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Review article

Stable isotope profiling and its potential applications to poultry sciences

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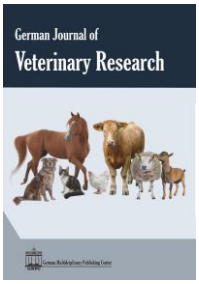

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Abstract

Metabolomics is a bottom-up approach to studying metabolism in living cells, tissues, organoids, or even whole organisms. In conjunction with genomics and proteomics, it is a crucial tool for better understanding complex biological systems. In comparison to genomics or proteomics, metabolomics more directly reflects the phenotype since it encompasses events that occur downstream of gene expression. In the field of poultry or avian diseases, this method is now well established, although it could have more innovative applications in the future. For example, ^{13}C-guided metabolomics and flux analysis could be used to identify biochemical pathways associated with heat or immune stress. It could also help to explore the effects of alternatives to antimicrobials on the intestinal health of poultry and their action on avian pathogens, including the mechanisms of resistance and persistence. In this review, we discuss some basic principles of metabolomics and its current and potential applications in poultry.

Keywords: Metabolism, Metabolomics, Isotopologue profiling, NMR, GC-MS

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Introduction

From a holistic perspective, "multi-omics" technologies (genomics, transcriptomics, proteomics, metabolomics, and ^{13}C-guided fluxomics) could provide new insights into emerging fields of biology and biochemistry, such as host/pathogen interactions, resistance development mechanisms, and drug targeting. The term "metabolomics" refers to the "metabolome," which describes the complete set of small metabolites (MW< about 1.500 Da) present in a cell, organ, tissue, or organism. Its regulation is complex and holds biological significance. These metabolites include small peptides, oligonucleotides, sugars, nucleosides, organic acids, ketones, aldehydes, amines, amino acids, lipids, and drug metabolites (Wishart et al., 2007; Nambiar et al., 2010). Metabolomics, also known as metabolic phenotyping, refers to the identification of the metabolome in a high-throughput manner. The related "metabonomics" is studying how metabolites interact over time in a biological system (Nicholson, 2006; Holmes et al., 2008). "Metabolite profiling" typically refers to the identification and quantification of a differentiated set of metabolites (known or unknown) belonging to a specific metabolic pathway or a specific class of substances (e.g., amino acids).

Metabolomics has various applications in several fields, including nutrition and medicine. For example, it can be used to study pathogenesis and pathogen-host interactions (Figure 1). Since pathogens hijack host cellular metabolism, limiting their growth and proliferation, studying metabolomics could help identify biomarkers for diagnosis or targets for drug development and understand host-pathogen interactions (Ghazal et al., 2000). In the present review, we will discuss the perspectives of metabolomics research and its potential applications in poultry science.

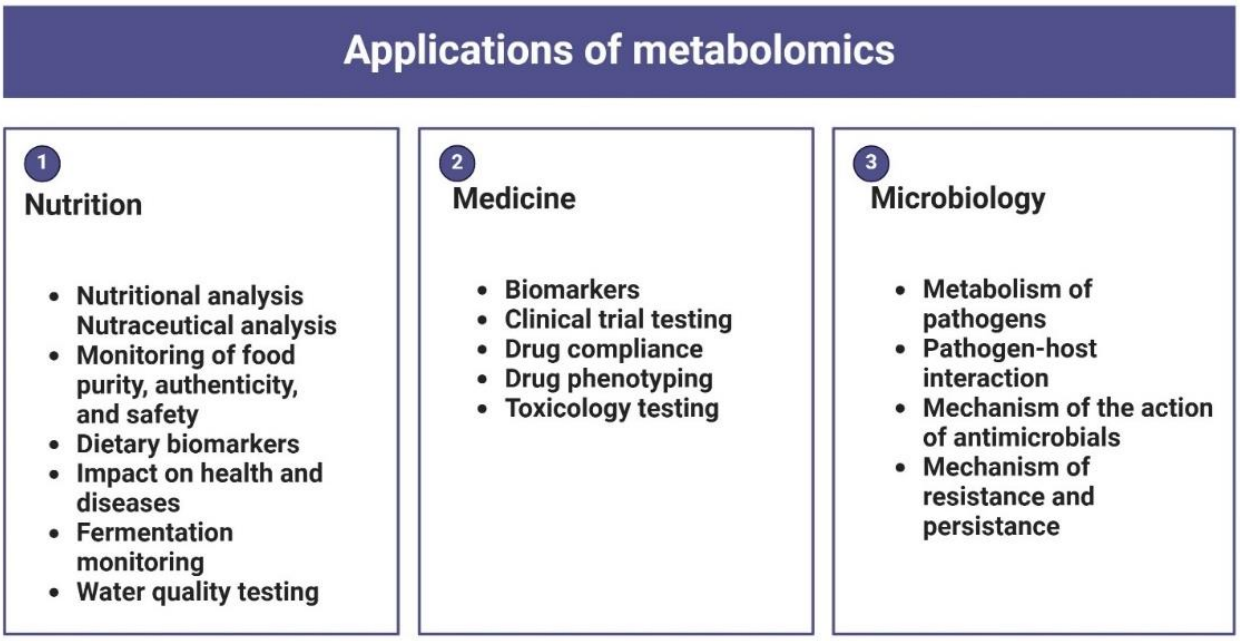

Figure 1: General applications of metabolomics.

Metabolomics - an overview

Metabolism

Metabolism refers to all metabolic reactions that take place in a cell or organism. It includes all the processes through which a cell or organism obtains energy and reproductive material from the environment to create macromolecules and the building blocks for their synthesis. In addition to the formation of macromolecules from simpler precursors, living systems need energy for mechanical activities and the active transport of molecules and ions. A vast network of interdependent chemical reactions is required for these processes. A particular series of reaction sequences that begin with a particular molecule and generate one or more molecules in a specific way is called a metabolic pathway. Enzymes that bind only very specific substrates in their active center and catalyze their conversion into very specific products ensure the high specificity of the reactions.

A distinction is made between chemotrophic and phototrophic organisms regarding the type of energy production. Phototrophs obtain energy from sunlight by carrying out photosynthesis; chemotrophs obtain energy from the oxidation of nutrients produced by phototrophs. The various metabolic pathways can be divided into two broad categories: catabolic and anabolic reactions. In catabolism, fuels such as carbohydrates or fats are converted into usable energy. In anabolism, this energy is used to synthesize macromolecules and other necessary compounds. The conversion of energy from food or light into adenosine triphosphate (ATP), which supplies the energy required to drive energetically unfavorable reactions through hydrolysis, is of central importance in this process.

As in other animals, birds produce energy through the oxidation of nutrients (carbohydrates, proteins, lipids). This process can be divided into three stages: i) Macromolecules of ingested nutrients are broken down into smaller units, whereby no significant energy is produced. Fats are broken down into fatty acids and glycerol, polysaccharides into glucose and other sugars, and proteins into individual amino acids. ii) Further degradation of glucose and most amino acids yields pyruvate, producing a relatively small amount of ATP via glycolysis in the case of sugars. Pyruvate, fatty acids, and some amino acids afford acetyl-CoA, which can be considered a common metabolic product of each nutrient in animals. iii) Acetyl-CoA is shuffled, e.g., to the mitochondrial tricarboxylic acid (TCA) cycle (citrate cycle) and, in the presence of electron acceptors such as oxygen, coupled to the subsequent oxidative phosphorylation, where a lot of ATP is generated.

