



## FUNCTIONING OF THE INTESTINAL ECOSYSTEM: FROM NEW TECHNOLOGIES IN MICROBIAL RESEARCH TO PRACTICAL POULTRY FEEDING – A REVIEW\*

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### Abstract

Unlike classical microbiology which focuses on bacteria capable of growing *in vitro*, metagenomics is a study of genetic information originating from microflora which aims to characterise the microbiome, namely the common genome of bacteria, archaea, fungi, protozoa and viruses living in the host. Metagenomics relies on next-generation sequencing (NGS), a large-scale sequencing technique which allows millions of sequential reactions to be carried out in parallel to decode entire communities of microorganisms. Metagenomic analyses support taxonomic analyses (involving gene fragments encoding ribosomal RNAs 5S and 16S in bacteria) or functional analyses for identifying genes encoding proteins that participate in the regulation of metabolic pathways in the body. New metagenomics technologies expand our knowledge of the phylogenetic structure of microflora in the gastrointestinal tract of poultry, and they support the identification of previously unknown groups of microbiota, mainly those occurring in small numbers. Next-generation sequencing also provides indirect information about the quantitative structure of the genes of gut microorganisms, but microbial activity and changes in the proportions of microbial metabolites that affect the host's intestinal integrity and metabolism remain insufficiently investigated. Therefore, research studies are undertaken to investigate the proportions of the key microbial metabolites in the intestinal contents of poultry relative to changes in the population size of the most important bacterial groups, including those determined by cheaper techniques.

**Key words:** intestinal microbiota, metagenomics, next-generation sequencing, intestinal ecosystem, poultry nutrition

“We are in the midst of what may, in retrospect, come to be referred to as the golden age of microbial ecology. The microorganisms and their genes associated with higher organisms (the microbiome) that were once viewed primarily as sources of human pathogens are now recognised as complex communities with an important influence on the health and disease status of the host”. This is how certain authors (Oakley et al., 2014) assess the effects of the five-year research programme into the human microbiome, funded by the US National Institute of Health in 2007–2012. The term “microbiome” was suggested by the Nobel Prize laureate Joshua Lederberg to describe the collective genome of all commensal, symbiotic and pathogenic microorganisms found in the human body, in the gastrointestinal tract, on the skin, and in urinary and respiratory systems (Hooper and Gordon, 2001). At present, this term denotes the collective genome of microorganisms, i.e. bacteria, archaea, fungi,

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protozoa and viruses living in the host's body, which undergo changes during the host's life (e.g. in response to the diet, environment, stress, medical interventions or medical conditions). The microbiome makes an important contribution to energy homeostasis, metabolism, health, immunological activity and neurodevelopment of the host's body (Turnbaugh et al., 2007; Cho and Blaser, 2012).

As at the end of 2018, over 20,000 scientific reports with the keyword "microbiome" had been published in the Web of Science. These reports have been published in the last 15 years, with 106, 2724 and 17357 works in subsequent five-year periods. This rapid increase in the number of scientific papers testifies to the growing interest in the microbiome. More than 100 papers focused on the microbiome of the gastrointestinal tract in poultry, whereas the remaining articles addressed diverse issues. The aim of this review article was to describe the potential scientific developments stemming from advances in metagenomics relative to classical microbiology, and to highlight the contribution of innovative research technologies to the acquisition of new knowledge about the composition and physiological impact of gastrointestinal microbiota on the body.

### **Metagenomics: a new chapter in microbiology**

Metagenomics studies genetic material from environmental or host-associated microbiota to describe microbial diversity and function (Choi et al., 2015). Metagenomics emerged as a separate field of research in the last decades of the 20th century when more effective methods of cloning and identifying the DNA of microbial samples from the environment, initially soil, had been developed (Handelsman et al., 1998). Technological progress has led to the development of next-generation sequencing (NGS), a massive parallel sequencing approach for decoding entire microbial communities (Thomas et al., 2012). Microbiology has been revolutionised by technological advancement and high-performance metagenomics technologies involving direct cloning, sequencing and functional analyses of genetic material, as well as by the progress in bioinformatics, including the development of computational methods for analysing the structure, functions and evolution of genes, genomes and proteins (Borda-Molina et al., 2018; Barko et al., 2018).

