.4 Results

Assignment of ^{1}H NMR Spectra Signals of Hepatic Extracts

^{1}H NMR spectra of liver extracts were inspected to investigate the characteristic resonances and gain an overview of the polar and non-polar compounds of pig liver.

The complete list of putatively annotated metabolites based on the chemical shifts, signal multiplicity and chemical formula is highlighted in **Tables 1** and **2** for polar and non-polar fractions, respectively. A representative 600 MHz ^{1}H NMR spectrum of the hepatic polar extract is shown in **Figure 6**.

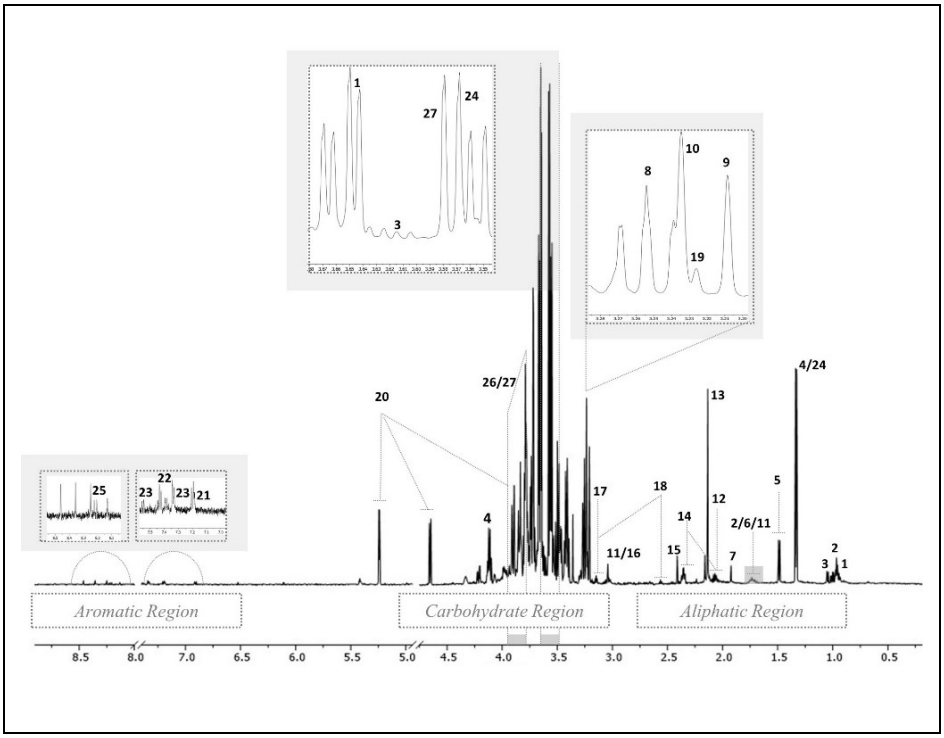


Figure 6. Representative 600 MHz 1D ^{1}H NMR spectrum of the polar extract of pig liver. For the signal numbering (1 to 27) indicated in the spectrum, refer to **Table 2**.

Table 2. Summary of the ^{1}H NMR spectra signals' assignment of the polar extract of pig liver.

BC*	N[a]	Metabolite name	Assigned	Δδ[b]	Formula
AA and derivatives	**1**	Isoleucine	γCH_3 δCH_3 αCH	0.96 (t) 1.04 (d) 3.65 (d)	$C_6H_{13}NO_2$
	2	Leucine	δCH_3 βCH_2 αCH γCH	0.96 (t) 0.99 (d) 3.72 (m) 1.72 (m)	$C_6H_{13}NO_2$
	3	Valine	γCH_3 γCH_3 βCH_2	0.98 (d) 1.04 (d) 2.25 (m)	$C_5H_{11}NO_2$

			αCH	3.61(d)	
AA and derivatives	**5**	Alanine	βCH_3	1.49 (d)	$C_3H_7NO_2$
	6	Lysine	δCH_2	1.72 (m)	$C_6H_{14}N_2O_2$
	8	Taurine	$N\text{-}CH_2$	3.25 (t)	$C_2H_7NO_3S$
	11	Ornithine	½ γCH_2 δCH_2 αCH	1.72 (m) 3.04(t) 3.77(t)	$C_5H_{12}N_2O_2$
	12	Proline	γCH_2	2.06 (m)	$C_5H_9NO_2$
	13	Methionine	δCH_3 αCH	2.13 (s) 3.78 (m)	$C_5H_{11}NO_2S$
	14	Glutamate	βCH_2 γCH_2	2.07 (m) 2.13(m) 2.35 (m)	$C_5H_8NO_4^-$
	16	Creatine	$N\text{-}CH_3$	3.04 (s)	$C_4H_9N_3O_2$
	18	β-Alanine	CH_2COOH $N\text{-}CH_2$	2.56 (t) 3.18 (t)	$C_3H_7NO_2$
	21	Tyrosine	C3H&C5H C2H&C6H	6.91 (d) 7.20 (d)	$C_9H_{11}NO_3$
	22	Phenylalanine	C3H&C5H C4H C2H&C6H	7.33 (m) 7.38 (m) 7.42 (m)	$C_9H_{11}NO_2$
	24	Threonine	γCH_3 αCH	1.33 (d) 3.57 (d)	$C_4H_9NO_3$
	17	Glutathione	αCH	3.77 (m)	$C_{10}H_{17}N_3O_6S$
	23	Tryptophan	αCH C1H C3H	4.06 (m) 7.33(s) 7.55(d)	$C_{11}H_{12}N_2O_2$
	26	Glutamine	βCH_2 αCH	2.13 (m) 3.77 (t)	$C_5H_{10}N_2O_3$
Organic Acids	**4**	Lactate	βCH_3 αCH	1.33 (d) 4.12 (q)	$C_3H_5O_3^-$

	7	Acetate	CH_3	1.92 (s)	$C_2H_3O_2^-$
	15	Succinate	CH_2	2.41 (s)	$C_4H_4O_4^{-2}$
Carbohydrates	**20**	α-Glucose	C_4H C_2H C_3H C_1H	3.41 (t) 3.54 (m) 3.72 (t) 5.24 (d)	$C_6H_{12}O_6$
		β-Glucose	C_2H C_5H C_6H C_1H	3.24 (m) 3.83 (m) 3.90 (dd) 4.65 (d)	
Choline and derivative	**9**	Choline	$N\text{-}(CH_3)_3$ βCH_2 αCH_2	3.21 (s) 3.53 (dd) 4.07 (m)	$C_5H_{14}NO^+$
	10	Glycerophosphocholine	$N\text{-}(CH_3)_3$	3.23 (s)	$C_8H_{21}NO_6P^+$
	19	Phosphorylcholine	$N\text{-}(CH_3)_3$	3.22 (s)	$C_5H_{14}NO_4P$
Alcohol	**27**	Glycerol	CH ½ CH_2 ½ CH_2	3.77 (m) 3.57 (m) 3.65 (m)	$C_3H_8O_3$
Nucleoside	**25**	Adenosine	Ring protons	8.25 (s)	$C_{10}H_{13}N_5O_4$

BC*= Biochemical Category; [a] N= numbering (1 to 27) of the signals indicated in Fig. 2; [b] s= singlet; d= doublet; t = triplet; q= quartet, m= multiplet, dd= double doublets. All chemical shifts were verified comparing experimental values with HMDB (https://hmdb.ca/), BMRB ((https://bmrb.io/), and literature (Wagner et al., 2014; Merrifield et al., 2011; Le Roy et al., 2016; Jiang et al., 2013; Fathi et al., 2017). IUPAC rules have been employed for molecules numbering system.

Although the overlapping of some signals was observed, several metabolites belonging to different biochemical categories were identified along the region of interest (δ 0–9 ppm). As can be seen, the spectrum of the polar extract was divided into three regions—aliphatic, middle, and aromatic—

described as follows. In the aliphatic region, the assignment of resonances at lower frequencies was easily conducted, since the signals and multiplicities (doublet, triplet, singlet) were well distinguishable. In the region from δ 0.9 to 2.7 ppm, the resonances were mainly attributable to amino acids (AA) and organic acids. Specifically, essential AA (isoleucine, leucine, valine, methionine), non-essential AA (alanine, proline, glutamate, tyrosine) and organic acids (lactate, acetate, and succinate) were identified. The identification in the middle region of the ^{1}H NMR polar spectrum from δ 3.0 to 5.2 ppm was very challenging due to the presence of many signals and the overlapping of peaks belonging to protons of different molecules. This region was mainly dominated by peaks attributable to carbohydrates (glucose), but choline and its derivatives (glycerophosphocholine and phosphorylcholine) were identified, too. The aromatic region from δ 6.5 to 8.5 ppm was mainly characterized by aromatic AA phenylalanine and tyrosine. **Figure 7** presents a representative ^{1}H NMR spectrum of the non-polar fraction of liver, which appeared less complex than that of the polar fraction, at a glance (**Figure 6**).

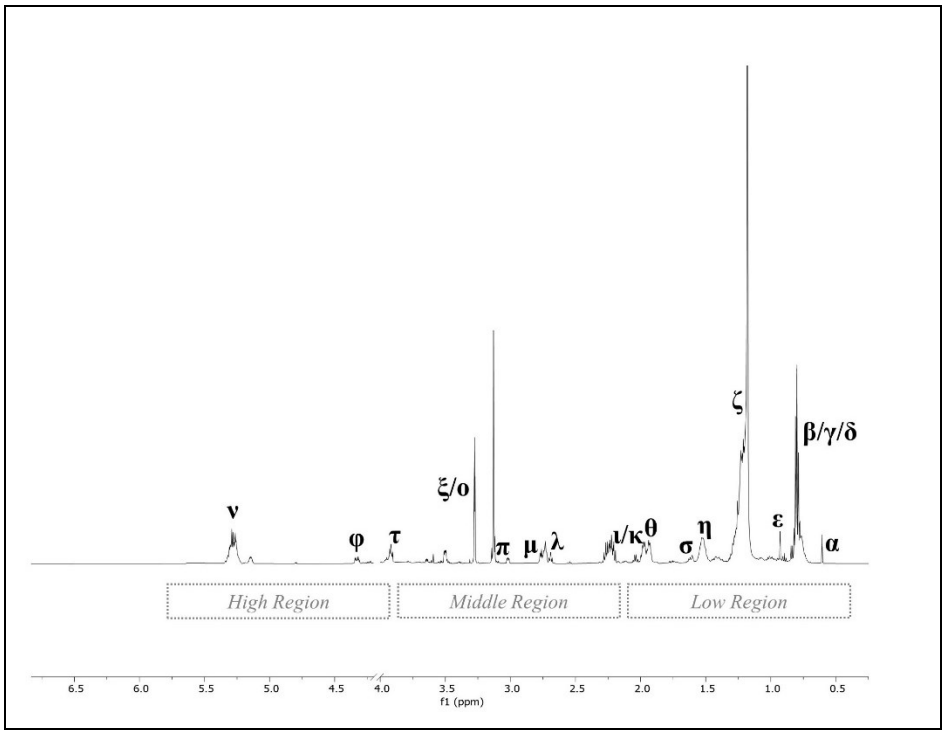


Figure 7. Representative 600 MHz 1D ^{1}H NMR spectrum of the non-polar extract of pig liver. For the signal notation (α to ϕ) indicated in the spectrum, refer to **Table 3**.

Table 3. Summary of the ^{1}H NMR spectra signals' assignment of the non-polar extract of pig liver.

BC*	N[a]	Metabolite name	Assignment	Δδ ppm[b]
Cholesterol	**α**	Total cholesterol	$C_{18}\mathbf{H}_3$	0.61 (s)
	β		$C_{26}\mathbf{H}_3$, $C_{27}\mathbf{H}_3$	0.84 (d)
	δ		$C_{21}\mathbf{H}_3$	0.82 (d)
	ε	Esterified cholesterol	$C_{19}\mathbf{H}_3$	0.93 (s)
Fatty acids	**γ**	Fatty acid residues	ω-C**H**$_3$	0.90 (t)
	ζ		(C**H**$_2$)n	1.19 (m)
	η		$COCH_2$-C**H**$_2$	1.52 (m) 1.61 (m)
	θ		C**H**$_2$-CH= (MUFA and PUFA)	1.97 (m) 2.04 (m)
	κ		-CO-C**H**$_2$	2.23 (m)
	λ		-CH=CH-C**H**$_2$-CH=CH- of linoleic acid	2.68 (t)
	ν		-C**H**=C**H**-	5.28 (m)
	σ		(-C**H**$_2$ all FA except ARA and EPA)	1.61 (m)
	μ	FA, PUFA	CH=CH-C**H**$_2$-(CH=CH-CH_2)n	2.75 (m)
MAG and TAG	**ι**	Monoglycerides (MAG)	FA, RH-C**H**$_2$-CO-O-C_2	2.20 (m)
	ϕ	Triglycerides (TAG)	C_1**H** and C_3**H** of glycerol backbone	4.22 (dd)
			C_1**H** and C_3**H** of glycerol backbone	4.26 (dd)
PL	**ξ**	Sphingomyelin	(-CH_2-N-(C**H**$_3$)$_3$) head group	3.27 (s)
	ο	Phosphatidylcholine (PC)	(-CH_2-N-(C**H**$_3$)$_3$) head group	3.31 (s)
	π	Phosphatidylethanolamine (PE)	-CH_2-C**H**$_2$-NH_2	3.13 (s)
	τ	Total phospholipids		3.92 (m)

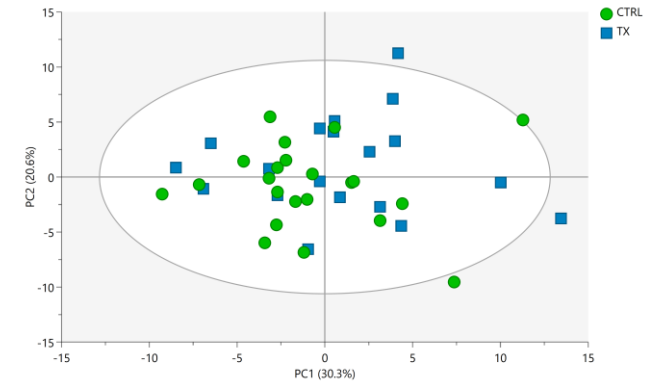
			Glycerol ($C_3\mathbf{H}_2$) of phospholipids	4.02 (m)

BC*= Biochemical category; [a] N= notation (α to ϕ) of the signals indicated in Fig. 3; Cluster midpoint and multiplicity [b] s= singlet; d= doublet; t = triplet; q= quartet, m= multiplet, dd= double doublets. MAG, Monoacylglycerol; TAG, Triacylglycerol; PL, Phospholipid. All chemical shifts were verified comparing experimental values with HMDB (https://hmdb.ca/), BMRB ((https://bmrb.io/), and literature (Wagner et al., 2014; Li et al., 2017; Jiang et al., 2013; Fathi et al., 2017). IUPAC rules have been employed for molecules numbering system.

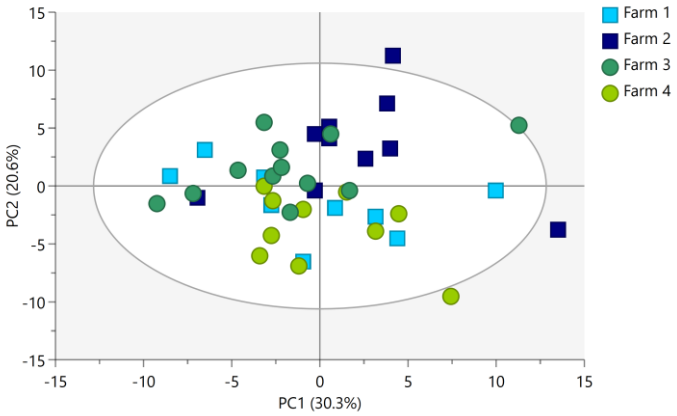
Three main regions were identified: low-, middle- and high-frequency region. Low chemical shift (δ 0.6–2.2 ppm) was predominantly characterized by the intrachain proton of fatty acids and total cholesterol containing the sum of low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and very low-density lipoprotein (VLDL) cholesterol. In the middle and high frequencies (δ > 2.2 ppm), the spectrum was characterized by peaks of protons mainly attributable to phospholipids.

Data Exploration

Unsupervised PCA was initially used as a preliminary tool to examine the structure and characteristics of both polar and non-polar extracts under investigation. For the polar fraction, 71.2% of the overall variance in the spectra was explained by a total of six extracted PCs, and the prediction ability was found to be around 51.2%. The two-dimensional score plot for PC1 and PC2 is reported in **Figure 8a**, where the samples are highlighted by group ID (TX and CTRL), and in **Figure 8b**, where the samples are colored according to Farm ID. Although the quality of the model was good, weak grouping behaviors emerged in samples based on group ID (**Figure 8a**) and Farm ID (**Figure 8b**), indicating a high within-groups variability existing in them.



(**a**) Group ID

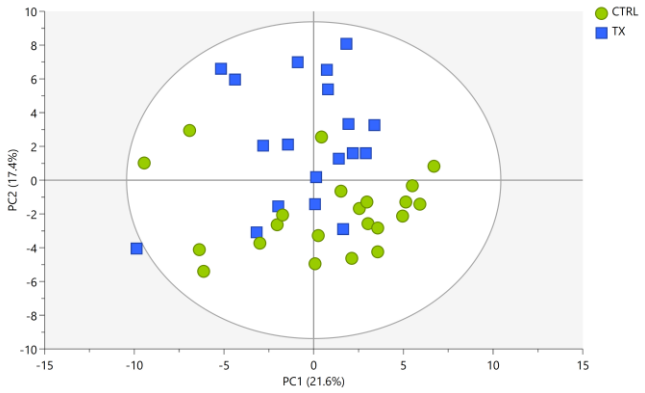


(**b**) Farm ID

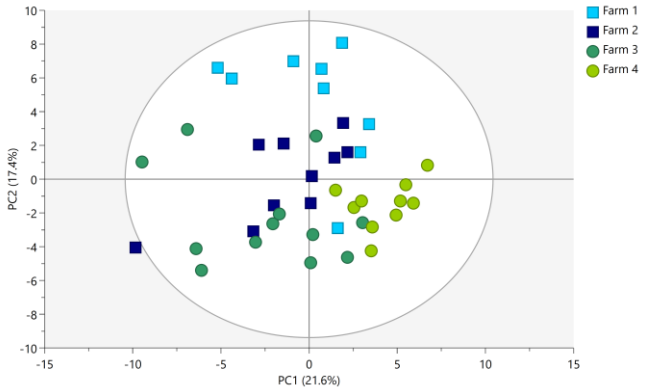
Figure 8. PCA score plots of pig liver polar extracts (R^2X = 0.712; Q^2 = 0.512) colored according to **(a)** group ID (CTRL, green circles; and TX, blue squares) and **(b)** Farm ID (Farm 1, blue squares; Farm 2, light blue squares; Farm 3, green circles; Farm 4, light green circles). The ellipse identifies the 95% confidence interval for Hotelling's T2.

For the non-polar fraction, a total of six PCs covering 72.5% of the total variance were extracted. The goodness of fit and the prediction ability of this PCA model were similar to those provided by the PCA model of the polar fraction. **Figure 9** shows the score plot for PC1 and PC2 of the non-polar extracts

of liver samples colored based on group ID (CTRL and TX; **Figure 9a**) and Farm ID (**Figure 9b**).



(**a**) Group ID



(**b**) Farm ID

Figure 9. PCA score plots of pig liver non-polar extracts (R^2X = 0.725; Q^2 = 0.419) colored according to **(a)** group ID (CTRL, green circles; and TX, blue squares) and **(b)** Farm ID (Farm 1, blue squares; Farm 2, light blue squares; Farm 3, green circles; Farm 4, light green circles). The ellipse identifies the 95% confidence interval for Hotelling's T2.

In the non-polar extracts, a slightly higher grouping trend was observed compared to the polar extracts. Indeed, PC2 seems to have an influence on

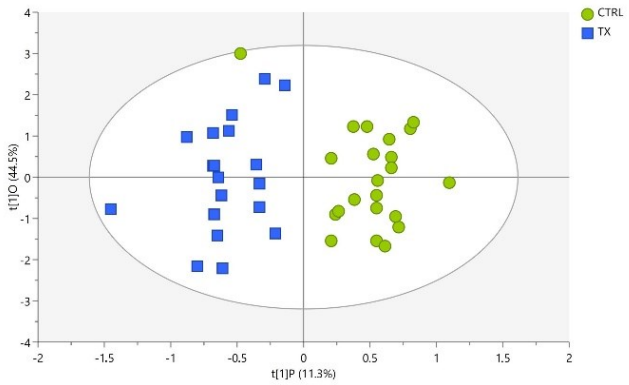
discriminating CTRL samples (PC2 negative values) from TX samples (PC2 positive values) (**Figure 9a**).

As illustrated in **Figure 8** (PCA score plots of polar extracts) and **Figure 9** (PCA score plots of non-polar extracts), the first two PCs collectively accounted for 50% and 39% of the overall extracted variability. The portion of the variability in the data matrices remaining unexplained by PC1 and PC2 alone should not be interpreted negatively for several reasons. Firstly, it is crucial to emphasize that the PCA was conducted using cross-validation to prevent overfitting. Consequently, the optimal number of PCs best suited for capturing the underlying data structure and the fraction of variance extracted by each of these components were automatically determined. As a result of cross-validation, the final PCA models for both polar and non-polar extracts were not limited to only two PCs but included six PCs, collectively explaining 71.2% and 72.5% of the total dataset variability. It is worth mentioning that the first two PCs, when considered individually, explain approximately ¾ of the overall variability for the polar extract (accounting for 70% (50% of a total of 71.2% in **Figure 8**)) and half of the overall variability (54% (39% of a total of 72.5% in **Figure 9**)) for the organic extract. This scenario is frequently encountered when applying multivariate statistical techniques in the analysis of high-dimensional omics data. The underlying factors contributing to the suboptimal cumulative score (PC1 + PC2) of explained variance may have included the natural complexity and high dimensionality of the data matrices. The dataset under analysis was highly complex and given the considerable number of original NMR variables subjected to PCA (i.e., 84 and 76 ppm buckets for polar and non-polar extract data matrices, respectively), reducing them to only two PCs, which capture almost all the variability, was highly unlikely. Another factor worthy of consideration is possible data noise. As is typical with omics data, the analysed dataset inevitably contained natural variability unrelated to the primary factors of interest and linked to the high biological variability of the samples. Thus, this noise may have reduced the proportion of variability explained by the first two PCs.

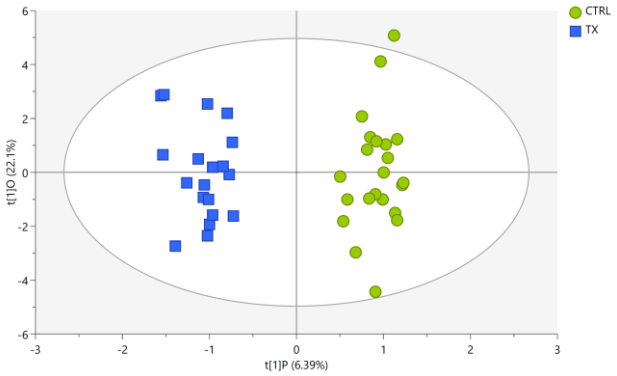
Considering the known high biological variability of the samples under investigation, this finding was deemed promising. Therefore, the ability of supervised multivariate analysis to discriminate liver samples was investigated.

Discriminant Models and Feature Selection

Following the encouraging outputs from the PCA, the supervised OPLS-DA was applied as a discriminant method able to distinguish the variation in the dataset with the predictive component, which is related to the class membership, and the orthogonal component describing the systematic variation within a class [44]. Two OPLS-DA models were built separately for polar and non-polar fractions aimed at distinguishing samples based on whether or not they were exposed to antibiotics and highlighting the driving forces among the variables most effective in discriminating between treated vs. untreated pigs. The unsupervised clustering trend became more pronounced, and the models' diagnostic tools revealed extremely good performances of the models of both fractions, particularly in the case of the non-polar liver extract. The score scatter plots of the polar and non-polar extracts are depicted in **Figure 10a** and **Figure 10b**, respectively.



(a) Polar Extracts



(b) Non-polar extracts

Figure 10. OPLS-DA score plots of pig liver **(a)** polar extracts and **(b)** non-polar extracts for the CTRL (green circles) and TX (blue squares) groups. The ellipse identifies the 95% confidence interval for Hotelling's T2.

For the liver polar fraction, the two groups of samples were perfectly separated in the bi-dimensional space, since samples from the CTRL group were distributed along the positive t[1]P component, and samples from the TX group were distributed along the negative component. Nevertheless, a mild degree of heterogeneity was observed within samples in the same group, which were not closely grouped along the vertical axis. The intra-class non-predictive variability, of which 44.5% was collected by the t[1]O component, was expected to be present but did not hinder the correct discrimination of untreated (CTRL group) vs. treated (TX group) pigs, except for one sample in the CTRL group, which fell into the left side of the plot, corresponding to the negative scores, where all the TX samples were located, but within the 95% confidence ellipse (**Figure 10a**). Satisfying statistical metrics were provided by internal cross-validation of the model: a fitting ability of about 89% (R^2X= 0.892), extracted predictive variation explaining around 77% of the information related to the specific class membership of the samples (R^2Y = 0.774) and a predictive power of approximately 61% (Q^2 = 0.613) proved the validity of the model. A classification accuracy of approximately 98% was obtained in the misclassification test, corresponding to only one sample being misclassified in the CTRL group.

Similar to the polar fraction, the OPLS-DA applied to the non-polar dataset returned very good quality parameters of the model: $R^2X = 0.939$, $R^2Y = 0.948$ and $Q^2 = 0.869$. A correct classification rate of 100% was obtained, revealing that all samples were correctly allocated in their group ID, as can be observed by the perfect separation between the two groups in the score scatter plot reported in **Figure 10b**.

Subsequently, the VIP analysis was applied in order to identify the NMR spectral signals, which mostly contributed to the successful separation of the liver samples achieved by both OPLS-DA models. Overall, 17 and 11 NMR signals were found to be characterized with VIP values > 1 in the polar and non-polar extracts, respectively. According to the results of the Mann–Whitney test and the two-sample t-test, 11 out of 17 selected features of the polar fraction and 6 out of 11 features of the non-polar fraction were statistically significantly different ($p < 0.05$).

Following the assignment of the resonances, the list of the identified metabolites, which were significantly different ($p < 0.05$) among the CTRL and TX groups—provided with their corresponding VIP score and FC value—was compiled and is reported in **Table 4**. As can be observed, unlike the NMR spectra of the polar extracts, in which the assignments of H resonances were unambiguously attributed to specific molecules, the NMR spectra of non-polar extracts were in some cases ascribed to classes of molecules (i.e., unsaturated fatty acids). According to the positive values of FC, all metabolites from the polar fraction appeared to be up-accumulated in the TX group of antibiotic treated pigs, while for the non-polar fraction, differences in metabolite accumulation were found.

Table 4. Discriminant metabolites between the antibiotic-treated (TX group) and untreated pigs (CTRL group).

Metabolite	**VIP score**[a]	**FC**[b]
Polar extract of pig liver		
Glucose	3.30	1.80

Glutathione	2.55	1.10
Proline	2.49	1.13
Tryptophan	2.29	1.13
Choline	1.30	1.23
Lactate	1.20	1.20
Non-polar extract of pig liver		
Fatty Acid	4.50	0.95
Phospholipids	2.92	1.52
Phosphatidylcholine	1.94	1.25
Total Cholesterol	2.88	0.89
Unsaturated Fatty Acid	1.08	0.88

[a] VIP=variable importance in projection; [b] FC= fold change

3.2.1.5 Discussion

As far as the authors know, this is the first NMR-based untargeted metabolomics study comparing the liver metabolome of antibiotic-treated vs. untreated heavy pigs. An NMR based analysis was used by Merrifield et al. [34] to provide a system overview of porcine metabolism via characterization of the liver and other organs and biofluid metabolomes to establish a metabolic framework from which pathology-or nutrition-based variations could be compared. In all living organisms, the liver is the organ in charge of the metabolism of major constituents (i.e., carbohydrates, proteins, and lipids), but it is also recognized as the most important site for metabolic clearance of xenobiotics, synthesis of molecules and vitamin storage, playing a crucial role in metabolic homeostasis [45]. Based on the findings of the present pilot study, the application of an untargeted metabolomics approach returned different metabolic fingerprinting of pigs' livers exposed to antimicrobial agents compared to untreated ones. Moreover, changes in the metabolism of the liver attributable to the differences in metabolite accumulation between TX and CTRL groups were observed, answering the starting biological question: *"Are there any metabolic differences between treated vs. untreated pigs?".*

In the ^{1}H NMR spectra of highly complex biological tissues, peak overlapping inevitably occurs, in particular for intact tissue sample analysis. In this case, the spectral assignment may be supported by 2D experiments [34]. In the present study, the polar and non-polar extracts of the liver were investigated separately, and 2D NMR experiments were not performed, as the resulting spectral signals were sufficiently resolved to enable the discrimination of resonances from the various compounds in both liver fractions. The investigation of separated aqueous and organic extracts of the liver is the approach commonly used in ^{1}H NMR-based metabolomics studies aimed at investigating hepatic metabolic changes; this strategy was used to study paracetamol-induced hepatotoxicity in pigs [36], hyperlipidemic hamsters [38], chicken and avian metabolism [37,39] and the impact of dietary sesamin on Atlantic salmon [33]. The metabolic atlas of porcine liver provided by Merrifield et al. [34] was particularly useful for the spectra signal assignment of the polar extract, whereas the assignment of the non-polar extract was mainly supported by studies addressing the lipophilic extract of the liver [33,38,39], the review by Li et al. [33], as well as online public databases [40,41].

Relevant ^{1}H NMR signals reported in **Table 4** were investigated based on their fold change ratio to opportunely check the nature of metabolites characterizing the discrimination between the two groups. In particular, the up-accumulation of glucose (FC = 1.80), glutathione (FC = 1.10), proline (FC = 1.13), tryptophan (FC = 1.13), choline (FC = 1.23) and lactate (FC = 1.20) recorded in the polar liver extract from pigs in the TX group is probably due to a wide array of factors, among which, the antimicrobial administration and all related life conditions in which the pigs lived. It is worth mentioning that the antibiotic treatment characterizing pigs in the TX group most likely underlies a pathology, which has been cured, implying that the animal has experienced a physiological state different from that of a healthy animal. As a general remark, antimicrobial administration should occur in the presence of proven disease for responsible and prudent usage of antimicrobials, leading veterinarians to only prescribe them under strict conditions (i.e., clinical signs and symptoms, laboratory results, costs) [46]; considering this scenario, it cannot be excluded that pigs from the TX group might have experienced a stress condition during their life cycle, which, in other words, means that the sphere of animal welfare was

threatened in the Health Domain—one Domain within the Five Domains model of animal welfare—and pigs were in a poor welfare condition for at least a well-defined time of their life (i.e., time of disease) [47]. In the field of animal-based measures, it is widely accepted that glucose represents the primary physiological indicator of stress [48]; during a stressful condition, the catecholamine released from the adrenal medulla regulates glycogenolysis in the liver, leading to maintenance of adequate glucose levels in blood circulation, entailing degradation of glycogen into glucose-6 phosphate, which is then hydrolyzed into glucose [49,50]. In addition to glycogenolysis, hepatic glucose production also relies on gluconeogenesis using glucose precursor molecules, such as amino acids, lactate, pyruvate, and glycerol, for de novo synthesis of glucose [50]. The up-accumulation of lactate in the liver of pigs in the TX group highly supports the link between carbohydrate metabolism and animals living in a poor welfare condition, which may have been represented by the state of disease. In the present study, pigs in the TX group had 1.2 times as much lactate as those in the CTRL group, suggesting that increased energy demand characterized the pigs exposed to antibiotics administration. In fact, lactate is highly involved in the Krebs cycle of generating energy from stored energy reserves [51], representing an alarm bell for metabolic disorders in a living organism [35,52]. In this context, the authors refer to the higher energy state of the TX group, in which the disease status may have solicited a higher energy request from the diseased organism than in the healthy animals. In particular, in the pathological state, the greater demand for energy for the immune and reparative processes takes place in the face of a reduced ingestion of food and often a lower oxygen saturation, which characterizes the frequent diseases affecting the respiratory system of the pig. Glutathione (GSH) is a tripeptide, particularly concentrated in the liver, playing a key role in the defense against oxidative stress. Liver is the organ most vulnerable to toxins and oxidative stress, and the concentration of GSH is greatly sensitive to environmental factors, heavy metals, glucose and xenobiotics [53]. In this study, the accumulation of GSH was slightly higher in the TX group than in the CTRL group, and we may suppose that a role could be played by the disease state, together with the antibiotic treatment. However, the literature investigating the relationship between GSH and antibiotic treatment in the liver

of pigs is poorly explored; therefore, only an assumption related to the biological role of metabolite in the investigated organ may be reported. Choline plays a key role in membrane integrity, lipid metabolism and methylation. Additionally, in this case, this metabolite was up-accumulated in the livers of pigs in the TX group. Similar to GSH, the literature is lacking for comparative purposes; however, the up-accumulation of choline seemed to follow the same direction as phosphatidylcholine—highly involved in regulating lipid, lipoprotein and energy metabolism [54]—whose accumulation was observed by assessing the non-polar extract of the liver. Proline, together with tryptophan, both up-accumulated in the TX group, are included in Aminoacyl-tRNA biosynthesis according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) [55]. This finding may be supported by the fact that most antibiotics are designed to specifically target ribosomal protein synthesis and aminoacyl-tRNA synthetases—a family of enzymes playing a central role in protein synthesis [56]. Intriguing findings were obtained when considering the non-polar fraction of the liver. In fact, the fold change in the TX pigs' group showed the down-accumulation of fatty acid residues ((CH_2)n in fatty acyl chain, FC = 0.95), total cholesterol ($C_{18}H_3$ total cholesterol, FC = 0.89) and unsaturated fatty acid (–$CHCH_2CH$ = in fatty acyl chain:20:4/22:6, FC = 0.88); however, phospholipids (>C_3H_2 in the glycerol backbone of PL, FC = 1.52) and phosphatidylcholine (–$CH_2N+(CH_3)_3$ in the PC head group, FC = 1.43) were up-accumulated in the TX pigs' group. In addition to liver metabolome, the transcriptomic analysis provided insights for the understanding of enzyme regulation in the case of antimicrobial treatment. According to Hu et al. (2020), the transcriptome and DNA methylome analysis of pigs' liver fed with low-dose of antibiotics displayed an increase in nicotinamide N-methyltransferase (NNMT) expression, a positive regulator of gluconeogenesis in primary hepatocytes, which could stabilize the Sirtuin 1 protein necessary for glucose and cholesterol metabolism to reduce the abundance of cholesterol in serum and liver [57,58]. The higher levels of glucose and the lower levels of cholesterol in the livers of pigs in the TX group could be supported by the role of NNMT, considering that liver is the organ characterized by the strongest expression of this enzyme [57,58]. Additionally, liver boasts of an excellent production of metabolic substrates for energy metabolism (i.e., fatty acids), which are made available during stressful

situations to support the organism [49]. The proof of this might be found in the lower level of fatty acids in the liver characterizing the TX pigs' group. Liver hepatocytes synthesize bile acids via cholesterol metabolism, and the decreasing level of total cholesterol ($C_{18}H_3$) observed in the TX group may suggest that high conversion activity in the liver is performed to promote the synthesis of bile acid. In addition, the up-accumulation of phosphatidylcholine in the TX group was found to be in agreement with the higher level of choline in the treated group, considering that choline undergoes phosphorylation in order to build the phospholipids.