The intermediate metabolites from the second stage (e.g., acetyl-CoA and pyruvate), but also the intermediate products of the TCA cycle (e.g., oxaloacetate, α-ketoglutarate (α-KG), also play a role as precursors in the anabolism of all organisms. These and other central metabolic

intermediates shown in Figure 2 also provide the starting materials for the synthesis of versatile and vital compounds such as amino acids, lipids, and nucleotides. This is important for metabolic flux analysis, where frequently the isotope labeling pattern in amino acids is taken as a kind of fingerprint left behind by the conversion of the labeled intermediates, such as pyruvate or oxaloacetate, into the more stable and abundant amino acids. This approach can be done since the biosynthesis of amino acids proceeds via these central metabolic intermediates such as pyruvate, oxaloacetate, α-KG, acetyl-CoA, 3-phosphoglycerate, phosphoribosyl-pyrophosphate, and erythrose-4-phosphate (see also Figure 2). The investigation of these core processes and their products can provide important information about an organism's way of life.

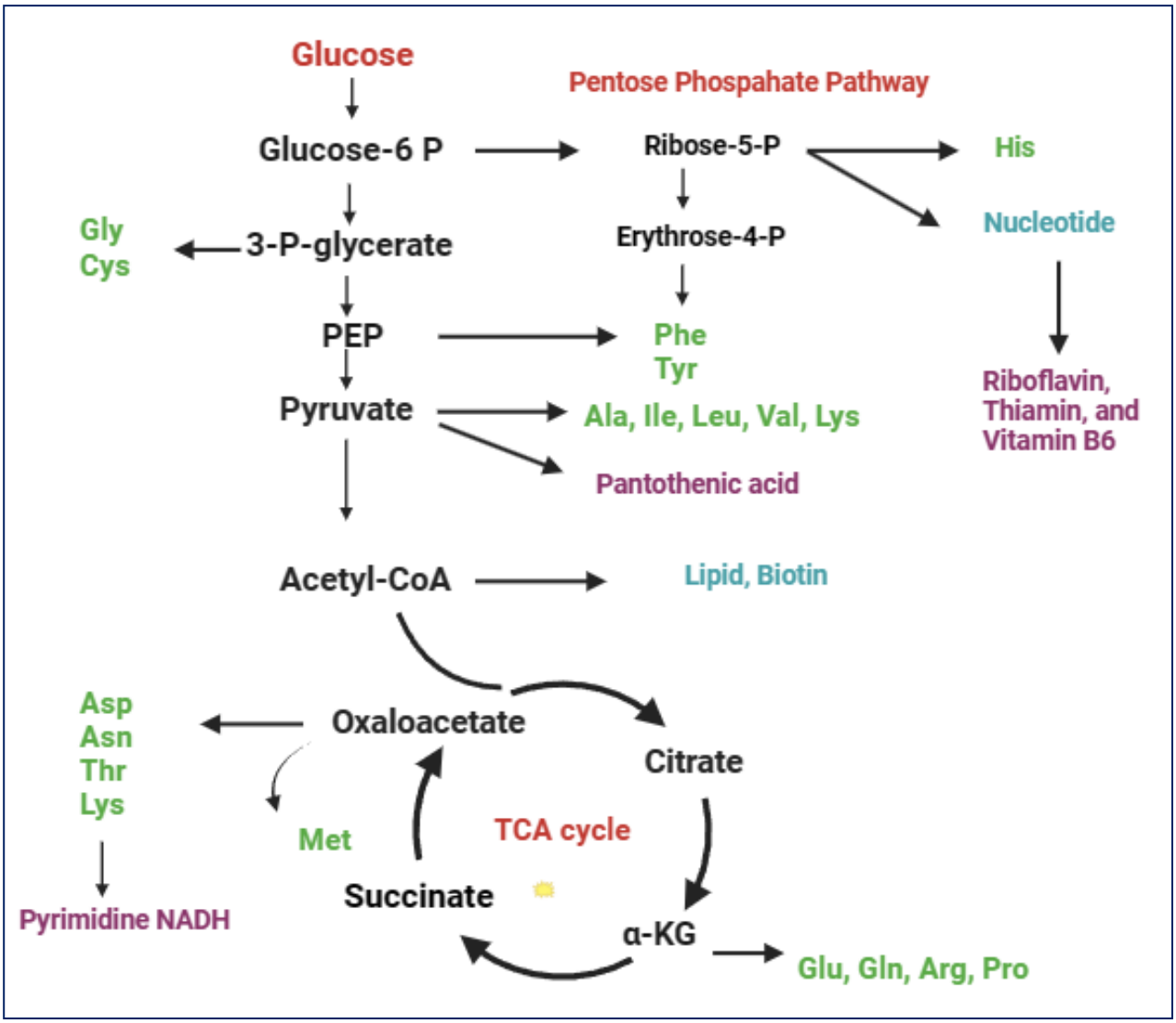

Figure 2: Scheme for the interconnections of glycolysis, TCA cycle, the pentose phosphate pathway, nucleotide biosynthesis, coenzymes, and amino acids biosynthesis. In ^{13}C-guided metabolic flux analysis, the analysis of the isotope composition in metabolic end products such as amino acids allows reconstruction of how a labeled starting substance such as glucose is distributed throughout the network. The figure was modified after (Eisenreich et al., 2016) and was generated by Biorender.

Metabolomics approaches

The two main metabolomics approaches, non-targeted and targeted, have been described frequently (Selamat et al., 2021) (Figure 3). Briefly, the non-targeted strategy aims to identify a broad range of metabolites without prior knowledge of their properties. This approach involves qualitative or semi-quantitative analysis of many metabolites. The main objectives are often to detect qualitative differences between two sample sets and to gain a comprehensive overview of various types of metabolites. The non-

targeted analysis aims to identify metabolites in a biological system, but due to the lack of many standards, it cannot provide absolute concentrations of all metabolites (Akyol et al., 2023).

Two terms are related to non-targeted metabolomics: metabolite fingerprinting and metabolite foot printing. Fingerprinting refers to intracellular metabolites. Metabolite foot printing is analogous to metabolite fingerprinting, but only extracellular metabolites are examined here.

Targeted metabolomics is a method that involves the analysis of specific molecules or a subset of metabolites or metabolic pathways mainly based on known standards. This analysis is quantitative or semi-quantitative in nature and does not require additional and extensive work for data processing (Fiehn, 2016; Ribbenstedt et al., 2018; Nalbantoglu, 2019; Cao et al., 2020; Wu et al., 2022; Chen et al., 2023). The focus is on specific metabolites, which makes the analysis more precise and easier. Metabolite profiling refers to the identification and quantification of a differentiated set of metabolites (known or unknown), often belonging to a specific metabolic pathway or a specific class of substances (e.g., amino acids) (Wu et al., 2022).