Metagenomics researchers seek answers to the following questions: "Who is there?", which corresponds to phylogenetic profiling (taxonomic analyses), and "What are they doing?", which relates to genetic analyses (functional analyses) (Chistoserdova, 2009). The sequences of the genes encoding 5S and 16S ribosomal RNA, the molecular determinants of microbial phylogenesis, play a crucial role in taxonomic analyses of bacteria and archaea (Schloss and Handelsman, 2003). Different fragments of the ribosomal gene are amplified in other microorganisms, including the 18S rRNA gene in eukaryotic species and the nuclear ribosomal internal transcribed spacer regions in fungi (Meyer et al., 2010). In comparison with traditional microbiological techniques, microbial genera and species can be identified much more accurately based on detailed analyses of nucleotide subunits and comparisons with 16S rRNA gene sequences deposited in public databases (Deusch et al., 2015). Metagenomic functional analyses are an essential element of phylogenetic research and support the search for functional interactions between microbial species colonising specific environments (Campanaro et al., 2016). Functional analyses of the

metagenome enable the determination of protein-encoding genes which participate in the control of the host's metabolic pathways. Gene functions are identified with the involvement of traditional methods, including analyses of gene homology (predicting gene functions by comparison with the structure of the recognised genes), gene inactivation and overexpression, as well as micromatrices and analyses of DNA microcracks with the use of bioinformatic tools (Sitnicka et al., 2010). Despite continuous progress in research, the acquisition of complete single genomes from metagenomic sequences still poses a challenge (Nielsen et al., 2014). Functional groups and bacterial taxa can be identified with the use of basic tools databases, such as the Kyoto Encyclopaedia of Genes and Genomes (KEGG). One of the limitations of this procedure stems from the fact that the diversity of genomes in nature is far greater than in limited sequence databases, which is why not all predicted genes will exhibit homology with known sequences (Sharpton, 2014).

Two distinct metagenomics approaches are commonly used: the first is referred to as marker-gene metagenomics or targeted metagenomics, and the second is known as shotgun metagenomics. In the past, the method of sequencing entire microbial DNA was more common. Shotgun metagenomics ("random firing pattern") offers a simpler solution by sequencing a large number of random sections of fragmented genomic DNA which are subsequently combined into continuous threads based on overlaps (overlapping regions) (Sharpton, 2014). This approach requires innovative computer-based computational methods to combine hundreds of thousands of short, randomly-obtained DNA sequences into longer, continuous fragments. Metagenomic analyses of the DNA pool of microorganisms colonising the bovine gut in a given environment also support the identification of genes encoding proteins that participate in the regulation of host metabolic pathways. Shotgun DNA sampling supports more efficient determination of functional metabolic profiles in bacterial communities than targeted metagenomics (Deusch et al., 2015).

The most important differences between the methods and results of classical microbiology and metagenomics are presented in Table 1. In classical microbiology, the microflora is regarded as a population of live microorganisms (colony forming units), whereas the term microbiome refers to the collective content of genomic microflora, where species-equivalent operational taxonomic units (OTUs) can be distinguished (Cole et al., 2014). An OTU is an organizational proxy for a species created by statistical clustering of units with high sequence similarity, typically higher than 97%. The number of OTUs is disproportionately larger because OTUs account for more complex microflora (fungi, viruses as well as microbial expression products), including anaerobic microflora.

Several types of NGS techniques can be identified, depending on the applied analytical system (e.g. Illumina, ThermoFisher, and Bioscience) with different combinations of read length and read numbers that determine the system's throughput and the number of bases per run. Therefore, various analytical platforms can produce different results (Stanley et al., 2013; Ranjitkar et al., 2016). This problem has been discussed in other review articles (Liu et al., 2012; Scholz et al., 2012; Mohinudeen et al., 2017; Vincent et al., 2017). According to Nielsen et al. (2014), bacterial genomes from different isolates of the same species usually show considerable genetic

heterogeneity. The results of microbiome analyses are also influenced by the method of sampling intestinal contents where samples can be collected individually from chickens or pooled from several birds. Errors are also encountered during sample preparation and storage, DNA extraction and read sequencing (Kunin et al., 2010; Cruaud et al., 2014; Hang et al., 2014). These issues are not discussed in this article.

Table 1. A brief comparison of conventional microbiological methods and metagenomics techniques (Salanitro et al., 1974; Tremaroli and Bäckhed, 2012; Wang et al., 2015; Barko et al., 2018; Borda-Molina et al., 2018)

