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Improving gut health by modulating the digestive microbiota of chickens? How metagenomics can help.

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Abstract

High throughput sequencing technologies led to a tremendous progress in the knowledge of gut microbiota in human as well as in livestock and model animal species. Now considered as a symbiont, this microbial ecosystem realizes essential functions for its host through a complex functional dialog. Gut health in particular depends on the composition and functions of the gut microbiota through the regulation of digestion and innate and adaptive immunity. Nutritional strategies making use of feed composition and additives have been used for a long time in poultry production to improve chicken gut health, but their mode of action is generally poorly understood beyond their effect on microbiota composition. Metagenomic approaches, by giving access to the functional potential of the gut microbiota, might become a decisive tool to decipher the complex functional interactions at stake in these effects on gut health. The access to the chicken intestinal metagenome will first allow us to perform in-depth diversity analyses to describe the microbes present in chicken production conditions in relation to the many factors influencing the intestinal microbiota ecosystem. Research is needed to understand what can be considered a healthy microbiota at the functional level and to understand how the different tools available to influence it interfere with the complex host-microbiota molecular crosstalk, in particular during the early intestinal colonisation by micro-organisms. Finally, we will have to relate individual microbiota characteristics with fine descriptors of gut health, related host phenotypes of interest, and host genotypes, to identify key functional elements and putative biomarkers predicting the ability of animals to cope with different kinds of health challenges. Only a multidisciplinary research relying on a multi-omics data integration approach will allow us to take up this challenge. This research will allow us to fine-tune prevention or healing nutritional strategies to optimize the gut microbiota and the related phenotypes, in particular gut health, by acting on the holobiont, i.e. both on the host and its gut microbiota.

Keywords

Gallus gallus, metagenome, gut health, functional metagenomics

1. Introduction

The impressive progress of sequencing technologies during the last decades has led to a huge progress in microbial ecology, by giving a rapid and much more exhaustive access to the many microbes of each microbial ecosystem. The intestinal microbiota in particular, comprised of a majority of anaerobic, uncultivable microorganisms, is now considered as a symbiotic partner for its host, realising essential functions that the host is unable to achieve and contributing to the regulation of its whole physiology and metabolism (Blottière *et al.* 2013, Sommer and Bäckhed 2013). It is now very clear that the host and its intestinal microbiota both contribute to the expression of phenotypic traits of interest (Hanning and Diaz-sanchez 2015). Health in

particular, and especially intestinal health, is the result of complex functional interactions between intestinal microbes and host immunity (Broom and Kogut 2018). Disruptions of the intestinal microbiota can lead to many kinds of non-infectious diseases by altering the host physiology and metabolism and triggering inflammation (Guchte *et al.* 2018). Conversely, alterations of the host immunity can disrupt the intestinal microbiota. Research led on the human intestinal microbiota is currently leading in therapeutic innovation in human medicine. It consists mainly in personalised nutrition or action of pre- or pro-biotics to direct the microbiota to a composition considered as more favourable to its host, development of diagnostic tools for complex diseases such as Crohn's disease or the response to cancer treatments, and fecal transplants to treat critical infections by the bacterium *Clostridium difficile* (Aziz *et al.* 2013).

These observations are true for animal species as well, although potential applications to heal or prevent diseases differ. In poultry production, many diseases cause gut dysfunction, or originate from the gut (Kogut and Arsenault 2016). Gut health is therefore at the centre of animal health. The adult gut microbiota in chicken was identified a long time ago for its protective effect toward the colonisation of intestines of young chicks by *Salmonella* sp. through a mechanism called competitive exclusion (Clavijo *et al.* 2018). It has since been proven effective toward other enteric pathogens, in chicken as well as in other livestock species and in human. The gut microbiota also largely contributes to digestion by the production of essential vitamins and metabolites such as short chain fatty acids (SCFA) and the digestion of complex molecules such as non-starch polysaccharides. It also contributes to host metabolism and physiology by the production of metabolites circulating in the entire organism, and it interacts with host immunity permanently through the intestinal epithelium.