3.2.1.6 Conclusions

The present study is the first step in broader research, which has the ultimate goal of identifying and validating biomarkers as authentication tools for antibiotic-free pork supply chains. As a first attempt to gain an insight into the metabolome of pigs, NMR spectroscopy was chosen as the instrumental analytical platform. This exploratory investigation demonstrated the feasibility of the NMR-based metabolomics approach for detecting metabolomic fingerprinting useful for the discrimination of livers of pigs on the basis of antibiotic treatment exposure. The untargeted approach was powerful in screening samples and detecting molecular signatures and changes in the liver metabolome, both polar metabolites and lipidome of pigs in the two considered conditions. This preliminary outcome encourages a more in-depth investigation via other analytical techniques widely used in metabolomics studies. Indeed, MS-based methods offer higher performance than NMR in terms of sensitivity—which is extremely useful for measuring species with low abundance but potentially valuable information—and specificity, helping the elucidation of the chemical structures of potential metabolites of interest.

This finding is very promising, taking into account the broad variability in the animals from commercial farms included in the study. Because the main objective of this pilot study was to ascertain the performances of an untargeted analytical strategy as a tool to answer the question, "Has this pig ever been treated with antibiotics?", no specific focus was given to the classes of

antibiotics the pigs were administered in the present study. The authors are of the opinion that a fit-for-purpose experimental design and the application of MS-based metabolomics platforms should enable the acquisition of more in-depth information and identification of putative biomarkers enabling the confirmation or rejection of an antibiotic treatment, bearing in mind that this issue serves the authenticity and health purposes in the pig chain.

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Case Study II

3.2.2 Case Study 2

Research Article

NMR-based untargeted metabolomic to explore the role of kidney and muscle for Antibiotic Free pig meat authentication

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Manuscript in preparation

Keywords: pigmeat authentication, untargeted metabolomics, Antibiotic Free, label claim, NMR

3.2.2.1 Abstract

Over the years, consumers' attention towards food safety, quality, and authenticity aspects has increased affecting the meat supply chain. This trend resulted in the introduction of new label claims assuring aspects related to the protection of animal welfare (i.e. rearing practices) and the attention to antibiotics exposure. Therefore, the adoption of voluntary certification schemes together with the declaration of new label claims provided new value-added meat and meat products to the meat supply chain. In the present study, a 1D ^{1}H NMR-based metabolomics approach coupled with the multivariate data analysis was performed on the kidney and diaphragm of pigs to differentiate antibiotic-free (ABF) *versus* antibiotic-treated (ABT) animals. The results revealed a significant discrimination of ABF from ABT animals, particularly when considering the aqueous extract of muscle and diaphragm. Conversely, the results obtained from the non-polar extract for both cases were less informative about the discrimination between ABF *vs* ABT. Finally, the aqueous extract was further investigated by identifying all those metabolites responsible for the discrimination between ABF *vs* ABT, mainly belonging to the following biochemical categories: carbohydrates, amino acids and derivatives, organic acids, alcohol, choline and derivatives, and nucleosides. The role of the kidney and diaphragm was discussed in light of the metabolic differences highlighted by the comparison between two groups of pigs differently exposed to antibiotics administration.

3.2.2.2 Introduction

By 2032, global pigmeat consumption is projected to grow 11% considering an increase of 0.7 kg/year/person on an edible retail weight equivalent basis (OECD/FAO, 2023) confirming the importance of the meat sector despite a plethora of meat-alternatives diets have emerged.

Through the years, the timeline of the events related to the meat sector was negatively marked by numerous food scandals that led the meat chain to end up in the crosshairs of media and journals. In this regard, some of the best-known food scandals to mention are Bovine Spongiform Encephalopathy (BSE) in cows or the horse meat in beef, known as “horse gate”, and the Melamine in milk that caused great concern in public opinion on a worldwide scale (Bradford et al., 2022). Raising consumer’s attention to food safety, quality, and authenticity has led to the growth of antibiotic-free (ABF) meat production eliciting several producers and retail chains to move their meat production systems toward a “free” direction (Mohammadi et al., 2023). Currently, the ABF claim for pigmeat production primarily relies on voluntary certification schemes which allow the food business operators to implement good management practices in farms to pursue animal welfare and consequently avoid the exposure of pigs to antibiotics treatment.

To date, the usage of antibiotics is tightly ruled by multiple European regulations that cover several aspects of antibiotic usage - Regulation (EU) 1944/2022, Regulation (UE) 1946/2022, and Regulation (UE) 625/2017- given that antimicrobial resistance is still recognized as a severe public health risk. For the protection of the public health, a decisive legislative action was undertaken to ban antibiotics as growth promoter – Regulation (EC) No 1831/2003- and other legislative measures to promote the lower usage of antibiotics. Among mandatory EU laws, a legislative pillar in the field of pharmacologically active substances setting the maximum residue levels (MRLs) in foodstuff of animal origin is the Regulation (EU) No 37/2010 which determines the limits of defined substances to be found in food matrices to assure the safety of meat and meat-based products.

Trends in meat market highlight higher attention to the farming and production method (i.e. “free” foods chain: animal raised without antibiotics, feed OGM free) which inevitably affect the price and brand reputation (Cornish et al., 2020). Despite the severe EU regulatory framework and the voluntary certification schemes for ABF labelling, it results quite challenging assure that meat and meat-based products are truly derived from an ABF supply chain. This complication is mainly due to the fact that higher level of assurance may be demonstrated only with bureaucratic documentation for supporting the traceability of meat supply chain; however, a chemical traceability should be considered to check the compliance with the declared claim given that these vulnerability factors may encourage the food fraud within the entire supply systems. The abovementioned barriers in ABF meat detection could be overcame with more reliable analytical methods to prove the authenticity of food avoiding any mismatch between food product claims and food product characteristics (CWA 17369: Authentic and fraud in the feed and food chain-Concepts, terms and definitions, 2019).

In the meat sector, cases of frauds usually involve the origin (i.e. sex, breed, feed, organic/conventional), the substitution of meat, fat and protein, the processing (i.e. irradiation, fresh/thawed), and the addition of non-meat ingredient (i.e. water) (Ballin, 2010). For antibiotic-free label claims authentication, the scientific literature displayed that most of the researchers are carried out to prove compliance with legislation - this mainly relies on the quantification of antibiotics residues to test if the substance residues are below the MRLs (Ramatla et al., 2017; Patyra et al., 2020). However, according to Ballin this approach is not conclusive to test the absence of antibiotic treatment and, therefore there is not a guarantee of ABF meat authentication (Ballin, 2010). As a result, the compliance with EU legislation in the field of pharmacologically active substances satisfactorily assure the food safety with the adoption in most of the cases of targeted approaches that fulfil the requests to identify and quantify the antibiotic residues. For label claims authentication purposes, following this approach may limit the investigations since unknown or unexpected markers might be unavoidably excluded because not considered during the investigation leading to serious scandals (Cavin et al., 2016; Mialon et al., 2023). In this sense, these limitations may affect ABF meat authentication

since focusing the research on pre-defined substances or related metabolites causes a priori exclusion of unknow interesting compounds. As a proof of the importance of the untargeted applications, the melamine scandal in milk in 2008 was not detected by the targeted methodologies adopted at the time (Mialon et al., 2023). All these premises lead to suppose that the need for more sensitive approaches is highly encouraged in the field of food authenticity.

The untargeted metabolomics approaches allow to identify novel biomarkers in ABF meat authentication (Mialon et al., 2023). Within the hierarchy of -omics sciences, metabolomics is the most sensitive to external variations (i.e. nutrition, environment, antibiotics administration) and this peculiar property allows to successfully explain the relationship between the organisms (i.e., animal, human) and all the external factors (i.e. antibiotics administration) by measuring metabolites levels. Generally, the study of metabolites, small molecular weight molecules (< 1000-2000 Da) (Cajka et al., 2016), is performed with NMR- or MS-based techniques coupled to chemometric techniques to investigate if two or more groups of samples can be discriminated and the nature of variables having this discriminating power. This analytical platform makes the metabolomics untargeted investigations versatile enabling to perform retrospective analysis (Jongedijk et al., 2023; Liesenfeld et al., 2020). Up to date, the untargeted metabolomics workflow follows a block scheme - experimental design, sample analysis, data analysis, and biological interpretation- which can be modelled according to the needs and the equipment of laboratories making the approach far from being standardized.

In the current study, an untargeted metabolomics approach based upon the 1D ^{1}H NMR spectroscopy (^{1}H NMR) was employed for samples fingerprinting in combination with chemometrics techniques to investigate changes in metabolome due to antibiotics exposure in relevant pig matrices, kidney, and diaphragm. This work is a part of a wider research project aiming to identify novel biomarkers to prove the authenticity of ABF label claims in relevant pig matrices. The optimistic results obtained from the exploration of liver metabolome of pigs (Fabrile et al., 2023) elicited the need to investigate other food matrices for the same authenticity purposes. Therefore, kidney (aqueous and organic fraction) and diaphragm (aqueous and organic fraction) were

investigated to understand which kind of food matrix can better explain the discrimination and if it is possible focusing the research just on metabolome or lipidome for authentication purposes with the aim to enhance sampling strategy and focusing on the most informative extract for differentiate ABF from ABT meat samples.

3.2.2.3 Material and Methods

Chemical and Reagents

HPLC grade methanol, chloroform, and water were purchased from Labscan (Dublin, Ireland). Analytica grade sodium dihydrogen phosphate monohydrate and di-sodium hydrogen phosphate dihydrate were supplied by Merck (Darmstadt, Germany). Deuterium oxide (99.9% D), methanol-d4 (99.8% D), and chloroform-d4 (99.8% D) were obtained from VWR International BVBA (Geldenaakseban, Leuven Belgium) and 3-(trimethylsilyl)-propionate-d4 (TSP) from Sigma-Aldrich (Milano, Italy).

Experimental design

A total of 103 heavy pigs were randomly selected to investigate metabolome and lipidome of kidney and diaphragm belongings to Antibiotic Free group (ABF) and Antibiotic Treated (ABT) group of pigs. The experimental plan was formulated considering pigs reared according to specific requirements to produce PDO Parma ham. Therefore, the pigs were slaughtered at 270 days of age (9 months about) experiencing the same environmental condition and livestock management practices (lactation, weaning and, growing finishing) complying with the animal welfare law requirements before they were slaughtered. Also, the pigs involved in this study were characterized by the same genetic factors (*Italian Large white*, *landrace and duroc*). For this experimental plan, the selected pigs were reared in different farms of northern Italy and collectively transported to the same commercial abattoir to be slaughtered under the supervision of the veterinary team. As general remark, pigs were divided in two groups according to their exposure to antibiotics administration during their entire lifecycle. In details, group classification was designed

considering the AMU estimated by calculating a treatment incidence 100 (TI_{100}) using the Defined Daily Dose Animal for Italy ($DDDA_{it}$) as standard, as described in a previous study on Italian fattening farms (Scali et al. 2020). The schematic representation of the experimental design is displayed in **Figure 11**.

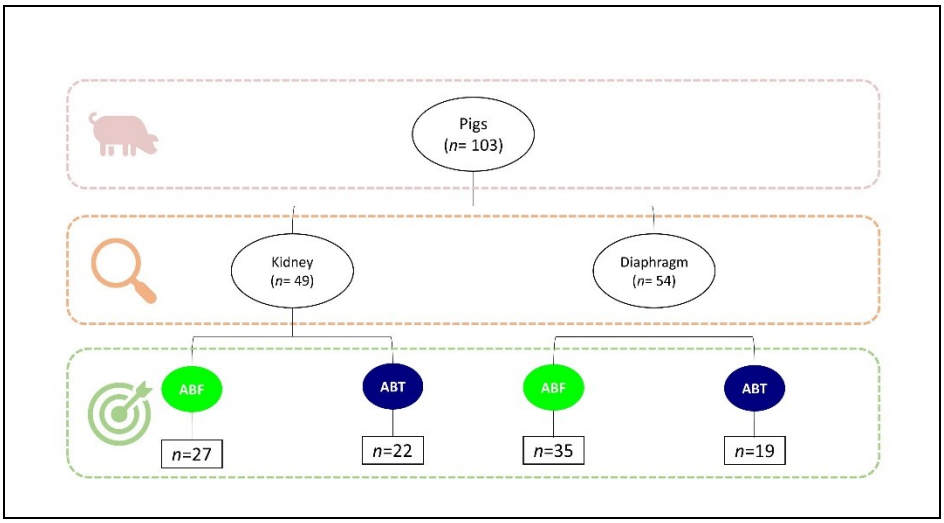


Figure 11. Schematic representation of experimental design. A total amount of 103 heavy pigs were selected to collect 49 kidney and 54 diaphragm samples of pig. Within each targeted sample, a comparison between ABF (control group) in green and ABT (treated group) in blue was performed according to the following structure: 27 ABF *vs* 22 ABT samples of pig's kidney and 35 *vs* 19 samples of pig's diaphragm.

For a greater reliability of the experimental design, kidney and diaphragm were sampled from different pigs to capture the highest biological variability trying to simulate a real meat market condition. As concerning the ethical statement, this investigation was performed on meat samples intended for human consumption and for this reason no animal was sacrificed for experimental purposes.

Kidney

49 samples of kidney were collected and labelled ABF and ABT, as follows: kidney collected from pig that were never exposed to antibiotic administration (ABF_K, *n*=27) and kidney collected from pigs that during their life experienced the antibiotic administration (ABT_K, *n*= 22). All samples were collected during the same day and immediately frozen at -20°C until the analysis. For the sample

preparation, medulla (B) and cortex (A) were considered as representative region of kidney, as displayed in **Figure 12**. Briefly, the kidney was divided in two specular part and just one side was considered for the subsequent sample preparation.

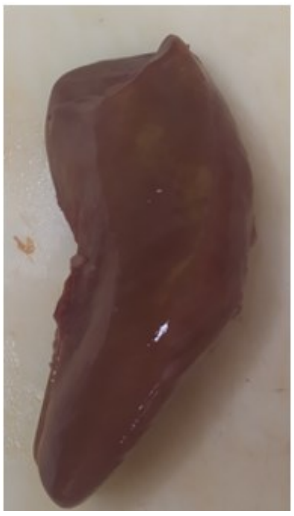
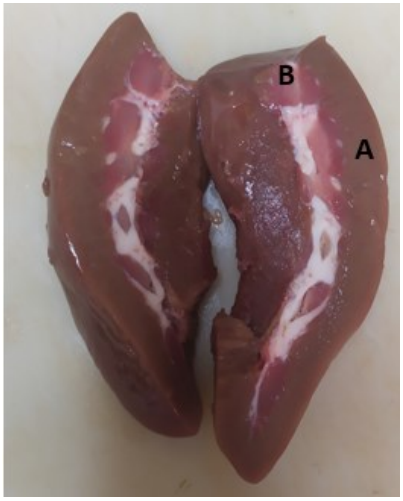
Whole kidney	Divided kidney
	

Figure 12. Longitudinal section of kidney of pig. A pigs kidney section containing (A) Cortex and (B) Medulla was considered as targeted sample to be analysed.

Diaphragm

54 samples of muscle tissue were collected in the diaphragmatic area of pigs and accordingly to their classification were labelled as ABF and ABT, as follows: muscle collected from pigs that were never exposed to antibiotic administration (ABF_M, n =35) and kidney collected from pigs that during their life experienced the antibiotic administration (ABT_M, n = 19). All samples were collected during the same day and immediately frozen at -20°C until the analysis. Concerning sample representativeness, no assumption was made since a small portion of diaphragm was cut off from casings. However, the connective tissue was removed from muscle sample to consider only the muscular tissue.

Sample preparation and metabolites extraction

Kidney and diaphragm samples were extracted following the readaptation of Bligh & Dyer protocols, a biphasic extraction procedure (BLIGH EG, DYER WJ, 1959). 100 mg of frozen manually grinded samples were extracted in 3 mL of a Methanol/Chloroform solution (2:1, *v/v*) and vortexed for 30 s. To improve the extraction yield, all samples were sonicated in a water-ice bath for 30 min and newly vortexed after adding an equal volume of chloroform and water, 1 mL per solvent. To optimize phase separation, samples were centrifuged (4.500 rpm, 35 minutes, 4°C). Up to this point, lipophilic and hydrophilic metabolites were in the same falcon. For both kidney and diaphragm, the metabolites dissolved in extraction solution were split into two different Eppendorf according to their polarity; therefore, the upper hydrophilic extract and the lower lipophilic extract were separately transferred in two different Eppendorf. To summarize, a polar and non-polar extract were collected from each type of sample – kidney and diaphragm. All extracts were dried in a sample concentrator using a nitrogen flow at room temperature. Prior ^{1}H NMR analysis, the concentrated hydrophilic extracts were dissolved in 700 µl of phosphate buffer (pH= 7.0; Na_2HPO_4/NaH_2PO_4 0.25 M, 100% D_2O) and transferred into 5-mm NMR tubes while lipophilic extracts were resuspended with Methanol-d_4 and Chloroform-d_4 and collected into 5-mm NMR tubes. All the resuspended extracts were stored at -80 °C until the NMR analysis.

^{1}H NMR fingerprinting of muscle and kidney

1D ^{1}H NMR spectra of the lipophilic and hydrophilic extracts of kidney and diaphragm were recorded on a NMR spectrometer operating at magnetic field of 600.17 MHz which was equipped with a 5 mm ROYAL probe (JEOL ECZ 600, JEOL Ltd., Tokyo, Japan). The ^{1}H NMR acquisition was preceded by a shimming phase conducted on both axial and radial components for each extract. Delta software package (ver. 5.3) was used to optimize settings for lipophilic and hydrophilic analysis, as follows. For hydrophilic analysis- for both kidney and diaphragm- proton acquisition was performed at 298 K, with 32 768 data points using a 30° pulse length and 5 s of relaxation delay. A total of 128 scans were

collected. The aqueous extract spectrum was collected by employing the basic shape pulse obs_DANTE_presaturation (JEOL ECZ600R database) for the suppression of water signal in the spectral region between 4.5-4.8 ppm. The analysis of lipophilic extract - for both kidney and diaphragm- was conducted at 298 K, with 65 536 data points using 30° pulse length and 10 s of relaxation delay. A total of 32 scans were recorded. In both cases, the spectral width recorded was set to 24 ppm to gain a lower distortion of the baseline (Musio et al., 2020). Finally, the raw spectra were obtained and collected.

Preprocessing of ^{1}H NMR spectra and data

All spectra were exported to MestReNova 14.2.1 software (Escondido, CA, USA) and visually inspected for the correction of phase and baseline. These preliminary operations on ^{1}H NMR spectra were easily conducted since the spectral width of 24 ppm allowed to obtain lower distortion of the base line or in some cases it was present at the extreme chemical shift of spectra. In agreed with this, larger spectral width is recorded, and the distortion will characterize the lowest and highest part of the spectrum where usually no relevant signals are found. Subsequently, all ^{1}H NMR overlapped spectra – categorized by type of sample and type of extract - were visually inspected to manually perform the selection of buckets of relevant signal. For kidney and diaphragm, the spectral region containing relevant proton resonances was between 0-9 ppm in both aqueous and organic extracts. For aqueous extracts of kidney and diaphragm, all ^{1}H NMR spectra were referenced to TSP resonance (δ= 0 ppm); while for organic extracts all spectra were referenced to the resonance at δ= 0 ppm since a perfect overlapping of all spectra was gained. A perfect overlapping of all spectra is highly pursued since the integration pattern was manually performed by visually inspecting the spectra to select relevant peaks. Therefore, data matrix was built considering the area of peak for each integrated bucket.

For aqueous extracts, the area of integrated peak was normalized to TSP signal while for organic extract the area of integrated peaks was normalized according to the relative percentage. Finally, a *N x M* (N = number of samples; M= ppm buckets) raw data matrix was obtained for both samples and kind of extracts.

Univariate and multivariate statistical analysis

The generated datasets for both extracts and kind of samples were separately imported to SIMCA 17 software package (v. 17.0.0, Sartorius Stedim Data Analytics AB, Umea, Sweden) for the multivariate statistical analysis.

For screening purposes, the unsupervised Principal Component Analysis (PCA) was performed to evaluate the presence of strong and moderate outliers and to visualize the putative grouping trends among all samples. The quality of the models was described considering the goodness-of-fit (R^2X) and the predictive ability parameter (Q^2) by using the default internal SIMCA seven-fold cross validation (CV-ANOVA p-value < 0.05 for model significance), by permutation tests (200 permutation) on data scaled to unit variance to exclude model overfitting.

For classification purposes, the Orthogonal Partial Least Squares- Discriminant Analysis (OPLS-DA) was performed on data scaled to unit variance and the models were validated in the same way described before. In this case, R^2X, the proportion of variance of the response variable that is explained by the model (R^2Y), and Q^2 were used as descriptive parameters for the quality of the model. The validated OPLS-DA models were used to predict the ABF condition of the samples and the correct classification rate was considered to verify the model predictive ability. OPLS-DA together with VIP (variable importance in projection) approach were carried out considering the most discriminant variables all those buckets with VIP score ≥ 1.0.

The list of relevant buckets was imported to OriginPro 2023 software (OriginLab Corporation, Northhampton, MA, USA) to perform univariate data analysis. The Shapiro-Wilk test was performed to test the normal distribution of populations, as fundamental requirement prior parametric statistics (Vinaixa et al., 2012). For all those relevant buckets which satisfied the normality condition, the parametric two sample T test was performed to check statistical significance ($p < 0.05$) between two groups. For the cases in which the normality condition was rejected, the non-parametric statistical test Mann Whitney was performed to check the statistical significance between two groups. All the buckets resulted

discriminant by confirming both the VIP ≥ 1.0 and resulting statistically significant $p < 0.05$ with both parametric and non-parametric statistics were considered for the identification and discussion. Although this study is far from being quantitative, the differences in metabolites levels between the two different conditions under investigation were calculated as the fold change (FC) ratio of the median intensities related to ABT/ABF to capture the magnitude of the differences on metabolites level. Relevant resonances were annotated by searching in literature (Merrifield et al., 2011; Le Roy et al., 2017; Dargue et al., 2020) and databases the Human Metabolome Data Base (HMDB) and Biological Magnetic Resonance Bank (BMRB).

3.2.2.4 Results and discussion

In this work, the untargeted NMR-based metabolomics approach was used to investigate putative differences in metabolome of pigs' kidney and diaphragm accordingly to the exposure of animals to antibiotic administration with the final aim to discriminate ABF from ABT pigmeat for authenticity purposes. The experimental set up allowed to investigate both the metabolome and lipidome for each food matrix, obtaining aqueous and organic extracts respectively, for a cross-comparison study. In the following paragraphs the results obtained for both food matrices will be presented and discussed.

Unsupervised statistical model for kidney and diaphragm

As first evaluation, the unsupervised multivariate statistics PCA was conducted on the NMR spectral data to obtain an insight into the effect of antibiotic administration on metabolic profile of pig's kidney and diaphragm. As general remark, the PCA was performed to assess samples clustering and evaluate the variance of datasets without providing any priori information regarding classes. Each built dataset was individually processed to characterize and describe allegedly trends withing samples and/or kind of extract.

The PCA model built for the aqueous extract of kidney resulted in 7 principal components (PCs) cumulatively explaining the 78.6% of the total

variance (R^2X) with a coefficient of predictive ability of Q^2=50.2%. The PCA firstly modelled highlighted the presence of a strong outlier located in the T2 Crit (99%) of the Hotelling's T2Range Line plot. The assessment of the suspected outlier was performed by visually inspecting the ^{1}H NMR spectrum and the conclusion was that an error occurred during the acquisition phase. All information related to the PCA model calculated on the original dataset were reported in Annex I. Hence, a second PCA model was recalculated filtering the original dataset by the analytical outlier. Therefore, the second model (*n*=48) was performed on data scaled to unit variance giving a model described by 6 PCs explaining R^2X= 75.9% and Q^2=60.1%. Although the total number of PCs and the score of R^2X of the recalculated model decreased compared to the original dataset, the score for Q^2 increased. Graphically, the PCA score plot of the recalculated model showed in **Figure 13 A(1)** did not emphasized a clear separation between ABF and ABT group of pigs, at a glance; however, a slight tendency of ABF samples group to internally clustered emerged.

The dataset related to the organic counterpart of kidney was also submitted to the same data workflow. Thus, the first PCA algorithm initially returned two strong outliers located in the T2 Crit (99%) of the Hotelling's T2Range Line plot and similarly to the water-soluble extract of kidney, a second PCA model was recalculated by excluding the outliers. For transparency, all information related to the PCA model calculated on the original dataset were reported in Annex I. Overall, the quality of the recalculated PCA model built for organic extracts of kidney was not so optimal compared to the aqueous extract. In fact, the recalculated PCA model (*n*= 47) resulted in 6 PCs with a coefficient of R^2X= 66.9% and Q^2= 34.8%. Although the recalculated model returned better value of Q^2 than the first model, the performance of the model described by the scores of both R^2X and Q^2 was considered not so good, in this screening phase. According to this, the PCA score plot was not able to provide a clear distinction between two groups of samples as shown in **Figure 13 A(2)**.

Similarly, the data processing pipeline carried out for muscle's dataset was based upon the same structure above described. In the case of polar extracts of muscle, the PCA algorithm applied to the original datasets returned a strong outlier located in the T2 Crit (99%) of the Hotelling's T2Range Line, referring to

Annex I. All the preliminary models were collected and shown in Annex I since original datasets were filtered by the analytical outliers; thus, all the recalculated models were discussed in the following sections. The second PCA model (*n*=53) was performed on polar spectral data scaled to unit variance giving a model described by 8 PCs explaining R^2X= 79.3% and Q^2=57.9%. By selecting the first two PCs, the recalculated model explained more than half (R^2X cum = 45.1 %) of the total explained variance. Finally, the PCA score plot showed **Figure 13 B(1)** clearly highlighted an optimal discrimination between ABF and ABT pigs.

For the organic extract of diaphragm, the recalculated PCA model (*n*=53) was performed on data scaled to unit variance giving a model described by 7 PCs explaining R^2X= 77.2% and Q^2=46.0%. The first two components were considered to plot the data explaining the 52.8% of the variance within the dataset (42.8% in PC1 and 10% in PC2). In this case, the score plot showed in **Figure 13 B(2)** suggested that two groups were not able to separate perfectly along the two PCs selected.

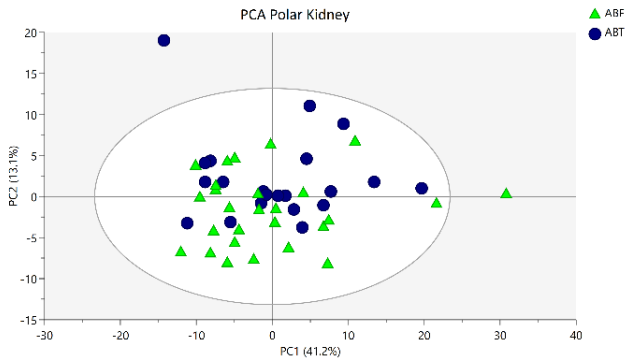
As a general remark, the unsupervised methods are typically carried out to evaluate the presence of trends within a dataset without using class membership information (Granato et al., 2018). In the present study, the PCA models built for water-soluble extracts of both food matrices, kidney, and diaphragm, displayed a better performance compared to the organic extracts of the same food matrix. This assumption was postulated not only by the evaluation of the objective performance parameters (R^2X and Q^2) but also through the graphical visualization of the score plots showing the discrimination between ABF *vs.* ABT samples, as illustrated in the **Figure 13**. In **Table 5** all values related to the performance of the unsupervised PCA models were summarized to easily compare the models.

Table 5. Overview of the performance parameter (R^2X and Q^2) of PCA models built for kidney (Aqueous and Organic Extracts) and Diaphragm (Aqueous and Organic Extracts) of pigs.			
Food matrix	**Extract**	**R^2X**	**Q^2**
Kidney	Aqueous extract	0.759	0.601
	Organic extract	0.669	0.348
Diaphragm	Aqueous extract	0.793	0.579
	Organic extract	0.772	0.46

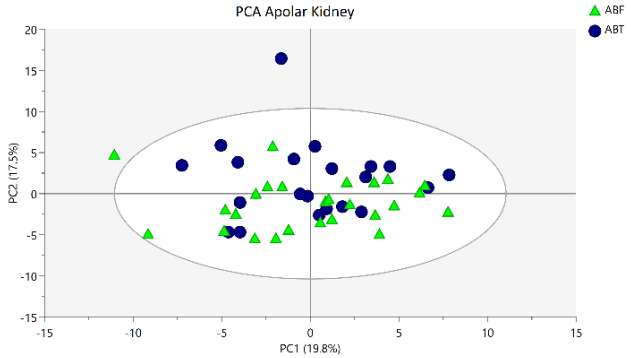
Comparing all the PCA score plots, the best performance was observed for the polar extracts of pigs' muscle. According to this finding, these unsupervised models suggested various assumptions about the role of metabolome and lipidome of kidney and diaphragm in relation to the variable under observation, the antibiotic administration. In this sense, the organic extracts for both kidney and diaphragm were not exhaustive in displaying the discrimination between ABF *vs.* ABT pigs. This outcome can be preliminary discussed by taking into consideration different perspectives. Firstly, the absence of evident differences in both organic extracts between two groups may be attributable to confounding factors other than antibiotic administration. In other terms, it might be possible that other external factors, such diet, can affect lipidome by masking the antibiotic administration variable. In this sense, distinct factors such as genes, feeding systems, and geographical location were proved to affect lipidome of the skeletal muscle (Li et al., 2022). Among all factors, literature research displayed a significant emphasis on the impact of feeding condition on lipidome of different animal species and type of samples (Wang et al., 2021; Wang et al., 2022; Rocchetti et al., 2022). Additionally, the preliminary outcomes for both muscle and kidney lipidome lead to pose attention to the choice of NMR as analytical platform. In fact, the relatively low NMR sensitivity may not allow to detect discriminant metabolites peculiar for this object of investigation, given that NMR lacks sensitivity to detect metabolites in the sub-micromolar range (Bhinderwala et al., 2018). Another potential explanation may be related to the experimental design and the choice of samples type. In

fact, the experimental design for this investigation was set considering the pivotal role of biological replicates and, thus, the inherent variability in each pig's biological answer. Additionally, the significant of biological role for each sampled organ and tissue must be considered for a thorough discussion of the results. The Kidney is well-established for its function in the excretion of substances within the organism and for all those clearance reactions during drug exposure (Dhondt et al., 2020). On the other hand, the diaphragm is the main muscle involved in the in respiratory act, located in the abdominal cavity. Therefore, the biological role should be taken into consideration when different results emerge by each organ. Nevertheless, this study was intended to get a molecular snapshot of the current meat market. In other terms, no priori hypothesis was postulated concerning the putative biological answer characterizing each organ/tissue since the main goal was investigating, on experimental level, marketed meat samples.

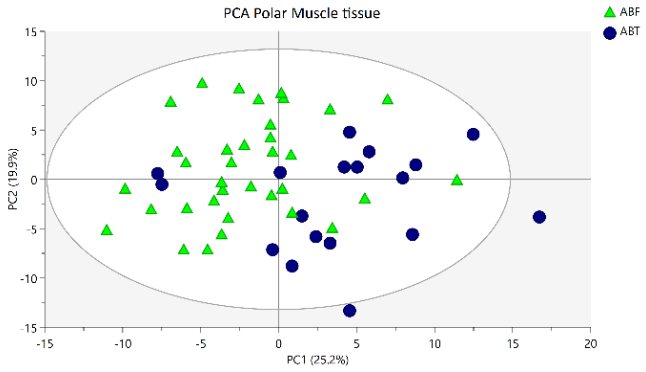
To summarize, the unsupervised PCA highlighted a great natural grouping trend in samples of muscle for the polar extract, while a discrete performance was highlighted for polar extract of kidney. On the contrary, the PCA for organic extracts of both matrices was not able to discriminate samples of animals treated with antibiotics from a control animals' population.



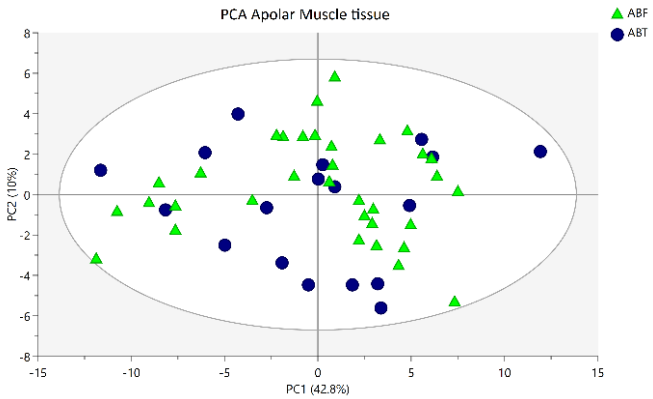
A(1) Polar extract of kidney



A(2) Apolar extract of kidney



B(1) Polar extract of muscle tissue



B(2) Apolar extract of muscle tissue

Figure 13. Score plots of PCA models built for kidney and muscle tissue considering polar (A1-B1) and apolar (A2-B2) extracts of each sample, respectively. The first two PCs were taken into consideration for all PCA models displayed. A) Kidney (A1-R^2Xcum=54.3%; A2- R^2Xcum=37.3%); B) Muscle tissue (B1-R^2Xcum=45.1%; B2- R^2Xcum=52.8%)

Discriminant analysis and important features: aqueous extracts

In the initial chemometric phase conducted for screening purposes, all PCA models were preliminarily discussed considering three essential elements, as follows: the role of each sample, the role of analytical platform, and the role of biological variability. Based on the encouraging unsupervised outputs, the supervised statistics was carried out for classification purposes, focusing exclusively on the polar extracts from both kind of samples. In this section of the work, the results obtained from the polar extracts of both food matrices will be presented and discussed to gain a deeper understanding of the discrimination between two groups – ABT and ABF – and the discriminant metabolites responsible for samples classification. The selection of putative discriminant metabolites was performed by considering only the aqueous extract since the preliminary results from unsupervised statistics did not yield satisfactorily insights concerning the lipophilic extract.

Finally, the multivariate supervised statistics OPLS-DA was employed as classification model to distinguish between ABT from ABF pigmeat. In the

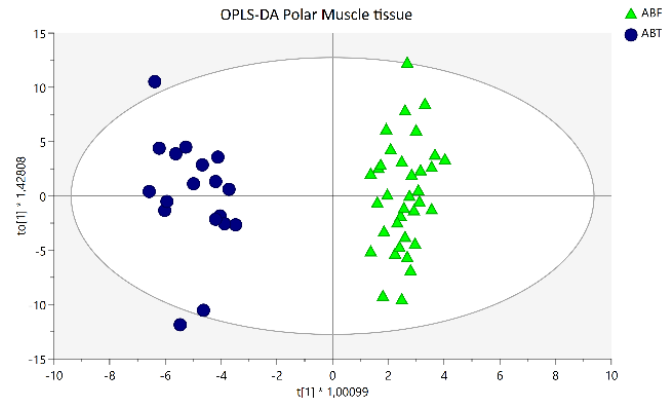
context of authentication, the OPLS-DA models are often performed to investigate the classification of samples and the discriminant metabolites (Rivera-Pérez et al., 2023; Mandrone et al., 2021).

In the case of polar extract of muscle, ^{1}H NMR spectral data were scaled to unit variance and a 7-fold cross validation was employed to carry out the OPLS-DA models. The quality of the OPLS-DA model was deemed satisfactory, considering the high score of 0.955 (R^2Y) and the 0.729 (R^2X). Also, the score of the predictivity ability of the model was Q^2=0.763 indicating that the metabolic phenotype univocally identifies the class of samples, ABF and ABT, respectively. The OPLS-DA score plot showed in **Figure 14(a)** clearly revealed a discrimination based on the variable under investigation: the antibiotic treatment. Indeed, two main clusters of samples can be differentiated along the predictive component: the antibiotic-free samples were located along the positive values of *x-axis*, whereas the antibiotic-treated samples populated the negative values of *x-axis*. The score plot revealed a slight degree of heterogeneity within each class, as samples for each distinct group were distributed on both the positive and negative sides of *y-axis*.