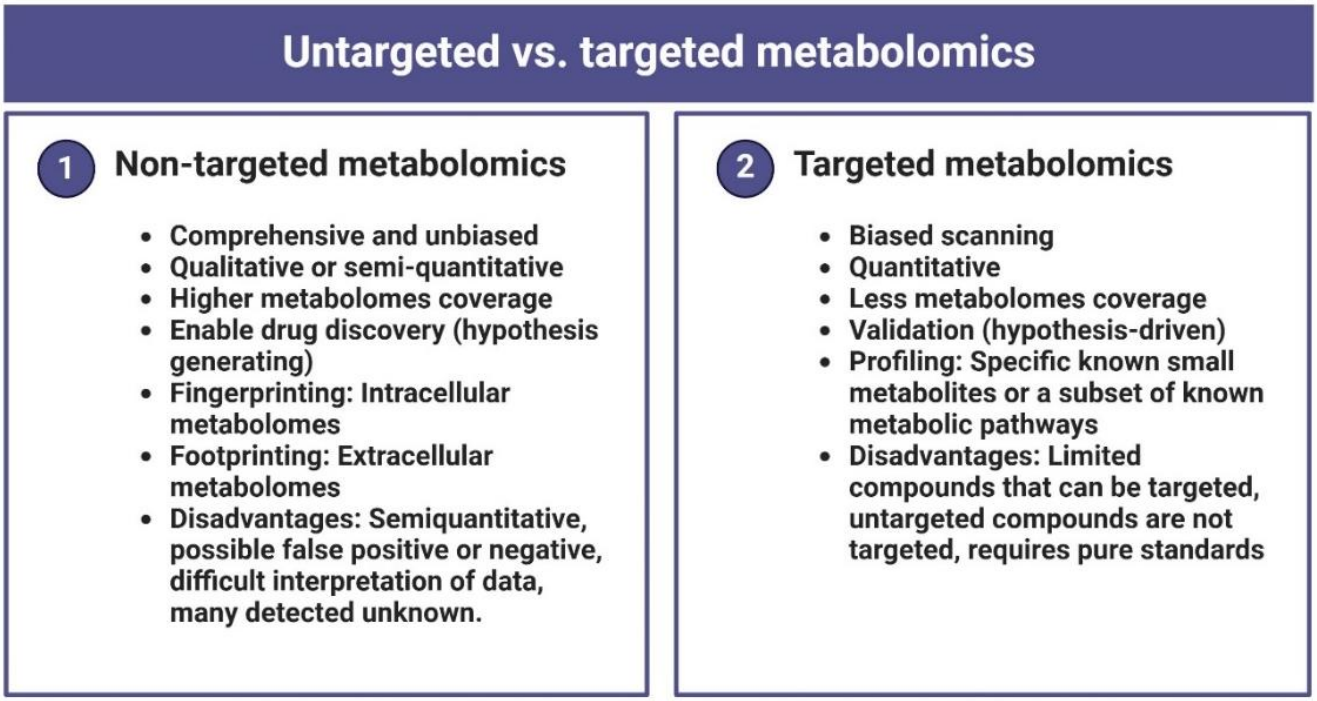

Figure 3: Untargeted vs. targeted metabolomics. The figure was modified after (Puchades-Carrasco and Pineda-Lucena, 2015; Selamat et al., 2021).

^{13}C-Guided metabolic flux analysis (MFA) and isotopologue profiling

In the area of MFA (Chen et al., 2017; Steiner and Eisenreich, 2022; Kaste and Shachar-Hill, 2024), isotopologue profiling is a technology that helps to identify metabolic pathways and fluxes by using direct and quantitative experimental parameters. It is based on the benefits of stable isotopes, which can be diagnosed by spectroscopic methods such as mass spectrometry or NMR spectroscopy. A stable isotope that is commonly used to analyze carbon metabolism is ^{13}C. This heavy carbon is only present in nature in small quantities of approx. 1% compared to the predominant ^{12}C isotope (99%). Therefore, the dynamic range in compounds carrying ^{13}C at natural abundance (i.e., about 1%) and ^{13}C-enriched compounds (i.e., up to 100 %) is high. Even more specificity can be introduced by positional ^{13}C-enrichments, i.e., ^{13}C-atoms at specific carbon positions of the molecules under study. This aspect refers to the term “isotopologue” which describes molecules having different isotopic compositions. For example, $^{12}CH_3$-$^{12}COOH$ and $^{13}CH_3$-$^{12}COOH$ are carbon isotopologues of acetic acid. A subgroup of isotopologues is defined by “isotopomers”, which carry the same number of isotopes but at different positions. For example, ^{13}CH-^{12}COOH

and $^{12}CH_3$-$^{13}COOH$ are carbon isotopomers of acetic acid. Theoretically, a compound with n C-atoms has n^2 carbon isotopologues. This means that the number of isotopologues can be high for molecules carrying many carbon atoms. For example, the six-carbon compound glucose has 64 carbon isotopologues. Starting from appropriate tracers in incorporation experiments (many ^{13}C-enriched organic compounds are commercially available), the formation of isotopologues in metabolic intermediates and products is not random but highly specific due to the active pathways between the tracer (i.e., the supplied isotope labeled substrate) and each metabolite in the cell or organism under study. The large number of isotopologues behind every metabolite in the metabolome of an organism thus adds a new dimension of data, enabling to description of the metabolic system under study at unprecedented resolution, even when only a limited number of metabolites is used for analysis.

The principles of ^{13}C- labeling and isotopologue profiling include i) labeling (pulsing) with the stable (i.e., non-radioactive) tracer compound into living cells, tissues, organoids, or whole organisms, ii) a chase period for metabolic and isotopic equilibrium in the biological system, resulting in specific labeling patterns of the newly synthesized metabolites, and iii) analysis using metabolomics tools such as GC -MS and/or NMR.

As an example, isotopologue profiling can be done by cultivating bacteria in a medium supplemented with the ^{13}C-labeled tracer (for example [U-$^{13}C_6$]glucose), leading to a specific distribution of the ^{13}C atoms in all downstream metabolites due to the individual chemical reactions and pathways involved in the metabolic conversion of the tracer down to the final products (Eisenreich et al., 2013; Steiner and Eisenreich, 2022). This approach can be used to track labeled molecules in metabolism, giving precise information about the reaction pathways in the network. On this basis, the structure and dynamics of the metabolic network can be reconstructed using experimental data, such as the rates of nutrient uptake or the fluxes in catabolism and anabolism.

Tools of metabolomics

Two analytical methods are typically utilized in metabolomics studies**.** The first method is the more sensitive mass spectrometry (MS) coupled with GC or LC. For example, the precise GC-MS analysis of individual N-tert-butyldimethylsilyl derivatives of amino acids has become a standard procedure. This method can clearly distinguish between molecules with different numbers of ^{13}C and ^{12}C atoms, making it possible to determine which amino acids have incorporated ^{13}C atoms and to what extent, i.e., the ^{13}C enrichments. The proportions of the corresponding differently labeled isotopomers can be quantified from the signal intensities in the mass spectra; however, their positions in the molecules can only be partly determined from the mass fragments generated during ionization. As an example, for N-tert-butyldimethylsilyl derivatives of amino acids, the mass values of the molecules having lost a methyl group from the silyl moieties and of fragments having lost the carboxylic atom of the original amino acid. In comparison to the mass fragment carrying all carbon atoms of the amino acid, the ^{13}C enrichment of the carboxylic atom can, therefore, be determined.

The second important but less sensitive method for determining isotopologue profiles is NMR spectroscopy, which uses frequency signals for each non-equivalent carbon atom in a magnetic field, especially to detect the positions within a molecule that are occupied by the ^{13}C-atoms. NMR, therefore, has a high positional resolution for detecting the isotopes but requires significantly more material (i.e., mg amounts) for measurement than GC-MS or LC-MS (i.e., mg- ng amounts).

Applications of metabolomics in poultry research

In this section, we present some recent examples of how implementing “classical” metabolomics (targeted or non-targeted) studies has helped poultry research.