The composition of the gut microbiota in bacterial species in poultry is highly dynamic. In current production conditions, eggs are separated from laying hens and chicks hatch without contact with their mothers, so that the first microorganisms colonising the digestive tract are found in the immediate environment: eggshell, surfaces in the hatchery, air/ water supply and the first feed ingested. For this reason, chicken production is unique since, without the maternal influence, in theory the gut microbiota composition can be modulated very early, which offers the possibility to influence the development of host immunity and digestion with presumably long-lasting effects throughout the animal life. Beyond this early influence of the immediate environmental micro-organisms, gut microbiota varies according to the animal age, the intestinal segment, the genetic breed, and is also influenced by many environmental parameters (Kers *et al.* 2018).

The feed composition and the use of additives is probably the most important factor influencing gut microbiota composition in poultry, as the primary source of nutrients for microbes themselves (Oakley *et al.* 2014, Pan and Yu 2014). Many studies report the positive effect on mainly feed efficiency or resistance to intestinal colonisation by bacterial pathogens of a vast array of feed additives, sometimes in relation to variations in gut microbiota composition. Nevertheless, their mode of action is generally not understood, or only poorly, which limits the possibility to improve their efficiency. Taxonomic information gathered about the ecosystem composition through high-throughput sequencing of the 16S rRNA gene of bacterial DNA, although very informative, is not sufficient to understand the functions at stake in the interactions between gut microbiota and host phenotypes of interest, among which gut health. For this, studies of whole metagenomes are needed. After an overview of the existing

knowledge about the effect of feed and additives in poultry production on gut microbiota and gut health, this paper reviews the whole metagenomics studies applied to chicken available. Finally, it will discuss the directions research should take to improve our knowledge of functional host-microbiota interactions based on metagenomics approaches and thus implement successful nutritional approaches to improve gut health in poultry production.

2. The chicken digestive microbiota: main features and effects of feed and additives on gut health

a. Current knowledge of the digestive microbiota

The digestive microbiota of chicken has been studied for a long time, first using cultivation techniques and, like other microbial ecosystems using DNA-based approaches and eventually next-generation DNA sequencing approaches targeting the 16S rRNA bacterial gene as a phylogenetic marker.

We know that with current breeding practices, the maternal influence on the early colonisation of the chick's digestive tract is much reduced compared to Mammals. Recent results though suggest that the embryo is colonised by bacteria from the maternal microbiota (Ding *et al.* 2017), which means that there might be a maternal influence, and that host genetics might have an impact on the early crosstalk between the embryo and its microbiota. Chicks hatch with no, or very few bacteria in their gut, which is quickly colonised by micro-organisms from the immediate environment: surfaces, eggshell, water, litter, and of course, feed. Very few studies focus on the identity of these primo-colonising micro-organisms, probably because their low abundance makes their isolation difficult, even through DNA amplification. It is nevertheless worth of interest, because their functional importance might be fundamental, by priming the immune system and the first molecular cross-talk occurring between host immunity and microbiota, with long-lasting consequences over the entire lifetime of the animal.

After this primo-colonisation, bacterial populations grow rapidly, reaching 10^9 to 10^{11} bacteria per gram the third day after hatching to stay relatively stable until 30 days of age. Qualitative changes also occur in the different digestive compartments according to the animal's age, with an increase of diversity over time (Ocejo *et al.* 2019). Segments of the upper part of the digestive tract: crop, gizzard and ileum contain mostly *Lactobacillus* species. Caeca, at the distal part of the digestive tract, are the most favourable site for bacterial development and are actually the more diverse and the richest in bacteria, due to a longer retention time than in other parts of the GI tract. Major phylas in caeca are *Bacteroidetes*, *Protebacteria* and *Firmicutes*, with for the latter *Clostridiales* more represented. The long retention time favours fermentations and the production of short chain fatty acids (SCFA) from the degradation of polysaccharides and interactions of bacteria with their host across the epithelium, in particular with host immunity due to the presence of secondary immune tissues like caecal tonsils. It is also, for the same reasons, the segment where pathogens are the most abundant. For all of these reasons, it is certainly the most studied intestinal segment in relation with gut health.

Microbiota composition of luminal content and intestinal mucosa also differ (Awad *et al.* 2016). The mucosal microbiota is of great functional importance since it directly interacts with the host cells and immunity. Nevertheless, it is not often studied, probably for practical reasons, being less easily collected and difficult to dissociate from the luminal content. Research should certainly focus on this microbiota since its role is most probably essential in the crosstalk

occurring with the host through the intestinal epithelium, thus contributing to the immune homeostasis allowing the control of pathogenic bacteria while maintaining beneficial microbes.

b. Effects of feed and additives on gut health through modifications of the gut microbiota: what do we know?