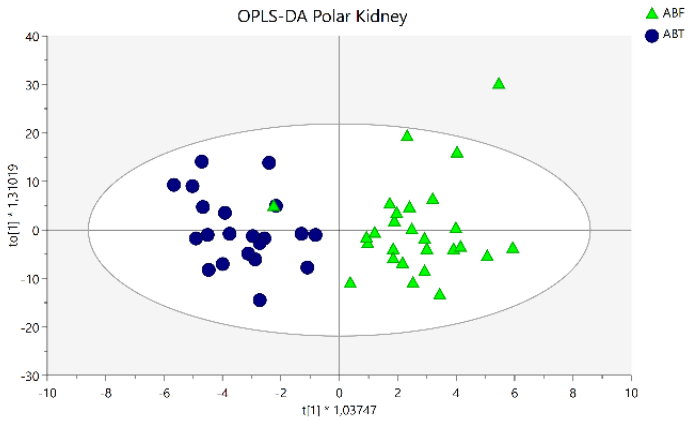
In the opinion of the authors, this finding was both expected and desired for two main reasons. On the one hand, it was expected because the biological replicates were considered to test the hypothesis. On the other hand, it was desired to capture the highest variability in biological responses from pigs. This approach aimed to provide a comprehensive understanding of whether antibiotic administration unequivocally affects the metabolome of the targeted tissue.

The same data treatment pipeline was applied to the dataset related to the polar extracts of kidney. In the case of polar extract of kidney, ^{1}H NMR spectral data were pareto scaled and a 7-fold cross validation was employed to carry out the OPLS-DA models. In this case, the quality of the OPLS-DA model for polar kidney samples was considered discrete in comparison with the results obtained from muscle. The model was described by 0.729 (R^2Y) and the 0.514 (R^2X). Additionally, the score of the predictivity ability of the model was considered good Q^2=0.466.

In both cases, the OPLS-DA score plots clearly distinguished samples classified as ABF from samples classified as ABT.



(a) Polar extracts of muscle tissue



(b) Polar extracts of kidney

Figure 14. Supervised OPLS-DA score plot showing the discrimination between ABF samples in green triangle *versus* ABT samples in dot blue for (a) the polar extract of muscle tissue and (b) the polar extract of kidney.

The role of muscle tissue: a focus on polar extract

For the selection of the discriminant buckets, all metabolites with a VIP score ≥ 1 were chosen and considered. Among the resulting VIP metabolites, only those exhibiting statistically significant differences between two groups were selected through the evaluation with parametric and non-parametric statistics. Consequently, all ^{1}H NMR signals deemed relevant through the VIP approach were assessed with univariate statistics. Firstly, the normal distribution of data was assessed with the Shapiro-Wilk test. For normal distributed data, the two-sample t-test (p-values < 0.05) was performed while when normal distribution condition was rejected, the Mann-Whitney U was performed. Also, the Welch Correction was applied in case of the equal variance condition was not assumed. Finally, the identification of ^{1}H NMR relevant buckets was performed when both VIP ≥ 1 and p-values < 0.05 conditions were satisfied. Also, the FC was calculated as the ratio of the median value of ABT samples to the median value of ABF: all positive values of FC indicated that the metabolites under observation were up accumulated. All data related to the annotated metabolites, univariate statistics and FC were summarised in **Table 6**.

Table 6. List of all relevant metabolites (VIP > 1.0, p < 0.05) involved in the discrimination of polar extract of muscle tissue related to pigs ABF versus ABT.

N.	Annotated Metabolites	VIP	FC	R*	p-value < */**/***	Biochemical category
1	β- Glucose	1.76	1.23	↑	6.448E-9**	Carbohydrates
2	α-Glucose	1.36	1.53	↑	1.093E-4**	
3	Myo-inositol	1.57	1.20	↑	2.108E-6**	
4	Glycerol	1.59	1.37	↑	1.361E-5*	Alcohol

5	Glycerophosphocholine	1.25	1.28	↑	3.458E-4*	Choline and derivatives
6	Phosphorylcholine	1.31	1.25	↑	2.792E-4**	
7	β-hydroxyibutyrate	1.29	1.13	↑	4.826E-4**	Organic acids
8	Acetate	1.11	1.12	↑	0.025*	
9	Phenylalanine	1.24	1.16	↑	0.003*	AA and derivatives
10	Leucine	1.20	1.07	↑	0.002**	
11	Alanine	1.22	1.05	↑	0.002**	
12	Arginine	1.07	1.07	↑	0.019**	
13	Valine	1.03	1.09	↑	0.034**	
14	Proline	1.24	1.09	↑	0.002**	
15	Tyrosine	1.25	1.23	↑	0.002*	
16	Threonine	1.42	1.13	↑	4.153E-5**	
17	Asparagine	1.07	1.32	↑	0.028***	
18	Isoleucine	1.35	1.20	↑	0.001*	
19	Lysine	1.15	1.08	↑	0.003**	
20	Aspartate	1.07	0.88	↓	0.043**	
21	Cysteine	1.54	1.23	↑	6.027E-6**	
22	Glutamate	1.12	1.13	↑	0.017*	
23	Ornithine	1.07	1.00	↑	0.044*	
24	Inosine	1.19	1.06	↑	0.003**	Nucleoside
25	LDL	1.36	1.29	↑	0.461E-4*	Lipid-based molecule

26	*Unknown*	1.26	1.06	↑	0.001**	*Unknown*

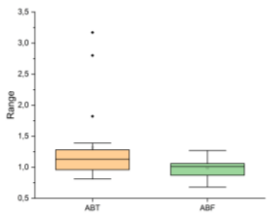
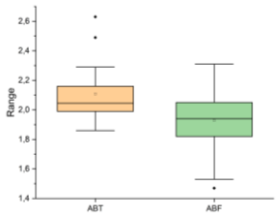
R*: Regulation

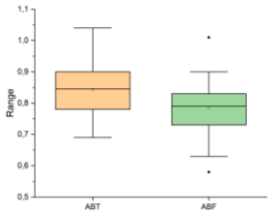
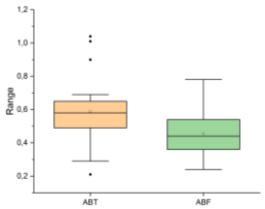
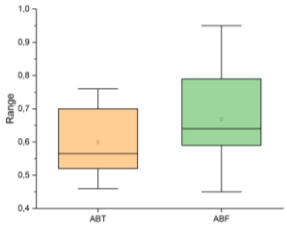
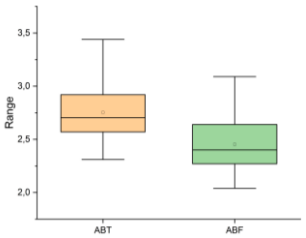
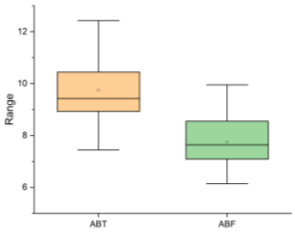
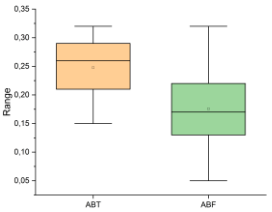
***The exact p-value obtained from Mann-Whitney Test at 0.05 level of significance.

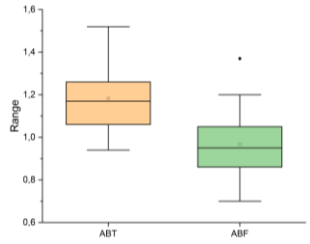
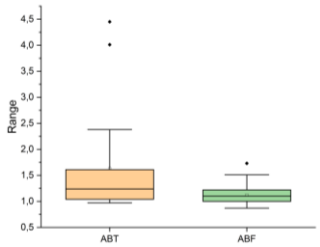
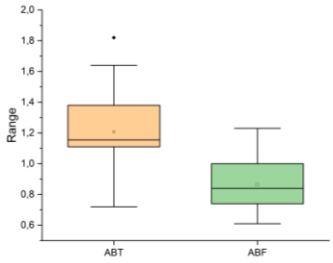
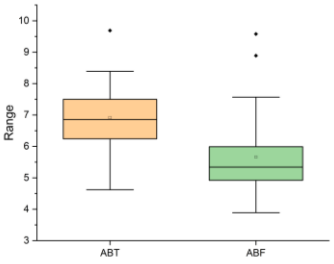
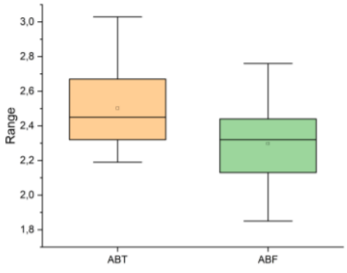
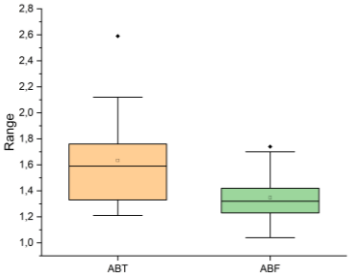
** The p-value obtained from Two Sample T test at 0.05 level of significance.

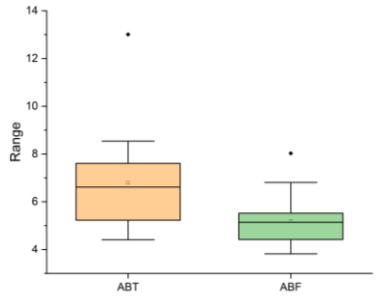
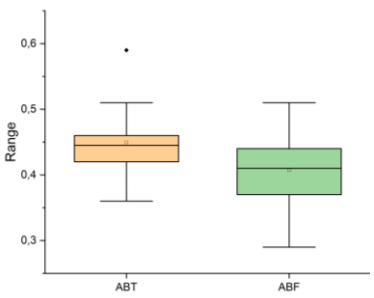
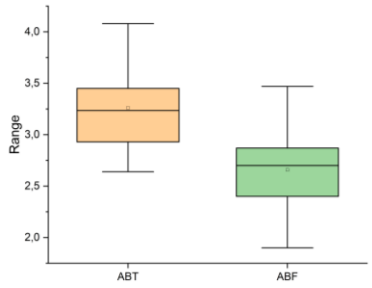
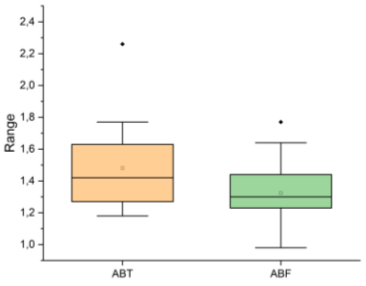
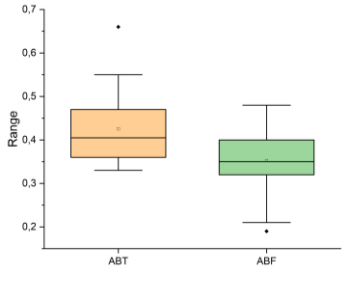
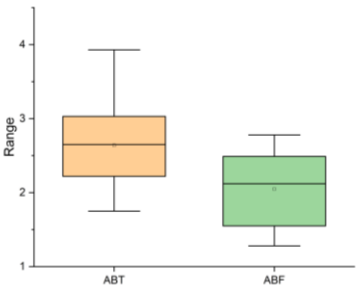
***When normality is assumed, and the equal variance is not assumed the Welch Correction was applied.

According to the **Table 6**, the discriminant annotated metabolites were grouped under different biochemical categories ranging from carbohydrates (α-Glucose, β-Glucose, and Myo-Inositol), Ammino Acids and derivatives (Phenylalanine, Leucine, Alanine, Arginine, Valine, Proline, Tyrosine, Threonine, Asparagine, Isoleucine, Lysine, Aspartate, Cysteine, Glutamate, and Ornithine), Organic Acids (β-hydroxyisobutyrate and Acetate), Alcohols (Glycerol), Choline and Derivatives (Glycerol, Glycerophosphocholine, and Phosphorylcholine), Nucleoside (Inosine) and lipid-based molecule (LDL). Differences in the accumulation of metabolites between ABT and ABF was displayed in the box plots in **Figure 15**. All annotated metabolites were up-accumulated in ABT phenotype compared to ABF phenotype except for the aspartate that resulted down-accumulated in ABF phenotype.

Acetate	**Alanine**
	

Arginine	Asparagine
	
Aspartate	**β-hydroxyibutyrate**
	
β-Glucose	**α-Glucose**
	

Cysteine	Glutamate
	
Glycerol	**Glycerophosphocholine**
	
Inosine	**Isoleucine**
	

LDL	Lysine
	
Myo-Inositol	**Ornithine**
	
Phenylalanine	**Phosphorilcholine**
	

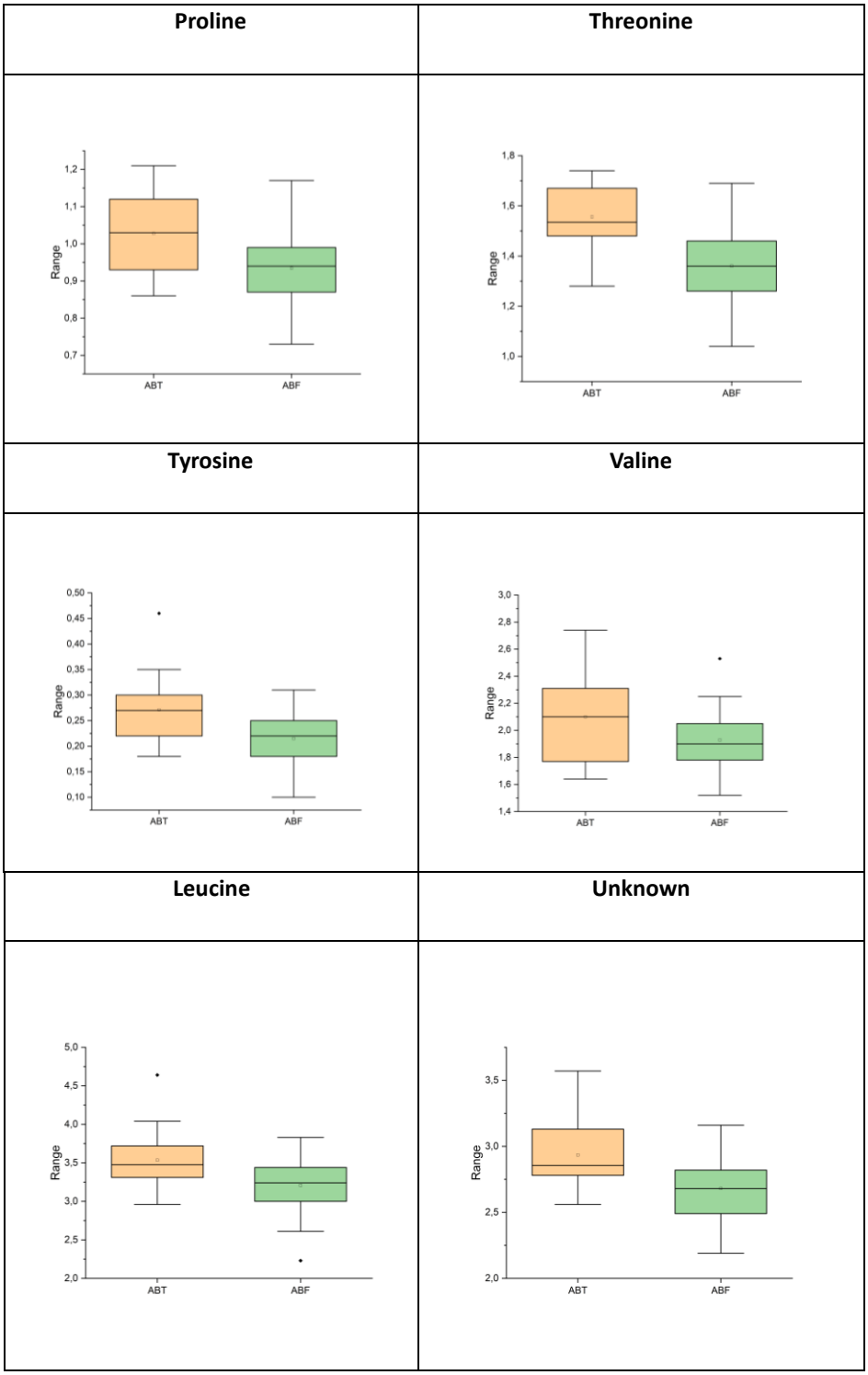


Figure 15. Box plots of annotated metabolites of polar extract of muscle tissue from ABT (orange) and ABF samples (green).

Among all the annotated metabolites, the most populated biochemical category was related to amino acids. This may suggest that amino acids metabolism may play a substantial role in replying to antibiotic administration. In fact, it was not surprising the fact that most of the annotated metabolites were amino acids – about the 50% of all annotated metabolites- given that muscle is a significant source of amino acids in meat (Ma et al., 2020); the noteworthy finding lies in the fact that these discriminant metabolites were differentially regulated between the two phenotypes, mainly showing an up-accumulation in ABT pigs group.

In agreement with our findings, Mu et al. explored the impact of in-feed antibiotics on metabolic profiles in cecum, colon, and feces of piglets, concluding that the main metabolism affected was related to amino acids (Mu et al., 2017). Additionally, Antunes et al. reported that the antibiotic administration affected sugar, amino acid, bile acid, and lipid metabolism (Antunes et al., 2011). Even when considering other types of non-edible samples, alterations in metabolome of pigs induced by the administration of antibiotics were detected. In the literature, the role of muscle was also investigated by Yan et al. to test the effect of antibiotics administration on myofiber types of skeletal muscle and the authors concluded that the antibiotic treatment increased muscle growth mainly due to the shift in fiber-type composition (Yan et al., 2020).

In the present study, the experimental design was set up to avoid prior knowledge about the specific antibiotics treatment used and instead focused on discriminating samples according to the statement declared on meat label, particularly the “Antibiotic Free” claim. Therefore, no particular assumptions were made concerning a specific class of antibiotics affecting the metabolism of diaphragm, muscle tissue, or kidney. However, literature proved that muscular metabolism may be indirectly affected. In fact, some antibiotics may alter gut microbiota composition which is strictly related to growth and alterations of muscular metabolism in piglets (Yan et al., 2020). Overall, the main focus of the current investigation was based on the exploration of two phenotypes, antibiotic-treated vs. antibiotic-free pigs, considering the key role of inter-

animal variability for the reliability, recognized essential for robust metabolomic models (Kieken et al., 2009)

The role of kidney: a focus on polar extract

The same work pipeline above described was followed for data handling related to the polar extracts of kidney. Briefly, OPLS-DA coupled with VIP approach (VIP ≥ 1.0) was carried out to select metabolites relevant in the classification and discrimination of ABT *vs.* ABF samples. After, the univariate data analysis was performed to test the statistically significance of the discriminant metabolites. Finally, all metabolites with VIP ≥ 1.0 and p-value < 0.5 were considered and annotated. The annotated metabolites mainly belonged to three different biochemical categories: carbohydrate (myo-inositol), choline and derivatives (Glycerophosphocholine) and amino acids (Creatine, Aspartate, and Proline). After the evaluation of FC, all metabolites resulted up-accumulated in Antibiotic Treated phenotype as summarized in **Table 7**.

Table 7. List of all relevant metabolites (VIP > 1.0, p < 0.05) involved in the discrimination of polar extract of kidney related to pigs ABF versus ABT.

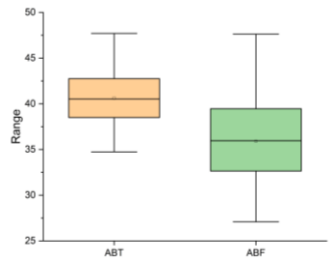
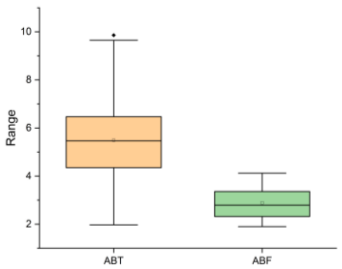
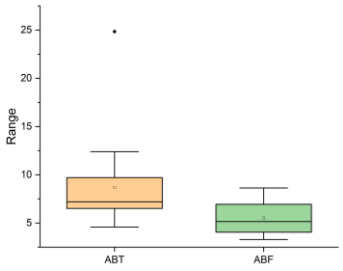
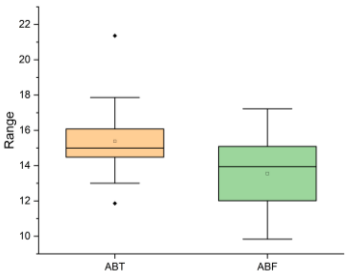
N	Annotated Metabolites	VIP	FC	R*	p value < */**	Biochemical category
1	Myo-inositol	3.08	1.07	↑	0.003**	Carbohydrates
2	Creatine	4.16	1.96	↑	1.43E-07*	AA and derivatives
3	Aspartate	3.61	1.40	↑	1.48E-04*	
4	Proline	1.09	1.20	↑	0.018*	
5	Glycerophosphocholine	5.42	1.13	↑	3.31E-04**	Choline and derivatives

R*: Regulation

***The exact p-value obtained from Mann-Whitney Test at 0.05 level of significance.

**** The p-value obtained from Two Sample T test at 0.05 level of significance.

Differences in metabolites accumulation between ABT and ABF were graphically displayed in the box plots in **Figure 16**.

Glycerophosphocholine	**Creatinine**
	
Aspartate	**Myo-Inositol**
	

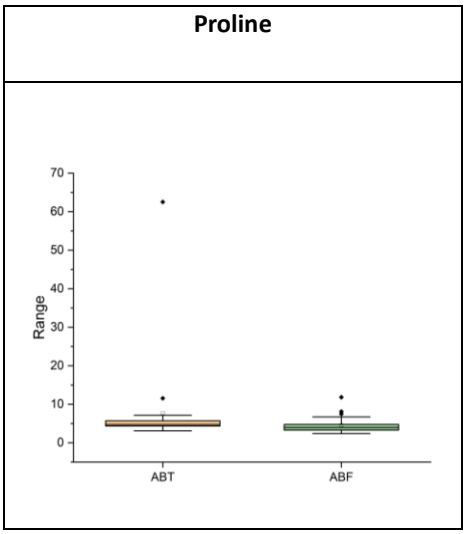


Figure 16. Box plots of annotated metabolites in polar extract of kidney from ABT (orange) and ABF samples (green).

To the best of the authors knowledge, this study represents the first investigation of kidney and diaphragm, as representative of muscular tissue, with the aim to clarify antibiotic-induced alteration in metabolome and lipidome of pigs. The obtained findings are consistent with those discussed in the previous study in which the metabolome of liver of pigs resulted markedly affected by antibiotic administration (Fabrile et al., 2023). In contrast, further investigations of the non-polar extracts of both kidney and diaphragm were avoided since considered not relevant for further discussion.

3.2.2.5 Conclusion

The present study has confirmed the feasibility of employing an untargeted metabolomics approach based on NMR fingerprinting to investigate metabolic differences in pigs exposed to antibiotic administration as opposed to those never exposed to such treatment. Utilizing NMR spectroscopy combined with chemometric techniques allowed to classify organ and tissue and their extracts accordingly to the object under investigation. Additionally, the cross-comparison between different type of samples allowed to highlight differences by comparing the results obtained from muscle with the results of kidney.

Notably, the number of relevant metabolites differently regulated in muscle were higher compared to kidney. According to the authors, this finding may suggest a key role of muscle as samples to be targeted for authentication purposes concerning the antibiotic administration.

In summary, the results suggested that the aqueous extracts from both type of samples exhibited greater sensitivity to the antibiotic administration compared to the organic counterparts from the same type of sample. For screening purposes, the untargeted metabolomics approach proved to be a robust and sensitive tool for exploring the cause-effect relationship between the antibiotics administration and changes in metabolome of organ and tissue of pigs. Consistent with this, the mere presence or absence of antibiotic treatment can be effectively verified following this approach before any potential quantitative confirmatory tests. This screening approach is valuable when a plethora of compounds needs to be investigated within a specific sample or when there is a lack information regarding the type of treatment to which each pig was exposed.

In the light of prominent findings, further investigation based on a targeted workflow may be encouraged since the research of biomarkers molecules-proving meat authenticity is of priority importance. Despite the low sensitivity of NMR spectroscopy compared to High Resolution Mass Spectrometry (HRMS), differences between two groups were evident, suggesting that changes may not be limited solely to the sub-micromolar range. However, in the opinion of the authors a data fusion strategy coupling NMR to HRMS may be implemented to gain a comprehensive overview of the metabolites responsible for the discrimination.

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Case Study III

3.3.3 Case Study 3

Research Article

UHPLC-HRMS untargeted metabolomic and lipidomic coupled to chemometric techniques to discriminate Antibiotic-Free from Antibiotic-treated animals: a study on liver, kidney, and muscle of pig.

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3.3.3.1 Abstract

Consumers' demand increased for meat and meat-based products obtained from production systems based on high ethical standards for rearing animals. This shift in purchasing choice reflected an increased sensitivity toward the protection of animal welfare and health. In this context, novel invisible attributes, not taste-based, are communicated through voluntary label claims to fulfil novel consumer requirements. The antibiotic-free claim widely spread among the labels of meat and meat-based products embedding not only the protection of animal welfare but also the attention toward the antimicrobial resistance. In the -omics sciences cascade, exploring changes in metabolome can be informative of exposure to antibiotics during the rearing periods of animals. In this study, an ultra-high performance liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) for metabolomic and lipidomic analysis of the liver, kidney, and diaphragm of pigs was exploited. The untargeted approach coupled with supervised and unsupervised multivariate data analysis was carried out to capture differences in metabolite levels attributable to antibiotic administration. Differences between two phenotypes – antibiotic-free *vs.* antibiotic-treated – were highlighted in the polar extracts of all organs and tissue analysed. A clear difference between the biological answer of liver, kidney, and diaphragm was highlighted with the unsupervised models Principal Component Analysis (PCA) considering liver for the polar extract and kidney for the organic extract. More than one thousands of features were selected resulting in the ability to discriminate Antibiotic Free from Antibiotic Treated Group.

3.3.3.2 Introduction

Consumer approach concerning the purchasing choice for meat and meat-based products moved from the experienced behaviour to the credence behaviour, coupling taste, and appearance with invisible attributes that assure and promote respect for animal welfare and health (Grunert et al., 2018). Nowadays, all these invisible attributes are shared with consumers through the introduction of further voluntary information on labels complementing the mandatory information (Regulation EU No 1169/2011). This inevitably affects price and brand reputation. The field of voluntary label claims encompasses numerous declarations e communicated to consumers through various formats, graphical representations, and/or in-text formats. To this aim, meat producers can embrace a voluntary standard by implementing the specified requirements outlined in the voluntary certification scheme. Once all requirements have been satisfied, compliance with the standards enables the application of a peculiar voluntary label claim on meat products. In the context of the officially recognized certification bodies, the Italian scenario provides different options in terms of certification schemes available to assure a high standard of quality in meat production systems. For example, the halal status of meat is often certified through an official certification with an associated logo (Maritha et al., 2022).

In the swine sector, there are a lot of issues to which consumers are sensitive such as production systems (intensive/extensive, outdoor/indoor) mainly related both to the protection of animal welfare and the usage of antibiotics. EU legislation promoted sustainable production systems with the introduction of EU organic production rules (EU Organic production). In this framework, it should be specified that meat obtained from organic production respond also to antibiotic-free statements, but antibiotic free-meat is not always included in organic production (Mohammadi et al., 2023).

An illustrative example is given by some existing definitions behind the statement “no antibiotics chicken” by USDA-FSIS which stated that the claim is related to “chickens never treated with antibiotics”. In contrast, the USDA-FSIS definition for organic chicken refers to the following “chickens can be treated with antibiotics during the first day, before the hatching” (Cervantes, 2015).

When limited to the assurance of antibiotic-free *status*, one of the greatest challenges in these production systems is related to the requirements adopted for introducing a product with specified attributes in the meat market, ensuring accurate communication to the consumers. To achieve this goal, numerous voluntary label claims currently exist referring to antibiotic-free production, but a clear guideline is still lacking, leading to potential misinterpretation of the attributes.

The antibiotic-free statement along the pig's production can be applied throughout the entire life cycle of pigs or for a defined period of life (i.e., from the fifth month of life or in the last three months) depending on the requirements outlined in the voluntary standard implemented by the producers. Within this voluntary framework, the formulation of technical specifications is peculiar and different for each certification organism. Moreover, within the same certification organism, requirements and key points may differ from one animal species to another. This trend of highly assured meat products emphasizes the lack of harmonization in the field of voluntary label claims from terminology, methodology, and requirements points of view. The absence of strict rules governing voluntary statements on meat labels can complicate the traceability of the meat supply chain, making necessary the exploration of new tools for meat authentication.

Currently, all producers must comply with EU regulations concerning the withdrawal period when pharmacologically active substances are administered. This involves that, in the case of antibiotic treatment, all food matrices should respect the maximum residues limits stipulated by the law (EC Regulation n. 37/2010). To date, the analytical methods mostly employed for the detection of antibiotic residues are often based on those officially used within the national control plan for the monitoring of antibiotic usage. These methods primarily follow a targeted strategy that allows to test specific antibiotic residues (either individually or with multi-class monitoring) in meat or tissues (Bouhlel et al., 2017). Given the need for a quantitative to describe the results of antibiotic residues monitoring as mandated by the EU Regulation 37/2010, the extensively validated targeted approaches are considered the most suitable option. However, new formulations/mixtures of veterinary drugs based upon a lower

dosage of the active substance (or the combination of lower dosages of multiple active substances with synergistic effects) can reverse the situation, especially in the case of "antibiotic-free production". In such cases, if the antibiotic administration occurred in the early stage of the pig's life, it might be possible that, after several months, these substances may have been metabolized becoming not detectable in subsequent analytical investigations.

In recent years, the introduction of high-throughput analytical platforms has fostered the development of the untargeted metabolomics approach, widely exploited across various fields (Gaugain et al., 2019) and for different purposes. In this regard, literature search provided a lot of research papers dealing with the employment of metabolomics for food authentication, spanning plant matrices (Rivera-Pérez et al., 2023), milk and dairy products (Bellassi et al., 2021; Salzano et al., 2020), wine (Tzachristas et al., 2021), olive oil (Gil-Solsona et al., 2016), meat (Maritha et al., 2022), and fish (Kaltenbach et al., 2023).

In the omics cascade, the metabolomics approach represents the closest point to the phenotypic outcome, allowing to collection of biological information about low-molecular weight (< 1000 Da) metabolites providing information about the physiological state of an organism (Suzuki et al., 2014). Recently, metabolome analysis has been proposed as a novel strategy in the field of veterinary drugs to investigate potential antibiotic exposure during the life of pigs; in detail, Hurtaud-Pessel et al described a post-targeted screening in full scan enabling the acquisition of all analytes and then analysing them retrospectively (Hurtaud-Pessel et al., 2011).

In the present study, the investigation of metabolome and lipidome was performed to comprehensively highlight the impact of antibiotic treatment (any potential presence or absence) on the liver, kidney, and diaphragm of pigs. For this purpose, an untargeted UHPLC-HRMS coupled with multivariate statistics approaches was conducted to capture differences in metabolite levels attributable to antibiotic administration. The first two discussed investigations aimed at the same aim by exploiting NMR; in this study, the UHPLC-HRMS was chosen as an analytical platform to compare the results with those obtained by

employing a 1D ^{1}H NMR spectrometer. Additionally, both water-soluble and organic extracts were analysed for each organ and tissue for a cross-comparison among all food matrices.

3.3.3.3 Material and methods

Chemical and reagents

LC-MS grade (≥ 99.9%) methanol, dichloromethane, acetonitrile, and formic acid were purchased from Honeywell Burdick & Jackson (Charlotte, NC, USA). Leucine enkephalin standard was obtained from the Waters TOF G2-S Sample Kit-1 (Waters, Milford, MA, USA). Water was purified by Milli-Q purification system (Millipore, Bedford, MA, USA).

Study design and Animals

A total of ninety-one samples, comprising 29 livers, 31 kidneys, and 31 diaphragms of heavy pigs were randomly collected for this study. The selected pigs were reared in four distinct farms in northern Italy, specialised in the production of Parma ham PDO from heavy pigs. All the pigs selected were nine months old, weighing approximately 170 kg, which is a typical requirement for the Italian heavy pigs. The primary focus of this investigation was the comparison between the antibiotic-treated (TX) versus untreated pigs (CTRL) considering as targeted samples livers, kidneys, and diaphragms for the following reasons. Although this study did not specifically aim to detect pharmacologically active substances, the targeted samples matched those listed in the EU Regulation 37/2010. Additionally, the liver of pigs was considered since a key role in the biotransformation of drugs and other xenobiotics – phase 1 and phase 2 metabolism- is recognized (Donato et al., 1999). The kidney of pigs was considered to be involved in the excretion of drugs from the organism (Dhondt et al., 2020), while the diaphragm was considered representative of muscle tissue.

Samples classification was based on metadata collected through ClassyFarm according to the score of TI_{100} calculated by the Istituto Zooprofilattico "Bruno Ubertini". The current study design for comparing TX *vs.*

CTRL was structured according to the following scheme: *n*=10 livers of pigs classified as CTRL group and n=19 livers of pigs classified as TX group were sampled; *n*=12 diaphragms of pigs for the CTRL group and *n*=19 diaphragms of pigs for the TX group were compared; *n*=9 kidneys of pigs for the CTRL group and *n*=22 kidneys of pigs for the TX group were compared.

All samples were collected on the same day at a commercial abattoir under veterinary supervision with the selection performed randomly online. Immediately after collection, all samples were stored at -20°C until the sample preparation.

Sample preparation

Sample preparation followed the slightly modified Bligh&Dyer methods (BLIGH EG, DYER WJ, 1959). Specifically, 100 mg of samples were isolated from the central lobe of the liver, the cortex and medulla of the kidney, and the diaphragm of pig. Therefore, 100 mg were manually ground and placed in a sterile glass tube for each type of sample. A cold solution of 3 mL of methanol: dichlorometane (2:1, *v/v*) was added to the sample and then vortexed for 30 s. Subsequently, a sonication step of 30 min in a water-ice bath was performed to enhance the recovery of as many metabolites as possible. In the second step of the biphasic procedure, 1 mL of water and 1 mL of dichlorometane were further added, and samples were vortexed for 30 s. All samples reaching a total ratio of 100mg/5 mL (100 mg of samples in a total mixture of 5 mL of extraction solvents) were centrifuged at 2220 g x 35 min and refrigerated at 4 °C to achieve phase separation.

A total of 500 µl of the polar supernatant and 500 µl of the lower lipophilic phase were separately transferred with a 1mL Hamilton syringe in glass vials. Also, quality control (QC) samples were prepared by pooling an aliquot of equal volume of all extracts to obtain two different QC according to the collected extract; therefore, a QC related to the polar extract of each sample and a QC related to the lipophilic extract of each sample, respectively. All polar extracts were ready to be submitted to metabolomic analysis while all lipophilic extracts were dried under a gentle stream of nitrogen flow at room temperature. Finally,

300 µL of an acetonitrile/methanol solution 1:1 (*v:v*) were added to the lipophilic samples as a resuspension mixture. Finally, all lipophilic samples were ready to be submitted for lipidomic analysis. All resuspended samples were stored at -80°C until the analysis.

Untargeted UHPLC-IMS-QTOF metabolomic and lipidomic analyses

The untargeted metabolomic and lipidomic analyses were performed on a binary Acquity UHPLC I-Class system (Waters) coupled with Synapt G2-Si HDMS QTOF mass spectrometer (Waters SpA, Milan, Italy) furnished with an electrospray ionization (ESI) Zspray™ (Waters) by operating both in positive ESI (+) and negative ESI (-) ionization modes. The experimental setup was optimized according to Riboni et al., 2023. Agreed with this, the reversed phase chromatographic separation was performed using a Kinetex 2.6 μm PS C18 100 Å (100 x 2.1 mm^2) column (Phenomenex, Torrance, CA), maintained at 40°C.