Identification of new biomarkers of dietary intake

Metabolomics can add knowledge about the changes that occur in the host's physiology and metabolism after having consumed special nutrients, diets, prebiotics, or probiotics (Afrouziyeh et al., 2022). It also provided insights into the poultry's metabolic circumstances and patterns, which can be helpful in assessing the benefits of specific dietary intakes and identifying or deciphering biological mechanisms (Cevallos-

Cevallos et al., 2009; Mozzi et al., 2013; Wu et al., 2022).

Prebiotics have a positive impact on the gut health of poultry by modulating beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*. This leads to a decrease in inflammatory mediators, promoting a healthy gut. Metabolomics analysis can provide valuable insights into the mechanisms of prebiotics. For instance, intake of fructooligosaccharides (FOS) was found to modulate 93 different metabolites, including amino acids, organic heterocyclic compounds, nucleosides, nucleotides and analogues, phenylpropionic acid and polyketides, benzenes, organic oxygen compounds, organic acids, and their derivatives, lipids and lipid-related molecules, and organic nitrogen compounds (Ma et al., 2022). More specifically, FOS intake affected L-lysine, L-methionine, L-valine, and L-histidine (precursors of metabolic proteins and involved in cell signaling), glycerophospholipids (components of cell membranes and precursors of lipid mediators in signal transduction), phenylpropanoids and polyketides (antioxidants, antimicrobials, and anti-inflammatory agents), as well as phosphatidylcholine and phosphatidylethanolamine (the most abundant phospholipids in cell membranes and plays a crucial role in lipid metabolism and overall health). A summary of the intestinal metabolites detected in chickens supplemented with prebiotics is shown in Figure 4.

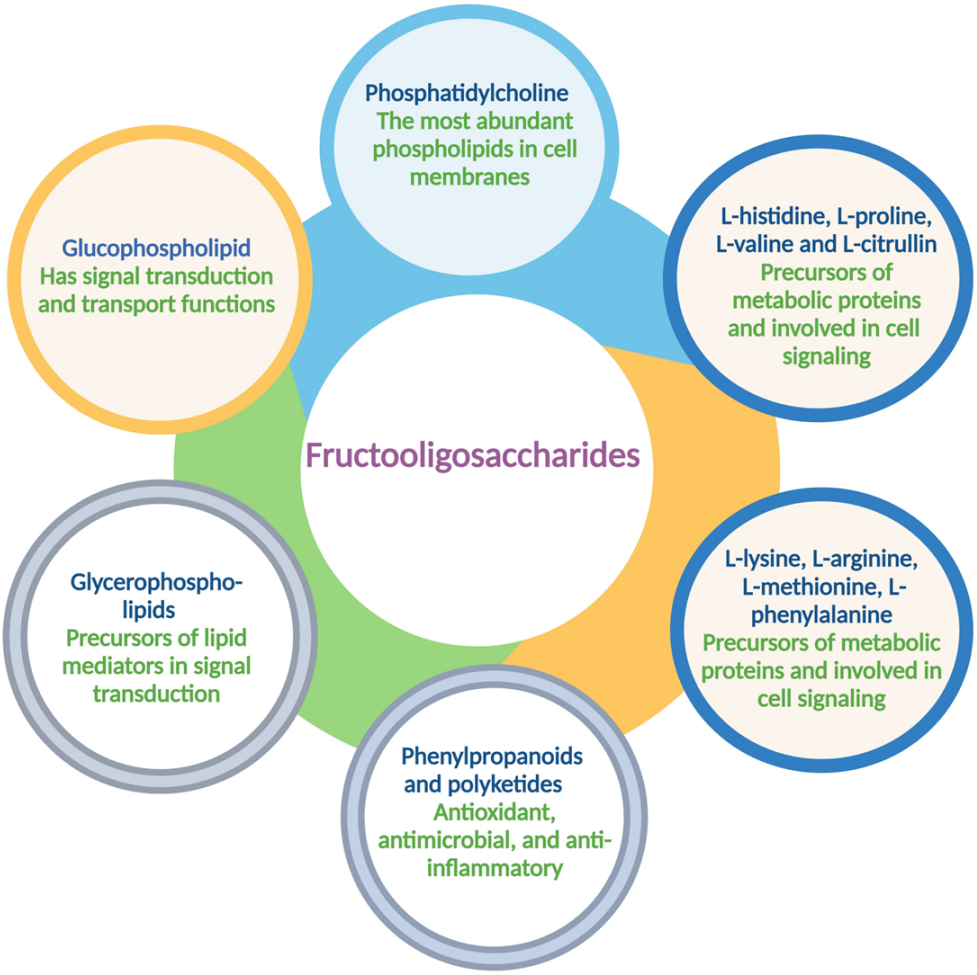

Figure 4: Intestinal metabolites detected in chickens supplemented with fructooligosaccharides and their potential biological functions. The figure was generated using BioRender after (Wu et al., 2022).

Probiotics have the ability to increase various metabolites, including vitamins, amino acids, short-chain fatty acids, choline, bile acid, and lipids, which can have several positive effects on the body reviewed by (Wu et al., 2022). These metabolites have different functions, such as: i) Amino acids are involved in protein synthesis and play a role in cell signaling; ii) short-chain fatty acids, such as acetate, propionate, and butyrate, are a source of energy and help in cholesterol

synthesis, promoting absorption, immune regulation, and reducing harmful bacteria, iii) bile acid promotes absorption, participates in gut immunity, and acts as an antimicrobial (Vavassori et al., 2009), and iv) choline is an essential dietary nutrient that regulates lipid metabolism and maintains glucose homeostasis. Choline is mainly metabolized in the liver, but intestinal microbial enzymes can convert it into trimethylamine. The latter is further oxidized in the liver to produce trimethylamine N-oxide, a marker metabolite related to liver and cardiovascular diseases (Schugar and Brown, 2015). Using MS analysis, Park et al. (2020) studied the intestinal metabolome of chickens supplemented with *B. subtilis* strain 1781 or strain 747. *B. subtilis* 1781 modulated a total of 83 metabolites (25 increased, 58 decreased), while *B. subtilis* 747 affected only 50 metabolites (12 increased, 38 decreased) related to amino acids (e.g., upregulation of the dipeptides alanylleucine, glutaminylleucine, valylleucine, and glycylisoleucine); purine metabolism (upregulation of N1-methyladenosine, N6-methyladenosine, guanine, and 2-deoxyguanosine), pyrimidine metabolism (downregulation of uridine-5′-monophosphate and cytidine), fatty acids (upregulation of sebacate, valerylglycine, and linoleoylcholine), sterol and bile acids (downregulation of bile acids, cholesterol, chenodeoxycholate, and 3-dehydrodeoxycholate), benzoate metabolism (downregulation of 2-(4-hydroxyphenyl)propionate and upregulation of salicylate-glucoside), nicotinamide metabolism (upregulation of nicotinamide ribonucleotide and nicotinamide adenine dinucleotide, and carbohydrate metabolism (upregulation of fructose and downregulation of lactate levels) (Park et al., 2020).

Studying the metabolic pathways of pathological conditions in chickens

The metabolomics approach was also used to study the metabolome and related metabolic pathways of chickens in response to pathological conditions such as heat stress (Sutton, 2021; Wu et al., 2022), immunosuppression (Sutton, 2021; Li et al., 2022), and immune stress (Bi et al., 2022a).