Classical microbiology	Metagenomics
<b>Methods</b>	
<ul style="list-style-type: none"> <li>• Enumeration of selected bacterial groups cultured on selective media</li> <li>• Determination of bacterial properties:               <ul style="list-style-type: none"> <li>– morphology and structure of the cell wall (Gram+/-),</li> <li>– responses to oxygen, antibiotic resistance, antibacterial activity immunomodulatory effects, etc.,</li> <li>– fermentation activity: substrates and main metabolites</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>• Analyses of nucleic acids – selection of genetic material for the entire microbial population</li> <li>• Determination of the structure and physiological functions of microbiota:               <ul style="list-style-type: none"> <li>– taxonomic analyses of bacterial populations (sequences encoding 16S rRNA)</li> <li>– functional analyses to determine the presence of genes encoding specific enzymatic/metabolic activity</li> </ul> </li> </ul>
<b>Results</b>	
<ul style="list-style-type: none"> <li>• Visible but only culturable bacterial colonies (approximately 1–10% of bacteria in the gastrointestinal tract)</li> <li>• Bacterial counts expressed in CFU/g or CFU/ml</li> </ul>	<ul style="list-style-type: none"> <li>• All bacteria, fungi, archaea, viruses and their gene products present in the environment</li> <li>• Composition of microbiota expressed by the number of species-equivalent operational taxonomic units (OTUs)</li> </ul>
<b>Applications</b>	
<ul style="list-style-type: none"> <li>• Isolation of target bacterial colonies</li> <li>• Clinical diagnosis</li> </ul>	<ul style="list-style-type: none"> <li>• Determination of bacterial diversity</li> <li>• Determination of microbial dysbiosis</li> </ul>

### Metagenomic analyses of poultry gut microbiota

Earlier studies show that bacterial counts in the ileum and caeca of day-old chicks can reach  $10^8$  and  $10^{10}$  CFU per g of intestinal contents, respectively (Apajalahti et al., 2004). Fifty species of bacteria were identified in day-old chicks, and the number of bacterial species in caecal digesta increased to 200 in 42-day-old chicks (Oakley et al., 2014). The rate of microbial colonisation in the gastrointestinal tract is determined by hygiene standards, diet composition and feed intake. In three-week-old chicks, the proportion of lactic acid bacteria in ileal microbiota exceeded 70%, whereas the proportion of other bacterial groups, including *Clostridium* spp., *Streptococcus* spp. and *Enterobacteriaceae* spp., was considerably smaller. In the final rearing stage, significant variations were noted in the composition of the microbiota colonising the small intestine and the caeca. In quantitative terms, *Lactobacillus* bacteria were predominant in the ileum, whereas the proportion of *Clostridium* did not exceed 10%. The reverse proportions of *Lactobacillus* and *Clostridium* were observed in the caeca (7.75 vs 39.3%) (Lu et al., 2003). Both intestinal segments generally differ in microbial counts, with  $10^8$ – $10^9$  CFU in the ileum, and  $10^{10}$ – $10^{11}$  CFU in the caeca (Yeoman et al., 2012).

Table 2. Examples of the use of 16S rRNA for profiling gut microbiota in view of the host's age, body weight and environmental conditions

Research focus	Animals	Dietary treatments	Main results	Reference
Diversity and succession of the intestinal bacterial community	Ross hybrid chickens at 35 days	Corn-soybean-based diet without animal protein or antibiotics	The following differences were determined in ileal and caecal microbiota: – ileum: <i>Lactobacillus</i> -related sequences (70%), <i>Clostridiaceae</i> (11%), <i>Streptococcus</i> (6.5%) and <i>Enterococcus</i> (6.5%); – caeca: <i>Clostridiaceae</i> -related sequences (65%), <i>Fusobacterium</i> (14%), <i>Lactobacillus</i> (8%) and <i>Bacteroides</i> (5%).	Lu et al., 2003
Composition of bacterial communities relative to feeding strategy and age	Ross 308 broilers at 8–36 days	Soybean-based diets	<i>Lactobacillaceae</i> were the predominant Firmicutes bacteria in all age groups and in all segments of the gut, excluding the caeca. In broilers, gut microorganisms “matured” between days 15 and 22.	Ranjitkar et al., 2016
Caecal bacterial communities and functional predictions by sex and body weight	Ross 308 broilers at 35 days	Equally fed chickens with high, medium and low final body weight	Male vs. female chickens: Bacteroides ↑ <i>Clostridium</i> ↓ <i>Shigella</i> ↓ Male chickens with high BW vs. low BW: <i>Faecalibacterium</i> ↑ <i>Shuttleworthia</i> ↑	Lee et al., 2017
Composition of caecal microflora in chickens reared under identical conditions	Cobb 500 broilers at 25 days	Wheat-soybean based diet applied in three trials	The flocks differed in feed conversion ratios and caecal microbiota profiles. Considerable variations in broiler microbiota were reported in one trial.	Stanley et al., 2013

Table 3. Diversity of microbiota in chickens selected for feed efficiency, body weight and body composition