For metabolomic analysis, the mobile phase, solvent A, consisted of water containing 0.1% (v/v) formic acid, while the solvent B consisted of acetonitrile containing 0.1% (v/v) formic acid. The flow rate was 0.4 mL min^{-1} and the injection volume was 2 µL. A multistep linear gradient elution was carried out starting with the solvent B set at 2% for 2 min, followed by a linear gradient to 50% within 9 min, then to 85% in 13 min, to 95% in 19.5 maintained for 0.5 min before column re-equilibration (4 min).

For lipidomic analysis, the mobile phases consisted of (A) 10 mM ammonium formate and 0.1% formic acid in water/acetonitrile 60:40 (*v/v*) and (B) 10 mM ammonium formate and 0.1% formic acid in isopropanol/acetonitrile 90:10 (*v/v*). The flow rate was 0.4 mL min^{-1} and the injection volume was 2 µL. The column was maintained at 55°C. A multistep gradient elution was performed starting with the solvent B set at 40 % (0.0 min) and reaching 65% (7.0 min), followed by an increase at 85% (15.50 min), and reaching 100% (15.60 min) maintained for 0.4 min before column re-equilibration (4 min).

The HRMS was set to operate in both positive and negative ionization modes, considering a capillary voltage 0.80 and 0.50 kV in ESI (+) and ESI (-),

respectively; the cone voltage, 50 V; source temperature, 150°C; source offset, 80 V; desolvatation temperature, 600°C; cone gas, 50 L h^{-1}; desolvation gas, 800 L h^{-1}; nebulizer pressure, 6.5 bar. IMS-HRMS analyses were performed at a mass resolution of 20 000 fwhm (full width at half-maximum) and using a travelling wave (TWIM) ad a drift cell with an ion mobility resolution of~45 (Ω/ΔΩ) (Giles et al., 2011). Nitrogen was employed as the drift gas at flow rate of 90 mL min^{-1}, transfer wave velocity set at 215 m s^{-1}, wave velocity at 650 m s^{-1}, and wave height at 40 V. The Major mix IMS/ToF Calibration Kit (Waters) -mass range: 151.1 – 1966.9 Da; CCS: 130.4 – 372.6 $Å^2$ was used for the CCS calibration in both ESI (+) and ESI (-). CCS calibration was automatically performed by the IntelliStart software using MassLynx platform. A leucine enkephaline solution (50 ng mL-1 in acetonitrile/water, 50:50 (*v/v*) with 0.1% of formic acid) was used as the lock mass. For both lipidomic and metabolomic analyses, spectra were acquired operating in the data-independent High-Definition MS^E acquisition mode using dynamic range enhancement and a collision energy ramp from 25 to 45 V for the high energy profile.

Data processing

The UHPLC-IMS-HRMS data were recorded in raw files by using the MassLynx (v4.2) sofware (Waters). Data analysis was performed by processing the raw data in Progenesis QI sofware (Waters, Milford, MA, USA). The program provides auto-alignment of signals, peak peaking, deconvolution, and normalization (Wang, J. et al., 2017; Progenesis QI). The following adducts were considered: $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$, $[M+H_2O+H]^+$, $[M-H_2O+H]^+$, $[M+2H]^{2+}$, $[M+2Na]^{2+}$, $[M+ 2Na-H]^+$, $[2M+H]^+$, $[2M+Na]^+$, $[M+H+Na]^{2+}$in positive ionization ESI (+); $[M-H]^-$, $[M+H_2O-H]^-$, $[M-H_2O-H]^-$,$[M+HCOO]^-$, $[2M-H]^-$,$[M+Na-2H]^-$, $[M+K-2H]^-$ in negative ionization ESI (-). The data were filtered by setting a minimum fold change of 3 compared to method blank.

For the current study, a multi-step data processing pipeline was implemented to ensure high data quality and reliability (Kirwan et al., 2014). By the expectations, the raw data need to be managed before the submission to statistical analysis. The preliminary step of data handling was related to the evaluation of relative standard deviation (RSD), also called coefficient of

variation, within QCs samples and the management of missing values. The RSD was calculated considering pooled QC samples for each feature by setting the cut-off RSD> 30% to have an acceptable level of precision (Kirwan et al., 2014; Naz et al., 2014). Additionally, the missing intensity value is a common issue thet needs to be considered and managed in the metabolomic dataset (Webb-Robertson et al., 2015). The threshold value for missing data was set at 40%, all those features having more than 40% of missing values were excluded from the dataset. According to the literature, all these precautions need to be implemented for metabolomics data since the high sensitivity of modern analytical platforms allows the detection of thousands of signals and it may be possible that within the detections a lot of interferent signals or noise could be included.

Multivariate data analysis: supervised and unsupervised models

As the initial descriptive stage, the principal component analysis (PCA) was carried out in the SIMCA 17 software package (v. 17.0.0, Sartorius Stedim Data Analytics AB, Umea, Sweden) to summarize the large number of variables into a small number of latent variables aiming to capture any clustering and to detect possible outliers. Subsequently, the supervised partial least squares-discriminant analysis (PLS-DA) models were implemented to highlight features allegedly involved in the discrimination between ABF and ABT samples. PLS-DA was chosen as the regression method given that this model works efficiently when the number of variables and the number of samples are not balanced, as often happens for metabolomics studies (Gaugain et al., 2019). Both PCA models and PLS-DA models were obtained after unit variance scaling. The quality of statistical models was evaluated by inspecting the goodness-of-fit (R^2X), which gives information about the explained variance by the model, and the predictive ability (Q^2), which gives information about the predictive ability of the model, according to cross-validation (Godzien et al., 2013). All features involved in the discrimination between ABF and ABT were selected using the variable important into projections (VIP) with different threshold according to the metabolomic dataset considered.

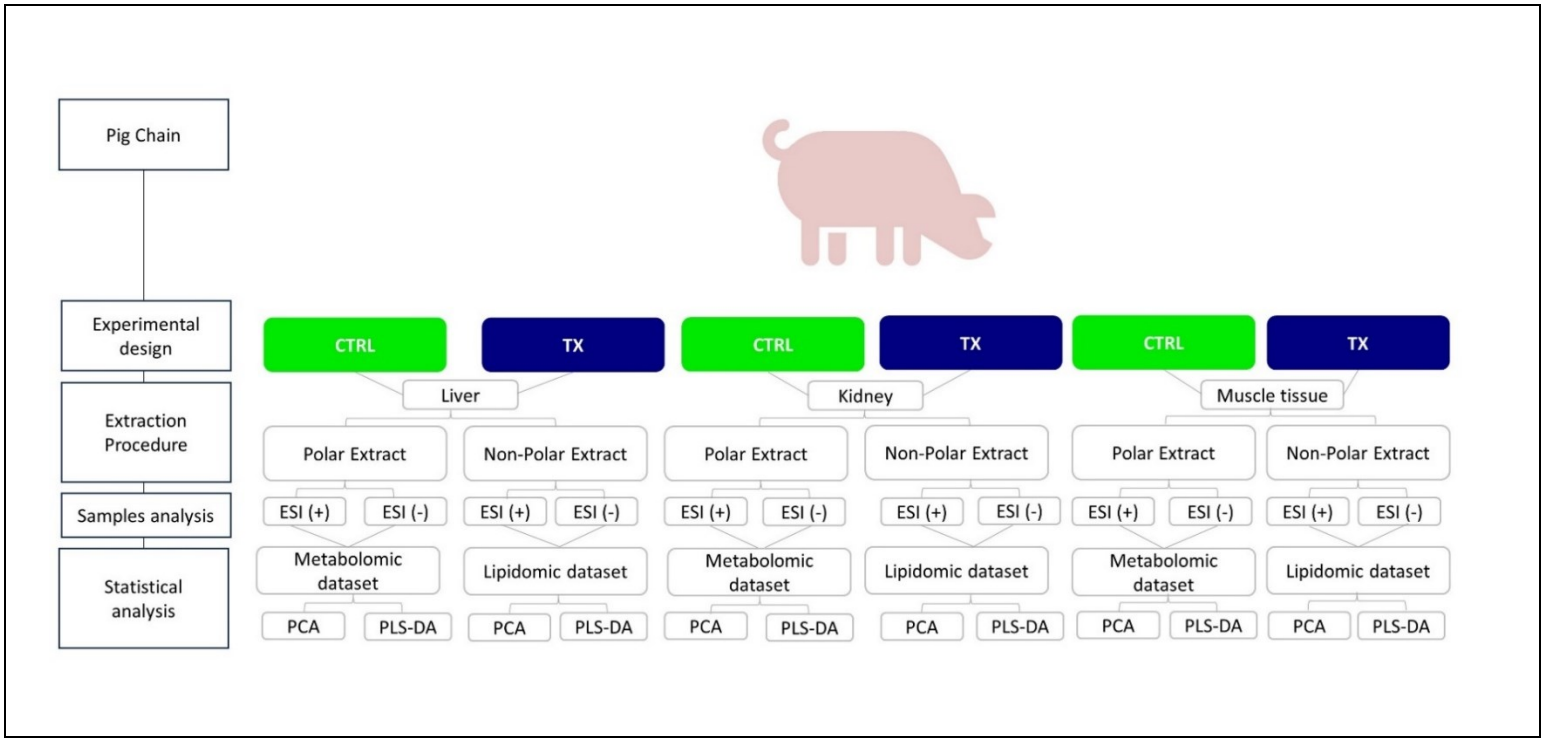

Figure 17. Graphical representation of the untargeted metabolomic and lipidomic workflow (From Experimental Design to Statistical Analysis)

3.3.3.4 Results and Discussion

A conventional workflow for statistical analysis (Anwardeen et al., 2023) based upon the use of both unsupervised and supervised models was employed for data handling. An untargeted HRMS-based metabolomics approach was used to validate the hypothesis that antibiotic administration during the rearing period of pigs may affect the metabolome and lipidome of the liver, kidney, and muscle. As a general remark, the presented datasets resulted from the combination of the analysis performed in both ESI (+) and ESI (-). Therefore, in the following section, the results related to six different built datasets- metabolomic and lipidomic- will be discussed. For clarity, **Figure 17** displays the workflow employed for data handling and processing. Therefore, the following section will be organized as follows: in the first part, the output obtained from the unsupervised statistical analysis will be presented; in the second part, the output obtained from the supervised statistical analysis will be discussed.

Data integrity: assessment of QC, RSD, and missing data

Initially, the assessment of the analytical performance was carried out by performing the PCA on the full dataset, including both QC samples and food matrices samples. The resulting PCA score plot, built on the full dataset encompassing QCs and samples, displayed the absence of errors that occurred during the acquisition phase, as all QCs clustered together at the centre of the score plot. This preliminary test is standard practice for high-dimensional metabolomics datasets to prove that no instrumental drift occurred during the analysis (Lacalle-Bergeron et al., 2023). Samples were acquired in a randomized way and QCs were injected every eight samples. Once the positive performance of the analytical platforms was checked, each dataset was filtered by the QCs. This filtered dataset, described only by samples and related features, was submitted to the PCA algorithm.

To monitor the overall samples preparation and the following steps, various precautions were implemented including *(i)* the randomization of the sample during both the extraction procedure and acquisition sequence *(ii)* analysis of method blanks both experimental blanks, methanol blank, and a mix vion preparation (iii) also QCs were used to test the analysis retrospectively. Samples

were analysed in batch following a randomized sequence to avoid time-dependent change during the analysis, thus making the results untrue. Sample sequence of injection was built the regular injection of quality control (QC) at the beginning, end, and between every eight samples per groups to check the system stability (Naz et al., 2014).

A substantial number of features was initially detected in both negative and positive ionization modes, ESI (-) and ESI (+), respectively. The raw generated dataset was described by the three-dimensional data table containing the intensity of detected peak in relation to m/z value, retention time, and sample. Data dimensionality was reduced by following the work pipeline described below.

For the polar extract of the liver, an initial detection of 21 636 features in ESI (-) modes and 13 281 features in ESI (+) modes occurred. Generally, the full dataset was built by considering only those features with the percentage of the relative standard deviations below 30% and the percentage of missing data less than 40%. After data filtering, the dataset for the polar extract of liver yielded 5 301 features for ESI (+) and 8 897 in ESI (-) modes, retaining 40% and 41% of the initial features. Subsequently, the features retained in both ESI (+) and ESI (-) modes were combined in a single dataset, representing the metabolomics dataset (*N* x *M*; *N*= number of samples; *M*= features) for the polar extract of the liver of pigs. For the dataset of the organic extract of the liver, among the initially detected features, 4 843 in ESI (-) and 5 891 in ESI (+) mode, about 20% (1 039) and 30% (1920) of features were retained in ESI (-) and ESI (+) mode, respectively. All retained features were integrated into a single dataset, ready for submission to statistical analysis.

Similarly, the total number of detected features for the polar extract of the kidney was 14 353 in ESI (-) mode and 11 153 in ESI (+) mode. Following filtering data, a reduction of 60% from the original dataset occurred, resulting in 5 758 features in ESI (-) mode, and 4 839 in ESI (+) mode. Subsequently, the metabolomics dataset was built by combining both ionization modes-filtered datasets, resulting in a worksheet organized as *N* x *M* (*N*= number of samples; *M*= features) and described by 10 597 features per 31 kidney samples. For the

raw apolar dataset of the kidney, 13 449 were detected in ESI (+) mode and 7 064 in ESI (-) mode. After filtering, 3 977 features were retained in ESI (+) mode and 1 522 in ESI (-) mode. The final lipidomic dataset for the kidney was then generated by combining the features obtained in ESI (+) and ESI (-) modes for further statistical investigation.

The third metabolomics dataset related to the polar extract of the diaphragm was built following the previously outlined procedure for the liver and kidney datasets. In this case, the initial raw dataset contained 10 703 features for ESI (+) mode and 12 103 for ESI (-) mode. After filtering, 4 706 features were retained for ESI (+) mode and 3 912 for ESI (-) mode. Consequently, the metabolomic dataset for the aqueous extract of muscle was built by merging the ESI (+) and ESI (-) datasets, resulting in a total amount of 8 618 features. The same logic was applied to the lipophilic counterpart of the diaphragm for data filtering. Briefly, the total amount of 2 560 and 6 170 features initially detected in ESI (-) and ESI (+) mode were reduced to 414 and 2 137 in ESI (-) and ESI (+) mode, after data filtering. The complete dataset for the lipophilic diaphragm was then built by combining the retained features.

Unsupervised MVDA on metabolomic dataset of pigs' matrices

The unsupervised PCA analysis was performed on each dataset to explore potential trends, clusters, and outliers (Lacalle-Bergeron et al., 2023). All the metabolomic datasets for aqueous extract of pigs' liver, kidney, and diaphragm were statistically processed following the same pipeline described as follows.

The PCA model for the aqueous extract of the liver reduced the dataset to four latent variables, principal components (PCs), explaining 58.1 % (R^2X) of the total variance with a coefficient of predictive ability (Q^2) of 35.8%. The first two components describing the 39.6% (PC1=23.5% and PC2=16.1%) were considered and plotted, as displayed in **Figure 18**. The PCA score plot emphasized clear grouping trends between the TX group and the CTRL group of pig samples. This observation led to two assumptions: firstly, the discrimination between two groups was markedly obtained, and secondly, the score plot suggested a slight tendency for the samples of the treated group to cluster

together. Notably, samples belonging to the control group seem to exhibit higher variability compared to the TX group.

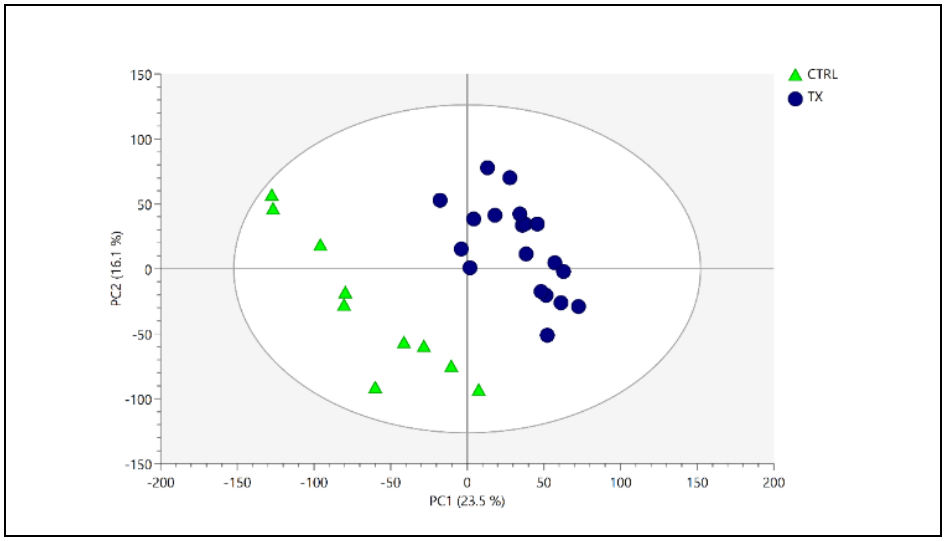

Figure 18. Unsupervised PCA (PC1 *vs* PC2) score plot of aqueous extract of pigs' liver. CTRL pig samples, green triangles; TX pig samples, blue dots. The PC1 (23.5%) and PC2 (16.1%) explained cumulatively the 39.6 %, approximately 70% the total explained variance (R^2X).

Similarly, the metabolomic dataset of aqueous kidneys was explored to test putative differences between TX and CTRL pigs. In this case, the PCA model was described by 5 PCs, explaining 52.2 % of the total variance (R^2X) with a coefficient of predictive ability (Q^2) of 20.6%. By plotting all possible combinations of the extracted principal components, the first two PCs were selected since returned the best model. Despite the cumulative variance explained by the first two components accounted for 28.9% (PC1=15.5%; PC2= 13.4%), approximately 55% of the total explained variance, this model was the best obtained among all alternatives. Contrary to the score plot displayed in **Figure 18** related to the polar extract of liver, the PCA score plot in **Figure 19** offers a completely different scenario. No discernible trend was displayed in discriminating treated samples (TX) from control samples (CTRL) as regards the polar extract of the kidney. However, it may be possible to visualize a slight

grouping trend within the control samples, a complete reversal compared to the results obtained in the liver.

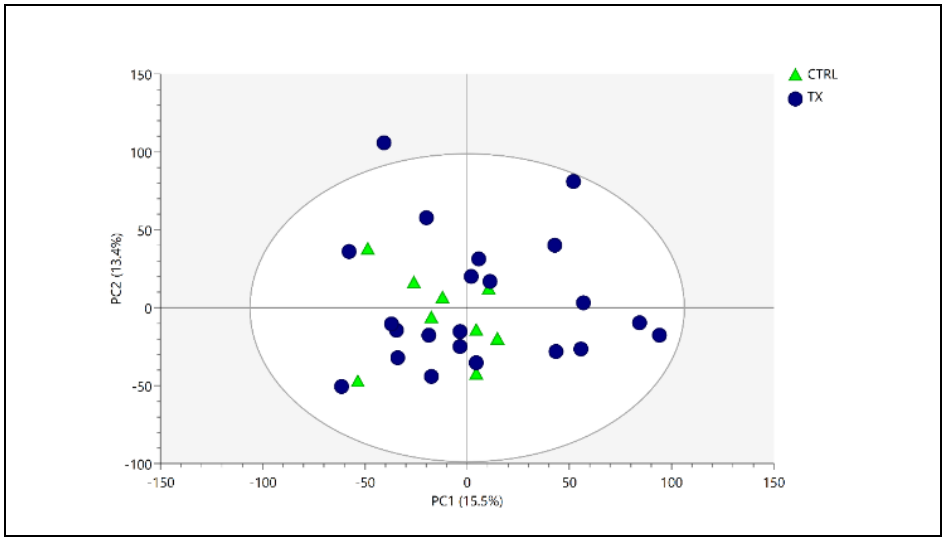

Figure 19. Unsupervised PCA (PC1 *vs* PC2) score plot of aqueous extract of pigs' kidney. CTRL pig samples, green triangles; TX pig samples, blue dots. The PC1 (15.5%) and PC2 (13.4%) explained cumulatively the 28.9 %, approximately 55% of the total variance (R^2X).

Lastly, the same pipeline of work was followed also for handling the metabolomics dataset of muscle tissue. In summary, all data were scaled to unit variance, and the PCA model was performed. Five PCs described the PCA model built that explained a goodness-of-fit score of 63% (R^2X) and a coefficient of predictive-ability of Q^2= 42.1%. Similarly, the first two PCs were considered, as the cumulative variance of the first two (PC1= 22.4% and PC2=17.7 %) covered more than half of the total variance explained by 40.1%. The PCA score plot was similar to the score plot obtained for the kidney, as displayed in **Figure 20**.

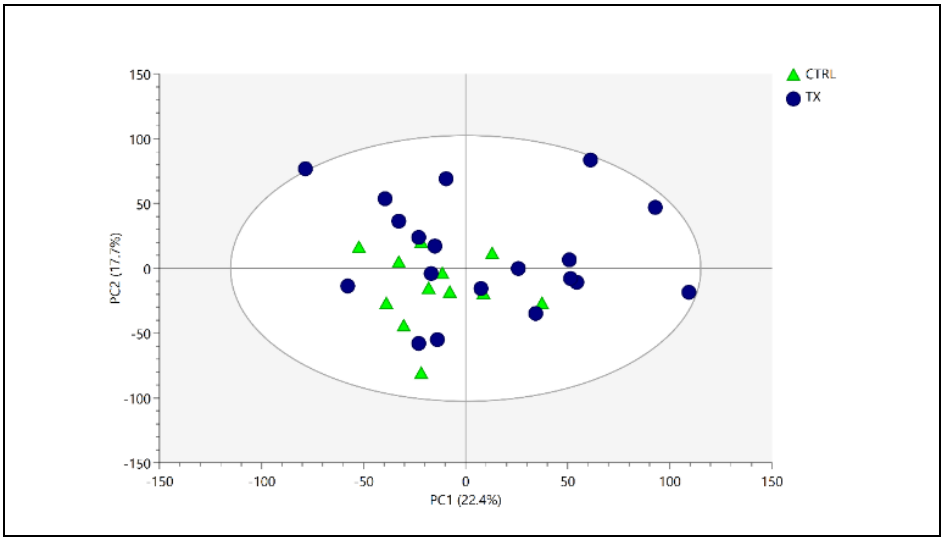

Figure 20. Unsupervised PCA (PC1 *vs* PC2) score plot of aqueous extract of pigs' muscle. CTRL pig samples, green triangles; TX pig samples, blue dots. The PC1 (22.4%) and PC2 (17.7%) explained cumulatively the 40.1 %, approximately 60 % of the total variance (R^2X).

Finally, all PCA models built for the samples suggested that different assumptions can be made. Notably, among the models, the polar extract of liver displayed the better results compared to those of the kidney and muscle. In detail, the PCA score plot displayed in **Figure 18** differentiated between the two groups of samples in the case of the liver. Yet in contrast to the expectations, both kidney and muscle tissue, as illustrated by the score plots in **Figures 19** and **20,** did not exhibit a clear discrimination between treated and control pig samples. In the case of the kidney, all samples were randomly distributed along the *x/y* axis, with considerable overlapping between samples from the TX and CTRL pig groups. In the muscle, CTRL pig samples clustered together, while TX pig samples were scattered randomly in plot hyperspace. In both cases, the only discernible pattern was that samples from the control group tended to cluster together, while samples from the treated group were randomly distributed along the *x/y* axis. Conversely, the polar extract of the liver displayed the two groups of samples.

Unsupervised MVDA on lipidomic dataset of pigs' matrices

The biphasic extraction procedure employed for the current study was considered both considering metabolomics literature (Cajka et al., 2016; Wagner et al., 2014) and the need to separately investigate the aqueous and lipophilic extracts. Hence, the generated datasets for lipophilic extracts were submitted to unsupervised statistical analysis following the workflow analogue to that previously described for the aqueous extract. The lipidomic dataset of the liver was the first to be explored by unsupervised statistics. The unsupervised PCA was modelled on data scaled to unit variance and the score plot displayed in **Figure 21** was obtained by plotting the first two PCs. The PCA model was described by 5 PCs with a total amount of explained variance of 69.8% (R^2X) and a score of predictive ability of 41.4% (Q^2). As a general remark, no relevant grouping trend was shown for the lipophilic extract of the liver. The PCA score plot in **Figure 21** resulted in stark contrast to the findings displayed in **Figure 18**, showing the results obtained from the aqueous extracts of the liver.

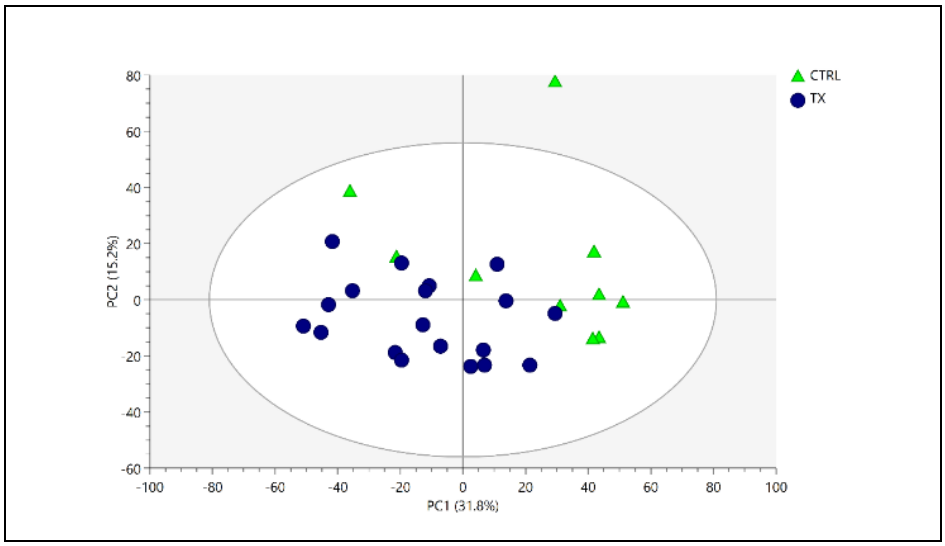

Figure 21. Unsupervised PCA (PC1 *vs* PC2) score plot of organic extract of pigs' liver. CTRL, green triangles; TX, blue dots. The PC1 (31.8%) and PC2 (15.5%) explained cumulatively the 47 %, approximately 70 % of the total variance (R^2X).

For the lipophilic extract of the kidney, the PCA algorithm described the dataset with 5 PCs with a total coefficient of explained variance of 58% (R^2X) and predictive ability of 23.5% (Q^2). The PCA score plot displayed in **Figure 22** combined the first two PCs explaining 39.3% of the total variance (PC1= 19% and PC2= 15.3%). Graphically, not only a perfect separation between samples from the TX and CTRL pig group occurred but also samples of each group internally clustered perfectly. The PCA score plot in **Figure 22** displayed encouraging results given the achieved optimal separation between the two groups. Also in this case, the polar extract of the same type of sample, the kidney, showed an opposite situation, as illustrated in **Figure 19.**

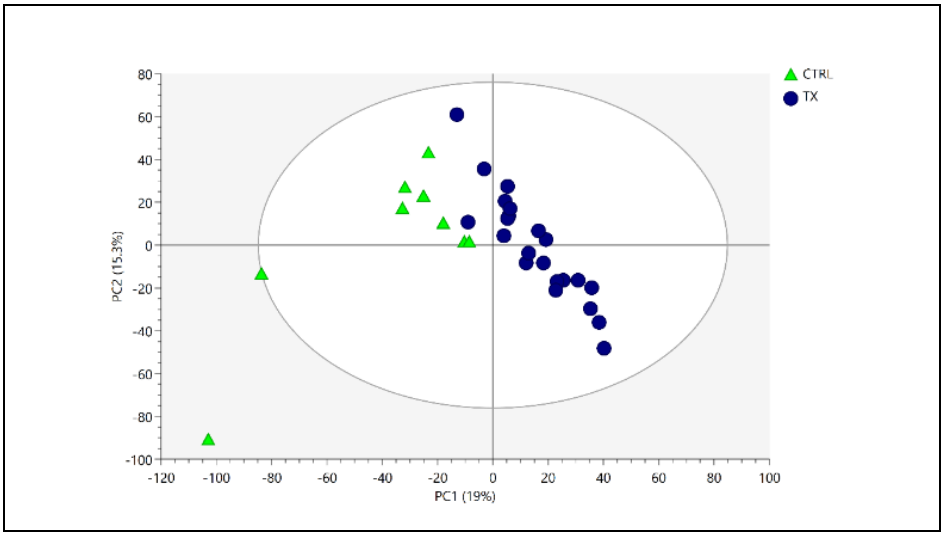

Figure 22. Unsupervised PCA (PC1 *vs* PC2) score plot of organic extract of pigs' kidney. CTRL, green triangles; TX, blue dots. The PC1 (19%) and PC2 (15.3%) explained cumulatively the 34.3 %, approximately 60% of the total variance (R^2X).

The unsupervised PCA model for the lipophilic extract of the diaphragm was described by 5 PCs explaining 69.5% of the total variance (R^2X) with a coefficient of predictive ability (Q^2) of 36.7%. In **Figure 23**, the first two PCs accounting for 39.3 of the cumulative explained variance (PC1= 22% and PC2= 17.3%) were plotted. The PCA score plot revealed a subtle grouping trend between pig samples from TX and CTRL groups; however, it is possible to

observe overlaps of samples belonging to the two distinct groups under investigation.

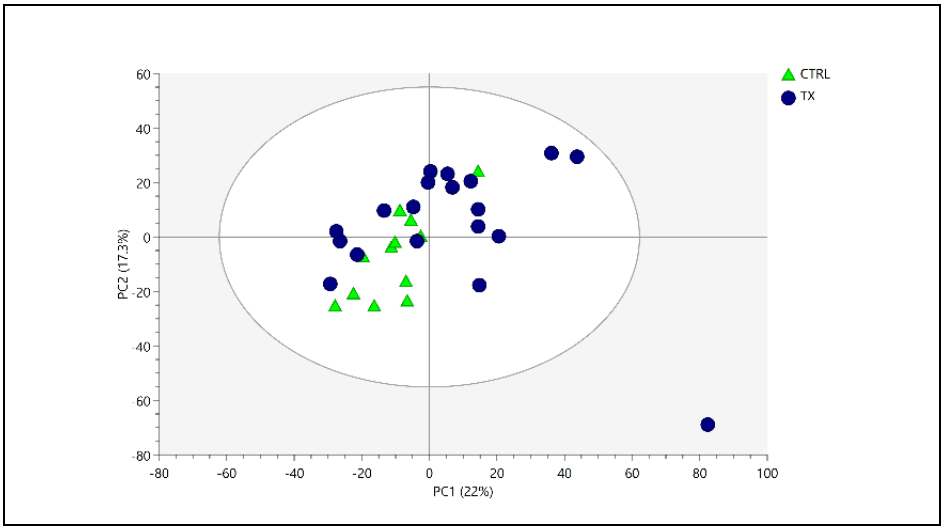

Figure 23. Unsupervised PCA (PC1 *vs* PC2) score plot of organic extract of pigs' muscle. CTRL, green triangles; TX, blue dots. The PC1 (22%) and PC2 (17.3%) explained cumulatively the 39.3 %, approximately 60% of the total variance(R^2X).

Overall, among all unsupervised models related to the lipophilic extracts, a clear discrimination between control and treated pig samples was observed in the kidney as displayed in **Figure 22**, a discrete performance in separation was achieved by the PCA model of diaphragm as displayed in **Figure 23** and by the liver, as shown in **Figure 21**.

Finally, all the unsupervised models were built for all types of samples and related extracts. Overall, the surprising result was dealing with completely divergent findings within the same type of sample. In agreement with this, the unsupervised statistics for metabolomics of the liver, as shown in **Figure 18**, displayed a great potential in differentiating samples according to the antibiotic's exposure while for the lipidome counterpart, no discrimination was achieved, as shown in **Figure 21**. On the contrary, the metabolomics PCA of kidney score plot, as shown in **Figure 27**, did not suggest a clear grouping trend within TX and CTRL samples while the lipidomic score plot for the same type of samples, as shown in **Figure 22**, unexpectedly showed a remarkable trend to

differentiate CTRL from TX samples. In the case of muscle, both metabolomic and lipidomic PCA score plots displayed in **Figures 20** and **33**, were not able to highlight a clear difference between two sample groups; however, better discrimination was highlighted by the lipidomic dataset of muscle.

For all described PCA models, the PC1 and PC2 were considered since this match was able to test the differences between the two phenotypes under investigation. It is generally recognized for metabolomics studies that values of $Q^2 > 0.5$ are indicative of good prediction performance of the model (Ramos et al., 2022), however, it is not a fixed rule. In the unsupervised model described above, all scores for the Q^2 were below 0.5 and this trend may be explained considering the high biological variability inter-animals of the investigated samples.

Supervised MVDA on metabolomic dataset of pigs' matrices

For classification purposes, the supervised PLS-DA models were built to explain differences between the considered groups of samples and the variability intra-group (Westerhuis et al., 2010). Therefore, three different models – metabolomics dataset of liver, kidney, and muscle- were built on data scaled to unit variance by performing a default option of *7-fold* cross validation. All 2D PLS-DA score plots related to the metabolomics dataset of liver, kidney, and muscle displayed evident clustering between TX and CTRL pig groups. As can be observed in **Figures 24**, **25**, and **26** a complete separation was achieved between two groups under investigation, thus the results obtained by the unsupervised PCA model were confirmed with the supervised PLS-DA.

The 2D PLS-DA score plot of the aqueous extract of the liver displayed samples of the TX group closely related compared to the samples of the CTRL group. In all cases, the goodness-of-fit of the Y variables (R^2Y), how the model fits the data, resulted in an optimal score of 99.4, 99.1, and 94.2% for liver, kidney, and muscle, respectively. Also, the predictive ability score Q^2, how the model predicts new data, resulted in high values for all displayed samples (Liver, Q^2= 92%; Kidney, Q^2= 90%; Muscle, Q^2= 94.2%).

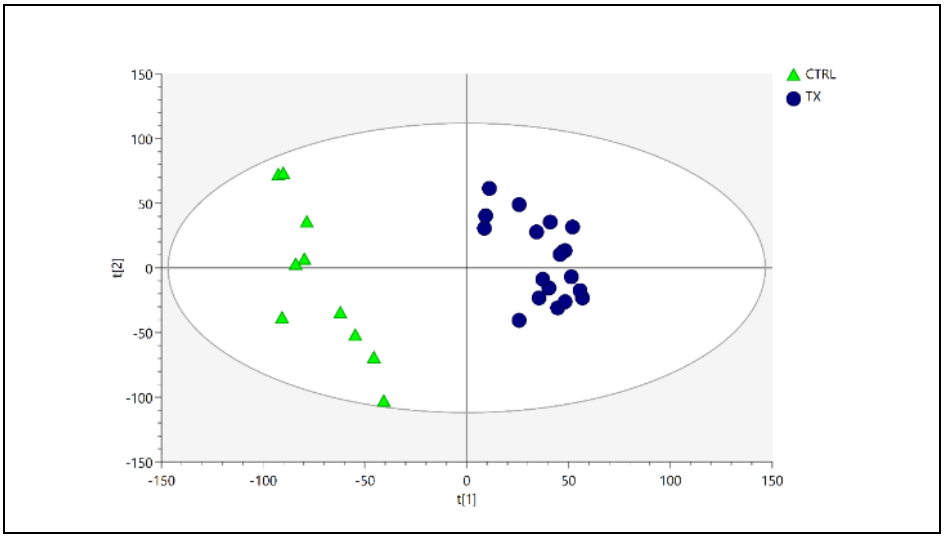

Figure 24. 2D PLS-DA score plot obtaining by plotting the aqueous extract of livers: control samples of pigs are in green triangle, treated samples of pigs are in blu dots. The diagnostic parameter of PLS-DA performance were R^2X= 49.9 %, R^2Y= 99.4%, and Q^2= 92%.

Instead, the 2D PLS-DA score plots of aqueous extract of kidney suggested that samples of CTRL were closely clustered compared to liver and muscle in which a certain degree of heterogeneity was displayed by the fact that samples were distributed along the positive and negative values of *t[2]*.