Micro-organisms living in the gut mainly depend on the feed ingested by animals to survive. Chicken feed consists in plants, mainly cereals, complemented with enzymes allowing an improved degradation of specific molecules. Many kinds of additives are also used: vitamins, specific amino acids, minerals, and prebiotics, probiotics and sometimes synbiotics or postbiotics supposed to stimulate the growth of beneficial bacteria with an expected improvement of productivity and health. Looking for new additives able to improve gut health has become even more important with the suppression of antibiotics as growth promoters. Many studies report the effect of the modification in each of these components on the taxonomic composition of the digestive microbiota. Their impact on the gut microbiota composition is huge. Some of these studies also document the correlated effect on host phenotypes of interest, mainly feed efficiency, and sometimes pathogen load or resistance to disease. This has been excellently reviewed (for instance (Oakley *et al.* 2014, Borda-molina *et al.* 2018)), and here is only a short survey of these studies. Given the amount of studies published, it is easy to get lost as to the most efficient additives and feeding strategies. This is further complicated by the lack of standard practices and the highly dynamic composition of the microbiota, which is influenced by many factors, among which environmental factors. Furthermore, the actual mechanisms by which these changes in composition occur, and how they improve (or degrade) host traits of interest are not well known, in general. Applying functional genomics approaches to know which bacterial genes change in abundance when using an additive would certainly improve our knowledge of the molecular mechanisms involved and improve our understanding of the host-microbiota crosstalk. At last, many of these studies focus on very specific host traits, whereas modifications of microbiota composition might have effects on multiple host phenotypes. For instance, increased fractions of NSP reduce nutrient digestibility (Choct *et al.* 1996), but also decrease the occurrence of necrotic enteritis and the colonisation by *Clostridium perfringens* (Pan and Yu 2014). We therefore advocate that integrated studies are needed, to document as completely as possible the effects of gut microbiota changes on its chicken host.

The cereal used as the main source of feed is known to affect the microbiota composition through the quantity of non-starch polysaccharides (NSP) available . Wheat thus comprises more NSP than maize, which is usually preferred as a source of cereal. NSP are not well digested by host enzymes; their digestion requires the intervention of bacteria equipped with enzymes such as glycosyl hydrolases. A higher proportion of NSP through a diet containing more wheat, for instance, is known to trigger an increased retention time of feed and a proliferation of slow-growing bacteria able to degrade NSP. More generally, the proportions of carbohydrates, proteins (Stanley *et al.* 2014)or lipids in the feed are known to affect gut microbiota composition. In addition, feed structure and technological processes applied such as the presence of whole grains also have an impact.

Several enzymes are used with success to improve feed digestibility. Their direct effects are well known since they target specific, well-known substrates. Furthermore, it is now clear that metabolites derived from the hydrolysis reactions they catalyse act as prebiotics, thus modifying the gut microbiota composition (Kiarie et al, 2013).

Prebiotics are molecules non-digestible by the host, which favour the growth of specific bacteria supposed to be beneficial to the host. The most used are oligo-saccharides such as fructo-oligosaccharides (FOS) or mannan-oligosaccharides (MOS), which both favour the growth of *Lactobacillus* species and reduce the quantity of *Clostridium* species (Pourabedin and Zhao 2015). Some studies demonstrate that they reduce the colonisation by pathogens. The study of xylo-oligosaccharides is more recent and promising. They are found in raw materials such as inuline extracted from endive or yeast growth medium. Prebiotics act through the increase of beneficial bacteria in the gut (*Lactobacillus*), a decrease in bacteria considered as detrimental (*Clostridia*), or the decrease in the colonisation by enteric pathogens.

Probiotics are selected live bacteria ingested by the animal, which are able to reach the intestine where they grow and bring benefits to their host. Their benefits on host health are documented (Clavijo *et al.* 2018), but their effects on performances are less obvious and depend on the strain used as a probiotic. Commercialised strains belong to ten to twenty genera, mainly *Lactobacillus*, *Bacillus*, *Bifidobacterium* and *Enterococcus*. These probiotic strains increase the population of beneficial bacteria, in particular *Lactobacilles* and *Bifidobacteria*, which inhibit the growth of pathogenic bacteria through the production of bacteriocins or organic acids (Bajagai et al, 2016).