Research focus	Experimental design	Main results	Reference
Metagenomics of the faecal microbiome in broilers with a low and high feed conversion ratio (FCR)	Broilers (♀ and ♂) with the highest body weight on day 49 were the parents of the analysed progeny. Four sibling pairs were selected, where one sibling in the pair had low FCR and the other sibling had high FCR between 35 and 49 days of age	Significant differences between the compared metagenomes suggest that the intestinal microbiome is linked with the low or high FCR phenotype	Singh et al., 2014
Impact of chicken selection for FCR on faecal microbiota composition	144 birds with extreme feed efficiency values at 3 weeks, and feed conversion values of $1.41 \pm 0.05$ and $2.02 \pm 0.04$ in the efficient and non-efficient groups, respectively, were used	The study revealed a clear link between host genetics, microbiota composition, and feed and digestive efficiency. <i>Lactobacillus</i> , <i>C. leptum</i> and <i>E. coli</i> were identified as the most important factors in this interaction	Mignon-Grasteau et al., 2015
Faecal microbiota in lean (LL) and fat (FL) line broilers divergently selected for fatness traits	The studied chicken lines originated from the Arbor Acres broiler which had undergone 15 generations of selection since 1996 based on the content of very-low-density-lipoprotein and abdominal fat percentage at 7 weeks of age	Significant differences between LL and FL chickens were found in the relative abundances of some energy metabolite-related bacteria (especially the SCFA-producers) and potential pathogens (e.g. <i>Enterococcus</i> ), and in numerous biochemical pathways relating to obesity, adiposity and energy balance	Hou et al., 2016
Composition and functional pathways of chicken faecal microbiota in relation to selection-induced obesity	Two chicken lines (fat and lean) divergently selected (for 15 generations at the same location and fed identical diets) for abdominal fat content (AFP) and plasma levels of very-low-density lipoprotein (VLDL) were used	Changes in the abundance and composition of gut microbiota in fat and lean chickens were induced by altering the frequencies of obesity-related alleles; microbiota changes led to differences in the citrate cycle and the PPAR signalling path of fat and lean chickens	Ding et al., 2016
Faecal microbiota composition and feed efficiency in chickens with different residual feed intake	Chickens of both sexes, aged 9 to 32 days, were randomly assigned to 2 different treatments: (1) with <i>ad libitum</i> access to feed, and (2) restricted to 90–95% of the average <i>ad libitum</i> feed intake	Chickens fed <i>ad libitum</i> vs. chickens fed a restricted diet: <i>Escherichia</i> ↑ <i>Shigella</i> ↑ <i>Turicibacter</i> ↑ <i>Lactobacillus</i> ↓	Siegerstetter et al., 2018

Table 4. Examples of the use of 16S rRNA sequences for profiling gut microbiota in view of dietary treatments

Experimental treatments	Animals	Main results	Reference
Barley-based diet with the addition of NSP-degrading enzymes	Cobb 500 broiler chickens	A positive correlation was observed between gut microbial communities and bird performance. Several species of bacteria that may have contributed to diet-induced differences in the composition of intestinal microbiota accounted for 1 to 5% of the population	Torok et al., 2008
<i>Per os</i> administration of <i>Campylobacter</i> and lupulone (as an antimicrobial factor; 125 mg/L)	Broiler chickens at 1 to 22 days	The effects of lupulone in challenged birds: <i>C. perfringens</i> ↓ (midgut and caeca), <i>Lactobacillus</i> ↓ (midgut), but without significant changes in the overall microbiota	Tillman et al., 2011
Diet without or with monensin sodium, virginiamycin and tylosin	Ross 308 broiler chickens	The administration of monensin alone or monensin/virginiamycin or monensin/tylosin exerted different modulatory effects on caecal microbiota. An explanation why the use of growth promoters and anticoccidials improved bird health and productivity was proposed	Danzeisen et al., 2011
Soybean-based diets with wheat, maize or maize silage	Ross 308 broiler chicken at 36 days	Wheat-based feed, maize-based feed, or maize-based concentrates supplemented with 15% or 30% crimped kernel maize silage had no significant influence on bacterial diversity in all segments of the gut	Ranjitkar et al., 2016
A diet without or with the addition of <i>Lactobacillus acidophilus</i> (LA) administered at 1x10 <sup>9</sup> cfu/kg feed	Ross 308 broiler chickens reared from 1–41 days	Supplementation of broiler diets with <i>Lactobacillus acidophilus</i> : final BW ↑, FCR ↓, a positive correlation with selected metabolic functions, in particular in relation to higher levels of β-glucosidase	De Cesare et al., 2017