Heat stress

Heat stress (HS) is a condition that affects the quality of meat by causing malfunction of the mitochondria and by altering aerobic metabolism (Lu et al., 2017). It was discovered that heat stress leads to metabolic disorders in the breast muscles, specifically affecting lipid metabolism. This was determined by analyzing 41 metabolites using LC-MS (Table 1). Additionally, HS can significantly affect the ATP binding cassette transporter transporters metabolic pathway (Li et al., 2024). There was also downregulation of the expression of peroxisome proliferator-activated receptor gamma coactivator-1-alpha (PGC-1α), ATP-binding cassette transporter A1 (ABCA1), and ATP-binding cassette transporter G1 (ABCG1) genes that could explain the impairment of fat metabolism in chickens kept under HS. These findings support the potential role of integrating a multi-omics approach to understand the pathogenesis of pathological conditions in poultry.

Several studies also reported the modulatory effect of HS on the serum metabolites in broiler chickens, including i) Increasing serum triglyceride and total cholesterol concentrations in AA broilers (Luo et al., 2018), ii) reduction of stearic, arachidonic, palmitic, and oleic acids (Zhang et al., 2018) and iii) modulation of serum lipid metabolites, such as downregulation of phospholipids (Guo et al., 2021). See also Table 1.

Immune stress

Chickens commonly lose immune homeostasis and experience immune stress due to high stocking density, mycotoxins, inappropriate management, and pathogen challenges, leading to a reduction in performance and severe economic losses (Zheng et al., 2021). Several studies were done to better understand the pathogenesis of immune stress in poultry and to identify potential therapeutic targets (Bi et al., 2022b, 2022a; Hu et al., 2024). It was found that immune stress changed metabolites related to amino acid and glycerophospholipid metabolism. Bi et al. 2022 induced a chronic model of immune stress using continuous interval intraperitoneally injection of LPS (250 μg/kg LPS at 12, 14, 33, and 35 d). Transcriptomics and metabolomics analysis of liver tissues using LC-MS/MS showed modulation of various metabolites (109 metabolites; 64 upregulated and 45 downregulated) compared with the control. The main findings in immune-stressed chickens can be summarized as follows: i) An increase of L-

threonine and a decrease in histamines (Bi et al., 2022a). L-threonine boosts the proliferation of T and B lymphocytes to enhance immune responses (Yuan et al., 2017). In contrast, histamine promotes dendritic cell migration, pro-inflammatory cytokine production, and Th2 cell activity (Coleman et al., 2020). ii) An increase of glycerophospholipids including LysoPC (18:1), citicoline, choline, PC (14:0/P-18:0), glycerylphosphorylethanolamine, glycerophosphocholine, and CDP-ethanolamine, suggesting that the level of glycerophospholipids could serve a potential biomarker for the early detection of LPS-induced immune stress (Bi et al., 2022a). Moreover, glycerophospholipids play a critical role in cell apoptosis and, thus, are also biomarkers of liver injury (Qiao et al., 2021). iii) Upregulation of 3-dehydrosphinganine, sphingosine in sphingolipid metabolism (Bi et al., 2022a). iv) Reduction of leucine (Bi et al., 2022a) activating the mammalian target of rapamycin (mTOR) signaling pathway (Neinast et al., 2019; Zheng et al., 2021). This highlights that the pathogenesis of immune stress in broilers is associated with the branched-chain amino acid metabolism through mTOR signaling.

Additionally, Bi and others found a modulation of serum metabolites of LPS-stressed chickens, including an increase of phenyllactic acid and α-KG (Bi et al., 2022b), potential anti-inflammatory metabolites (Asadi Shahmirzadi et al., 2020; Zhou et al., 2021) which might also be used as biomarkers for immune stress in poultry.

Table 1: Alteration of metabolites of breast muscles of heat-stressed broilers.

	Upregulation	Downregulation	Reference
Breast muscles	**Phosphatidylethanolamine** [P-18:1(9Z)/16:1(9Z); 18:2(9Z,12Z)/P-16:0; 18:2(9Z,12Z)/18:0; 15:0/22:2(13Z,16Z)]	**Phosphatidylethanolamine** [P-16:0/20:4(6E,8Z,11Z,14Z)(5OH[S]); NMe(18:0/22:5(4Z,7Z,10Z,13Z,16Z))]	Li et al. (2024)
	Phosphatidylcholine [18:2(9Z,12Z)/P-16:0; glycerophosphocholine; persicaxanthin; delphinidin3-rutinoside; d-glucuronicacid; fructosamine; 1-(11Z,14Z-eicosadienoyl)-glycero-3-phosphate; 1-arachidonoylglycerophosphoinositol; N-(1-deoxy-1-fructosyl)glycine; sedoheptulose1-phosphate]	**Phosphatidylcholine** [16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z); SM(d18:1/16:0); alanyl-dl-leucine; alanyl-tryptophan; linoelaidylcarnitine; 1,2-dipalmitoyl-sn-glycero-3-PC; pctadecenoylcarnitine; spermidine; alanyl-dl-phenylalanine; decanoyl-L-carnitine; 3-hydroxyhexadecanoylcarnitine; 25-hydroxyvitaminD3-26,23-lactol; rockogenin; pipericine; 2-methylbutyroylcarnitine; cis-andtrans-L-mercapto-p-menthan-3-one; histamine; alanyl-tyrosine; palmitoylsphingomyelin; alanyl-arginine; 3-phenylpropylglucosinolate; nicotinamideadeninedinucleotide; phenylalanyl-gamma-glutamate; 9(Z),11(E)-conjugatedLinoleicA acid; taurine]	
Serum lipid	**Phosphatidylcholine** (17:0/17:0)	**Phosphatidylcholine** [(18:0/20:4); PC (15:0/23:4); PC (18:0/22:6); PC (18:2/18:2)]	Guo et al. (2021)
		phosphatidylethanolamine (PE) (18:1/18:1)	
		polyethylene terephthalate (PEt) (37:3/8:0)	
		phosphatidylglycerol (PG) (32:1/16:2)	
		phosphatidyl methyl ethanolamine (PMe) (19:3/13:0); PMe (26:1/9:0)	
		ceramide (cer) (d18:1/22:0); cer (d18:1/24:1); cer (d20:2/22:2)	
		lyso-phosphatidylcholine (LPC) (18:0)	
		triglycerides (TG) (18:0/18:1/18:2); TG (19:4/21:6/21:6) levels	
		sphingomyelin (SM) (d16:0/18:1)	

Studying the metabolic pathways of avian pathogens

Metabolomics has been used to identify biomarkers for avian pathogens but also, more generally to provide a deeper understanding of host-pathogen interactions. For instance, metabolomics analyses have been conducted with chickens infected by several avian viral pathogens, including infectious bronchitis virus (IBV) (Xu et al., 2019; Kuang et al., 2020), avian leukosis virus (ALV) (Chen et al., 2021), infectious laryngotracheitis virus (ILTV) (Xu et al., 2022), infectious bursal disease virus (IBDV) (Lin et al., 2020; Dai et al., 2022), and Newcastle disease virus (NDV) (Liu et al., 2019; Dai et al., 2024).