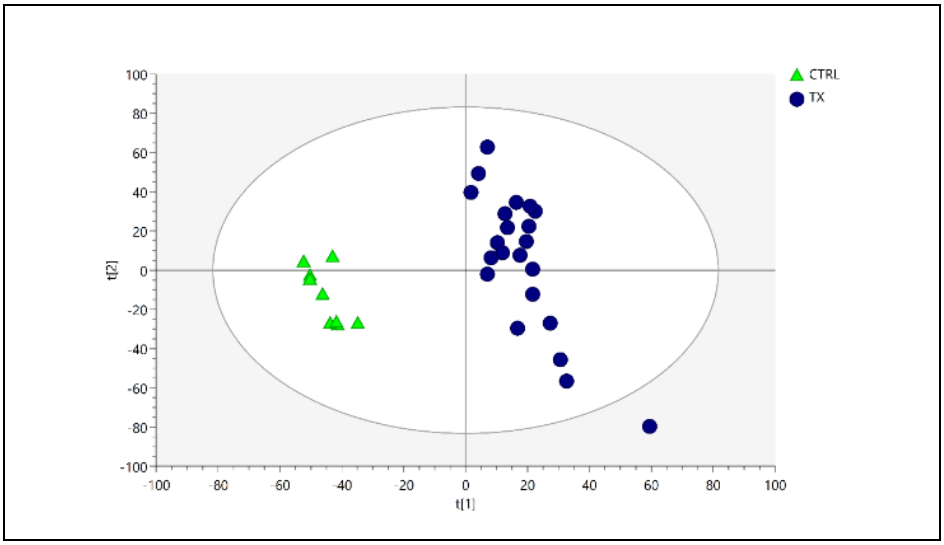

Figure 25. 2D PLS-DA score plot obtaining by plotting the aqueous extract of kidney: control samples of pigs are in green triangle, treated samples of pigs are in blu dots. The diagnostic parameter of PLS-DA performance were R^2X= 28.7 %, R^2Y= 99.1, and Q^2= 90%.

These results agreed with the results obtained previously in the application of NMR-based untargeted metabolomics performed on the same kind of sample shown in the 1st and 2nd Case Studies described above (Fabrile et al., 2023).

All these optimistic results may highlight the potential role of metabolomics as sensitive to antibiotic administration becoming a relevant object of investigation in this field. Also, to validate the results obtained from PLS-DA, as a double check, the misclassification table was used to understand the proportion of correctly classified samples. A correct classification, 100%, was achieved for all analysed samples. In other terms, all observations were correctly classified in their belonging groups, CTRL or TX and the related Fisher's probability was 5e-08 for liver and kidney and 7.1e-09 for diaphragm.

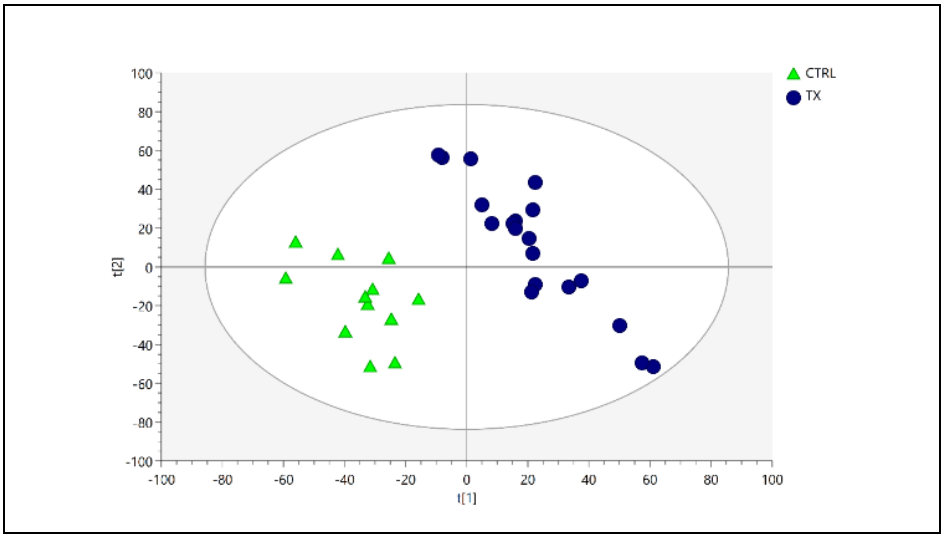

Figure 26. 2D PLS-DA score plot obtaining by plotting the aqueous extract of muscle: control samples of pigs are in green triangle, treated samples of pigs are in blu dots. The diagnostic parameter of PLS-DA performance were R^2X= 51.3 %, R^2Y= 99%, and Q^2= 94.2%.

The PLS-DA together with Variable Importance in Projection (VIP) was used to classify samples and identify the molecules responsible for discriminating treated from control pig groups. With the aim to select only those features with the most discriminating power in the case of TX *vs.* CTRL, different cut-offs of VIP scores were fixed according to the considered samples. As a general rule, the VIP score > 1.0 is considered in most cases to select and identify the relevant biomarkers (Rocchetti et al., 2018; Chatterjee et al., 2019). All relevant features selected were listed in the Annex II, referring to **Tables 8**, **9**, and **10**. A substantial number of features resulted relevant in the discrimination of TX *vs.* CTRL phenotype considering the metabolomics dataset. Overall, the VIP cut-off was set to values higher than 1.5 for all metabolomics samples with some exceptions. For the metabolomics dataset of the kidney, the VIP score > 1.80 resulted in a list of 650 relevant features; for the metabolomics dataset of both liver and muscle, the VIP score > 1.70 returned 348 and 271 important features, respectively. The list of features contains features detected in both negative ESI (-) and positive ESI (+) ionization mode since the metabolomic dataset was built by combining the results obtained from both ionization modes.

Supervised MVDA on lipidomic dataset of pigs' matrices

As regards the organic extracts, the results obtained by the unsupervised statistics were further investigated by supervised PLS-DA statistics for samples classification. Therefore, the same pipeline of work applied to the metabolomics counterpart was adopted. Briefly, data were scaled to unit variance, and an internal SIMCA default option 7-fold cross-validation was applied to all lipidomic datasets. Finally, the classification of samples was achieved as illustrated in **Figure 27** and **Figure 28**. For the muscle, the PLS-DA algorithm was not able to provide a model for the sample classification. Instead, for liver and kidney the 2D PLS-DA score plots clearly showed a discrimination between samples collected from antibiotic-treated pigs and samples from the control group. Additionally, the results obtained from the previous unsupervised statistics were enhanced in the supervised statistics.

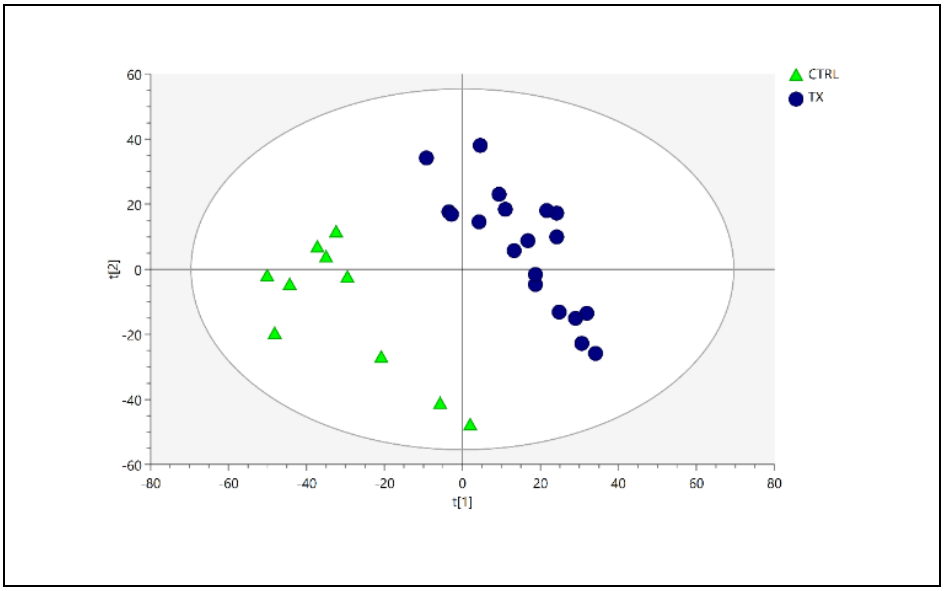

Figure 27. 2D PLS-DA score plot obtaining by plotting the organic extract of liver: control samples of pigs are in green triangle, treated samples of pigs are in blu dots. The diagnostic parameter of PLS-DA performance were R^2X= 66.1 %, R^2Y= 99.5 %, and Q^2= 94 %.

The quality of the PLS-DA model was also inspected in this case by considering the goodness of fit (R^2Y) of the Y variables and the predictive ability of the model (Q^2). In the case of the lipidomic dataset of the liver, the scores obtained for R^2Y= 99.5% and Q^2=94% were considered good; also, as displayed in **Figure 27**, an evident discrimination between two groups was highlighted since samples of the CTRL group mainly populated the negative values of *t[1*] while samples of TX group were located in positive area of *t[1]*. Also, the high variability intragroup was displayed since samples were vertically distributed along the *t[2]* axis, for both CTRL and TX cases.

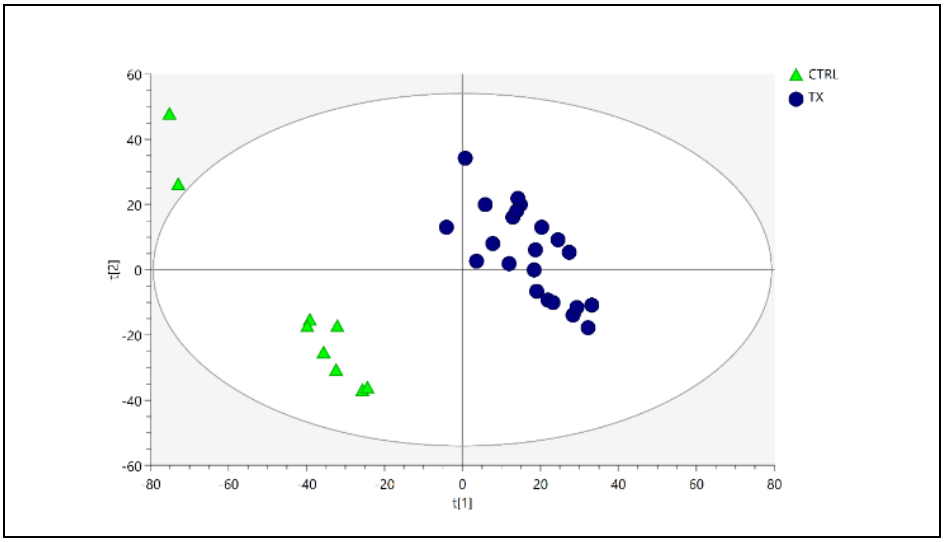

Figure 28. 2D PLS-DA score plot obtaining by plotting the organic extract of kidney: control samples of pigs are in green triangle, treated samples of pigs are in blu dots. The diagnostic parameter of PLS-DA performance were R^2X= 26.3 %, R^2Y= 96.1%, and Q^2= 86.9 %.

For the lipidomic dataset of kidneys, the grouping trends between TX and CTRL pig groups enhanced with the supervised PLS-DA compared to the unsupervised PCA. The performance was considered good given the high score of R^2Y= 96.1%, and Q^2= 86.9 %. Also, the classification of the observations within the prediction set was tested with a misclassification table. For both lipidomic extracts of the kidney and the liver, the correct classification rate of the observations was 100% with Fisher's probability of 5e-08.

Finally, a VIP approach was also used to select relevant features to discriminate TX from CTRL pig samples, as previously described for the metabolomics dataset. All resulting relevant features were summarized in **Tables 11** and **12**, as shown in Annex II. However, the VIP score > 1.50 returned a list of 169 relevant features for the lipidomic dataset of the liver and for the kidney, the VIP score > 1.75 resulted in 210 relevant features. As a general remark, the list of features contains features detected in both negative and positive ionization modes since the lipidomic dataset was built by combining the results obtained from ESI (-) and ESI (+) modes.

Overall, the findings that emerged from both metabolomics and lipidomic datasets displayed that the exposure of pigs to antibiotic administration can be highlighted besides the knowledge of information concerning the presence/absence of antibiotic residues in meat. In fact, no targeted analysis was designed and performed to previously detect the presence of antibiotics in the selected samples. According to this, no targeted information about samples was investigated to support the untargeted approach along the entire workflow. The only objective assumption was that samples from the CTRL and TX pig groups were different according to data collected by the Italian monitoring systems ClassyFarm. Notwithstanding, these findings displayed the discrimination between pig samples from the CTRL and the TX groups.

3.3.3.5 Conclusion

The current study proved that the investigation of both the metabolome and lipidome of the liver, kidney, and diaphragm of pigs may be useful in elucidating if pigs were exposed to antibiotics treatment during their life cycle. The untargeted metabolomics and lipidomic carried out with HRMS coupled with multivariate data analysis proved to be a successful tool when no *a priori* information about the samples was available.

These findings allowed to validate the hypothesis that some kinds of samples may be more informative about the exposure to antibiotics administration during the rearing period in pigs. The polar extract of the liver was able to distinguish the two groups of samples with unsupervised statistics, where the model was not trained with the class information of the sample. Also, the organic extract of the kidney displayed a greater difference between the two phenotypes under investigation. In the opinion of the authors, the higher sensitivity of the analytical platforms employed, UHPLC-QTOF, allowed to detect small molecules metabolites relevant for the discrimination between treated *versus* untreated pigs compared to the NMR. Further steps will be conducted to identify the resulting relevant features from the VIP approach. Also, more studies will be needed to validate the hypothesis by considering a higher number of samples and more detailed metadata. However, these

findings are promising leading to suggest that -omics sciences may significantly support the authentication framework avoiding that allegedly meat frauds can treat the integrity of the pig supply chain.

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Chapter 4

Discussion

Summary

In this section, a critical comment concerning the work discussed in the previous chapter will be presented. Also, the main advantages and drawbacks of the methods employed will be outlined to make a comparison among all studies presented in the experimental section. A brief discussion will be also conducted on the feasibility of metabolomics for authentication purposes considering the interface between the application of this methodology and the current legislative scenario.

In the present Ph.D. thesis, the feasibility of metabolomics for meat authentication purposes was investigated to assess the possibility of considering and officially recognizing new tools capable of detecting food fraud in the supply chain with the ultimate goal to preserve the integrity of the pig production chain.

The introduction of new voluntary label claims complicates the authentication framework, as it brings highly assured meat products into the meat market (Abrams et al., 2010; Bradford et al., 2022). Broadly speaking, food labelling deals with any information declared on food packaging that is mandatorily regulated by EU Regulation No. 1169/2011. Besides the respect to the mandatory requirements, some dedicated spaces on the food packaging can be covered by voluntary label claims. Meat producers can adopt voluntary standards and freely share with consumers the peculiar characteristics of the food products (Kaczorowska et al., 2021). To date, the availability of various label claims for meat and meat-based products (i.e., antibiotic free or raised without antibiotics) may represent a field in which the mandatory legislation meets the voluntary standards. In the case of the Antibiotic Free claim, greater attention by EU legislation is posed to investigate the usage of antibiotics in livestock by monitoring the antibiotic residues in animal-origin commodities.

Several EU regulations covering distinct aspects have been implemented to commercialize meat and meat-based products to both monitor the usage of antibiotics in livestock and to protect the health of consumers, as summarized in **Table 8**.

Table 8. European legal frameworks antibiotics related.

Legal Reference	Main Antibiotics-related topic covered
Regulation (EU) 470/2009	This regulation lays down the rules and procedure to properly establish residue limits of pharmacologically active substances in food of animal origin
Regulation (EU) 37/2010*	This regulation classifies the pharmacologically active substances and establish their maximum residues limit to be found in the food of animal.

Regulation (EU) 625/2017	This regulation lays down rules for performing official controls activity to ensure the application established laws, the protection of animal welfare and health and plant.
Regulation (EU) 6/2019*	This regulation lays down the rule for placing on the market (i.e. marketing authorisations), manufacturing, import and export, and related activity to commercialize and distribute the veterinary medicinal products.
Regulation (EU) 4/2019	This regulation set out the rule for the manufacture, marketing, and use of medicated animal feed.
Regulation (EU) 1871/2019*	This regulation lays down rules to define the reference point of action (RPA) for residues of pharmacologically active substances
Regulation (EU) 808/2021*	This regulation lays down the rule for analytical methods and results interpretation related to the analyses of the residues of pharmacologically active substances performed by the laboratory analysis.
Regulation (EU) 1644/2022	These regulations establish criteria to be followed in the control plans relating to the use of pharmacologically active substances in animal-origin commodities.
Regulation (EU) 1646/2022	
Regulation (EU) 1255/2022	This regulation lays down the list of antimicrobials or groups of antimicrobials reserved for treatment of human infections

This table summarizes the timeline of the key dates for the regulations referring to distinct aspects of the usage, monitoring, and commercialization of the pharmacologically active substances, veterinary medicinal products, and antimicrobials. All listed regulations are in forced. *New consolidated versions are available for Regulation (EU) 37/2010, Regulation (EU) 6/2019, Regulation (EU) 1871/2019, Regulation (EU) 808/2021.

All these restrictive legislative measures have been implemented within the European Union to limit, monitor, and detect the usage of antibiotics. Antimicrobial resistance represents one of the main problems affecting human and animal health and this threat has made necessary to implement all those contrasting measures given that a global action plan is required (Wagenlehner et al., 2022; WHO, 2023). Among the actions, it is worth mentioning the main goal set by the European Union which is related to the reduction of the overall sales of antimicrobials by 50% by 2030, as declared within the Farm to Fork strategy (European Commission, Farm to Fork strategy).

All the implemented measures highlighted that a significant action is asked to be performed by the member states toward the reduction of veterinary medicines- mainly antibiotics- within the European livestock scenario (Baudoin et al., 2021). This attention to the reduction of antibiotics usage combined with the needs of consumers (Centner et al., 2016) affected the meat market, retail, and grocery with the introduction on the shelves of novel label claims assuring "free from antibiotics".

The current state-of-the-art in the field of antibiotic's detection highlighted that different methodologies, mainly targeted, exist to verify the compliance of antibiotic residues within maximum residue limits in foodstuffs of animal origin (Chiesa et al., 2017; Mompelat et al., 2015; Gaudin et al., 2009).

Nevertheless, the analysis of residues in meat may not detect all antibiotic classes, especially if carried out after the withdrawal period or when residues are below the limit of detection (Whelan et al., 2023). In agreement with this, the "absence of antibiotics residues" is a different concept from the "absence of treatments" supported by the assumption that absence of evidence is not evidence of absence. The residue testing in meat can successfully prove the presence or absence of antibiotics in meat and meat-based products to check compliance with the mandatory legislation; however, several questions for the rearing period emerge since certification marks, and related label claims, pose greater attention to the specification of the life period during which animals is considered "Free from" antibiotics (e.g., Antibiotic Free throughout the whole life cycle, starting from the weaning phase or in the last 120 days), as reported

in the information publicly available on the Italian certification organism's website CSQA. Therefore, antibiotic-free production can be performed and communicated on various levels with different label claims according to the established criteria used to assure the antibiotic-free chain. In this scenario, the need for chemical traceability for the integrity of the meat chain emerged.

New meat frauds around the corner, needs and requests.

Food fraud is an old problem dating back to Rome and the Greek population (Sumar & Ismail, 1995) still threatening the integrity of the food chain (Robson et al., 2021). Despite the long historical tradition, fraudulent practices under different shapes and in unusual ways are a constant present negatively affecting the current food systems network. Why this so ancient phenomenon is still actual? Why is not possible to eradicate it?

In agreement with some authors, the creativity behind the fraudulent practices is the real achievement of food fraudsters allowing them to survive over the years (Bannor et. al, 2023). It is not a case that some of the most important food scandals were isolated phenomena given that once the frauds have been detected, new food frauds emerge, as in the case of the Melamine milk scandal (Gossner et al., 2009). Behind this assumption, new advanced technologies lead to suppose that food frauds still occur since a failure within the food systems exists. In fact, in most of the cases the approach adopted is based on acclaimed food fraud events by implementing reactive countermeasures rather than the assessment of food fraud vulnerability factors (Rezazade et al., 2022). The opportunities, the motivations, and the control measures are the key elements defining the vulnerability of food systems to fraudulent practices (van Ruth et al., 2017).

In the current Ph.D. thesis, new voluntary label claims in meat production were recognized as a putative vulnerability factor of the entire meat systems network given that producers can certify their meat products by fulfilling certification scheme requirements – a list of requirements needs to be implemented to declare label claims (Easy to be implemented- Opportunity). Up to date, the voluntary label claim can be mainly demonstrated with bureaucratic documents since specific monitoring systems do not exist to check the

truthfulness of the label claim (Control Measures). And also, the emerging niche meat markets define higher prices compared to the traditional market, the economic motivations.

In addition, being the nature of the certification scheme voluntary, each country has a list of different certification schemes – thus label claims- available making the European scenario complex. To this aim, an informal survey was conducted to gain an overview of the Italian scenario concerning antibiotic-free claims. All information collected during the survey was not intended to be published, it was for informative purposes. Hence, the survey was performed by using a quite simple approach: visually inspect the labels at the local stores, visit the producer's website to understand the transparency in the shared information, and directly contact certification bodies. The main focus was on pig meat products and a spiced scenario emerged from the information collected (Kiwa) (CSQA) (RINA). Firstly, under the umbrella topic of Antibiotic Free, really different production processes can be included (i.e. Antibiotic Free from the birth of the pig, Antibiotic Free from the post-weaning of the pig, Antibiotic Free in the last 120 days of life). Also, the concomitance assurance of Animal Welfare and Antibiotic Free occurs. By this, two potential drawbacks need to be taken into consideration for the certification schemes: the transparency of the scheme requirements and the possibility of misleading consumers (Commission Communication, 2010). Concerning the Antibiotic Free claims, the lack of harmonization of certification scheme requirements affects the authentication framework since different tools should be considered to prove and test the truthfulness of the claim. The legislative section previously described displays greater attention in the field of antibiotics administration in animal husbandry, however, some limitations emerged concerning the performance of the analytical tool when the hypothesis to be tested is related to the certification schemes. In this sense, the following example can be explicative. As highlighted from the survey, consumers can find in the meat market meat labelled as "No antibiotics ever" and "No antibiotics in the last three months" or "Animal Welfare and Antibiotic Free". Supposing a positive compliance with legislation from all samples, however different statements require different approaches. In other terms, approaches should be refined and customized according to the level of assurance declared by the producers: if

different labels exist, also different approaches to verify the truthfulness of the claim should exist to avoid false declaration.

The study design: a coin with two sides

The lack of harmonization about the requirements of the certification schemes introduced in the previous section can be also discussed on the experimental level. Designing an experimental strategy able to test multiple hypotheses simultaneously can be challenging. It is widely accepted that the experimental workflow starts with the formulation of the hypothesis and/or biological question. Thus, the object under investigation should be clearly defined for the satisfactory performance of the investigation and to properly discuss the results (Scalbert et al., 2009). As previously highlighted, different label claims exist under the umbrella of the "antibiotic free" meat chain. This multitude of statements may require a customized analytical workflow to properly test different levels of assurance.

Therefore, the emphasized lack of harmonization among certification schemes also affects the design of the experimental study making the definition of a univocal testing approach challenging. The experimental setup may differ for testing two different label claims, i.e. "no antibiotics ever" *vs.* "no antibiotics in the last three months" for authenticity purposes. Going through what is officially declared on the meat label, the statement "No antibiotics ever" refers to animals never treated with antibiotics. Besides what is officially declared by the label claim, further deductible assumptions can be made concerning the animal's husbandry condition. Firstly, it is possible to assume that animals never treated with antibiotics were animals that never experienced health diseases that need to be treated with pharmacologically active substances, considering that the prescription of antimicrobial medicinal products occurs based on evident epidemiological situation (Schmerold et al., 2023). Briefly, the label claim "No antibiotics ever" may be intended considering a direct and a deductible meaning: the direct meaning is related to the fact that the animals were reared under the antibiotic-free production rules; the deductible meaning is related to the assumption that animals were in good health status. This first

example proves the close interdependence of animal welfare and the usage of antibiotics.

The second example related to the label claim “no antibiotics in the last four months or 120 days” may highlight a completely reversed situation. In this case, animals were treated with antibiotics in the early stage of life at least, according to their life cycle. In the present Ph.D. thesis, the animal species under investigation were pigs, therefore a detailed discussion will be provided considering pigs as targeted animals. Briefly, for pigs intended for human consumption with an average life of nine months, “no antibiotics in the last four months” means that pigs were exposed to antibiotics during the first five months of their life. In this case, it may be possible to deduce that animals also experienced pain, discomfort, and disease need to be treated with antibiotics. This last example displayed how two different label claims grouped in the same category – Antibiotic Free production – may hide two different situations on the animal husbandry level.

Based on the description of these two recurring label claims, it is evident that the condition to be tested is completely different. Also, the deductible assumptions for both cases highlighted the close relationship between the animal welfare and the antibiotic administration. These differences can be discussed on experimental levels by considering the comparison between two groups of animals, a classic control *vs*. treated experimental design

The role of the treated group when the investigation between Antibiotic Free *vs.* Antibiotic Treated is performed becomes relevant. In the experimental studies described in Chapter 3, the treated group of pigs was identified with the following definition “groups of pigs not included in the Antibiotic Free production chain and certainly exposed to antibiotics administration”.

To the aim of the present Ph.D. thesis, the main premise was assuming the compliance of considered samples with the EU legislation without considering the case of unappropriated medication with banned substances. As previously introduced, antibiotic-treated animals are animals who experience disease, pain, and stress, thus poor welfare conditions. This non-direct correlation

highlights that animals were antibiotics-treated as well as affected by a disease, for a defined life period at least.

Therefore, the study design results in a coin with two sides: the designed comparison between two groups of animals can be intended as "antibiotic-free *vs.* antibiotic-treated animals" or "positive animal welfare *vs*. poor animal welfare".

Did these two sides affect the performance of the investigation?

In an untargeted study, this mismatch can have a positive and negative effect. Considering the broader meaning of the label claims, both direct and deductible messages, the untargeted metabolomics approach can help simultaneously to test multiple hypotheses since no restrictions have to be applied to the experimental workflow – it allows the analysis without limitations by collecting as many metabolites as possible. However, the drawbacks can be related to the specificity of the results. In other terms, the main question is related to the results intended to be pursued. For screening purposes, the untargeted approach works with high performance returning a huge number of data to be discussed while for more specific and accurate results, it may be challenging starting from a broader assumption as a biological question given that once the identification of molecules is performed could be challenging the discussion of the meaning of the biomarkers. In other words, the biomarkers can be related to both conditions: the experienced disease and the antibiotic assumption. For discrimination purposes, the untargeted metabolomics approach results the best option for testing putative differences on metabolites level for two distinct groups of animals. In the opinion of the authors, further targeted analysis – both on the experimental design levels and sample analysis- may be useful for detecting molecules as clear proof of antibiotic-free label claim.

The eternal struggle in metabolomics: NMR vs. HRMS

The metabolomics approach allows the comprehensive study of small molecules present in cells, tissue, and biofluids able to provide information on the metabolic state of an organism on metabolite levels (Beger et al., 2016).

Starting from the late '90s, different strategies, analytical platforms, and methodologies have populated the scientific facilities making this approach flexible and dynamic. The two major analytical platforms employed in metabolomics analysis are Nuclear Magnetic Resonance and Mass Spectrometry.

In all cases, the applications described in Chapter 3, both analytical platforms have been used to analyse the same kind of samples- liver, kidney, and muscle tissue. According to the studies, different critical aspects emerged during the investigation. For case studies 1 and 2, the NMR spectroscopy operating at high frequencies (600.17 MHz) was selected and used to analyse samples. On the other hand, the UHPLC-HRMS was employed for the third case study. Both analytical platforms were able to discriminate liver, kidney, and diaphragm from antibiotic-free and antibiotic-treated pig groups, considering also the splitting into aqueous and organic extract for each type of sample. The supervised discriminant models (OPLS-DA for Case Studies 1 and 2; PLS-DA for Case Study 3) coupled with the VIP approach allowed to selection of the list of features relevant to the discrimination of two groups. However, merely considering the number of features, the comparison between those resulted relevant in NMR-based metabolomics studies and HRMS-based metabolomics study highlighted a stark difference. The magnitude of selected and identified features was of the order of tens for NMR-based applications, while hundreds of features were relevant for discrimination within the HRMS-based application. As aforementioned, the sensitivity of the two analytical platforms is different, thus these results were not surprising. Despite the remarkable results achieved with NMR for discrimination purposes, some assumptions emerged when the research of specific biomarkers must be performed. Label claims assuring detailed invisible attributes may require a more sensitive approach for biomarker selection. However, for screening purposes, NMR displayed great potential supported by the fact that it is non-destructive, a key advantage.

Harmonization in the experimental workflow

The untargeted metabolomics approach proved to be feasible in the detection of differences between antibiotic-treated *vs.* antibiotic-free pigs'

samples. In the first chapter, all available options to customize the untargeted workflow were discussed ranging from the experimental design to the biological interpretation (Fabrile et al., 2023). Literature displays metabolomics explored in different food matrices for authenticity purposes (Cajka et al., 2010; Amalia et al., 2023; Osorio et al., 2012) and these encouraging trends lead to suppose that shortly this kind of approach may be officially recognized as official tool by regulatory bodies. However, in a long-term perspective, it may be useful to harmonise rules for data reporting aiming to officially recognize metabolomics as a prominent tool within a regulatory framework. In this sense, an analogue work is ongoing related to the OECD reporting framework for omics in regulatory toxicology (Harrill et al., 2021).

Animal Welfare and Antibiotic Free: a univocal biomarker

Although different domains (Nutrition, Environment, Health, Behaviour, and Mental State) can be used to define animal welfare, animal health is *a priori* condition for the definition of animal welfare. Label claims covering both animal welfare and antibiotic-free are currently available in the meat market; however, it is not a general rule since in most cases they are individually declared according to the type of certification scheme adopted by the producers. Antibiotic Free and Animal Welfare are closely related topics. In pig production systems, producers adopting Antibiotic-Free production need to avoid pigs experiencing disease and stress, otherwise, pigs should be treated with antibiotics and then excluded from the antibiotic-free channel. In agreement, literature shows how some diseases, such as respiratory or enteric, can be prevented by managing environmental stressors (Albernaz-Gonçalves et al., 2022). This may lead to the assumption that a cause-consequence relationship exists between Animal Welfare and Antibiotics usage. This relationship exists also on experimental levels, as discussed before, the comparison between two groups of animals can be seen as "antibiotic-free *vs.* antibiotic-treated animals" or "positive animal welfare *vs*. poor animal welfare". All these assumptions lead to ask if two label claims should be separated given that a univocal biomarker assuring both attributes is expected to be found.

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Chapter 5

Conclusion and outlook

Summary

In this section, a general conclusion will be presented, and future perspectives will be also outlined.

In this Ph.D. thesis, the untargeted metabolomics approach was exploited to discriminate pigs exposed to antibiotics administration from untread based on potential differences affecting metabolome and lipidome. To this aim, three experimental studies were presented. For each performed study, different biological questions were postulated. The first application was aimed at gaining molecular insight into the liver metabolome of pigs for comparing Antibiotic Free vs. Antibiotic treated samples. Therefore, the following biological question was formulated:

Are there any metabolic differences between treated vs. untreated pigs?

The ^{1}H NMR-based untargeted metabolomic approach successfully detected differences in molecular signatures in the liver metabolome, both polar and non-polar metabolites, of pigs in the two considered conditions. Therefore, the feasibility of the NMR-based metabolomics approach for screening purposes was proved. For the polar extract of the liver, the results highlighted the up-accumulation of glucose, glutathione, proline, tryptophan, choline, and lactate; while for the non-polar extract of the liver, protons attributable to fatty acid, total cholesterol, and unsaturated fatty acid were down-accumulated; while phospholipids and phosphatidylcholine were up-accumulated. Although different confounding factors were considered as potentially affecting metabolome, among these, the antimicrobial administration and related husbandry conditions played a significant role. Metabolic differences were displayed characterizing the two different phenotypic outcomes considering the liver as a targeted sample. Liver, both aqueous and organic extract, differentiated antibiotic-free from antibiotic-treated samples.

Being the untargeted metabolomic approach hypothesis-generating, the biological question for Case Study 2 was postulated considering the following premise. Currently, liver, kidney, and muscle tissue are among the relevant food matrices to check the compliance of antibiotic residues. Starting from the encouraging outputs obtained by the liver, the following question was formulated:

Is it possible to capture metabolic differences in kidney and muscle from treated vs. untreated pigs?

Firstly, the feasibility of NMR-untargeted metabolomics to investigate metabolic differences in pigs exposed to antibiotic administration *versus* pigs never exposed to antibiotic treatment was confirmed. For the kidney and diaphragm, remarkable results were obtained for the aqueous extract compared to the discrete performances of both organic datasets. The amount of relevant and statistically significant metabolites differently accumulated in muscle was higher (26) compared to the kidney (5). Metabolism mainly affected in muscle were those related to carbohydrates, Ammino Acids and derivatives, Organic Acids, Alcohols, Choline and Derivatives, Nucleosides, and lipid-based molecules. On the contrary, carbohydrates, choline and derivatives, and amino acids were the biochemical category mostly involved in discriminating antibiotic treated from untreated pigs. These findings may highlight the key role of muscle as samples to be targeted for authentication purposes concerning the antibiotic administration. Also, the aqueous extracts for both food matrices were more sensitive to antibiotic administration compared to the organic counterpart of the same sample.

The prominent results highlighted by the first two case studies confirmed the potential of the approach in investigating differences between two pigs' groups, underling the significant role of the aqueous extracts for each type of sample. Assuming to preserve the experimental design, a shift towards the HRMS was performed. Therefore, the third biological question was postulated:

Does the HRMS-based metabolomic approach provide different molecular insights able to differentiate antibiotic-treated vs. untreated pigs?

The HRMS-based investigation of both the metabolome and lipidome of the liver, kidney, and diaphragm of pigs may be useful in discriminating pigs exposed to antibiotics treatment during their life cycle from untreated. Finally, further details emerged from the results. The aqueous extract of the liver differentiated samples according to the antibiotic's exposure while for the organic counterpart, no discrimination was achieved. On the opposite, the aqueous extract of the liver did not differentiate samples according to the

antibiotic's exposure while in the organic counterpart a perfect discrimination was achieved. In the case of muscle, the aqueous extract was not able to discriminate antibiotic-treated from untreated pig samples while a discrete separation was achieved for the organic extracts. The magnitude of relevant features increased in HRMS-study of the order of thousands suggesting that the shift from NMR to HRMS allowed to detection more relevant features.

In summary, the liver – investigated with both analytical platforms- displayed a great discrimination power when the aqueous extract was considered. For the kidney as well as the muscle, the results varied according to the analytical platforms: in the NMR-application, the aqueous extract performed better while in the HRMS-application an optimal separation was achieved within the organic extract. Overall, the antibiotic-free claim authentication can be successfully investigated within the targeted samples.

This study allowed to perform the validation of the hypothesis since at the beginning of the project only biological questions were formulated considering an objective difference in the history of the animals from which the targeted samples were collected; however, no assumption related to the hypothesis was made. The driving force was testing and validating the hypothesis. This task was carried out on both theoretical and experimental framework. Being the untargeted metabolomics hypothesis-generating approach, as previously stated, the hypothesis generated:

Assuming that metabolomics differences have been detected between two groups of samples, by testing different samples and analytical platforms. May these differences be attributable only to antibiotic administration?

This new hypothesis opens the window to future outlooks considering both the theoretical and the experimental framework. Although there are still pending doubts that should be experimentally clarified, however, further investigations should start with the formulation of a more accurate experimental design. This mainly refers to the management of all confounding factors that may define changes in the metabolome and lipidome of pigs. The awareness of multiple factors can facilitate the biomarker interpretation and

discussion in light of the identification of univocal biomarkers able to prove the authenticity of meat labelled as antibiotic free.