New analytical methods are more effective than traditional microbiological techniques in determining the variations in intestinal microbiota. Recent research indicates that digestive tract microbiota consists of more than 500 phylotypes or around 1 million bacterial genes, which is 40–50 times higher in comparison with the chicken genome (Wei et al., 2013; Sergeant et al., 2014; Oakley et al., 2014). According to estimates, the gastrointestinal microbiota in poultry is composed of 640 (Apajalahti et al., 2004) or even 900 (Wei et al., 2013) identified species, but only 10% of these species have been cultured and characterised under laboratory conditions (Torok et al., 2011). The sequences identified in the gastrointestinal tracts of both poultry and humans represented 13 bacterial phyla (Wei et al., 2013) with a predominance of Firmicutes and Bacteroidetes, of which 10 accounted for a small part of the community (Oakley et al., 2014). Firmicutes are predominant in the upper gastrointestinal tract (approx. 90% of the microbial community), and are represented mostly by *Lactobacillus*, *Clostridium* and *Streptococcus* (Wei et al., 2013). In the gut, the proportion of Firmicutes was estimated at 44–55%, the proportion of Bacteroidetes, represented by the genus *Bacteroides* was estimated at 22–45%, whereas Actinobacteria (including *Bifidobacterium*) and Proteobacteria (including *Escherichia* and *Shigella*) were far less abundant (Qu et al., 2008). Other authors give different values. In a study by De Cesare et al. (2017), the caecal digesta in chickens was also dominated by Firmicutes (93.9%), whereas the proportions of Proteobacteria (1.74%), Actinobacteria (0.92%), Bacteroidetes (0.35%) and Tenericutes (1.18%) were considerably smaller. In the work of Borda-Molina et al. (2016), six bacterial families, including five members of the phylum Firmicutes and one member of the phylum Actinobacteria, accounted for 1% of the bacterial community in caecal samples.

According to Borda-Molina et al. (2018), the majority of metagenomics studies investigating the gastrointestinal tract of chickens have focused on caecal function, responses to pathogen challenge, the prominent role of microbiota in growth performance, comparisons of fat and lean lines, virulome and of antibiotic resistance genes. The experimental design and the results of the reviewed studies are presented in Tables 2–4. The studies cited in Table 2 revealed differences in the composition of ileal and caecal microbiota (Lu et al., 2003), demonstrated that microbial “maturation” takes place in the third week of a chicken’s life (Ranjitkar et al., 2016), reported differences in the composition of microbiota in male and female broiler chickens (Lee et al., 2017), and demonstrated that environmental conditions and individual characteristics influenced the gut microbiota profile (Stanley et al., 2013). Recent studies have investigated intestinal microbiota in the context of biosecurity, housing conditions, litter, feed access and climate (Kers et al., 2018). Wei et al. (2018) compared the microbiomes of chickens and turkeys and reported differences in the species composition of their microbiota. The turkey microbiome was less diverse than the chicken microbiome, but in both bird species, Firmicutes, Bacteroidetes and Proteobacteria were the largest phyla that accounted for >90% of all sequences. The predominant genera in both chicken and turkey sequences were *Clostridium*, *Ruminococcus*, *Lactobacillus* and *Bacteroides*, but their distribution differed in the analysed bird species. In turkeys, *Campylobacter* resided predominately in the caeca, and they were more abundant at 10 weeks of age and less abundant at slaughter (Wilkinson et al., 2017).

The effect of intestinal microbiota on feed conversion in meat-type poultry is also a widely discussed problem in the literature. Poultry diets are composed mainly of cereals and high-protein components, and feed efficiency significantly affects the profitability of meat production. Feed accounts for 70% of total costs in the production of poultry meat (Willems et al., 2013), which is why the effect of intestinal microbiota on feed efficiency in poultry farming has been extensively studied. The results of experiments analysing the diversity of caecal microbiota in chickens with different feed efficiency, body weight and body composition are presented in Table 3. In a study by Singh et al. (2014), chickens with a high feed conversion ratio (FCR) were characterised by a higher abundance of the genera *Acinetobacter*, *Bacteroides*, *Streptococcus*, *Clostridium* and *Lactobacillus*, whereas birds with low FCR were colonised predominantly by *Escherichia*, *Shigella* and *Salmonella*. In an experiment by Mignon-Grasteau et al. (2015), the faecal digestibility coefficient of dry matter was genetically and positively correlated with *L. crispatus*, *C. leptum* and *C. coccoides*, and negatively correlated with *E. coli*. Lipid digestibility was negatively correlated with *E. coli*, and apparent metabolisable energy (AMEn) was positively correlated with *C. coccoides* and the *C. coccoides* to *Lactobacillus* ratio. Hou et al. (2016), Ding et al. (2016) and Siegerstetter et al. (2018) found that long-term selection for body weight, a lower content of abdominal fat, lower plasma levels of very-low-density lipoproteins and restricted feed intake influenced the composition of caecal microbiota. However, Hou et al. (2016) stressed that their results did not support the formulation of concrete conclusions regarding the influence of chicken gut microbiota on the metabolism of lean and fat line chickens. Further research is required to determine whether these bacteria are the cause or the consequence of the differences in feed utilisation (Pan and Yu, 2014). One of the consequences of the selection for feed intake in poultry can be an increased amount of readily fermentable components in the caeca (Walugembe et al., 2014), which can improve feed utilisation, increase intestinal weight and prolong caecal transit time (Rougière et al., 2002). For this reason, changes in the gut microbiota may result from feed intake and transit time in the gastrointestinal tract. Studies of humans demonstrated that population size of Firmicutes in faeces was positively correlated with the ability to recover energy from the diet (Jumpertz et al., 2011; Oakley et al., 2014) and that the Firmicutes to Bacteroidetes ratio could have important implications for health and nutrition. Studies of chickens revealed that a lower proportion of Firmicutes relative to Bacteroidetes in the caeca can promote effective utilisation of dietary energy. Sergeant et al. (2014) identified the sequences of more than 200 enzymes degrading non-starch polysaccharides in Bacteroidetes, which could be responsible for the utilisation of dietary energy in the lower gastrointestinal tract. Taxonomic analyses provide detailed information about the structure of microbial communities, but this knowledge is difficult to apply in evaluations of microbiota's influence on the host's body. A total of 59 bacterial genera were identified in the microbiome of chicken caeca, including 39 genera of the phylum Firmicutes, where the three most prevalent genera (*Ruminococcus*, *Clostridium* and *Eubacterium*) accounted for 5% and the remaining genera represented up to 1% of the phylum Firmicutes (Wei et al., 2013). Bacteroidetes, the second most abundant phylum, was represented by 6 genera, including the