Indeed, this knowledge could provide new insights into the diagnosis, prevention, and treatment of avian pathogens. The most recent studies on the potential use of metabolomics to understand pathogen-host interactions in poultry are summarized as follows:

Poultry infected by the leukosis virus subgroup J (ALV-J). ALV-J is a highly pathogenic and transmissible subgroup of ALV, which causes myeloid leukosis in broiler breeders. Unfortunately, there are no vaccines or effective drugs available against ALV. LC-MS was used to identify potential biomarkers of ALV-J in chicken plasma. It was found that 3-phosphoglycerol-glutathione, bis-4-nitrophenyl phosphate, 4-ketocyclophosphamide, oxidized photinus luciferin, phenyl sulfate, and aryl sulfate were significantly decreased. However, 2-methylthiobenzothiazole, irinotecan, methadone, 3-o-ethyl-l-ascorbic acid, and o-acetylneuraminic acid were significantly increased in ALV-J infected chickens (Chen et al., 2021). In 2024, untargeted metabolomics was performed based on ultrahigh-performance liquid chromatography–quadrupole time-of-flight tandem mass spectrometry (UHPLC-QTOF-MS) in chicken embryo fibroblast (CEF) cells infected by ALV-J. The authors found a modulation of lipid metabolism, including glycerol phospholipid metabolism and sphingolipids metabolism (Xu et al., 2024). I

Poultry infected by the Newcastle disease virus (NDV). Several metabolomics studies have been done to understand the pathogenesis of NDV on the level of metabolites (Dai et al., 2024)**:** i) NDV decreased the level of GSH and the activity of SOD, CAT, GST, GPx, and GR in the brain and liver of chickens (Subbaiah et al., 2011; Rehman et al., 2018). The impairment of the oxidative phosphorylation process might be a result of NDV-induced mitochondrial injury. ii) NDV increased the level of NO (Kristeen-Teo et al., 2017), which might be associated with an increase in arginine metabolism (Liu et al., 2019). iii) NDV increased the levels of amino acids, including tyrosine, isoleucine, threonine, methionine, serine, and alanine, which may play a role in virus replication. iv) NDV increased purine and pyrimidine biosynthesis pathways, which may also have a role in virus replication (Liu et al., 2019). v) NDV altered the levels of plasma metabolites, including lowering glycerophospholipids, sphingolipids, and amino acid metabolites. However, further studies are still needed to explore the impacts of these lipid metabolism on the virus replication and identifications of specific biomarkers.

Poultry infected by the nephropathogenic infectious bronchitis virus (NIBV). Metabolomics analysis of the bursa of Fabricius in chickens was done using gas chromatography time-of-flight/mass spectrometry (GC-TOF/MS) (Kuang et al., 2020). Twelve potential biomarkers were identified: 3-Phenyllactic acid, 2-deoxytetronic acid, aminomalonic acid, malonamide, uric acid, arachidonic acid, 2-methylglutaric acid, linoleic acid, ethanolamine, stearic acid, N-alpha-acetyl-l-ornithine, and O-acetylserine.

Poultry infected by the Cryptosporidium baileyi.At five days- post-infection of chickens with *C. bailey*, 138 differential serum metabolites were described, including 115 upregulated and 23 downregulated compounds (Wu et al., 2021). These metabolites were significantly enriched into six pathways, of which two pathways were most affected. These pathways are associated with energy and lipid metabolism, namely glycerophospholipid metabolism and sphingolipid metabolism. Additionally, choline, sirolimus, all-trans retinoic acid, PC(14:0/22:1(13Z)), PC(15:0/22:6(4Z,7Z,10Z, 13Z,16Z,19Z)), PE(16:1(9Z)/24:1(15Z)), phos-phorcholine, SM(d18:0/16:1(9Z)(OH)) and sphinganine were identified as *C. baileyi* metabolites. These metabolites are associated with lipid metabolism and immunity-associated pathways, highlighting that metabolomics profiling could provide new insights into the host-parasite relationships and could be useful in developing early diagnostic biomarkers for *Cryptosporidium*.

Biomarkers for Salmonella Enteritidis in chicken meat. Chen et al. 2023 identified five potential biomarkers, namely acetylcholine, L-methionine, L-proline, L-valine, and L-norleucine, for *Salmonella* Enteritidis contamination in chicken meat, using ultra-high-performance liquid chromatography coupled to triple-quadrupole mass spectrometry (UHPLC-QqQ-MS)-based targeted metabolomics (Chen et al., 2023). Further studies are required to evaluate the potential application of these biomarkers for indirect detection of *Salmonella* in

chicken meat.

Biomarkers for intestinal nematodes

Oladosu and colleagues studied the plasma metabolome of chickens infected with *Ascaridia galli* and *Heterakis gallinarum* using ^{1}H-NMR spectroscopy (Oladosu et al., 2023). The authors found several metabolites that are associated with different metabolic pathways, including the phenylalanine-tyrosine-tryptophan, alanine-aspartate-glutamate, and arginine-proline axes in the pathogenesis of intestinal nematodes. A total of 20 metabolites, among them glutamate, succinate, trimethylamine-N-oxide, myo-inositol, and acetate, were significantly increased at 2-, 6-, and 10 weeks post-infection. These findings highlight that the metabolomics approach could expand our comprehension of host-nematode interactions.

Metabolomics for studying the mechanism of antimicrobial resistance

Over the past decade, the global population has significantly increased, leading to a rise in the demand for food and feed. Poultry is a popular meat globally and is the second most produced and consumed meat in the EU after pork (EuroStat, 2022). Intensive poultry production increases the risk of infection, including zoonotic diseases. This harms animal health and productivity, raising concerns about food safety and sustainability. Additionally, the extensive use of antibiotics in agriculture, humans, and the food industry has led to the emergence of multidrug resistance (MDR), which the WHO recently identified as among the most important problems threatening human health globally. Therefore, there is an urgent need to identify new targets for drug candidates, including enzymes in the metabolic pathways of avian pathogens. As a novel methodological approach, metabolomics could contribute to this endeavor to develop new drugs, such as alternatives to classical antimicrobials.

Numerous mechanisms could explain the emergence of antibiotic resistance. i) Genetic-based mechanism, whereby the acquisition of mobile genetic elements and mutations in chromosomal genes that confer antibiotic resistance result in a permanent alteration in antimicrobial sensitivity (Blair et al., 2015). ii) Phenotypic-based mechanism, in which reductions in antibiotic sensitivity can happen either homogeneously (tolerance) or heterogeneously (heteroresistance, persistence) (Brauner et al., 2016; Lázár and Kishony, 2019; Westblade et al., 2020). Persister cells undergo three crucial steps to survive antibiotic treatment: i) Shutting down or silence essential cell functions to avoid damage by antibiotics or other stressors, ii) maintaining viability in a state of stasis, and iii) growing again once the stressor has subsided. Differences between resistance, heteroresistance, and persistence are shown in Figure 5. Microbial biofilm formation is one example of a phenotypic-based mechanism that reduces antibiotic sensitivity (Crabbé et al., 2019).