Additionally, the collection of both tissue/organ and biofluids from the same animal may be useful to gain an overview of the role of all biological samples in replying to antibiotic administration and to select the proper targeted sample for authentication purposes. In this Ph.D. thesis, the liver, kidney, and diaphragm showed their great potential to be recognized as official samples to be targeted for antibiotic-free authentication. However, further investigations on other type of samples that can be collected in a non-invasive way (such as saliva) may be encouraged; this may allow to perform analysis also time-dependent (i.e. monitoring the changes in metabolome every two months life in pigs exposed and/or unexposed to antibiotics administration).

Annex I

Unsupervised statics results of diaphragm: water-soluble and organic extracts.

The first PCA model built with the full dataset (*n*=54) of polar extract of diaphragm highlighted the presence of one strong outlier belonging to ABT group of samples. As first evaluation, all samples not included in the ellipse (95%) were investigated as suspected outliers. Then, the Hotelling's T2 Range line plot highlighted the presence of two outliers in T2Critic (99%) and (95%), as shown in **Figure 30**. However, only the outlier located in T2Critic (99%) was excluded since the observation resulted far away from the model given that an error occurred during sample preparation and analysis.

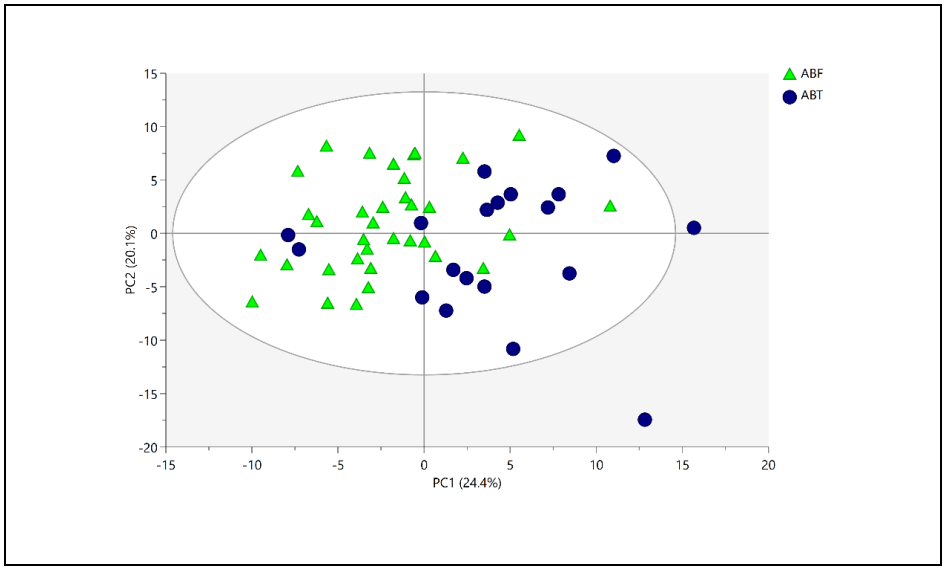

Figure 29. PCA score plot of polar extract of muscle of pigs displaying in green triangle Antibiotic Free samples and blue dot Antibiotic Treated samples. The first two components PC1= 24.4% and PC2= 20.1% explaining the 44.5% were considered and plotted.

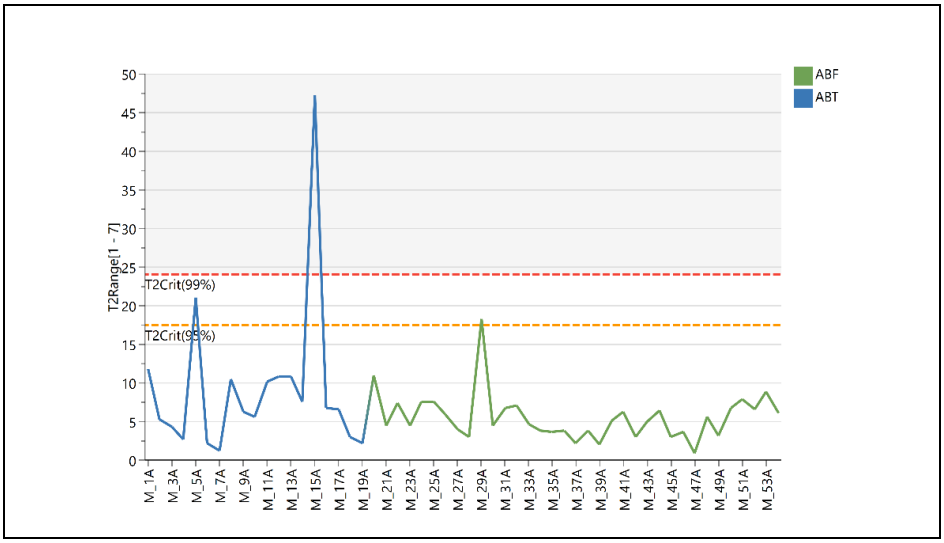

Figure 30. Hotelling's T2 Range line plot of polar extract of muscle of pigs highlights the presence of two outliers T2Crit (99%) and (95%) both belonging to the ABT group. ABT and ABF are both displayed on the same line: ABT samples in light blue line and ABF samples in green.

The analytical outlier detected in the polar extract of diaphragm was also excluded a priori from the organic datasets. Therefore, the original organic dataset was filtered by the detected samples (*n*=53). The PCA model was built for the organic extract and another strong outlier emerged. The Hotelling's T2 Range line plot displayed a strong outlier located in the T2Critic (99%) belonging to ABF samples group. A total of 7 PC described the original organic dataset with an R^2X of 76.5% and Q^2 of 42.6%.

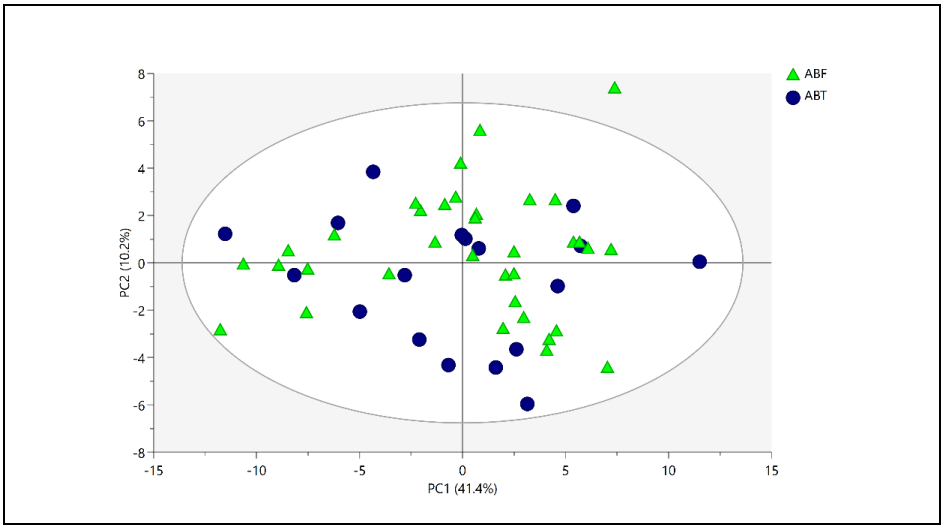

Figure 31. PCA score plot of organic extract of muscle of pigs displaying in green triangle Antibiotic Free samples and blue dot Antibiotic Treated samples. The first two components PC1= 41.4% and PC2= 10.2% explaining the 51.6% were considered and plotted.

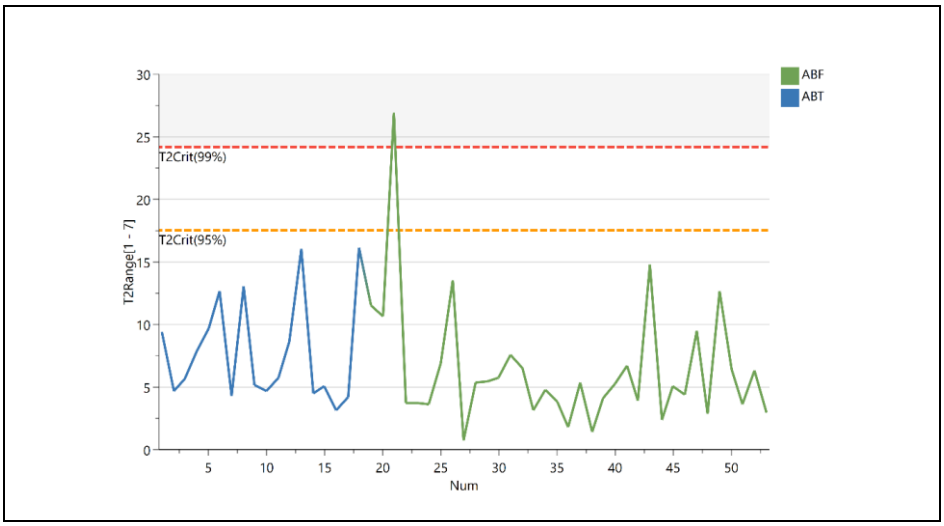

Figure 32. Hotelling's T2 Range line plot of organic extract of muscle of pigs highlights the presence of one outlier T2Crit(99%) belonging to the ABF group. ABT and ABF are both displayed on the same line: ABT samples in light blue line and ABF samples in green.

Unsupervised statics results of kidney: water-soluble and organic extracts.

The first PCA model built with the full dataset (*n*=49) of polar extract of kidney highlighted the presence of one strong outlier. As first evaluation, all samples not included in the ellipse (95%) were investigated as suspected outliers. Then, the Hotelling's T2 Range line plot highlighted the presence of a strong outlier in T2Critic (99%), as shown in **Figure 34**. This model was discarded for further discussion since the presence of a strong outlier was confirmed. However, the original PCA model was described by 7 PCs with R^2X of 78.6% and Q^2 of 50.2%.

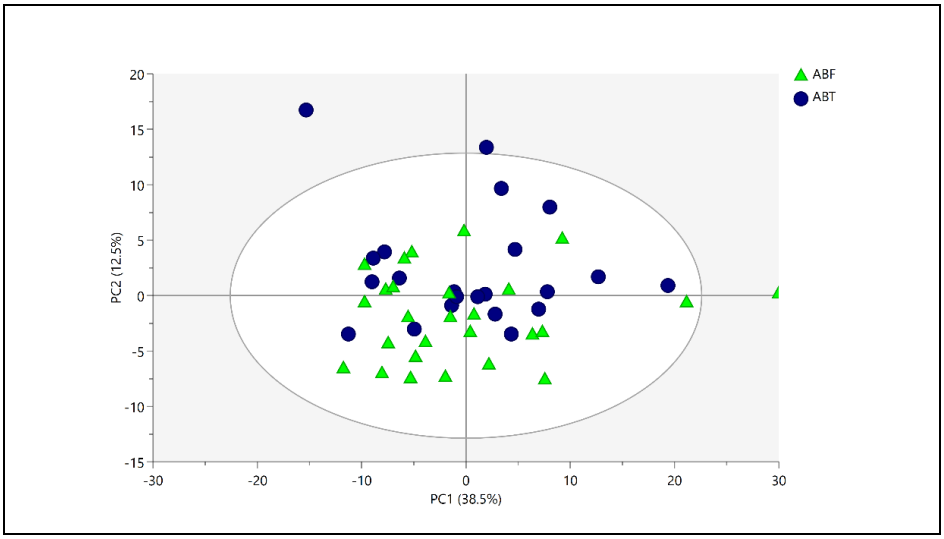

Figure 33. PCA score plot of polar extract of kidney of pigs displaying in green triangle Antibiotic Free samples and blue dot Antibiotic Treated samples. The first two components PC1= 38.5% and PC2= 12.5% explaining the 51% were considered and plotted.

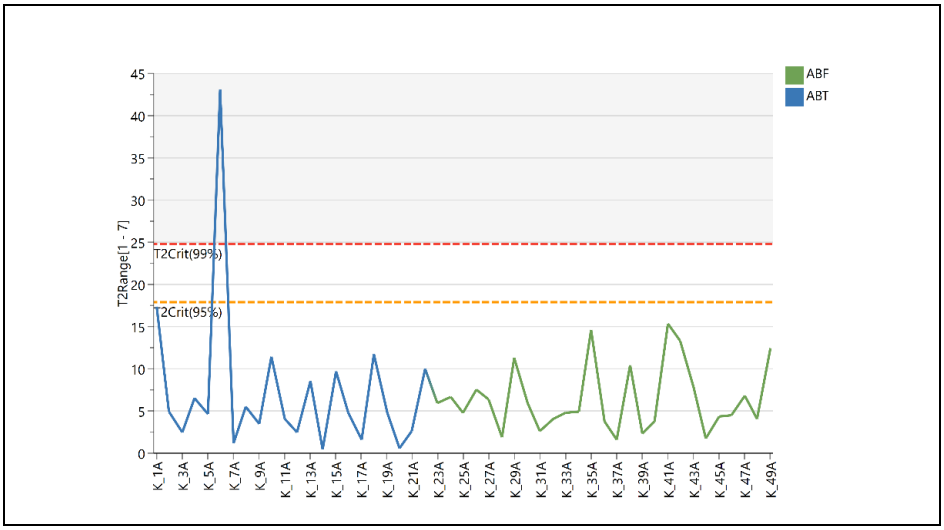

Figure 34. Hotelling's T2 Range line plot of polar extract of kidney of pigs highlights the presence of a strong outlier T2Crit (99%). ABT and ABF are both displayed on the same line: ABT samples in light blue line and ABF samples in green.

For the organic extract of kidney, the first PCA algorithm returned 7 principal components (PCs) and the first two explaining 35.1% of the variance (18.5% in PC1 and 16.6% in PC2) were considered. The model was characterized by discrete values of R^2X= 70.9% and Q^2= 23.6%. Given the presence of two strong outliers, the Hotelling's T2 Range line plot was performed and both suspected outliers were confirmed being located in T2Critic (99%), as shown in **Figure 36**. This model was discarded for further discussion.

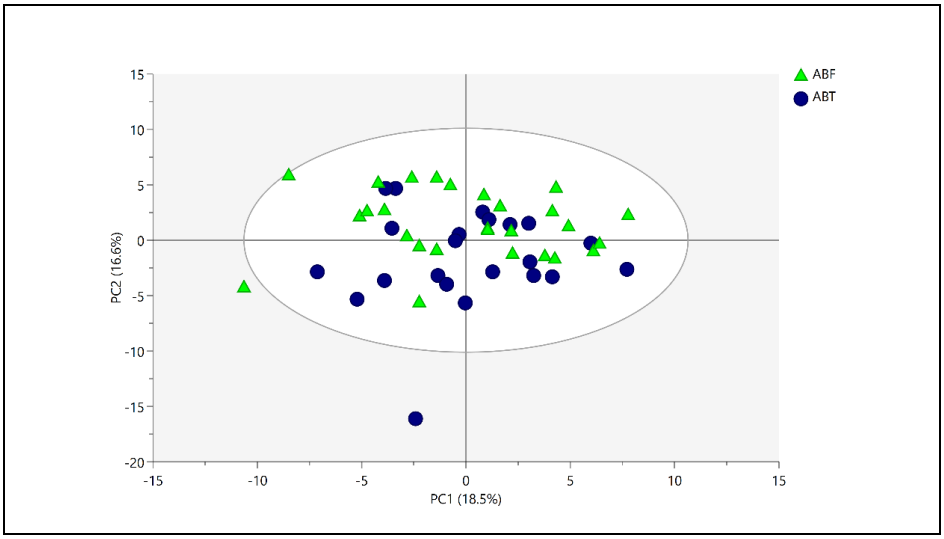

Figure 35. PCA score plot of apolar extract of kidney of pigs displaying in green triangle Antibiotic Free samples and blue dot Antibiotic Treated samples. The first two components PC1= 38.5% and PC2= 12.5% explaining the 51% were considered and plotted.

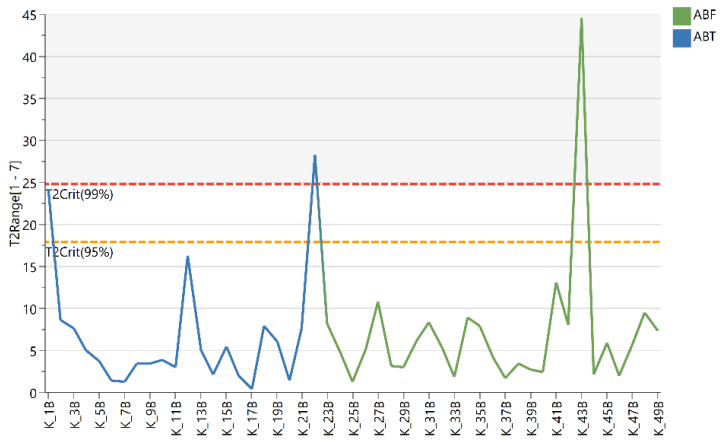

Figure 36. Hotelling's T2 Range line plot of apolar extract of pigs highlighted the presence of two strong outliers in T2Crit (99%), one sample classified as ABF and the other sample belonging to ABT. ABT and ABF are both displayed on the same line: ABT samples in light blue line and ABF samples in green.

^{1}N NMR representative spectrum of the polar extract of kidney and muscle

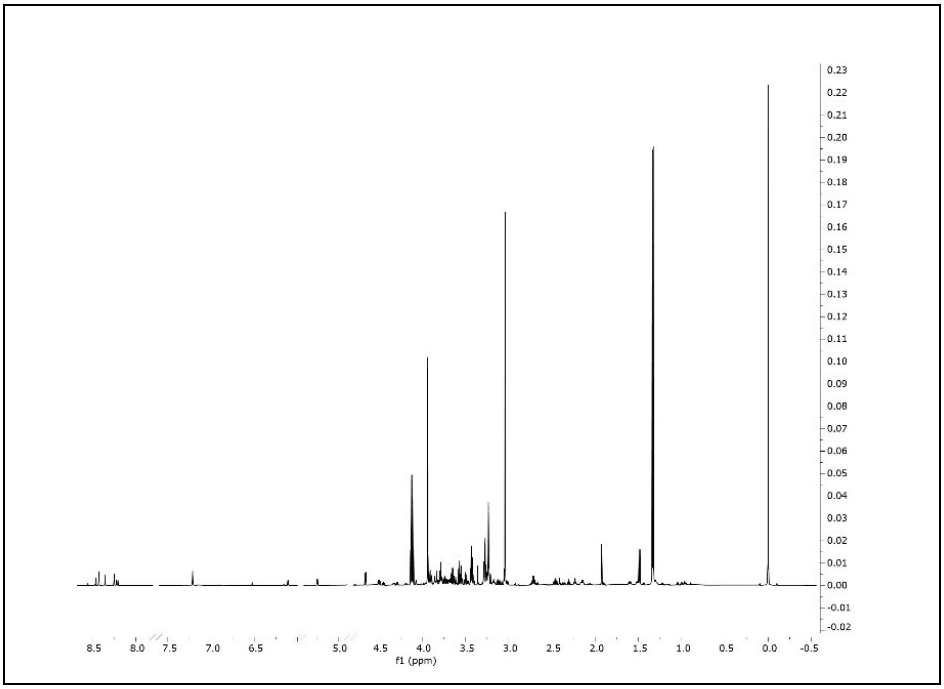

Figure 37. ^{1}H NMR representative spectrum of the polar extract of muscle

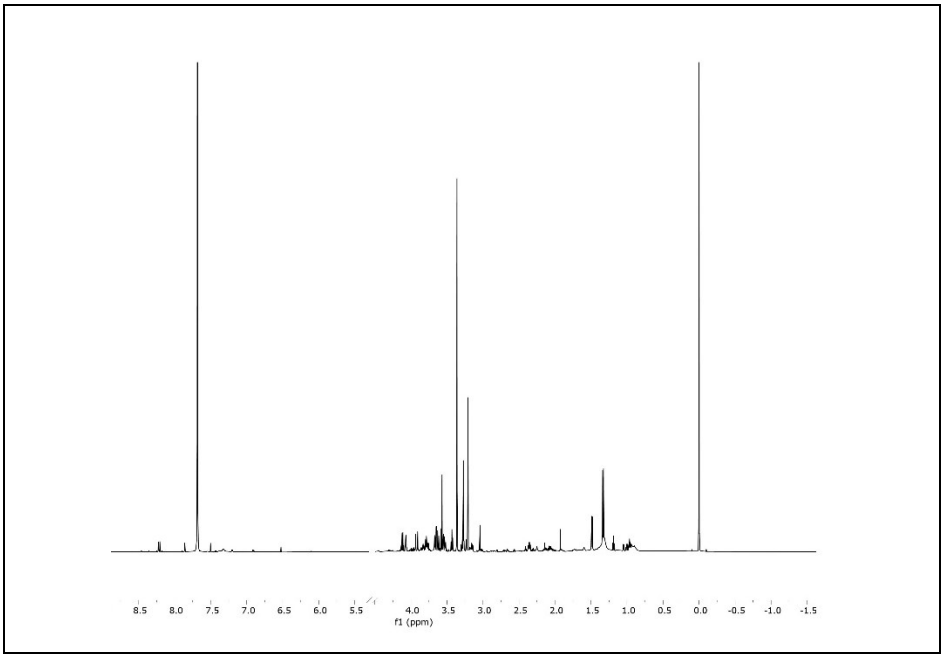

Figure 38. ^{1}H NMR representative spectrum of the polar extract of kidney

Annex II

Selection of discriminating features in metabolome of pigs' matrices

PLS-DA model together with Variable importance in Projection (VIP) analysis were performed to select relevant features contributing to the discrimination between two groups. As general rule, VIP score > 1.0 is considered related to discriminant features.

Table 9. List of relevant features resulting from the metabolomic dataset of kidney by filtering data with VIP score >1.80.

N.	PRIMARY ID	VIP	N.	PRIMARY ID	VIP
1	8.24_257.1747m/z	3.016	**23**	6.79_363.2152m/z	2.617
2	1.24_166.0504m/z	2.866	**24**	5.22_201.1118m/z	2.616
3	7.86_257.1745m/z	2.853	**25**	6.69_541.2645m/z	2.609
4	9.16_302.3046m/z	2.820	**26**	1.51_320.0020n	2.608
5	0.56_1034.9292n	2.808	**27**	6.68_300.2086n	2.606
6	5.60_203.1275m/z	2.805	**28**	6.93_434.2140n	2.598
7	0.65_363.3143m/z	2.799	**29**	0.63_229.2364m/z	2.592
8	6.51_400.2687m/z	2.787	**30**	7.39_392.2041n	2.570
9	6.28_387.1646m/z	2.775	**31**	0.56_869.9617n	2.551
10	0.57_561.9927n	2.733	**32**	0.56_871.9588n	2.550
11	1.58_165.0428n	2.732	**33**	0.63_134.0800n	2.547
12	4.94_315.1552m/z	2.730	**34**	8.16_254.2476m/z	2.544
13	0.55_344.1415m/z	2.675	**35**	8.00_507.1813m/z	2.543
14	0.63_229.1545m/z	2.660	**36**	6.70_541.5081m/z	2.543
15	6.69_609.2515m/z	2.651	**37**	6.49_387.1645m/z	2.534
16	0.56_559.9962n	2.646	**38**	1.15_229.1544m/z	2.531
17	0.56_722.9663n	2.634	**39**	6.26_364.1722n	2.527
18	0.67_376.0389n	2.632	**40**	4.93_339.1523m/z	2.520
19	0.80_166.0726m/z	2.630	**41**	6.43_543.2801m/z	2.513
20	14.89_334.2044m/z	2.628	**42**	1.09_307.1225m/z	2.509
21	0.57_957.0198n	2.625	**43**	7.27_455.1932m/z	2.504
22	0.78_230.1574m/z	2.618	**44**	4.33_325.1758m/z	2.497

45	1.06_196.1743m/z	2.495	**77**	0.61_151.0500n	2.359
46	13.02_578.4168m/z	2.492	**78**	0.57_397.0251n	2.359
47	8.97_299.2823n	2.489	**79**	1.51_271.5565m/z	2.357
48	0.79_222.1370n	2.484	**80**	0.61_502.1484m/z	2.354
49	1.07_152.0017m/z	2.480	**81**	1.37_232.1306m/z	2.354
50	6.25_387.1622m/z	2.475	**82**	10.79_547.3576m/z	2.347
51	1.16_229.2363m/z	2.473	**83**	1.65_272.1258n	2.343
52	1.06_216.0475n	2.470	**84**	0.79_131.0588n	2.343
53	8.97_252.2681m/z	2.465	**85**	10.53_297.2058m/z	2.340
54	8.97_282.4572m/z	2.462	**86**	0.90_351.1561m/z	2.340
55	1.58_138.0553m/z	2.450	**87**	0.67_474.9993m/z	2.337
56	9.46_241.1795m/z	2.444	**88**	0.63_152.2349m/z	2.335
57	0.79_241.3200m/z	2.444	**89**	5.19_335.1335m/z	2.329
58	13.06_450.1530n	2.443	**90**	0.90_351.0557m/z	2.327
59	1.16_229.3147m/z	2.443	**91**	12.69_452.1713m/z	2.327
60	0.62_601.1375m/z	2.442	**92**	0.57_1082.0330n	2.324
61	4.51_359.0905m/z	2.435	**93**	0.56_564.9963m/z	2.324
62	9.20_447.2575m/z	2.434	**94**	7.38_415.1945m/z	2.323
63	0.56_691.0185n	2.434	**95**	4.31_162.0477m/z	2.317
64	12.10_403.2452m/z	2.433	**96**	0.52_257.1105m/z	2.310
65	3.07_318.2155m/z	2.423	**97**	0.56_250.0283n	2.308
66	12.11_601.2311n	2.397	**98**	8.01_418.2192n	2.307
67	1.50_92.0243m/z	2.391	**99**	0.57_271.1755m/z	2.307
68	0.78_229.2361m/z	2.388	**100**	8.29_221.1092n	2.298
69	0.78_184.3442n	2.377	**101**	0.55_251.0309m/z	2.297
70	1.60_108.0445m/z	2.374	**102**	10.94_590.4753m/z	2.295
71	10.93_305.2471m/z	2.374	**103**	11.74_466.1883m/z	2.295
72	2.99_194.0481m/z	2.370	**104**	11.40_461.2623m/z	2.294
73	0.56_1973.1157n	2.365	**105**	7.27_433.2065m/z	2.291
74	0.79_158.1174m/z	2.365	**106**	0.56_832.0048n	2.290
75	12.91_552.4022m/z	2.363	**107**	11.39_393.2746m/z	2.284
76	12.16_551.3892m/z	2.362	**108**	10.93_658.3325m/z	2.284

109	0.63_144.0482m/z	2.274	**141**	0.57_1003.1230m/z	2.227
110	11.74_434.2522m/z	2.272	**142**	18.02_480.2390m/z	2.226
111	12.59_413.1899m/z	2.272	**143**	0.67_446.0016n	2.226
112	7.60_916.2837m/z	2.269	**144**	12.64_340.2858m/z	2.223
113	10.93_545.5967n	2.268	**145**	0.90_351.3214m/z	2.222
114	0.79_282.1194m/z	2.267	**146**	10.94_530.3180m/z	2.221
115	6.90_539.2488m/z	2.267	**147**	0.63_250.0873m/z	2.220
116	0.90_351.2512m/z	2.266	**148**	0.63_169.0355m/z	2.219
117	10.03_284.1976n	2.262	**149**	10.86_635.2506n	2.218
118	11.74_366.3666m/z	2.260	**150**	3.75_181.0495m/z	2.217
119	11.61_530.3232m/z	2.258	**151**	12.00_613.3949n	2.217
120	11.71_368.2780m/z	2.258	**152**	0.56_397.0267n	2.214
121	6.89_347.2198m/z	2.257	**153**	10.94_530.4465m/z	2.212
122	0.56_877.9686n	2.255	**154**	11.74_385.2821n	2.212
123	10.85_612.5190m/z	2.252	**155**	0.56_625.0746n	2.211
124	1.51_268.4751m/z	2.249	**156**	0.61_135.1280n	2.209
125	8.30_420.2331n	2.249	**157**	12.09_500.2297m/z	2.206
126	13.06_239.0582m/z	2.248	**158**	7.91_462.2446n	2.206
127	5.19_229.1422m/z	2.246	**159**	0.72_948.2578m/z	2.205
128	0.80_123.9543m/z	2.244	**160**	0.56_750.0903n	2.203
129	9.68_387.2479m/z	2.244	**161**	3.06_318.0279m/z	2.203
130	10.85_631.4100n	2.243	**162**	1.02_289.9679m/z	2.202
131	1.48_234.9543m/z	2.243	**163**	9.68_424.3423m/z	2.196
132	4.04_110.0164n	2.239	**164**	1.06_196.1024m/z	2.192
133	10.93_546.4820m/z	2.238	**165**	4.77_332.0433m/z	2.192
134	0.63_152.4003m/z	2.237	**166**	1.30_210.0431m/z	2.192
135	10.94_590.5998m/z	2.237	**167**	18.02_412.2511m/z	2.191
136	10.93_590.3452m/z	2.235	**168**	8.49_441.2095m/z	2.190
137	10.93_545.3456n	2.235	**169**	14.11_382.3315m/z	2.187
138	10.94_726.3187m/z	2.234	**170**	1.50_368.0048n	2.185
139	5.54_204.0653m/z	2.230	**171**	12.04_306.1734m/z	2.184
140	13.10_1019.6481m/z	2.227	**172**	8.01_417.3207m/z	2.180

173	8.20_418.2190n	2.178	**205**	0.82_124.0436m/z	2.143
174	0.80_261.0567m/z	2.176	**206**	1.55_454.1330m/z	2.141
175	0.65_290.1572m/z	2.174	**207**	0.55_244.0714n	2.139
176	0.53_102.0912m/z	2.174	**208**	9.49_471.2558m/z	2.137
177	11.75_451.2510m/z	2.173	**209**	10.85_610.2659m/z	2.137
178	0.56_1125.1323n	2.173	**210**	5.00_612.4690m/z	2.133
179	10.56_640.2593m/z	2.173	**211**	0.53_162.1812m/z	2.132
180	13.06_541.1289m/z	2.169	**212**	12.24_948.1371m/z	2.132
181	5.91_770.7324m/z	2.169	**213**	10.82_498.1967n	2.130
182	10.93_636.3261m/z	2.169	**214**	5.49_298.2021m/z	2.129
183	2.70_149.0453m/z	2.165	**215**	12.87_596.3920m/z	2.128
184	12.84_523.2900n	2.165	**216**	3.51_180.0878m/z	2.125
185	8.01_417.4269m/z	2.163	**217**	10.94_730.2616m/z	2.124
186	0.63_223.1209n	2.162	**218**	1.51_270.3407m/z	2.124
187	0.56_669.0374n	2.160	**219**	10.84_502.4124m/z	2.124
188	11.74_336.2522m/z	2.160	**220**	5.98_803.1850m/z	2.123
189	12.84_591.2758n	2.156	**221**	0.61_152.1878m/z	2.121
190	1.51_291.1619m/z	2.156	**222**	0.79_494.2109n	2.120
191	12.23_608.2036m/z	2.153	**223**	11.74_366.4650m/z	2.120
192	1.60_183.0515n	2.153	**224**	0.56_810.0273n	2.119
193	10.69_680.2468m/z	2.152	**225**	1.48_94.0404m/z	2.118
194	4.32_369.1147m/z	2.151	**226**	12.69_312.2538m/z	2.115
195	15.77_699.5614n	2.150	**227**	10.82_363.2884m/z	2.115
196	12.69_312.4381m/z	2.149	**228**	13.10_717.4286m/z	2.112
197	7.26_457.2035m/z	2.148	**229**	0.56_1023.0460n	2.111
198	0.56_500.0596n	2.147	**230**	0.86_227.1171n	2.110
199	0.97_210.9564m/z	2.146	**231**	12.68_268.2629m/z	2.110
200	14.11_450.3187m/z	2.145	**232**	6.11_688.2718m/z	2.109
201	3.53_310.1148m/z	2.145	**233**	8.89_489.2688m/z	2.109
202	8.66_443.2260m/z	2.144	**234**	0.57_875.1046n	2.108
203	0.59_139.9834m/z	2.143	**235**	13.06_335.1561m/z	2.107
204	1.52_135.1526m/z	2.143	**236**	13.06_574.3842m/z	2.105

237	11.85_534.3525m/z	2.105	**269**	0.67_534.0235n	2.070
238	5.89_661.2001m/z	2.105	**270**	6.18_288.2164m/z	2.068
239	10.82_504.5485m/z	2.103	**271**	13.80_305.2468m/z	2.067
240	12.37_487.2222n	2.101	**272**	5.81_275.1028m/z	2.066
241	10.85_502.5284m/z	2.100	**273**	14.35_464.3338m/z	2.066
242	5.91_659.4782n	2.100	**274**	0.80_124.9901m/z	2.065
243	0.48_266.1590m/z	2.099	**275**	13.08_379.1571m/z	2.064
244	0.62_209.0289m/z	2.098	**276**	13.06_337.3620m/z	2.064
245	7.96_250.1107m/z	2.097	**277**	13.10_649.4414m/z	2.064
246	12.11_442.2955m/z	2.094	**278**	10.09_784.3461m/z	2.062
247	1.50_135.0935m/z	2.093	**279**	11.98_397.3035m/z	2.062
248	8.99_264.1262m/z	2.093	**280**	7.75_525.2693m/z	2.062
249	1.72_229.1542m/z	2.093	**281**	5.91_660.6290m/z	2.061
250	10.84_505.4100m/z	2.090	**282**	12.78_529.2139n	2.061
251	10.93_668.2627m/z	2.089	**283**	4.29_281.0996m/z	2.061
252	13.10_380.6205m/z	2.089	**284**	10.82_539.3216n	2.059
253	8.46_443.2245m/z	2.089	**285**	0.78_365.0283m/z	2.059
254	11.62_487.0615m/z	2.088	**286**	13.10_380.7257m/z	2.059
255	1.52_270.2787m/z	2.087	**287**	0.53_144.2137n	2.058
256	1.48_137.3214m/z	2.086	**288**	0.53_163.0564m/z	2.055
257	0.56_838.0131n	2.085	**289**	13.06_707.3761m/z	2.054
258	3.51_180.1602m/z	2.084	**290**	7.90_485.2350m/z	2.053
259	13.06_314.1781n	2.082	**291**	10.85_484.6872m/z	2.053
260	4.69_178.0495m/z	2.080	**292**	12.12_442.4085m/z	2.052
261	13.10_381.8580m/z	2.080	**293**	0.49_1040.8167m/z	2.051
262	10.92_292.6549m/z	2.079	**294**	10.88_533.3317n	2.049
263	13.10_379.3608m/z	2.078	**295**	10.66_612.3294m/z	2.044
264	0.67_413.0745m/z	2.078	**296**	12.12_532.2659m/z	2.043
265	7.34_316.2476m/z	2.078	**297**	11.31_408.2930n	2.043
266	8.92_469.2400m/z	2.077	**298**	0.50_213.2376m/z	2.043
267	1.51_269.4540m/z	2.075	**299**	0.56_544.2755n	2.043
268	1.73_330.0451m/z	2.072	**300**	0.56_559.9969n	2.040