predominant genus *Bacteroides* (approx. 40% of the population). The cited studies analysing the effect of microbiota on the utilisation of dietary energy in humans and chickens did not account for such considerable quantitative differences in bacterial genera, but compared only quantitative differences between the phyla Firmicutes and *Bacteroides* (Jumpertz et al., 2011; Bervoets et al., 2013; Oakley et al., 2014).

### Metagenomics from the physiological perspective

Complex and cost-intensive metagenomics technologies enable the characterisation of microbial composition, but they fail to provide new information on the metabolites produced by gut microorganisms. Metagenomics procedures describe the accumulation of genomes and the corresponding genes in a given ecosystem, as well as the potential functionality of bacteria in specific environments (Marchesi and Ravel, 2015). According to estimates, around 20% of the genes in the metagenome of chicken intestinal microbiota control carbohydrate metabolism, 10% control protein and amino acid metabolism, and 1–2% are responsible for lipid metabolism (Qu et al., 2008). Microbial pathogens of the genera *Salmonella*, *Clostridium*, *Campylobacter*, *Staphylococcus* and *E. coli* pose a potential health risk for poultry (Oakley et al., 2014). Therefore, the mechanisms of competition between beneficial and pathogenic microflora, which involve competitive exclusion and the production of bacteriocins, are an important consideration (Yeoman et al., 2012). The composition of gut microbiota determines the functioning of the intestinal ecosystem (Table 5), and the composition and the physicochemical properties of the intestinal contents as well as the effects of microbial metabolites also play an important role (Zduńczyk et al., 2015). Polysaccharide fermentation processes play a crucial role in poultry caeca and in the colons of humans and monogastric animals, and they affect not only intestinal health, but the entire body (Stanley et al., 2014; Choi et al., 2015).

Table 5. Components of the intestinal ecosystem, factors affecting gut function, main metabolites and their physiological effects

Ecosystem components	Impact factors
Intestinal contents	Diet: chemical composition and physicochemical properties (viscosity, water content) Enzymatic activity of the microbiota
Intestinal microbiota	Feed composition, mainly the content of non-starch polysaccharides (NSP) Bird genotype Environmental conditions
Metabolites	Effects
Short-chain fatty acids (SCFAs): acetic, propionic, butyric and other	Local – intestinal integrity, inhibition of pathogen development, absorption of Ca, Fe and Mg, stimulation of the intestinal immune system Systemic – influence on lipid and glucose metabolism
Lactic acid	Reduction in the pH of intestinal contents
Bacteriocins	Bacteriostatic or bactericidal effects
Peptides and neurotransmitters	Stimulation of enteroendocrine cells (EECs), communication with the central nervous system, influence on metabolism
Ammonia, amines, bile acid degradation products and other	Possible damage to intestinal mucosa, mucosal disorders, initiation of neoplastic processes

Table 6. The effect of dietary treatments on gastrointestinal function and microbiota composition