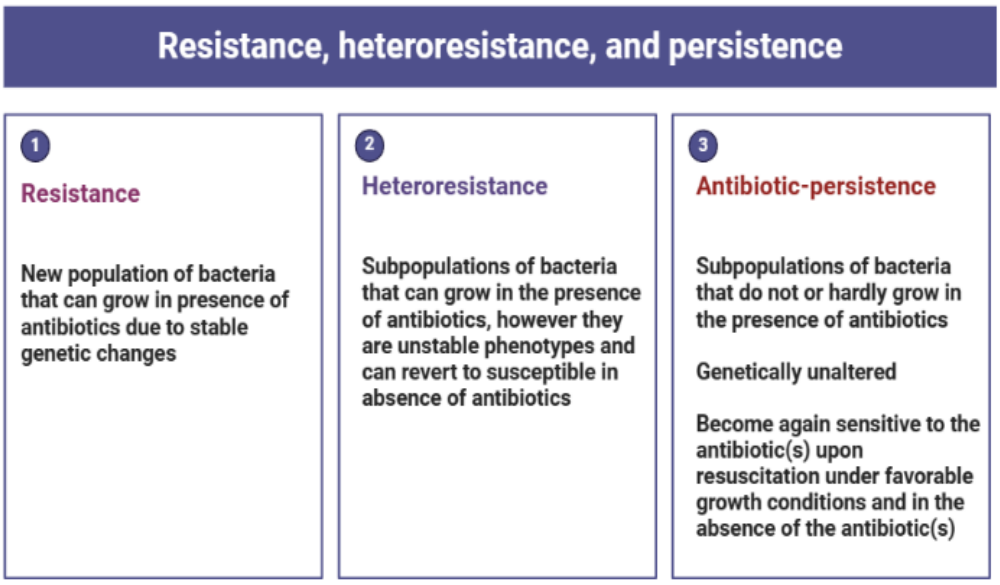

Figure 5: Differences between resistance, heteroresistance, and persistence. Genomics might not explain phenotypic-based resistance mechanisms, including heteroresistance and persistence.

Genetic antimicrobial resistance (AMR) may develop more easily as a result of phenotypically reduced metabolism subpopulations of bacteria that survive longer on antibiotics (persisters) by having a reduced metabolism, including antibiotic uptake (Radlinski et al., 2019; Windels et al., 2019; Eisenreich et al., 2022). However, in persisters, some metabolic reactions are still crucial to provide the required energy for maintaining DNA stability and homeostasis of osmolarity, cell-wall modifications, and, in some examples, overexpression of efflux pumps (Pacheco et al., 2017). During the state of persistence, mutation rates could also increase (Eisenreich et al., 2022), and, therefore, persistence could accelerate the occurrence of AMR. To combat AMR, it is therefore important to understand all mechanisms behind resistance development also elucidate the metabolic pathways that support persistence.

Bacteria's metabolic state is an important factor contributing to their susceptibility or resistance to antibiotics (Allison et al., 2011; Zhao et al., 2021; Zheng et al., 2022). Any drugs modulating or even inhibiting essential metabolic reactions could, therefore, open new therapies. However, also the metabolic state of the host organism during antibiotic therapy could increase its effectiveness (Tacconelli and Pezzani, 2019). The incorporation of new therapeutics to modulate host metabolism could, therefore, improve antibiotic treatments, even using current antibiotics.

Metabolomics has identified biomarkers differentiating antibiotic-resistant and antibiotic-sensitive bacteria. These biomarkers from antibiotic-sensitive bacteria could, therefore, help to reprogram the antibiotic-resistant metabolism into a more sensitive one (Peng et al., 2015; Deng et al., 2020; Jiang et al., 2020). During the last 20 years, the metabolism of pathogenic bacteria has been studied based on “omics” technologies. However, the metabolic pathways and fluxes depend on the environmental conditions. For example, infected host cells and organisms provide multiple substrates for bacterial replication. Therefore, the metabolism of pathogenic bacteria grown under controlled conditions (i.e., in the lab on defined media) can be quite different from the metabolism of the same bacterium thriving in a host cell or organism during infection. Due to this complex interplay between the host and pathogen, the metabolic routes and fluxes of pathogenic bacteria are not fully understood during infections.

Stable-isotope labeling experiments have been shown to be well-qualified for the analysis of bacterial pathogens in special media mimicking host environments or in host-pathogen situations, including intracellular conditions. Table 2 shows some findings of ^{13}C-labelling experiments for several pathogens, including intracellular and extracellular bacteria, some of them being also relevant for avian infections.

Isotopologue profiling approach for understanding the mechanism of alternatives to antimicrobials

Whereas metabolite profiling is well established in poultry research (see above), MFA based on ^{13}C-labels (isotopologue profiling) has not been introduced into poultry research to the best of our knowledge. This is surprising since isotopologue profiling approaches could help develop alternatives to antimicrobials against chronic stress and avian pathogens. In the following chapters, we will, therefore, focus in more detail on this promising technology.

There is no doubt that metabolism, particularly central carbon metabolism, can provide a promising alternative field for drug discovery (Murima et al., 2014). It is also quite obvious that the conversion of nutrients into energy (ATP) is crucial for pathogen virulence and bacterial infection persistence (Eisenreich et al., 2019, 2020). As another benefit, bacteria can use different metabolic pathways than the infected host cells. This presents an interesting opportunity to develop drugs that target the unique metabolic enzymes of the bacterial pathways only. The idea is that inhibiting these reactions would not cause severe side effects in hosts.

As a simple experiment to address the question of how a pathogenic bacterium modulates its metabolism due to the presence of an anti-infective (at sublethal concentrations), isotopologue profiling was used with *E. coli* growing in medium containing sublethal concentrations of hypochlorite. The modulation of fluxes due to hypochlorite, i.e., the adaptation of *E. coli* to the exogenous stress factor, could be determined at high resolution by NMR and GC/MS (Drazic et al., 2015).

Table 2: Examples of isotopologue profiling analyses of several pathogens.