Synbiotics are the combination of specific probiotics and prebiotics chosen to favour their growth once they have reached the intestine. Currently, post-biotics are also used: they are specific metabolites produced by known probiotic bacteria (Kareem *et al.* 2016). The reasoning behind their development is that probiotic bacteria act on their host through the metabolites they produce, so that beneficial effects on gut health can be delivered by directly administering these metabolites to animals. Butyrate is one of them. This SCFA is a major source of energy for host cells, and it inhibits pathogen growth in the chicken gut (Gonzalez-Ortiz *et al.* 2017).

3. Metagenomic approaches applied to chicken intestinal microbiota: where are we?

a. Definition and methods: metagenomics/ metaproteomics/ metatranscriptomics (and metabolomics?)

Most published studies of the chicken digestive microbiota made use of the targeted sequencing of the bacterial 16S rRNA gene to perform an inventory of the bacteria present. This gene is present with at least one copy in every bacterial genome and possesses highly conserved domains surrounding highly variable domains, which allows the definition of conserved primers for PCR amplifications of the variable regions. Amplicons are sequenced and assigned to known taxons through bio-informatic analyses. This allows to perform an inventory of the dominant bacteria present in any ecosystem, without the need to cultivate bacteria. Although very informative, this approach has many limitations, like a probable bias in the quantification of some types of bacteria due to the unequal number of 16S rRNA gene according to bacterial species. It also rarely allows the identification of bacteria at the species level, and furnishes a list of OTU (operational taxonomic units) identified generally at the family and sometimes at the genus level, while many OTUs remain without annotation.

True metagenomic approaches, i.e. whole metagenomics approaches relying on the full sequencing of the metagenome are both more difficult and much more expensive. They are also many times more informative, because they give access not only to one gene used as a phylogenetic marker, but to all the genes of the bacteria of a given ecosystem. These approaches

rely on the massive sequencing of fragments of bacterial DNA from the ecosystem, followed by bioinformatics analyses to assemble the reads produced into genes. It leads to lists of hundreds of thousands of genes for every sample sequenced. This allows not only a taxonomic inventory of the bacteria present, but also a functional inventory of the genomic potential of the ecosystem. The building of reference metagenomes greatly facilitates such studies by allowing a lower sequencing depth in subsequent analyses and a much improved annotation of the genes. Such reference metagenomes have been first produced for human (Qin *et al.* 2010) and then for model and livestock species: in mice (Xiao *et al.* 2018), pig (Xiao *et al.* 2016), cows (Stewart *et al.* 2018), dog (Coelho *et al.* 2018), rat (Pan *et al.* 2018) and recently for chicken as well (Huang *et al.* 2018).

Most studies conducted until know looked at the impact of feed and additives on gut microbiota on the taxonomic composition. Nevertheless, taxonomic composition might not always be the more relevant criterion to follow: changes might occur at the functional level without visible changes at the taxonomical level, because bacterial genes could display differential expression levels, and because some slight and hence undetectable changes in abundances could nevertheless have important functional consequences detectable through a functional metagenomics approach. Furthermore, understanding the functional mechanisms at stake with the help of functional metagenomics approaches, could allow us to identify the pathways responsible for the multiple effects of gut microbiota changes on host phenotypes and hence to more efficiently improve the desirable host traits without degrading other traits.