Aim	Experimental design	Main results	Reference
The effect of wheat grain on GIT function in growing turkeys, including microflora composition	Control basal diet was diluted with 22.5% ground and pelleted wheat or whole wheat grain	Inclusion of 22.5% whole wheat grain: <i>Bacteroidia</i> domain ↑, <i>Salmonella</i> ↓, butyric acid ↑, total SCFA ↑ in caecal digesta	Zduńczyk et al., 2013
The effect of whole wheat feeding on caecal microbiota in growing turkeys	Diets containing whole wheat grain as a substitute for 25, 50, 75 and 100% of the total dietary content of wheat were fed to growing turkeys at 5–12 weeks	Dietary whole wheat led to a linear decrease in the caecal counts of <i>Escherichia coli</i> , <i>Clostridiaceae</i> and <i>Clostridium perfringens</i> , but had no effect on <i>Bacteria</i> , <i>Lactobacillus</i> , <i>Enterococcus</i> , <i>Bifidobacterium</i> , <i>Salmonella</i> and <i>Bacteroides</i>	Jankowski et al., 2013
The effect of dietary blue lupine seeds on intestinal microbiota in laying hens	A control soybean-based diet and two diets supplemented with 10% or 20% of blue lupine seed	Diet containing 20% lupine seeds: caecal <i>Bifidobacterium</i> sp. ↑, <i>Lactobacillus</i> ↑, <i>Enterococcus</i> ↑, <i>Bacteroides</i> ↓, <i>Prevotella</i> ↓, <i>E. coli</i> ↓	Zduńczyk et al., 2014
The effect of diets with yellow lupine seeds (YL) on caecal function in young turkeys	Three dietary inclusion levels of YL (8, 16 and 24%) as a substitute for soybean meal and wheat	Higher YL inclusion rates led to a linear increase in total bacterial counts and a simultaneous linear decrease in the counts of <i>Escherichia coli</i> , <i>Clostridiaceae</i> and <i>Bacteroides</i>	Zduńczyk et al., 2016
The effect of dietary faba bean (FB) seeds with high or low tannin content on the gastrointestinal function of turkeys at 13–18 weeks of age	Turkeys were fed a control wheat-soybean meal-based diet and 6 diets where SBM was partially replaced with HT or LT seeds at 10, 20 and 30%	Dietary FB seeds: caecal fermentation process ↑, HT vs. LT seeds: caecal <i>Salmonella</i> ↓, caecal SCFA production (including butyrate) ↑, pH of caecal digesta ↓	Zduńczyk et al., 2018

The metabolites produced by intestinal microbiota belong to three main groups of products: nutrients that are utilised by the host's body, including by the intestinal epithelium, to promote growth and enhance the function of short-chain fatty acids (SCFAs), including acetic acid, propionic acid and butyric acid; biologically active components (mainly B vitamins), and harmful and toxic substances which compromise nutritional benefits and pose a hazard to the host's health. These products exert local effects on competitive bacterial groups in the intestinal epithelium and the intestinal immune system (GALT – gut-associated lymphoid tissue) as well as systemic effects by influencing the host's metabolism (Table 5). Local effects include competitive inhibition of pathogenic microflora, protection of the intestinal mucosa and stimulation of the GALT response. In the group of SCFAs that exert systemic effects, acetic acid plays a key role by acting as a substrate in metabolic reactions, including the synthesis of cholesterol and long-chain fatty acids which regulate lipid metabolism (Millet et al., 2010). Butyric acid is a biomarker of intestinal health which delivers important local effects (De Maesschalck et al., 2015; Ducatelle et al., 2018).

The end products of protein fermentation by bacteria, including ammonia, phenols, indols, and amines, compromise gut health (Millet et al., 2010). Intestinal bacteria such as *Clostridium*, *Enterococci* and *Bacteroides* rely on proteins as an energy source, in particular when carbohydrates are not available in sufficient quantities in the intestinal contents. Therefore, high-protein diets can potentiate potentially pathogenic and pro-inflammatory microbiota (Yao et al., 2016). The mechanisms by which gut microbiota exert systemic and local effects, including competitive inhibition of pathogenic microflora, protection of intestinal mucosa, and GALT stimulation, have been described by other authors (Rehman et al., 2007; Den Besten et al., 2013; Pan and Yu, 2014; Stanley et al., 2014; Clavijo and Florez, 2018).

### **Dietary methods for controlling the activity of intestinal microbiota**

In view of the potential benefits and risks of intestinal microbiota, numerous attempts have been made in the literature to control the development and activity of gut microorganisms by modifying poultry diets or feeding regimes (Zduńczyk et al., 2015). Various approaches and feed additives have been evaluated to inhibit or stimulate fermentation processes in the gastrointestinal tract of chickens, including a reduction in the dietary content of non-starch-polysaccharide (NSP)-degrading enzymes, addition of NSP-degrading enzymes, improvements in the physical structure of chicken diets, addition of readily fermentable oligosaccharides and polysaccharides (prebiotics), and the addition of viable bacterial cultures (available on the market as prebiotics). The effectiveness of these treatments has been assessed by numerous review articles in recent years (Huyghebaert et al., 2011; Hume, 2011; Oakley et al., 2014; Olnood et al., 2015; Ruiz et al., 2015; Clavijo and Florez, 2018). The results of few experiments where NGS methods were used to evaluate the effects of dietary modifications on poultry gut microbiota are briefly summarised in Table 6. The composition of the chicken microbiome was less influenced by the incorporation of various cereals into poultry diets (Ranjitkar et al., 2016) and the addition of degrading enzymes (Torok et al., 2008) than by antibiotics (Danzeisen et al., 2011) and *Campylobacter* infection (Tillman et al., 2011).