Pathogens	Experiment	Reference
Campylobacter jejuni	For the first time, metabolomics using ^{13}C-labeled glucose proved that *Campylobacter* spp could catabolize glucose via the pentose phosphate and Entner-Doudoroff pathways to synthesize amino acids and cell surface carbohydrates.	Vorwerk et al. (2015)
	The mechanism of colonization in mice was studied using 13C isotopologue profiling and a transposon insertion library. It was found that serine is the major carbon source and is essential for infection. This explains why serine has been used as an additive to the growth medium for *C jejuni*.	Gao et al. (2017)
Chlamydia trachomatis	Metabolomics of *Chlamydia trachomatis* cultivated on HeLa cells was investigated. The main findings were: i) Incorporation of [U-^{13}C6]glucose at high rates into chlamydial lipopolysaccharide. ii) Bacterial alanine, aspartate, and glutamate are biosynthesized from glucose 6-phosphate of the host cell using dicarboxylates from the host cell, such as malate or α-KG (from host glutamine), highlighting the importance of host glutamine for *C. trachomatis* pathogenesis.	Mehlitz et al. (2017)
Coxiella burnetii	Metabolomics was studied using ^{13}C-glucose, ^{13}C-serine or ^{13}C-glycerol. It was found that serine mainly contributes to energy generation through the TCA cycle, while glycerol enters gluconeogenesis (Bipartite-type metabolism). However, glucose was used for cell wall biosynthesis, pyruvate production through glycolysis, or erythrose 4-phosphate synthesis via the pentose phosphate pathway for tyrosine biosynthesis. These results highlight the multi-substrate utilization of *C. burnetii* in a bipartite metabolic, which can benefit its survival.	Häuslein et al. (2017a)
	The carbon metabolism of both intracellular and axenically cultivated *C. burnetii* was investigated using [^{13}C]glucose or [^{13}C]glutamate. It was found that *C. burnetii* may use multiple carbon sources, including glucose and glutamate, highlighting the role of isotopologue profiling in identifying gaps related to metabolic pathway data.	Kuba et al. (2019)
	The mechanism of lactate biosynthesis within *C. burnetii* was analyzed using [^{13}C]glucose labeling. It was found that two *C. burnetii* (CBU1241 and CBU0823) strains are unlikely to be responsible for lactate production. Neither CBU0823 nor CBU1241 exhibited any lactate dehydrogenase activity in vitro enzyme activity assays, and CBU0823 did not produce lactate from a malolactic enzyme reaction.	Hofmann et al., (2021)
E. coli	The growth of *E. coli* BL21 in minimal media with different gluconeogenic carbon sources was assessed. The authors found that adding ferrous sulphate minimal medium enhanced the growth of *E. coli*.	Paliy and Gunaseker a (2007)
	Isotopologue profiling (^{13}C-glucose) of enterohemorrhagic *E. coli* was investigated. A relationship between metabolism governed by the respective ecological niche and virulence could be obtained. This was explained by obtaining different metabolic phenotypes as well as different expressions of virulence factors.	Polzin et al. (2013)
	The study evaluated the production of resveratrol by engineered *E. coli* through ^{13}C metabolic flux analysis. It was found that a balance between the malonyl-CoA supply node and the citric acid cycle was crucial for resveratrol production.	Hong et al. (2020)
Listeria monocytogenes	Isotopologue profiling was analyzed using [U-$^{13}C_6$]glucose. The incomplete TCA cycle and the importance of pyruvate carboxylation to oxaloacetate were identified as essential reactions to afford aspartate or diaminopimelate (mDAP).	Eisenreich et al. (2006)
	The fuel source for CD8+ T cells in mice infected with *Listeria monocytogenes* was evaluated. Using ^{13}C-labeled metabolites (glucose, glutamine, acetate), it was found that at early infection, CD8+ T cells rely on glutamic-oxaloacetic transaminase 1. In the late stage of infection, CD8+ T cells switch fuel preference to acetate-dependent TCA cycle metabolism.	Ma et al. (2023)
Pseudomonas aeruginosa	Isotopologue profiling was done to determine the potential reasons for different biofilm formation among clinical isolates. Biofilm formation in *P. aeruginosa* was attributed to the higher activities of the TCA cycle and Entner-Doudoroff pathway and the lower activity of PPP.	Opperman and Shachar-Hill (2016)
Staphylococcus aureus	Metabolomics of wild-type strains and small colony-variant (persistent infection) using [U-$^{13}C_6$]glucose together with transcriptome analysis. Small-colony variants had a reduced aconitase activity, reflected by a reduction of ^{13}C-excess in TCA cycle-related amino acids.	Kriegeskort e et al. (2014)
	Studied the surface proteins and the formation of biofilms by *Staphylococcus aureus* using stable isotopes, [^{15}N]glycine and [1-^{13}C]threonine, or [2-^{13}C,^{15}N]leucine. It was found that 20% of pentaglycyl bridges are not cross-linked and are, therefore, potential attachment sites for surface proteins. In the planktonic cells, none of these sites have a surface protein attached, but in mature biofilms, one-fourth of these sites have a surface protein attached. Additionally, it was observed that the concentration of β-strands in leucine-rich regions doubles in the mature biofilm, as shown by the leucine label.	Kim et al. (2018)
Salmonella	Metabolomics of *Salmonella* cultivated on CaCo-2 cells. Glucose was found to be a nutrient source during intracellular replication.	Götz et al. (2010)

Similarly, the carbon fluxes in other avian pathogens could be studied in the presence of antimicrobials or potential alternatives. In addition, these experiments could be expanded to infected cells, tissues, or birds to describe the metabolic adaptations under more relevant conditions. It can be foreseen that these experiments could help to identify unique metabolic targets for further drug development in poultry sciences. Based on these findings, essential reactions in the metabolic adaptations could be targeted by innovative therapies and preventive measures.

Challenges and future perspectives related to isotopologue profiling

Several challenges should be followed when designing isotopologue profiling experiments. These include i) ensuring isotopic and metabolic steady, ii) dealing with a sufficiently large number of metabolites, iii) choosing an appropriate ^{13}C-supplement, iv) optimizing culture conditions, and v) considering conditions for harvesting and metabolic quenching. In the following section, a summary of potential pitfalls and suggested potential solutions will be highlighted.

Isotopic and metabolic steady

The metabolic steady state is a condition in which the isotopic composition of all metabolites remains constant, and the fluxes are not affected by the concentration of metabolites (Buescher et al., 2015). The type of metabolites affects this steady state condition: soluble metabolic intermediates equilibrate their isotopic composition within minutes after uptake of ^{13}C-tracers, while it can take hours and more for metabolites bound in proteins or membranes. When time issues are a challenge, qualitative analysis addressing relative carbon fluxes in wild-type vs. mutants is more appropriate rather than a quantitative analysis (providing absolute values for metabolic fluxes). The isotopic steady state can be verified by assessing the isotopic compositions of a metabolite at several time points after having added a tracer (Beste et al., 2013).

The presence of a huge number of metabolites (thousands of compounds)

Non-targeted metabolomics studies should focus on highly abundant and stable compounds, such as amino acids, organic acids, fatty acids, sugars, etc. While LC-MS-based methods are being developed to analyze a high number of metabolites, including low-abundant labile intermediates (Shi et al., 2020), GC-MS-based analysis of amino acids is one of the most reliable and robust ways to perform isotopologue profiling of bacteria (Eisenreich et al., 2010; Long and Antoniewicz, 2019). As described above, the labeling pattern of amino acids provides the data for many central metabolic intermediates, and, therefore, the analysis of amino acids can provide a high coverage of reactions in the core metabolic network.

Selection of the ^{13}C-isotope

Generally, [U-$^{13}C_6$]glucose is an effective tracer for evaluating the overall performance of an MFA experiment. However, it may not distinguish between similar metabolic pathways (for example, glycolysis vs. pentose phosphate pathway vs. Entner-Doudoroff pathway). For analyzing the usage of co-substrates in some pathogens, [U-$^{13}C_6$]glucose may also allow only limited analysis of the TCA cycle activity. In many pathogens growing under complex conditions, the lower part of the central carbon metabolism, including the TCA cycle, is not or only less supplied by glucose (Eisenreich et al., 2013; Steiner and Eisenreich, 2022). Rather, these bacteria (mainly intracellular bacterial pathogens) use multiple substrates in a “bipartite metabolism”. Here, several carbon substrates from the hosts are utilized from the hosts lowering the metabolic burden of an infected host cell or organism. Indeed, the withdrawal of glucose alone from the host cell can lead to undesirable outcomes such as autophagy and apoptosis. Withdrawing several substrates from the host cell has less detrimental effects. In those examples, potentially labeled tracers could include glycerol, lactate, acetate, serine, or cysteine (Chen et al., 2017; Häuslein et al., 2017b; Mehlitz et al., 2017).

Conclusion

Metabolomics and especially ^{13}C-guided metabolomics or metabolic flux analysis have great potential also in poultry sciences. These techniques can significantly enhance the discovery of new metabolites (biomarkers) or pathways related to dietary intake, the understanding of metabolic pathways in avian pathogens and their hosts, and the detailed study of pathological conditions such as heat stress or

immune distress. Emerging questions could be addressed on how novel therapeutics modulate the pathways in nutrient usage or how antimicrobial resistance during the farming of poultry is related to metabolism. For example, by identifying metabolic pathways influenced by specific nutrients and dietary patterns, researchers could develop targeted interventions for disease prevention and treatment in the future.

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