301	5.40_336.1668m/z	2.040	**333**	5.23_146.0601m/z	2.019
302	0.57_612.9186m/z	2.039	**334**	11.63_427.2926n	2.018
303	10.64_996.2522m/z	2.039	**335**	10.82_332.2937m/z	2.017
304	12.69_312.3490m/z	2.039	**336**	0.56_778.0397n	2.017
305	0.56_132.0769m/z	2.039	**337**	5.22_427.8716m/z	2.016
306	12.00_534.3533m/z	2.039	**338**	1.47_119.0123n	2.016
307	13.10_633.1219m/z	2.037	**339**	11.39_660.3477m/z	2.016
308	6.11_374.2527m/z	2.036	**340**	11.28_572.2926m/z	2.014
309	10.69_612.2603m/z	2.036	**341**	3.51_180.2305m/z	2.014
310	13.06_604.4481n	2.035	**342**	13.10_393.2116n	2.013
311	12.12_442.5150m/z	2.035	**343**	11.99_549.6298n	2.013
312	0.57_605.1978m/z	2.034	**344**	13.06_206.0203n	2.012
313	9.91_399.3349n	2.034	**345**	1.78_227.1387m/z	2.011
314	1.02_275.0072m/z	2.033	**346**	13.06_320.1467n	2.008
315	1.48_172.1838n	2.033	**347**	10.00_428.2784m/z	2.008
316	12.78_534.1979m/z	2.031	**348**	9.72_425.3528n	2.008
317	13.10_379.2618m/z	2.031	**349**	7.96_443.1728m/z	2.008
318	0.97_128.0343m/z	2.031	**350**	14.34_396.3471m/z	2.006
319	13.10_380.7019m/z	2.031	**351**	12.37_351.2771n	2.006
320	0.58_376.0583m/z	2.029	**352**	17.99_458.2295m/z	2.004
321	13.10_379.6138m/z	2.029	**353**	0.56_160.1330m/z	2.004
322	5.94_660.9140m/z	2.027	**354**	11.68_440.2798m/z	2.001
323	1.51_269.3926m/z	2.027	**355**	12.70_374.2681m/z	1.999
324	3.04_386.8788m/z	2.026	**356**	5.61_783.0752m/z	1.998
325	0.69_222.9826n	2.025	**357**	1.56_650.1419m/z	1.998
326	5.95_770.5661m/z	2.024	**358**	13.10_515.1320m/z	1.996
327	10.83_308.6039m/z	2.023	**359**	9.91_400.4505m/z	1.995
328	0.55_570.1280n	2.023	**360**	0.61_483.2131n	1.995
329	10.83_552.2594m/z	2.023	**361**	11.28_307.2629m/z	1.995
330	10.96_607.3367m/z	2.020	**362**	2.71_148.0388n	1.995
331	4.31_161.0466n	2.020	**363**	11.34_407.2900m/z	1.994
332	8.53_499.2883m/z	2.020	**364**	10.83_519.8714m/z	1.994

365	0.56_427.9568n	1.994	**397**	10.10_426.4695m/z	1.961
366	0.70_218.9061n	1.994	**398**	6.25_722.8897m/z	1.960
367	10.73_549.2159m/z	1.994	**399**	7.49_416.2049n	1.960
368	11.98_549.3797n	1.989	**400**	0.52_498.8917n	1.960
369	13.06_326.2155m/z	1.989	**401**	10.11_426.5763m/z	1.958
370	1.51_465.8248m/z	1.987	**402**	5.09_548.8067n	1.958
371	12.69_652.2000m/z	1.987	**403**	0.58_234.0719n	1.957
372	5.89_770.8993m/z	1.986	**404**	0.57_375.0375n	1.956
373	0.56_604.0173n	1.985	**405**	13.10_865.3590m/z	1.956
374	13.10_581.4539m/z	1.985	**406**	0.97_183.9694m/z	1.956
375	10.85_1175.5784m/z	1.984	**407**	13.08_426.1731n	1.956
376	4.05_339.0945n	1.981	**408**	10.69_610.2640m/z	1.954
377	4.29_413.1416m/z	1.980	**409**	9.91_400.5544m/z	1.954
378	6.92_457.2041m/z	1.979	**410**	11.61_477.2290m/z	1.954
379	0.89_300.0069m/z	1.978	**411**	11.39_532.3389m/z	1.951
380	14.75_597.3031m/z	1.977	**412**	1.51_268.3803m/z	1.951
381	10.69_363.2883m/z	1.977	**413**	10.00_278.1419m/z	1.949
382	10.92_549.3052m/z	1.976	**414**	11.66_669.2391n	1.949
383	0.56_399.0069n	1.976	**415**	10.51_465.3818n	1.948
384	0.97_280.9376n	1.975	**416**	1.02_324.0058m/z	1.948
385	11.61_485.5310m/z	1.974	**417**	1.50_267.3040m/z	1.946
386	1.48_137.9845m/z	1.973	**418**	0.95_318.0289n	1.945
387	12.23_336.5834m/z	1.971	**419**	4.90_146.0601m/z	1.944
388	10.11_425.3505n	1.971	**420**	0.52_860.9037n	1.943
389	5.09_468.3590m/z	1.971	**421**	10.83_1485.9747n	1.942
390	1.03_306.0128m/z	1.971	**422**	0.63_292.0268n	1.941
391	0.58_235.2415m/z	1.970	**423**	0.57_250.0211n	1.939
392	1.52_443.0886m/z	1.969	**424**	13.10_356.1877n	1.938
393	13.06_322.2691m/z	1.966	**425**	1.50_208.9733m/z	1.938
394	0.56_794.0141n	1.966	**426**	6.64_888.6693m/z	1.937
395	10.84_699.2653n	1.965	**427**	0.56_417.0174n	1.936
396	5.09_656.8655n	1.965	**428**	0.52_301.9441m/z	1.936

429	10.50_635.2130n	1.936	**461**	10.68_598.2658m/z	1.918
430	0.61_504.1688n	1.936	**462**	0.49_668.9337m/z	1.918
431	10.83_518.3085m/z	1.935	**463**	0.63_134.0498m/z	1.917
432	13.06_639.3925m/z	1.934	**464**	1.50_267.0725m/z	1.916
433	0.56_664.0137n	1.934	**465**	13.06_172.9898m/z	1.916
434	12.11_443.7982m/z	1.934	**466**	0.69_373.8800n	1.914
435	0.49_323.1747m/z	1.933	**467**	11.43_402.2647m/z	1.912
436	12.33_369.2851n	1.932	**468**	13.42_418.3311m/z	1.911
437	11.48_667.2390n	1.932	**469**	0.97_274.0473n	1.911
438	0.62_804.2156m/z	1.930	**470**	10.83_486.2973m/z	1.910
439	11.26_365.3035m/z	1.930	**471**	13.10_631.1250m/z	1.909
440	0.60_179.9408m/z	1.930	**472**	12.23_336.5131m/z	1.909
441	1.51_267.2438m/z	1.928	**473**	1.54_190.9654m/z	1.909
442	12.59_377.1414m/z	1.928	**474**	2.21_151.0252m/z	1.908
443	4.87_337.1064m/z	1.928	**475**	11.58_567.3535n	1.906
444	12.02_395.2903m/z	1.927	**476**	11.43_419.2877m/z	1.905
445	4.31_162.0549m/z	1.926	**477**	2.34_166.2092m/z	1.904
446	9.68_424.5606m/z	1.926	**478**	12.69_323.2574m/z	1.904
447	12.12_527.2737m/z	1.926	**479**	1.02_240.0120n	1.904
448	3.70_457.2437m/z	1.925	**480**	0.56_688.0156m/z	1.904
449	0.71_345.1060m/z	1.925	**481**	10.35_398.2648m/z	1.903
450	0.57_576.9732m/z	1.924	**482**	11.61_464.9259n	1.902
451	0.61_665.2113m/z	1.924	**483**	1.50_271.4982m/z	1.900
452	12.09_444.5354m/z	1.923	**484**	12.69_313.6758m/z	1.900
453	0.61_628.1763m/z	1.923	**485**	3.87_354.2384m/z	1.899
454	0.77_185.0112m/z	1.922	**486**	10.61_485.2059m/z	1.898
455	13.10_379.4322m/z	1.922	**487**	10.31_464.3135m/z	1.897
456	8.73_445.2388m/z	1.922	**488**	12.23_336.3519m/z	1.897
457	10.50_753.1773n	1.922	**489**	13.10_381.9825m/z	1.897
458	0.91_371.0303n	1.920	**490**	0.96_216.9254n	1.897
459	1.55_486.0695n	1.918	**491**	0.52_139.1235n	1.896
460	1.51_267.4557m/z	1.918	**492**	2.70_166.2095m/z	1.896

493	3.57_429.7407m/z	1.895	**525**	0.55_178.0531m/z	1.874
494	0.58_427.1691n	1.894	**526**	5.96_679.3509m/z	1.874
495	1.49_226.9864n	1.894	**527**	12.63_358.2331m/z	1.874
496	13.06_775.3646m/z	1.893	**528**	10.70_817.2295n	1.874
497	11.61_463.8211n	1.893	**529**	1.49_601.0892m/z	1.874
498	13.10_279.2320m/z	1.892	**530**	0.47_200.8794n	1.870
499	10.84_612.2608m/z	1.892	**531**	13.10_379.5078m/z	1.870
500	13.10_565.1348m/z	1.891	**532**	0.57_283.9186n	1.869
501	11.29_504.4292m/z	1.889	**533**	10.68_552.3227m/z	1.869
502	12.23_404.4515m/z	1.889	**534**	0.77_298.0192m/z	1.868
503	0.53_965.8353m/z	1.888	**535**	4.70_393.1524m/z	1.868
504	13.06_943.6058m/z	1.888	**536**	13.11_767.0988m/z	1.868
505	0.61_261.0715m/z	1.887	**537**	0.52_218.1176n	1.868
506	2.21_283.0673m/z	1.887	**538**	0.80_355.0419n	1.867
507	5.91_660.7714m/z	1.887	**539**	0.58_480.1825m/z	1.866
508	0.55_113.0344m/z	1.887	**540**	1.02_406.9919n	1.866
509	12.23_336.4454m/z	1.886	**541**	1.48_138.7977m/z	1.865
510	6.21_925.9225m/z	1.885	**542**	13.10_369.1571m/z	1.864
511	10.83_820.5095m/z	1.884	**543**	2.16_153.0183n	1.864
512	0.87_137.9845m/z	1.884	**544**	10.42_490.3886m/z	1.863
513	1.03_110.9755m/z	1.884	**545**	6.97_879.0606m/z	1.863
514	0.52_552.0314m/z	1.883	**546**	11.61_481.7171n	1.863
515	12.78_410.3811m/z	1.883	**547**	1.48_275.9939m/z	1.863
516	11.61_318.5810n	1.882	**548**	12.57_593.4954m/z	1.863
517	6.25_723.3920m/z	1.879	**549**	11.64_1050.1319m/z	1.859
518	0.57_185.0917m/z	1.879	**550**	0.49_688.0017m/z	1.859
519	1.04_258.0258n	1.878	**551**	0.58_287.9756n	1.858
520	13.07_241.0534m/z	1.878	**552**	13.10_381.6296m/z	1.858
521	12.23_455.1975n	1.878	**553**	1.27_242.0638m/z	1.858
522	0.88_167.0201m/z	1.877	**554**	0.52_453.8931m/z	1.858
523	13.10_464.1353m/z	1.877	**555**	11.62_626.2132m/z	1.857
524	2.35_148.0388n	1.877	**556**	12.23_336.2537m/z	1.857

557	0.96_242.9838n	1.856	**589**	6.09_287.1475m/z	1.839
558	9.14_437.3482m/z	1.856	**590**	5.67_149.9765m/z	1.838
559	12.12_398.3046m/z	1.856	**591**	11.66_541.2911n	1.837
560	0.56_775.2217m/z	1.856	**592**	13.10_694.4280m/z	1.837
561	12.25_351.1260m/z	1.856	**593**	0.96_317.9919m/z	1.836
562	13.44_452.3156m/z	1.855	**594**	10.64_1064.2394m/z	1.836
563	11.45_417.2745m/z	1.854	**595**	0.56_685.0113n	1.835
564	13.11_347.3189m/z	1.854	**596**	11.83_431.7941m/z	1.835
565	0.56_870.9724m/z	1.854	**597**	12.23_540.2163m/z	1.835
566	13.10_661.3966m/z	1.853	**598**	0.55_271.1033m/z	1.833
567	4.91_937.7890m/z	1.853	**599**	5.96_679.2082m/z	1.832
568	11.91_422.1989m/z	1.853	**600**	0.70_343.0899m/z	1.831
569	12.58_430.2562m/z	1.852	**601**	0.71_162.8990m/z	1.831
570	11.01_1023.6390m/z	1.849	**602**	11.33_475.2782m/z	1.830
571	0.67_379.0830m/z	1.848	**603**	0.57_668.0285m/z	1.829
572	2.35_296.0990m/z	1.848	**604**	9.50_448.2649n	1.829
573	6.30_265.2036n	1.848	**605**	3.07_318.1233m/z	1.829
574	7.46_285.1119m/z	1.848	**606**	18.97_362.2358m/z	1.828
575	10.25_454.3237m/z	1.847	**607**	10.68_618.2471m/z	1.828
576	0.70_234.9917m/z	1.847	**608**	13.10_797.3725m/z	1.828
577	12.40_350.4654m/z	1.846	**609**	4.98_254.0988m/z	1.828
578	1.36_233.0629m/z	1.846	**610**	12.23_292.2632m/z	1.827
579	12.18_382.2330m/z	1.845	**611**	5.19_508.1787m/z	1.827
580	0.57_935.0371n	1.845	**612**	13.10_699.1124m/z	1.827
581	12.78_495.2500m/z	1.844	**613**	10.67_680.3167m/z	1.826
582	9.02_897.1635m/z	1.843	**614**	0.62_472.0788n	1.826
583	0.80_123.1736m/z	1.843	**615**	12.40_350.3693m/z	1.825
584	6.63_888.9211m/z	1.842	**616**	10.92_540.3200m/z	1.825
585	1.56_298.0593m/z	1.841	**617**	8.61_308.2212m/z	1.825
586	10.72_492.2364m/z	1.841	**618**	12.78_410.4841m/z	1.825
587	10.64_303.6465m/z	1.840	**619**	13.06_122.9738m/z	1.825
588	0.71_228.9206n	1.839	**620**	6.69_142.0655m/z	1.824

621	1.50_434.8477m/z	1.823	**643**	12.23_338.5991n	1.805
622	10.31_399.2740n	1.822	**644**	0.56_413.1536n	1.804
623	12.39_486.2468m/z	1.822	**645**	0.63_136.0222m/z	1.803
624	1.54_206.9607m/z	1.821	**646**	0.82_153.0292m/z	1.802
625	11.28_505.3154n	1.821	**647**	13.10_563.1378m/z	1.801
626	13.06_542.4703n	1.820	**648**	1.04_347.8731m/z	1.801
627	3.07_347.9842m/z	1.820	**649**	13.42_454.3293m/z	1.801
628	2.72_296.0990m/z	1.820	**650**	11.66_757.2089n	1.800
629	13.10_891.1181m/z	1.820			
630	10.71_494.2895m/z	1.819			
631	1.03_267.9037m/z	1.819			
632	0.52_400.0832m/z	1.817			
633	11.59_533.2736m/z	1.817			
634	10.64_567.3286n	1.816			
635	10.06_542.2497m/z	1.814			
636	1.50_433.8491m/z	1.814			
637	12.23_812.1651m/z	1.811			
638	0.49_349.0569m/z	1.809			
639	2.35_164.0566m/z	1.807			
640	10.83_1084.5719n	1.807			
641	1.51_267.1603m/z	1.806			
642	10.17_253.2161m/z	1.806			

Features are collected in the column “Primary ID” in the Retention Time_Mass to Charge Ratio.

Table 10. List of relevant features resulting from the metabolomic dataset of liver by filtering data with VIP score >1.70.

N.	Primary ID	VIP	N.	Primary ID	VIP
1	4.46_556.2037m/z	1.970	**30**	12.41_350.2689m/z	1.874
2	0.53_111.0788n	1.943	**31**	3.08_135.0304m/z	1.874
3	1.13_451.9569m/z	1.943	**32**	12.21_632.2027m/z	1.872
4	12.41_374.3713m/z	1.942	**33**	0.59_509.1564m/z	1.872
5	1.62_183.0522n	1.938	**34**	12.21_429.7427m/z	1.872
6	10.42_603.3874m/z	1.936	**35**	0.74_171.0760m/z	1.872
7	5.23_593.9015n	1.929	**36**	0.54_258.0699m/z	1.871
8	1.13_400.0526n	1.929	**37**	12.20_428.2407m/z	1.870
9	12.41_374.2687m/z	1.926	**38**	10.38_513.2614n	1.870
10	12.41_442.2562m/z	1.923	**39**	12.41_418.2558m/z	1.870
11	3.56_314.1343m/z	1.921	**40**	10.61_520.5031m/z	1.868
12	0.86_230.0180n	1.911	**41**	0.82_152.9950m/z	1.867
13	12.93_388.2839m/z	1.909	**42**	0.80_245.1262m/z	1.862
14	3.55_432.1876m/z	1.908	**43**	0.74_171.1430m/z	1.858
15	13.10_607.4199m/z	1.907	**44**	7.53_640.3381m/z	1.856
16	0.54_240.1205n	1.906	**45**	12.21_700.1899m/z	1.855
17	12.21_365.3275m/z	1.904	**46**	12.21_360.2532m/z	1.855
18	1.13_399.1530m/z	1.899	**47**	3.81_254.1038m/z	1.854
19	6.59_1095.0620m/z	1.897	**48**	12.21_429.8185m/z	1.854
20	4.46_558.2200m/z	1.896	**49**	1.61_403.0940n	1.852
21	0.62_571.1274m/z	1.893	**50**	1.62_138.0553m/z	1.852
22	12.93_456.2708m/z	1.892	**51**	0.55_354.1407m/z	1.850
23	12.21_496.2283m/z	1.890	**52**	12.41_619.2865m/z	1.850
24	0.71_253.8694n	1.890	**53**	3.08_249.0618m/z	1.849
25	12.20_564.2164m/z	1.886	**54**	12.21_360.4519m/z	1.848
26	12.21_428.3517m/z	1.884	**55**	1.62_108.0444m/z	1.847
27	1.61_480.0336m/z	1.880	**56**	12.21_428.4567m/z	1.846
28	18.22_622.3092n	1.877	**57**	0.73_420.8796n	1.845
29	1.13_399.3284m/z	1.875	**58**	12.41_374.4715m/z	1.844

59	10.69_744.2910m/z	1.843	**91**	0.61_898.2288n	1.815
60	0.85_212.0221m/z	1.843	**92**	0.71_251.8741n	1.813
61	1.13_246.0495n	1.842	**93**	10.38_579.2460n	1.812
62	13.15_364.2840m/z	1.842	**94**	3.75_457.1498m/z	1.812
63	10.62_510.2342m/z	1.841	**95**	10.69_880.2656m/z	1.811
64	10.46_636.3932m/z	1.840	**96**	12.21_445.2304m/z	1.810
65	10.61_522.2676m/z	1.840	**97**	3.74_455.1343m/z	1.810
66	12.21_972.1396m/z	1.839	**98**	12.21_360.6979m/z	1.809
67	12.21_836.1640m/z	1.838	**99**	10.97_838.2942m/z	1.809
68	0.62_664.3892m/z	1.835	**100**	3.81_376.3677m/z	1.808
69	10.96_602.2946m/z	1.835	**101**	13.10_675.4073m/z	1.805
70	10.84_391.2241m/z	1.834	**102**	10.96_974.2687m/z	1.805
71	10.96_906.2815m/z	1.834	**103**	11.58_284.2216m/z	1.803
72	10.51_707.2721n	1.832	**104**	1.13_172.0128n	1.802
73	10.34_399.2737n	1.831	**105**	12.21_362.8383m/z	1.802
74	12.21_768.1772m/z	1.829	**106**	10.61_498.2562m/z	1.801
75	0.53_337.0325m/z	1.829	**107**	0.80_245.4088m/z	1.800
76	1.63_138.1788m/z	1.827	**108**	0.71_234.0446m/z	1.799
77	10.61_537.2548m/z	1.827	**109**	0.79_152.1883m/z	1.799
78	0.62_663.1096n	1.826	**110**	0.54_225.1793m/z	1.799
79	12.20_435.2116n	1.826	**111**	12.18_384.2524m/z	1.798
80	0.54_227.1056m/z	1.825	**112**	10.61_554.2030m/z	1.798
81	0.53_93.0448m/z	1.824	**113**	12.87_338.2691m/z	1.798
82	0.64_206.0798m/z	1.823	**114**	2.52_338.0907m/z	1.797
83	10.96_843.2434n	1.822	**115**	10.96_1042.2553m/z	1.796
84	0.80_246.0497n	1.821	**116**	6.64_526.2770m/z	1.795
85	12.91_390.2995m/z	1.820	**117**	6.75_599.2667m/z	1.794
86	4.90_266.1022m/z	1.819	**118**	12.21_363.0147m/z	1.794
87	12.73_593.4037m/z	1.819	**119**	1.07_278.1137m/z	1.794
88	6.34_250.1075m/z	1.817	**120**	10.35_398.2648m/z	1.793
89	12.44_386.2689m/z	1.817	**121**	10.97_641.2838n	1.793
90	10.29_475.2896m/z	1.815	**122**	0.73_223.0519n	1.792

123	1.66_243.1340m/z	1.792	**154**	8.80_518.3243m/z	1.771
124	10.97_749.3324n	1.790	**156**	10.97_1000.1927m/z	1.769
125	10.69_948.2520m/z	1.790	**155**	10.61_551.3021n	1.771
126	7.48_346.0970m/z	1.789	**157**	12.21_694.4092m/z	1.769
127	0.92_180.1375m/z	1.788	**158**	13.54_879.6617m/z	1.769
128	4.02_428.7491m/z	1.787	**159**	12.88_746.1929m/z	1.769
129	0.94_431.3274m/z	1.786	**160**	12.20_361.3649n	1.769
130	12.21_904.1516m/z	1.786	**161**	12.87_406.2561m/z	1.768
131	13.26_364.2839m/z	1.786	**162**	12.21_361.7847m/z	1.767
132	10.69_592.2997m/z	1.785	**163**	10.69_1152.2152m/z	1.767
133	6.40_672.3320m/z	1.784	**164**	0.52_155.1985n	1.767
134	12.88_610.2185m/z	1.784	**165**	10.38_805.1832n	1.766
135	12.20_474.2199m/z	1.783	**166**	13.17_416.3155m/z	1.766
136	0.53_249.0625n	1.782	**167**	5.55_594.2764m/z	1.765
137	0.79_492.0996n	1.782	**168**	12.21_649.1906m/z	1.764
138	1.13_399.5140m/z	1.780	**169**	10.35_466.2533m/z	1.764
139	1.13_540.8177m/z	1.780	**170**	12.21_360.3558m/z	1.764
140	12.66_380.2405m/z	1.780	**171**	1.85_221.0919m/z	1.764
141	0.54_226.1808m/z	1.779	**172**	11.56_688.4769m/z	1.764
142	10.46_636.5227m/z	1.777	**173**	0.54_113.1312n	1.763
143	8.80_296.2335n	1.777	**174**	13.03_352.2847m/z	1.763
144	12.91_402.3000m/z	1.776	**175**	12.23_359.2425n	1.762
145	12.87_406.3639m/z	1.776	**176**	6.64_528.2927m/z	1.762
146	10.30_498.2802n	1.776	**177**	10.62_453.5168n	1.761
147	6.21_925.9228m/z	1.774	**178**	11.73_366.2629m/z	1.761
148	3.81_376.1628m/z	1.774	**179**	6.99_614.3293m/z	1.760
149	1.31_199.9407m/z	1.773	**180**	10.97_1110.2426m/z	1.759
150	8.88_534.2869m/z	1.773	**181**	10.45_664.2548m/z	1.759
151	3.97_245.1860m/z	1.771	**182**	12.21_360.7949m/z	1.758
152	12.21_617.1724n	1.771	**183**	0.53_310.0216m/z	1.758
153	10.69_639.3551n	1.771	**184**	11.56_689.4860n	1.757

185	12.87_338.4614m/z	1.757	**216**	0.52_154.0613m/z	1.739
186	1.10_275.3006m/z	1.757	**217**	12.21_364.1113m/z	1.739
188	12.20_542.2073m/z	1.755	**218**	12.23_337.6627m/z	1.739
187	12.21_437.1971n	1.756	**219**	11.48_684.3514n	1.739
189	12.87_814.1804m/z	1.755	**220**	16.72_410.2359m/z	1.739
190	12.88_542.2315m/z	1.754	**221**	12.23_541.2229n	1.739
191	10.45_636.2584m/z	1.754	**222**	12.87_423.2460m/z	1.739
192	1.13_400.0529n	1.753	**223**	0.61_508.1405n	1.739
193	12.77_362.2685m/z	1.753	**224**	9.99_485.2940m/z	1.738
194	0.53_296.0064m/z	1.753	**225**	0.88_280.0268n	1.737
195	12.23_472.2283m/z	1.753	**226**	12.87_453.2697n	1.736
196	1.06_199.9410m/z	1.751	**227**	4.34_273.0663m/z	1.736
197	0.79_248.2231m/z	1.751	**228**	6.23_705.3448m/z	1.735
198	6.99_554.3076m/z	1.750	**229**	11.60_704.3356m/z	1.735
199	4.94_506.2423m/z	1.750	**230**	9.84_619.3828m/z	1.735
200	11.56_448.3053m/z	1.748	**231**	10.69_1016.2396m/z	1.735
201	0.53_241.0512n	1.748	**232**	8.80_560.3530n	1.735
202	12.21_479.1966n	1.748	**233**	7.04_534.3085m/z	1.735
203	13.98_571.2873m/z	1.747	**234**	0.62_134.0465m/z	1.734
204	12.87_406.4666m/z	1.746	**235**	8.55_536.3335m/z	1.734
205	11.06_602.2628m/z	1.746	**236**	0.80_245.2062m/z	1.734
206	10.61_656.2390m/z	1.746	**237**	10.43_568.9888m/z	1.733
207	12.88_339.6065n	1.745	**238**	12.21_547.1839n	1.733
208	0.54_225.0981m/z	1.744	**239**	3.97_243.1702m/z	1.733
209	10.61_510.2134m/z	1.744	**240**	11.87_378.2248m/z	1.733
210	12.87_950.1548m/z	1.743	**241**	5.55_393.1228m/z	1.732
211	0.82_153.1248m/z	1.743	**242**	10.38_338.7882m/z	1.731
212	10.61_476.3923m/z	1.742	**243**	13.53_436.3023m/z	1.731
213	13.29_354.2997m/z	1.741	**244**	15.53_585.3038m/z	1.731
214	5.55_593.2677n	1.741	**245**	11.88_395.2140m/z	1.731
215	3.97_243.2538m/z	1.740	**246**	8.80_475.3050n	1.730

247	13.17_484.3021m/z	1.730	**278**	6.67_682.3171m/z	1.719
248	10.81_570.2801m/z	1.730	**279**	1.88_137.0344m/z	1.718
249	1.65_147.0325n	1.730	**280**	12.23_608.2026m/z	1.718
250	0.57_272.1244m/z	1.729	**281**	10.51_656.5852m/z	1.718
251	0.80_247.3221m/z	1.728	**282**	1.63_93.0614m/z	1.718
252	8.76_259.1899m/z	1.728	**283**	10.97_1068.1787m/z	1.718
253	12.22_336.2532m/z	1.728	**284**	8.89_535.3275n	1.717
254	16.72_478.2231m/z	1.727	**285**	10.45_632.5800m/z	1.717
255	17.66_402.2131m/z	1.727	**286**	5.22_688.3290m/z	1.717
256	16.74_410.3437m/z	1.727	**287**	2.05_251.1027m/z	1.717
257	11.58_352.2089m/z	1.727	**288**	8.69_511.2524m/z	1.716
258	4.35_252.0869m/z	1.726	**289**	2.99_136.0390n	1.715
259	12.23_336.4450m/z	1.725	**290**	11.60_1044.2709m/z	1.715
260	12.92_378.2993m/z	1.724	**291**	3.93_385.2448m/z	1.715
261	8.93_578.3080m/z	1.724	**292**	1.63_260.1361n	1.715
262	8.93_533.3114n	1.723	**293**	8.57_520.2948m/z	1.715
263	10.78_527.2365n	1.723	**294**	0.98_229.9270m/z	1.714
264	12.20_241.1716n	1.723	**295**	12.20_180.1297n	1.714
265	0.91_290.0871m/z	1.723	**296**	12.23_376.2317n	1.714
266	10.38_337.2734m/z	1.723	**297**	4.07_730.3156m/z	1.714
267	10.51_724.3045m/z	1.723	**298**	12.04_418.2558m/z	1.713
268	14.15_382.3307m/z	1.722	**299**	10.45_632.4505m/z	1.713
269	4.88_384.1412m/z	1.721	**300**	1.72_235.1075m/z	1.713
270	0.80_463.8158m/z	1.721	**301**	0.61_353.0917m/z	1.713
271	12.87_338.3677m/z	1.721	**302**	10.80_503.3008n	1.712
272	0.53_137.0581n	1.721	**303**	17.49_803.2796m/z	1.712
273	16.61_456.2157m/z	1.721	**304**	6.71_541.2643m/z	1.711
274	1.61_350.9353m/z	1.720	**305**	0.73_343.0903m/z	1.711
275	3.97_243.3336m/z	1.720	**306**	10.97_702.3198m/z	1.711
276	12.20_361.4643n	1.720	**307**	12.20_383.2440n	1.710
277	2.51_336.0744m/z	1.719	**308**	10.38_479.9066m/z	1.710

309	10.54_298.2131n	1.710	**329**	8.80_580.3248m/z	1.703
310	10.61_1090.5479n	1.710	**330**	7.14_489.3405n	1.703
311	0.52_407.0239m/z	1.709	**331**	0.61_136.1863m/z	1.703
312	12.88_340.8028m/z	1.709	**332**	10.61_452.4999m/z	1.703
313	0.92_180.0655m/z	1.709	**333**	9.41_448.2626n	1.703
314	12.06_378.2267m/z	1.709	**334**	12.87_576.1686m/z	1.702
315	0.83_558.0627m/z	1.709	**335**	0.60_560.1361n	1.702
316	10.97_636.2740m/z	1.709	**336**	10.47_503.3909m/z	1.702
317	6.67_554.3085m/z	1.708	**337**	8.80_652.2541m/z	1.702
318	12.87_457.2119n	1.708	**338**	12.23_812.1644m/z	1.702
319	12.23_880.1518m/z	1.708	**339**	0.80_544.0040m/z	1.702
320	2.53_293.0903n	1.707	**340**	3.61_263.1386m/z	1.702
321	7.37_596.3173m/z	1.706	**341**	12.07_442.2560m/z	1.701
322	12.20_794.1017m/z	1.706	**342**	10.69_767.2802n	1.701
323	3.50_459.1855n	1.706	**343**	10.06_352.1852n	1.701
324	10.52_640.2994m/z	1.706	**344**	12.07_374.2687m/z	1.701
325	10.84_482.2335m/z	1.704	**345**	0.82_287.1509n	1.701
326	12.64_430.2551m/z	1.704	**347**	10.51_660.3955m/z	1.700
327	0.96_316.0343m/z	1.704	**348**	10.43_608.3534m/z	1.700
328	10.43_581.3193m/z	1.703			

Features are collected in the column “Primary ID” in the Retention Time_Mass to Charge Ratio.

Table 11. List of relevant features resulting from the metabolomic dataset of muscle by filtering data with VIP score >1.70.