b. Lessons learned from first metagenomics studies

In chicken, a few studies making use of true metagenomics approaches have been published. This has been reviewed recently (Borda-molina *et al.* 2018). The first study compared the microbiotas of one healthy animal, and one animal challenged with *Campylobacter jejuni*. The number of animals was too low to identify the functional elements associated with the increase in *Campylobacter* abundance. Nevertheless, this first study underlined the functional importance of the metavirulome, i.e. the virulence genes of a given microbiota, and identified a high percentage of mobile elements (tranposases) responsible for horizontal gene transfer among bacteria (Qu *et al.* 2008). The second study used a higher number of animals to understand how antibiotics treatment (chlortetracycline) modified the caecal microbiota. A whole metagenome approach applied to 16 pools of caecal microbiota differing according to animal age and antibiotic treatment received succeeded in identifying microbial genes potentially explaining the positive effect of antibiotic treatments on growth and health (Danzeisen *et al.* 2011); a modification of the availability of transport systems for a wide array of molecules was observed. A more recent study used the same antibiotics to compare the 16S rRNA gene based composition of the fecal microbiota with the abundance of antibiotic resistance genes identified through a whole-metagenome sequencing approach (Xiong *et al.* 2018). It concludes that chlortetracycline selected for resistant bacteria and inhibited the sensitive bacteria, thus causing a shift in bacterial abundances that in turn changed the resistome structure. Another study identified a high number of enzymes involved in the degradation of polysaccharides and in fermentation pathways leading to the production of beneficial SCFA, but also uptake hydrogenases. It hypothesizes that several of the most abundant genera present in the caecal microbiome (in this case *Megamonas*, *Helicobacter* and *Campylobacter*) act as hydrogen sinks, thus allowing a more productive fermentation to acetate and an increased production of SCFA benefiting the host (Sergeant *et al.* 2014). At last, other studies focused on

the fecal microbiota in relation to feed efficiency or lipid metabolism (Singh *et al.* 2014, Hou *et al.* 2016). The more recent study, which also produced the first reference intestinal metagenome in chicken, tested the effects of a plant derived growth promoter compared to the effects of an antibiotic on the microbial genes composition. It confirmed the predominant role of *Lactobacillus* in the observed favorable effect on growth of the plant growth promoter used, and the effect of the antibiotic on the growth of antibiotic-producing bacteria and on the abundance of antibiotic resistance genes, thus emphasizing the need for safe alternatives to antibiotics in poultry production (Huang *et al.* 2018).

These studies illustrate the interest of whole metagenomics approaches and emphasize the importance of the chicken gut metagenome for gut health. These research areas will soon all benefit from the existence of chicken reference metagenomes, which extraordinarily extend the identification of microbial genes and will allow much more precise studies of the functional potential of the gut microbiota. The first reference metagenome built used an Illumina HiSeq technology to produce a saturated catalog of 9.04 million genes and included all compartments of the digestive tract, with samples collected at different time points on mainly broiler chickens (Huang *et al.* 2018). Another initiative coordinated by INRA (the MetaChick consortium) is currently focusing on the comparison of chicken microbiotas in the many production systems encountered in chicken production in France, to perform an extensive assessment of the existing biodiversity of the caecal microbiota and to gain insight into the actual differences between farms and production systems. This knowledge will be for applied perspectives of nutritional modulations of the gut microbiota in chicken, since different strategies might be necessary in different production systems according to the microbiota encountered. It will soon lead to the publication of an extensive caecal reference metagenome for both laying hens and broilers, whereas most other studies conducted focus on broiler chickens.

c. Other –Omics approaches: metabolomics/ metaproteomics

A few published studies refer to other –omics approaches to study the chicken gut metagenome. No metatranscriptomics approach has been used in chicken, to our knowledge, which is understandable given the absence of reference metagenome until now and the technical difficulties of this approach with rapidly degraded mRNA in fecal or caecal contents. Its development would be of high interest, since metatranscriptomics gives access to the actually expressed genes of the metagenome in a given condition, whereas metagenomics furnishes the functional potential of the studied ecosystem. Just like host transcriptomics though, it only gives a transient image of the gene expression and several time points and conditions should be considered in carefully designed experimentations.

Metabolomics and metaproteomics furnish the metabolites and proteins actually produced and are thus highly interesting. These molecules are those that supposedly interact with the host and mediate the impact of gut microbiota on host traits of interest. A few studies made use of a metaproteomics approach to study the chicken gut microbiota. The first one made use of two pools of feces from 18 weeks old hens and identified abundant stress proteins as well as proteins involved in metabolic processes of carbohydrates, alcohol and protein (Tang *et al.* 2014). Another study used this approach on pools of three chickens at different time points as a way to identify bacteria species eligible as potential probiotics based on their proteic profiles (Polansky *et al.* 2016), in particular for enzymes leading to the production of the SCFAs acetate, propionate and butyrate. Eligible bacteria had also to be able to colonize newly hatched chicks

from donor microbiotas and to form spores, which finally led to the identification of *Anaerostipes, Anaerotruncus* and *Subdoligranulum* suitable probiotic candidates. The latest study reports the effects of a supplementation in phosphorus and microbial phytase on the microbial proteins using 24 pools of 2 animals (Tilocca *et al.* 2016). They showed the benefits of this supplementation on the gut microbiota, which thrives under supplementation and is stressed without it.