Metagenomics methods are expensive, which limits their availability for many researchers studying the gastrointestinal ecosystem (Borda-Molina et al., 2018). In the last decade, the effects of oligosaccharides, bacterial cultures and other factors on the composition of gut microbiota were investigated with the involvement of simpler techniques, including traditional culture methods, in many studies published in the literature, including in the renowned Poultry Science journal (Song et al., 2014; Zhao L. et al., 2013; Zhao P.Y. et al., 2016; Olmood et al., 2015). The results of own previous research into gut microbiota, including the major bacterial groups of *Lactobacillus*, *Bifidobacterium*, *Escherichia coli* and *Clostridium*, which relied on fluorescence in situ hybridization (FISH) and specific fluorescent probes for identifying DNA or RNA sequences, are presented in Table 5. In these studies, the activity of enzymes ( $\alpha$ - and  $\beta$ -glucosidase,  $\alpha$ - and  $\beta$ -galactosidase,  $\beta$ -glucuronidase,  $\alpha$ -arabinopyranosidase,  $\beta$ -xylosidase and other) and the concentrations of SCFAs and ammonia, the key indicators of microbial enzymatic activity in the caeca, were also determined.

In a review article by Zduńczyk et al. (2016), partial replacement of ground wheat with wheat grain in the diets fed to turkeys induced desirable changes in their gut microbiota. In a study by Zduńczyk et al. (2013), the inclusion of 22.5% wheat grain in turkey diets improved gastrointestinal function, increased the percentage of *Bacteria* and *Bifidobacterium* spp., decreased the counts of *Salmonella* spp. in intestinal microflora, and increased the content of butyric acid and total SCFAs in the caecal digesta. In an experiment conducted by Jankowski et al. (2013), gradual increase in the dietary content of wheat grain decreased the counts of *Escherichia coli*, *Clostridiaceae* and *Clostridium perfringens*. Favourable changes in the gut microbiota were also noted when soybean meal (SBM) was partially replaced with lupine or faba bean seeds, which decreased the content of raffinose family oligosaccharides and non-starch cereal polysaccharides. In laying hens, partial replacement of SBM with 20% narrow-leaved lupine seeds increased the population size of *Bifidobacterium* and *Clostridium* spp., and decreased the counts of *Escherichia coli* and bacteria of the genera *Bacteroides*, *Prevotella* and *Porphyromonas* in the caecal contents (Zduńczyk et al., 2014). Desirable changes in the composition and enzymatic activity of gut microbiota led to an increase in the concentration of SCFAs and a decrease in ammonia levels and the pH value of caecal contents. In young turkeys fed diets with increasing levels of lupine seeds (8, 16 and 24%), a linear increase in the activity of bacterial glycolytic enzymes, an increase in the concentrations of SCFAs, and a decrease in the pH of caecal contents were observed relative to the group fed a soybean-based diet (Zduńczyk et al., 2016). A linear increase in total bacterial counts and a linear decrease in the populations of *Escherichia coli*, *Clostridiaceae* and *Bacteroides* were noted. Turkeys whose diets were supplemented with faba beans where characterised by higher counts of gut bacteria than turkeys fed soybean-wheat-based diets. Only the addition of low-tannin faba beans decreased the counts of *Salmonella* bacteria and increased the concentrations of volatile fatty acids, including butyric acid, in the intestinal contents of turkeys (Zduńczyk et al., 2018).

As demonstrated by the reviewed literature, new metagenomic techniques expand our understanding of the phylogenetic structure of gastrointestinal flora in

poultry. In the cited studies, metagenomic methods supported the identification of previously unknown microorganisms that occur in small numbers. Next-generation sequencing provides indirect information about the quantitative structure of genes in gut microorganisms. There is a general scarcity of new information about microbial activity and the proportions of metabolites which affect intestinal integrity and the metabolism of the host organism. In this context, research studies are undertaken to obtain information about changes in the population size of the most important bacterial groups relative to the proportions of the key microbial metabolites in the intestinal contents of poultry, with the use of cheaper techniques. Further research is needed to characterise the composition of intestinal microbiota in greater detail based on complex, multi-faceted and dynamic relationships between the diet, the host organism and gut microbiota. The results will contribute to an improvement in feed efficiency and the welfare of birds in intensive poultry farming.

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