N.	Primary ID	VIP	N.	Primary ID	VIP
1	1.24_134.8933m/z	2.729	**31**	1.43_376.1181m/z	2.224
2	3.48_181.0712m/z	2.718	**32**	0.63_157.1107n	2.216
3	0.45_102.9434m/z	2.680	**33**	0.56_839.1263n	2.212
4	4.06_363.1122m/z	2.680	**34**	0.54_393.3576m/z	2.191
5	0.78_197.9131n	2.635	**35**	1.51_251.0359m/z	2.184
6	1.50_159.9971n	2.589	**36**	11.30_1006.1965m/z	2.179
7	10.43_279.2325m/z	2.552	**37**	3.44_246.1701m/z	2.177
8	1.26_162.8995m/z	2.538	**38**	0.73_102.9415m/z	2.175
9	10.43_279.3216m/z	2.508	**39**	1.52_859.1539m/z	2.158
10	9.88_444.3685m/z	2.505	**40**	11.62_507.5821m/z	2.148
11	0.90_193.0035m/z	2.464	**41**	1.51_689.0616m/z	2.138
12	10.43_279.4073m/z	2.461	**42**	0.45_216.9090m/z	2.135
13	1.50_299.0415n	2.457	**43**	0.55_388.0700m/z	2.128
14	7.22_226.0174m/z	2.414	**44**	0.55_386.1107m/z	2.123
15	0.90_225.9783m/z	2.397	**45**	1.54_818.9185m/z	2.119
16	1.51_253.9738m/z	2.392	**46**	0.56_250.0289n	2.114
17	4.18_435.1872m/z	2.383	**47**	0.45_411.8566n	2.112
18	0.45_126.9428n	2.368	**48**	0.79_383.9937m/z	2.107
19	3.44_246.2558m/z	2.344	**49**	11.14_436.8824m/z	2.106
20	1.51_497.9277m/z	2.342	**50**	0.47_443.1366n	2.090
21	5.28_274.2012m/z	2.310	**51**	12.15_274.6720m/z	2.087
22	1.08_285.0048m/z	2.281	**52**	8.00_417.2115m/z	2.082
23	11.40_739.2949n	2.280	**53**	1.03_462.8255m/z	2.081
24	3.44_246.3366m/z	2.278	**54**	0.91_177.0247m/z	2.077
25	0.58_228.0633m/z	2.268	**55**	0.89_257.1117m/z	2.073
26	0.45_349.8647m/z	2.267	**56**	0.56_288.9749m/z	2.072
27	10.83_273.0034m/z	2.256	**57**	0.92_368.8273m/z	2.068
28	0.55_335.1468n	2.237	**58**	0.57_160.1337m/z	2.067
29	10.52_507.4132n	2.232	**59**	1.49_210.0405n	2.066
30	1.27_524.9812m/z	2.232	**60**	0.51_348.9718m/z	2.064

61	9.10_416.3369m/z	2.064	**93**	1.28_236.9310m/z	1.956
62	1.49_239.0354m/z	2.063	**94**	1.52_589.0724m/z	1.955
63	0.56_276.1309n	2.062	**95**	0.56_980.0610n	1.955
64	1.36_481.7233m/z	2.059	**96**	1.54_332.0126m/z	1.955
65	5.51_330.2262m/z	2.055	**97**	0.59_295.0132n	1.948
66	0.54_437.1064m/z	2.037	**98**	6.69_541.2654m/z	1.944
67	0.47_149.0955n	2.034	**99**	4.23_302.3805m/z	1.942
68	11.30_938.2095m/z	2.033	**100**	0.72_260.3211m/z	1.941
69	5.22_330.2276m/z	2.031	**101**	0.66_228.1477n	1.940
70	9.23_384.3109m/z	2.031	**102**	0.47_584.2148m/z	1.934
71	11.02_436.2814m/z	2.028	**103**	0.85_155.9516m/z	1.933
72	0.73_357.9966m/z	2.023	**104**	0.90_340.9835n	1.933
73	0.94_314.0848m/z	2.019	**105**	0.54_408.1727n	1.930
74	0.45_254.4153m/z	2.018	**106**	1.02_891.0006m/z	1.925
75	11.04_476.2486m/z	2.018	**107**	1.52_820.2064n	1.925
76	0.46_79.9593n	2.017	**108**	1.02_374.2305m/z	1.917
77	0.81_261.1445m/z	2.008	**109**	11.13_377.2450m/z	1.916
78	0.70_254.8768m/z	2.007	**110**	10.87_535.2986m/z	1.916
79	0.81_178.0861m/z	1.997	**111**	11.61_482.6394m/z	1.916
80	0.54_394.1574n	1.995	**112**	0.72_260.4117m/z	1.915
81	0.56_670.1012n	1.993	**113**	11.39_612.3007m/z	1.910
82	1.52_177.0413m/z	1.991	**114**	10.93_543.2480m/z	1.906
83	0.45_326.8946n	1.991	**115**	9.16_302.3044m/z	1.900
84	1.52_1178.1434m/z	1.985	**116**	0.61_369.2142m/z	1.899
85	4.23_302.1965m/z	1.984	**117**	11.37_670.2741m/z	1.898
86	0.72_259.4001m/z	1.978	**118**	0.72_261.3561m/z	1.896
87	11.66_1024.2070m/z	1.973	**119**	1.25_305.9450m/z	1.896
88	7.93_386.2899m/z	1.972	**120**	9.91_299.2569m/z	1.894
89	0.79_158.1182m/z	1.971	**121**	0.47_241.2949m/z	1.891
90	0.87_222.9093m/z	1.967	**122**	0.47_212.2583m/z	1.890
91	1.52_135.3058m/z	1.959	**123**	0.90_196.0177n	1.889
92	0.45_391.8101n	1.959	**124**	0.50_357.1403m/z	1.889

125	11.40_671.3076n	1.888	**157**	0.71_337.0209n	1.830
126	11.63_283.4393m/z	1.887	**158**	11.63_283.6592m/z	1.830
127	0.90_275.9841n	1.885	**159**	4.23_302.2902m/z	1.828
128	0.48_484.1207m/z	1.883	**160**	0.72_259.0219m/z	1.825
129	1.50_335.0038m/z	1.879	**161**	3.67_450.0864m/z	1.825
130	6.92_358.2585m/z	1.878	**162**	0.90_322.9482n	1.824
131	11.63_283.2636m/z	1.878	**163**	0.54_447.0790m/z	1.823
132	1.02_347.0245m/z	1.877	**164**	9.62_468.3682m/z	1.821
133	0.56_412.9999n	1.873	**165**	0.47_765.1966m/z	1.820
134	11.96_564.3641m/z	1.868	**166**	11.16_626.2694m/z	1.818
135	0.60_357.1026m/z	1.867	**167**	0.72_259.1907m/z	1.818
136	10.84_255.2326m/z	1.867	**168**	3.67_382.3049m/z	1.818
137	11.27_195.9718n	1.866	**169**	11.29_870.2225m/z	1.816
138	1.52_92.0249m/z	1.865	**170**	11.63_799.2710n	1.816
139	1.52_135.1525m/z	1.863	**171**	0.73_327.9816n	1.816
140	10.84_255.3182m/z	1.862	**172**	0.62_388.0647m/z	1.814
141	0.72_259.2504m/z	1.862	**173**	0.47_714.2809n	1.808
142	0.52_334.1053n	1.858	**174**	11.63_283.3544m/z	1.808
143	9.68_423.3353n	1.855	**175**	1.48_181.0072n	1.805
144	1.52_135.0941m/z	1.852	**176**	1.20_304.0899n	1.800
145	1.54_190.9658m/z	1.848	**177**	0.62_259.1050n	1.799
146	11.64_888.2328m/z	1.846	**178**	1.51_189.9777m/z	1.798
147	10.84_255.4000m/z	1.840	**179**	11.29_713.2736n	1.797
148	0.71_567.7616m/z	1.840	**180**	3.43_298.0693m/z	1.795
149	0.71_232.8559m/z	1.839	**181**	0.63_241.6023m/z	1.792
150	1.32_319.0687n	1.839	**182**	0.64_453.0627m/z	1.792
151	0.83_121.9380n	1.838	**183**	11.02_195.9718n	1.789
152	0.69_290.0872m/z	1.838	**184**	0.61_245.3161m/z	1.789
153	10.25_496.3992m/z	1.833	**185**	11.09_549.2881m/z	1.788
154	11.29_1074.1830m/z	1.832	**186**	9.70_279.2320m/z	1.786
155	1.34_220.0978m/z	1.832	**187**	3.83_601.3319m/z	1.786
156	11.17_596.2660m/z	1.830	**188**	11.64_935.2452n	1.785

189	10.01_412.3418m/z	1.784	**221**	11.17_643.2795n	1.750
190	1.54_274.8008m/z	1.782	**222**	9.42_479.3577m/z	1.748
191	12.17_624.2963m/z	1.780	**223**	11.60_536.7385m/z	1.745
192	0.73_219.0055m/z	1.777	**224**	1.54_665.0353m/z	1.744
193	0.72_337.0232n	1.776	**225**	0.72_339.9955n	1.741
194	1.24_404.0074n	1.775	**226**	1.51_177.0241m/z	1.740
195	0.69_558.0438m/z	1.774	**227**	11.29_802.2356m/z	1.737
196	0.65_517.9672m/z	1.772	**228**	0.54_423.1138n	1.737
197	0.90_276.1444m/z	1.772	**229**	0.85_524.6733m/z	1.736
198	1.46_397.0308m/z	1.771	**230**	0.47_241.2140m/z	1.735
199	11.66_632.2537m/z	1.771	**231**	0.63_243.4674m/z	1.732
200	0.91_191.0189m/z	1.769	**232**	1.51_513.8902n	1.731
201	0.90_342.9992n	1.768	**233**	0.96_637.1192m/z	1.730
202	11.39_604.3357m/z	1.768	**234**	0.55_495.9283m/z	1.725
203	0.85_289.1261n	1.768	**235**	1.02_134.0467m/z	1.724
204	3.67_382.2047m/z	1.768	**236**	0.70_322.8655n	1.724
205	11.51_558.3458m/z	1.767	**237**	9.68_424.4541m/z	1.724
206	0.56_722.9556n	1.767	**238**	0.54_276.1325n	1.722
207	0.57_1071.3101m/z	1.765	**239**	0.90_223.9637m/z	1.720
208	11.16_467.2722m/z	1.764	**240**	1.20_327.0798m/z	1.720
209	1.52_135.1972m/z	1.764	**241**	5.33_459.7398m/z	1.719
210	4.15_208.1327m/z	1.761	**242**	0.46_153.9694n	1.718
211	11.02_153.8968n	1.760	**243**	11.17_596.3944m/z	1.718
212	11.63_1092.1936m/z	1.757	**244**	11.39_643.3130n	1.718
213	5.47_274.2017m/z	1.755	**245**	0.47_241.1303m/z	1.718
214	0.61_227.5673n	1.755	**246**	0.71_460.9254n	1.718
215	0.66_478.8716n	1.755	**247**	0.64_180.9760m/z	1.718
216	0.65_504.9006m/z	1.753	**248**	11.19_573.3565m/z	1.717
217	0.72_319.0583n	1.752	**249**	0.70_388.0645m/z	1.715
218	1.30_218.0667m/z	1.752	**250**	0.73_259.3116m/z	1.715
219	11.02_437.5911n	1.751	**251**	9.36_442.3528m/z	1.715
220	1.06_388.2074m/z	1.750	**252**	1.02_876.0386n	1.714

253	0.57_286.0599m/z	1.713	**266**	11.95_898.1621m/z	1.706
254	1.16_345.8577m/z	1.713	**267**	1.02_324.4657m/z	1.705
255	1.51_135.0309m/z	1.713	**268**	11.94_410.1228m/z	1.704
256	11.43_788.2580m/z	1.713	**269**	0.63_471.0537m/z	1.703
257	10.96_638.2756m/z	1.712	**270**	4.94_304.2121m/z	1.703
258	0.73_224.9250m/z	1.711	**271**	11.34_269.2473m/z	1.701
259	0.52_416.1642m/z	1.711			
260	0.55_378.1499m/z	1.711			
261	0.47_211.0959n	1.709			
262	0.47_344.0932m/z	1.708			
263	0.46_282.0649m/z	1.708			
264	12.15_510.5119m/z	1.707			
265	0.62_307.1364n	1.707			

Features are collected in the column “Primary ID” in the Retention Time_Mass to Charge Ratio

Selection of discriminating features in lipidome of pigs' matrices

Table 12. List of relevant features resulting from the lipidomic dataset of liver by filtering data with VIP score>1.50.

N.	PRIMARY ID	VIP	N.	PRIMARY ID	N.
1	9.94_703.5193m/z	1.871	**30**	9.51_607.5867n	1.695
2	9.92_645.2973m/z	1.828	**31**	10.40_681.3434m/z	1.695
3	8.20_647.4566m/z	1.816	**32**	6.45_509.4801n	1.692
4	0.49_248.9230m/z	1.808	**33**	8.21_565.6731n	1.690
5	0.82_414.3728m/z	1.803	**34**	10.34_264.2686m/z	1.689
6	6.85_827.5662n	1.791	**35**	5.69_747.5645m/z	1.682
7	7.35_619.4259m/z	1.788	**36**	10.35_658.8829m/z	1.679
8	9.95_621.6049n	1.770	**37**	9.05_264.2687m/z	1.675
9	9.91_621.6059n	1.770	**38**	9.52_607.5887n	1.673
10	9.95_666.8740m/z	1.769	**39**	8.20_610.8000m/z	1.673
11	9.07_675.4879m/z	1.765	**40**	0.53_121.1111n	1.673
12	9.03_593.5748n	1.756	**41**	9.95_666.7400m/z	1.671
13	9.91_644.7302m/z	1.753	**42**	2.21_325.2987n	1.671
14	5.00_700.5507n	1.746	**43**	9.08_593.5739n	1.670
15	0.90_416.3879m/z	1.746	**44**	6.82_759.5771n	1.670
16	10.39_717.5351m/z	1.745	**45**	9.06_616.6963m/z	1.667
17	10.34_659.3214m/z	1.742	**46**	5.69_702.5673n	1.665
18	10.35_635.6210n	1.738	**47**	8.19_565.8010n	1.663
19	9.91_644.8644m/z	1.733	**48**	6.23_722.5527m/z	1.662
20	10.39_635.6206n	1.731	**49**	1.75_347.2832n	1.662
21	8.17_565.5438n	1.728	**50**	8.18_264.2686m/z	1.654
22	6.28_682.5584m/z	1.723	**51**	10.82_711.6244m/z	1.650
23	10.39_680.7569m/z	1.722	**52**	9.09_593.8386n	1.649
24	6.47_548.4454m/z	1.713	**53**	8.20_565.5425n	1.649
25	0.49_250.9216m/z	1.709	**54**	7.76_551.5270n	1.647
26	8.30_491.2611m/z	1.704	**55**	2.06_349.2985n	1.644
27	10.39_680.8925m/z	1.700	**56**	10.82_649.7766n	1.640
28	10.35_658.7471m/z	1.700	**57**	9.95_720.5216n	1.638
29	2.26_269.2261m/z	1.696	**58**	10.82_731.5510m/z	1.638

59	10.78_649.6372n	1.637	**91**	7.35_560.6287m/z	1.588
60	10.82_696.7748m/z	1.636	**92**	10.78_264.2688m/z	1.585
61	6.86_851.3700m/z	1.635	**93**	5.79_745.4996m/z	1.584
62	5.69_748.3253m/z	1.632	**94**	10.73_134.1097n	1.582
63	0.78_412.3555m/z	1.631	**95**	0.72_535.3275n	1.581
64	9.31_619.5894n	1.630	**96**	1.13_398.3168m/z	1.580
65	8.66_579.5573n	1.627	**97**	7.13_221.0238m/z	1.579
66	0.99_430.4002m/z	1.622	**98**	6.86_850.8620m/z	1.579
67	9.94_316.3002n	1.621	**99**	7.36_264.2689m/z	1.578
68	2.03_299.2827n	1.620	**100**	7.35_537.5124n	1.578
69	3.30_410.1755m/z	1.618	**101**	7.34_561.1615m/z	1.576
70	5.01_745.5473m/z	1.611	**102**	8.18_587.6547n	1.575
71	10.82_649.6362n	1.611	**103**	5.69_747.8501m/z	1.575
72	10.83_649.9113n	1.609	**104**	9.94_622.5543n	1.575
73	9.32_619.5890n	1.607	**105**	9.94_264.2688m/z	1.573
74	1.57_297.2672n	1.607	**106**	10.40_667.6462n	1.572
75	9.03_593.8381n	1.607	**107**	7.13_162.9822m/z	1.571
76	5.70_951.5257m/z	1.605	**108**	7.08_832.8872m/z	1.571
77	9.52_684.6131m/z	1.605	**109**	1.11_390.3366m/z	1.568
78	9.08_593.7081n	1.602	**110**	10.39_734.5368n	1.568
79	5.69_687.5435m/z	1.598	**111**	8.65_562.5547m/z	1.568
80	10.78_650.3590n	1.597	**112**	7.77_551.5267n	1.566
81	11.24_663.6513n	1.597	**113**	6.40_723.4948m/z	1.565
82	10.72_199.1483m/z	1.596	**114**	6.86_700.5038n	1.564
83	6.63_841.5191m/z	1.596	**115**	1.74_307.2656m/z	1.562
84	10.82_695.3707m/z	1.595	**116**	6.42_747.3246m/z	1.561
85	5.69_838.5406n	1.594	**117**	7.35_537.7626n	1.561
86	1.74_245.2262m/z	1.593	**118**	1.75_347.4872n	1.559
87	1.75_323.2833n	1.593	**119**	7.09_754.4910n	1.559
88	10.80_649.9114n	1.591	**120**	5.69_725.6999m/z	1.556
89	1.75_286.2297n	1.590	**121**	6.86_782.8618m/z	1.555
90	7.15_89.9921n	1.589	**122**	5.69_815.5517m/z	1.554

123	5.69_704.7064n	1.553	**147**	0.90_405.3976n	1.519
124	5.69_793.8430m/z	1.551	**148**	6.40_1036.4703m/z	1.519
125	6.40_1172.4451m/z	1.548	**149**	6.91_523.4955n	1.518
126	10.78_649.7756n	1.548	**150**	6.63_773.5319m/z	1.518
127	7.27_864.5692m/z	1.547	**151**	10.81_673.0011m/z	1.517
128	6.85_210.9488m/z	1.545	**152**	7.28_774.8953m/z	1.515
129	1.44_345.2666n	1.544	**153**	7.35_561.0569m/z	1.515
130	7.28_758.5686m/z	1.544	**154**	6.87_777.5297n	1.515
131	5.70_749.7170m/z	1.543	**155**	5.69_702.8475n	1.513
132	5.69_702.9500n	1.540	**156**	6.86_760.8775m/z	1.512
133	6.86_178.9769m/z	1.539	**157**	10.15_269.2263m/z	1.512
134	3.65_269.2257m/z	1.538	**158**	5.69_703.7168m/z	1.511
135	11.19_663.6515n	1.538	**159**	6.86_918.5413m/z	1.511
136	6.62_604.5360m/z	1.538	**160**	7.28_775.3723m/z	1.511
137	6.45_509.4798n	1.535	**161**	6.86_762.8159m/z	1.511
138	0.54_239.1542n	1.534	**162**	5.69_542.4904m/z	1.510
139	7.36_520.7516m/z	1.531	**163**	6.40_1138.3544m/z	1.506
140	9.93_645.6768m/z	1.531	**164**	5.69_929.5174m/z	1.505
141	6.86_782.7165m/z	1.531	**165**	6.86_762.4397m/z	1.504
142	6.40_968.4833m/z	1.530	**166**	0.72_553.3369n	1.504
143	9.91_579.5335m/z	1.529	**167**	9.94_645.8139m/z	1.503
144	1.75_323.4804n	1.526	**168**	6.86_761.3559n	1.503
145	6.86_221.0238m/z	1.524	**169**	7.35_520.6310m/z	1.502
146	6.86_850.7097m/z	1.520			

Features are collected in the column “Primary ID” in the Retention Time_Mass to Charge Ratio

Table 13. List of relevant features resulting from the lipidomic dataset of kidney by filtering data with VIP score >1.75.

N.	Primary ID	VIP	N.	Primary ID	VIP
1	5.71_207.0487m/z	2.383	**31**	7.68_513.2919n	2.136
2	6.30_207.1236m/z	2.381	**32**	6.30_177.0336m/z	2.130
3	5.73_207.1235m/z	2.368	**33**	6.29_445.2401m/z	2.128
4	6.57_372.2135n	2.362	**34**	9.36_621.6050n	2.127
5	5.72_445.2401m/z	2.347	**35**	6.38_857.4991n	2.126
6	6.54_445.2407m/z	2.338	**36**	8.45_519.2977m/z	2.124
7	5.14_389.2109m/z	2.328	**37**	8.43_475.2168n	2.124
8	6.55_389.2109m/z	2.311	**38**	5.73_177.0334m/z	2.119
9	6.11_445.2406m/z	2.303	**39**	7.64_510.1860m/z	2.111
10	5.72_89.9924n	2.285	**40**	9.36_666.8748m/z	2.103
11	5.71_389.2109m/z	2.282	**41**	9.36_656.5746m/z	2.100
12	6.13_534.2668m/z	2.278	**42**	6.38_1072.4054m/z	2.097
13	6.11_389.2109m/z	2.260	**43**	6.30_1150.4707m/z	2.080
14	6.55_510.1862m/z	2.254	**44**	4.41_677.4985n	2.080
15	5.71_520.2152m/z	2.245	**45**	9.37_682.5904m/z	2.076
16	5.75_256.9542m/z	2.244	**46**	8.42_701.4914m/z	2.071
17	7.13_673.4925m/z	2.238	**47**	6.31_1021.4573n	2.067
18	6.26_417.2136m/z	2.217	**48**	6.29_389.2109m/z	2.066
19	6.62_673.4927m/z	2.215	**49**	6.30_418.2373m/z	2.061
20	6.31_183.9613n	2.213	**50**	6.58_431.2558m/z	2.061
21	6.29_520.2154m/z	2.189	**51**	4.61_704.5215m/z	2.059
22	5.73_179.0488m/z	2.179	**52**	6.57_534.2664m/z	2.053
23	9.78_697.6081m/z	2.164	**53**	5.72_534.2664m/z	2.049
24	5.72_417.2160m/z	2.163	**54**	9.76_635.6207n	2.047
25	6.38_884.4880m/z	2.160	**55**	6.26_510.1865m/z	2.042
26	6.11_510.1862m/z	2.160	**56**	10.61_708.6492m/z	2.038
27	5.14_408.2799n	2.157	**57**	5.72_493.2244n	2.037
28	7.11_534.2664m/z	2.146	**58**	5.13_437.2597n	2.035
29	5.72_137.9559n	2.138	**59**	9.37_647.6205n	2.033
30	6.31_179.0487m/z	2.138	**60**	8.45_463.3805m/z	2.033

61	9.37_692.8942m/z	2.032	**93**	7.66_541.2438m/z	1.930
62	8.44_534.2662m/z	2.031	**94**	6.14_810.5918n	1.928
63	8.95_710.6286m/z	2.029	**95**	9.76_670.5888m/z	1.927
64	6.82_341.1445m/z	2.018	**96**	8.52_628.5426m/z	1.926
65	7.37_886.6281m/z	2.018	**97**	2.43_413.2114m/z	1.916
66	6.87_652.4725m/z	2.018	**98**	6.30_431.2559m/z	1.916
67	5.90_251.0910m/z	2.012	**99**	6.12_841.5812n	1.916
68	8.40_885.7052m/z	2.012	**100**	2.45_725.4964m/z	1.915
69	9.38_264.2692m/z	2.009	**101**	6.88_625.4841n	1.913
70	8.62_870.6872m/z	2.009	**102**	6.88_634.4483n	1.909
71	9.31_683.6398n	2.003	**103**	2.43_404.2580n	1.909
72	6.55_493.2244n	2.000	**104**	9.39_872.7083m/z	1.909
73	6.56_467.1879m/z	2.000	**105**	4.47_826.5420n	1.907
74	2.36_465.2062m/z	2.000	**106**	7.67_1129.6231m/z	1.905
75	6.38_341.1447m/z	1.995	**107**	2.45_699.4972m/z	1.903
76	8.98_633.6044n	1.995	**108**	8.13_624.5556m/z	1.900
77	5.14_815.7025m/z	1.990	**109**	6.54_1058.5522m/z	1.897
78	8.83_640.5866m/z	1.983	**110**	6.07_673.4953m/z	1.894
79	5.14_687.5437m/z	1.976	**111**	10.18_694.7738m/z	1.889
80	6.30_534.2666m/z	1.975	**112**	10.18_694.9112m/z	1.889
81	5.63_519.3256m/z	1.974	**113**	5.14_684.4024n	1.885
82	9.37_692.7587m/z	1.968	**114**	6.18_854.4878m/z	1.879
83	10.10_711.6707n	1.965	**115**	5.37_728.5831n	1.875
84	8.96_652.5876m/z	1.957	**116**	10.18_649.6366n	1.874
85	8.53_593.5739n	1.953	**117**	10.18_684.6056m/z	1.874
86	8.44_445.2426m/z	1.947	**118**	5.70_1098.5076m/z	1.872
87	4.61_726.5038m/z	1.946	**119**	5.29_706.6802m/z	1.871
88	5.63_444.3382n	1.946	**120**	2.36_437.2115m/z	1.869
89	6.87_626.4733m/z	1.944	**121**	6.86_674.5106m/z	1.865
90	5.63_547.4441m/z	1.942	**122**	5.02_853.5612n	1.862
91	9.37_709.6089m/z	1.942	**123**	8.91_703.6307n	1.862
92	8.52_655.5618m/z	1.940	**124**	6.38_812.5783m/z	1.861

125	8.41_841.7134m/z	1.857	**157**	5.14_793.6949m/z	1.811
126	1.61_347.3792n	1.856	**158**	5.63_521.2469n	1.809
127	5.52_922.5528m/z	1.856	**159**	10.18_711.6246m/z	1.809
128	5.29_796.5080m/z	1.854	**160**	1.10_302.3048m/z	1.806
129	4.46_745.5486m/z	1.853	**161**	5.75_978.4957m/z	1.805
130	3.07_727.5118m/z	1.853	**162**	9.38_264.4416m/z	1.804
131	6.82_902.5855m/z	1.852	**163**	5.68_872.5394m/z	1.803
132	9.37_604.7333m/z	1.851	**164**	5.71_489.2708m/z	1.802
133	5.29_750.5284m/z	1.848	**165**	5.63_861.6695m/z	1.802
134	7.00_764.4995n	1.846	**166**	6.82_651.5289m/z	1.802
135	0.59_321.0776m/z	1.846	**167**	7.32_551.5260n	1.799
136	6.39_936.4304m/z	1.845	**168**	8.82_873.7038m/z	1.798
137	5.14_783.5153m/z	1.845	**169**	6.88_652.6073m/z	1.796
138	2.44_697.5009m/z	1.844	**170**	6.06_900.5664m/z	1.795
139	5.14_722.5473m/z	1.844	**171**	0.79_254.2476m/z	1.793
140	6.50_673.4934m/z	1.840	**172**	5.63_547.5667m/z	1.792
141	2.40_551.3947n	1.840	**173**	5.72_764.5422m/z	1.791
142	7.01_768.5155m/z	1.839	**174**	6.82_901.5247n	1.791
143	3.84_967.7913m/z	1.838	**175**	9.36_621.6059n	1.789
144	8.43_681.6247n	1.834	**176**	6.82_980.4581m/z	1.788
145	8.44_463.4934m/z	1.833	**177**	7.70_299.0014m/z	1.788
146	6.38_823.4415n	1.832	**178**	8.77_680.5738m/z	1.788
147	7.90_841.6531n	1.829	**179**	6.01_769.0369m/z	1.787
148	4.86_692.5216m/z	1.827	**180**	8.82_781.5032m/z	1.786
149	8.88_699.5874m/z	1.827	**181**	7.10_675.4944m/z	1.786
150	5.89_419.2438m/z	1.826	**182**	5.14_467.1882m/z	1.784
151	7.21_539.5267n	1.822	**183**	5.29_706.9222m/z	1.783
152	5.73_416.2336n	1.819	**184**	6.56_792.5709m/z	1.783
153	6.87_675.5187n	1.814	**185**	6.07_811.6038m/z	1.780
154	6.82_828.4909m/z	1.814	**186**	9.38_682.8655m/z	1.780
155	8.59_664.5868m/z	1.814	**187**	8.88_637.5992n	1.779
156	2.38_749.4964m/z	1.812	**188**	5.29_706.8195m/z	1.778

189	6.83_902.7448m/z	1.776	**200**	2.06_445.2387m/z	1.764
190	7.67_1197.6097m/z	1.776	**201**	6.37_767.5881n	1.764
191	12.52_930.6699m/z	1.773	**202**	9.38_252.2667m/z	1.763
192	6.69_728.5576m/z	1.771	**203**	2.36_466.1535m/z	1.761
193	6.66_804.5083m/z	1.769	**204**	5.91_874.7112m/z	1.758
194	5.63_354.2075n	1.768	**205**	8.78_645.6049n	1.755
195	5.14_815.8527m/z	1.767	**206**	6.77_623.3562m/z	1.755
196	6.31_125.0002m/z	1.766	**207**	4.47_701.8414m/z	1.754
197	3.06_699.5162m/z	1.766	**208**	1.01_282.3699m/z	1.753
198	6.82_897.4932m/z	1.766	**209**	6.71_818.5901m/z	1.753
199	2.99_492.1963m/z	1.765	**210**	9.38_631.3172m/z	1.752

Features are collected in the column “Primary ID” in the Retention Time_Mass to Charge Ratio.

Epilogue

In the first months of my Ph.D. project "New tools for authentication and traceability to assure the integrity of pig chain", a recurring question was in my mind: *Where do I start with this topic?* So, I thought to start from the simple things, the meaning of the words "Antibiotic Free" and "Animal Welfare".

Once I reviewed the literature, I realized that this topic required a holistic approach by integrating different disciplines – chemistry, veterinary, statistics, legislation, and biochemistry- to gain an overview of the issues. During these three years, I had the opportunity to refine my knowledge of metabolomics and chemometric techniques by working on my research project and collaborating with various research units. I am curious by nature, and I enjoy discovering new paths. Therefore, I ventured into hugely different activities, passing from the support of the veterinarian during the sampling process to the assignment of ^{1}H NMR signals in spectra. To date, I know the exciting potential of -omics approach by seeing "pros and cons" and "do and don'ts" at the experimental level.

And just like that,

while I am writing the last words of this experience, I am thinking about that girl at the beginning of the Ph.D. programme during a year marked by dramatic worldwide event and I am grateful to this experience for the awareness that science always means life. For this reason, I decided to leave here a personal reflection that I wrote on my diary in 2021 since it embodies the meaning of my untargeted work on my personal life.

On 11th February 2021

Life is made of memories and each memory is connected to another memory to build a network, as for the molecules. A group of memories may help us to face new experiences, challenges, and new experiences will be new memories, as a chain reaction. I mean, a past experience could affect our present life but our present life, one day, will be the past, like a second chain reaction. Now, let imagine that there are experiences that we have not lived yet, hidden molecules or secret pathways, and, probably, those will be the experiences that will change our life. We cannot see them, we do not know them, but they have the power

to upset our life. They have no name, no structure, but they have so vigorous power.

My favourite question: what I cannot see could be more important of what I can see?
Everything will be clear when we will be able to see every single molecule, to face every single experience, to connect every single pathway or memory. One day, being able to say "There was the reason why..." but, in the meanwhile, isn't so funny walking in the void?

As someone said:

E il naufragar m'è dolce in questo mare (L'infinito, G. Leopardi).

Acknowledgments

It is true that scientific research comes from hard work, study, and trials but it is also true that scientific research is possible thanks to human contribution. The support of professors, teammates, and family has been essential during my Ph.D. programme.
This thesis would not have been possible without the precious collaboration with different research units.

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Thank you for all those times in which: you deserve this, Pia.
Thank you for sharing with me the precious value of time since only with time and patience science will give you the results that you pursued.
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And now it is time for my family, who supported me since ever.

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Thank you to all my aunties, cousins, and grandparents for being always with me. Thank you to my second family Elisa, Mimmo, and Daniela for supporting me throughout the years. My heart is full of love thanks to you.

To my grandmother, Miella

To my great-grandmother, Giovanna

To my sister, Paola

You are not here anymore, but you are always with me, my angels.

A special place is reserved for you in my heart and my mind.

Thank you to my stepfather Rocco for supporting me and for standing always by my side. We are not family by blood, we are family by heart.

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Thank you for respecting me, my choices, and my thoughts.

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Countless times I thought i was not enough, while you remembered my greatest values countless times.

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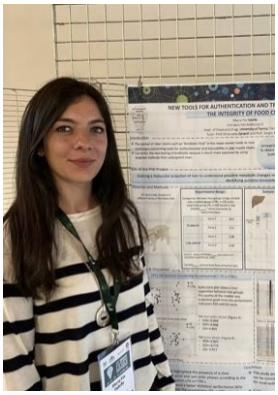
believe in myself. This path was possible thanks to your patience and your love. You are my soulmate.

And to the dreamer who lives in you, Pia

Dreams come true!

Author Information

Maria Pia Fabrile



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About me

Maria Pia got the bachelor's degree in food science and technology at the University of Foggia (Department of Agricultural Sciences, Food, Natural Resources, and Engineering (DAFNE) with a thesis titled "BRC: quality standards affecting HACCP, workplace and food processes and products"; then, she moved to University of Parma for the master's degree in Food Science and Technology (Department of Food and Drug) with a thesis titled "Fumonisin accumulation in maize: which metabolic pathways are involved in cross talk plant-pathogen?". In 2019, she worked for a food company operating in the field of canteen (schools, hospitals) as Health and Safety Specialist before and as food quality assurance officer later. In 2020, she started the PhD course in Food science of University of Parma working within the "Unit of hygiene and inspection of food of animal origin".

She is passionate about research, curious about life and motivated.

Scientific activity

Conference and Seminars

Can metabolomics fingerprinting detect the antibiotic administration in pigs?
Fabrile MP, Caligiani A, Ghidini S, Alborali GL, De Luca S, Scali F, Lolli V, Conter M, Varrà MO, Ianieri A, Zanardi E. (Poster Presentation)
69th International Congress of Meat Science and Technology, University of Padova, 20/08/2023 – 25/08/2023

An exploratory study on the use of antibiotics in the pig chain as an issue for public health and food authenticity
Fabrile MP, Ghidini S, Caligiani A, Scali F, Varrà MO, Alborali GL, Ianieri A, Zanardi E.
(Oral presentation, Non-presenting Author)
76° Convegno SISVET (Società Italiana delle Scienze Veterinarie), Villa Romanazzi-Carducci Bari, 21/06/2023 - 23/06/2023

Approccio innovativo per la rivelazione dell'uso di antibiotici nei suini
Fabrile MP, Caligiani A, Alborali GL, Scali F, De Luca S, Lolli V, Varrà MO, Zanardi E. (Poster Presentation)
XXXI Convegno Nazionale A.I.V.I. (Associazione Italiana Veterinari Igienisti), University of Teramo, 22/09/2022 –24/09/2024

New tools for authentication and traceability to assure the integrity of food chain
Fabrile MP, PhD candidate. Zanardi E, Ghidini S, Academic tutors. (Poster Presentation)
26th Workshop on the developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Torino, Asti, 19/09/2022 – 21/09/2022.

Exploiting NMR-based untargeted metabolomics approach to unravel the administration of antibiotics in pig liver
Fabrile MP, Caligiani A, Ghidini S, Alborali GL, De Luca S, Scali F, Lolli V, Varrà MO, Ianieri A, Zanardi E. (Poster Presentation)
68th International Congress of Meat Science and Technology, Kobe (Japan), Virtual Edition, 22/08/2022 –25/08/2022

New tools for authentication and traceability to assure the integrity of food chain
Fabrile MP, PhD candidate. Zanardi E, Ghidini S, Academic tutors. (Miniposter presentation)

Workshop PhDFood2021, Developments in the Italian PhD Research on Food Science, Technology and Biotechnology, University of Palermo, Virtual Edition, 14/09/2021 – 15/09/2021.

Application of a portable near-infrared spectroscopic device for on-field monitoring of the country of origin labelling of musky octopus (Eledone spp.)
Varrà MO*, Ghidini S, Fabrile MP, Ianieri A, Zanardi E (Oral presentation- Non-presenting author)
74° Convegno SISVet (Società Italiana delle Scienze Veterinarie), Virtual Edition, 23/06/2021 – 26/06/2021

Publications

Filling gaps in animal welfare assessment through metabolomics
[2023] Frontiers in Veterinary Science
Fabrile MP, Ghidini S, Conter M, Varrà MO, Ianieri A, Zanardi E.
[Review Article]

Country of origin label monitoring of musky and common octopuses (Eledone spp. and Octopus vulgaris) by means of a portable near-infrared spectroscopic device
[2022] Food Control
Varrà MO, Ghidini S, Fabrile MP, Ianieri A, Zanardi E.
[Research Article]

^{1}H NMR Metabolomics on Pigs' Liver Exposed to Antibiotics Administration: An Explorative Study.
[2023] Foods
Fabrile MP, Ghidini S, Caligiani A, Scali F, Varrà MO, Lolli V, Alborali GL, Ianieri A, Zanardi E.

Real-time and non-destructive control of the freshness and viability of live mussels through portable near-infrared spectroscopy
[Submitted manuscript] Food Control
Ghidini S, Varrà MO, Bersellini D, Conter M, Fabrile MP, Ianieri A, Zanardi E.

Awards

Masetti Prize 2023 – Europass, EFSA and University of Parma

The awards, in memory of Giordana Masetti, who directed the Europass Office for years, aim to support internship activities at EFSA and encourage interest in research and in-depth study.

Other experiences

Guest Scientist Integrated Data (IDATA) Unit – [Nov 2023- In progress]
Team Data Gateway and Outreach (DGO)
Team Data Management and Analysis (DMA)

Task 1: Mapping of Minimum Method Performance Requirements (MMPRs)/Reference Point of Action (RPAs)/Coccidiostats implemented in the 2024 Veterinary Medicinal Product National Control Plan in collaboration with European Union Reference Laboratories (EURLs) and European Commission (EC);

Task 2: Drafting of a guideline intended for Member States (MS), European Union Reference Laboratories (EURLs), and European Commission (EC) for navigating the validation dashboard related to the submission of plans (i) national risk-based control plan for production in the Member States, ii) national randomised surveillance plan for production in the Member States, and iii) national risk-based control plan for third-country imports' according to the EU Regulation No 1646/2022.

Task 3: Collaboration project between EFSA and European Medicines Agency (EMA) for a new coding interface.

Attended Courses, Seminars and Symposium

IT Gartner Symposium Replays sessions: the use of artificial intelligence (AI)

EFSA- 11/2023-Ongoing

Food authenticity PhD Excellence Academy
ER-PhooD, University of Parma, School of Advanced studies on food and nutrition,
25-27/09/2023

Meat Safety Assurance Systems (MSAS) PhD School
ER-PhooD, University of Parma, School of Advanced studies on food and nutrition,
5-8/09/2023

6th MS Lipidomic School
Unitech OMICs, University of Milano, 06/06/2023 – 07/06/2023

Analisi dei dati e riproducibilità della ricerca in R
University of Parma, Dipartimento di Scienze Medico-Veterinarie, 06/02/2023 – 17/02/2023

Parma Summer School 2022- Risk Assessment of Regulated Product
EFSA, University of Parma, School of Advanced Studies of Food and Nutrition and University Cattolica del Sacro Cuore, 28/09/2022 – 30/09/2022

La sostenibilità delle filiere degli alimenti di origine animale: il tema dei sottoprodotti
Workshop organizzato nell'ambito della Scuola di Studi Superiori in Alimenti e Nutrizione e realizzato grazie al finanziamento della Regione Emilia-Romagna attraverso il Progetto triennale di alta formazione in ambito culturale, economico e tecnologico "Sostenibilità alimentare: da problema globale a opportunità di sviluppo socioeconomico regionale", 20/05/2022

Gestione della contaminazione microbica in impianti di macellazione suinicola: criticità e approcci innovativi.
University of Parma, Virtual Edition, 12/2021

Towards the future of food: the cultured meat between food safety, sustainability, and public perception.
University of Parma, School of Advanced studies on food and nutrition, CSEIA and Regione Emilia-Romagna, 09/2021

Parma Summer School 2021- Food Safety Aspects of Integrated Food Systems
EFSA, University of Parma, School of Advanced Studies of Food and Nutrition and University Cattolica del Sacro Cuore, 28/09/2021 – 30/09/2021

L'importanza della proprietà intellettuale nella gestione dell'innovazione e la valorizzazione del know-how e i modelli di gestione collaborativa dell'innovazione
ART-ER, Attrattività Ricerca Territorio, Emilia-Romagna, 04/2021 – 05/2021

Design of Experiments course- Statistical experimental design
Sartorius Data Analytics, 04/2021 – 05/2021