At last, metabolomics approaches are complementary to metaproteomic approaches, by identifying the metabolites produced through the expressed enzymes. One difficulty though is the impossibility to distinguish metabolites from the host from metabolites resulting from the activity of the gut microbiota. On study made use of this approach and showed than modifications in metabolite profiles in birds fed with rice bran were associated with a reduction in the colonization of caeca by *Salmonella Typhimurium* (Rubinelli *et al.* 2017).

4. Discussion – perspectives : how metagenomics will assist microbiota modulation strategies

Metagenomics furnishes a detailed vision of the array of genes present in an ecosystem at the time of the sample collection and of the dominant bacterial genomes present, which allows both taxonomic and functional analyses of the studied ecosystem. Attempts at whole metagenomics approaches are more and more frequent, although they remain too complex and expensive to be used routinely by most laboratories. The 16S approach remains the reference method. Nevertheless, the publication of reference metagenomes should pave the way toward more studies making use of whole metagenomes. Many research domains can benefit from this approach, and we will only focus on those that can improve nutritional modulation strategies. In chicken production, nutritional strategies in relation to gut health mainly aim at preventing health challenges. In addition to the use of different cereals or additives, strategies such as early nutrition or *in-ovo* application of probiotics aim at accelerating the colonisation of the gut by a favourable microbiota and at efficiently priming the host immune system. The early days post-hatch, and even before hatch to some extent, are certainly a window of opportunity to apply nutritional strategies in chicken with long-term favourable consequences for the animal.

Whatever the strategy considered, knowing the bacterial genes potentially involved can improve our understanding of the molecular mechanisms at stake. Nevertheless, knowing the genes involved does not necessarily gives insight into the effects they cause in the host, and we advocate that integrative studies detailing with the same precision both the gut microbiota and the host phenotypes should be conducted. Host genetic variations should also be depicted, since microbiome and host contribute together to the expression of traits of interest such as feed efficiency and gut health. Fine descriptors of the host immunity should be described through transcriptomics in intestinal tissues or even systemic organs, genomics, observation of histological modifications and isolation of specific molecules such as for instance IgA. This will allow the identification of correlated phenotypes and changes in microbial gene abundances and give a comprehensive view of the modifications occurring in the holobiont. This will improve our knowledge of the early molecular interactions occurring in the perinatal period between host immunity and microbiota. In addition, such integrative strategies should allow the identification of early biomarkers of different kinds for gut health dysfunctions, which are highly needed in current poultry production (Ducatelle *et al.* 2018). Furthermore, all –omics

approaches are complementary and their simultaneous analysis using adapted bio-informatics tools could really improve our global understanding of how interactions between host and microbiota shape gut health in poultry. Although not adapted to every experiment due to their cost and complexity, such strategies applied to well designed experimental studies applied to very large cohorts to ensure statistical robustness should lead to a huge progress in the coming years. Such studies should help in the definition of a "healthy" microbiota, or rather of a "healthy" microbiota in a given environment. They might also lead to the identification of microbial genes of interest such as specific enzymes (Al-Darkazali et al, 2017) and help researchers to define sets of probiotics strains based on their functions (Brugiroux et al, 2016). The development of standard practices, from sample collect and DNA extraction to bio-informatic analyses, as in human research on the intestinal metagenome (Costea *et al.* 2017), will also be essential to compare results from distinct studies.

Finally, the field application of the knowledge acquired experimentally, be it diagnostic tools based on signature markers (genes, bacterial species, metabolites, enzymes, etc) or the application of an additive, might be difficult due to the highly dynamic nature of the gut microbiota and the many factors modifying its functioning. It is advisable to conduct studies in conditions very close to the field conditions, or even in field conditions when applicable. The key functional molecules at the heart of the host-gut microbiota interactions are probably less influenced by endogenous and environmental parameters than taxonomic compositions alone, which should be a help for applied perspectives.

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