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## **Digestive efficiency, Gut Microbiota and Genetics – Are they interrelated?**

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### **Introduction**

Digestive efficiency (DE) is a part of feed efficiency, and its improvement is a major goal in poultry production for economic, environmental and sociological objectives. It allows reducing production costs, using feedstuffs of low or variable quality or alternative feedstuffs and decreases the animal manure. Indeed, feed costs are the main part of production cost and shows high fluctuations (ITAVI, 2014). For meat-type chickens, it is ranging from 55 to 65 % depending on production type (Riffard *et al.*, 2011). Regarding social demands, an increased in digestibility would lead to an improved animal health and welfare, through a decreased nutrient content in the intestine and a lower microbiota development or a better equilibrium between favorable and unfavorable microorganisms (Crévieu-Gabriel and Naciri, 2001; Klis and Lensing, 2007; Timbermont *et al.*, 2011). Moreover, the undigested dietary compounds increase the quantity of fermentation substrates in the litter and the frequency of pododermatitis (Shepherd and Fairchild, 2010).

With increase of human population, an increase in need for poultry meat and for the crops required for both animal and human consumptions will follow. Moreover, feedstuffs are used by the industry for biofuel. To decrease the competition between these different uses of feedstuffs, for animal nutrition conventional crop vegetal resources are replaced by unconventional feedstuffs as by-products. Moreover in several countries, the use of local feedstuffs is needed to decrease the dependence toward soya as protein source, as it mainly comes from importation, and other cereal than corn is introduce in diet. However most of these alternatives feedstuffs have variable or low nutritional values as wheat or oilseed a rapeseed meal used in many countries throughout the world, thus leading to reduced digestibility and increased animal wastages.

It has been shown from several years that nutrient digestibility depends on animal development, diet composition and feed technology. Moreover, to improve digestibility of low quality feedstuffs, additives as enzyme have been proposed to increase hydrolysis of macromolecules of the diet, and also additives to control digestive microbiota that is involved in this physiological function of the host. However, this microbiota has been shown to present a high individual variability, whatever the animal species. Thus, in human, although main bacteria are shared between individuals (the core microbiome), a very high variability of abundance has been observed for the most common species (Li *et al.*, 2013; Qin *et al.*, 2010; Salonen *et al.*, 2012; Tap *et al.*, 2009; Turnbaugh and Gordon, 2009). In animal such as chicken, a considerable variation is also observed between individual microbiota although under carefully controlled environmental conditions, as feed and rearing conditions (Gabriel *et al.*, 2007; Stanley *et al.*, 2013b ; Torok *et al.*, 2008; Zhu *et al.*, 2002). Understanding the origin of this individual variability is required to allow manipulating digestive microbial communities in order to improve DE. This high individual variability is probably due to the fact that after their first contact, the host and its digestive commensal microbiota co-evolve. These latest years, several studies have search to evaluate the relative role of the potential contributing factors to this variation, environment origin and also genetics.

In this review, after reminding how can be defined and measured DE in chicken and what is at the present day known in its digestive microbiota, we will present relationships observed between these two entities. In a second part, the present knowledge on genetic control of these two entities and the availability of new approaches to study it will be displayed. In this review, a tentative has been made to gather present knowledge on this topic, particularly in chicken, however it can not be considered as exhaustive due to the huge number of studies, and new proposed concept by research teams involved in this topic. In the last part, we will present which further studies are needed to increase our knowledge to improve DE in chicken.

## **1. Digestive efficiency and gut microbiota**

### **1.1. Digestive efficiency and digestive microbiota : some reminders**

#### **1.1.1. Digestive efficiency for animal : difference between the measure at the ileal and faecal level**

Faecal digestibility has been the most used method to determine digestibility in chicken as observed by published studies on Web of Knowledge. This measure of digestibility may be considered as the global use of nutrients by the digestive tract (Fuller *et al.*, 2012). It can be considered that animal digestive capacity correspond to its capacity to extract compounds from the diet for it. It is performed by hydrolyzing macromolecules to absorbable molecules and absorbing them, with its enzymatic / absorbent system, but also with the help or competition of its digestive microbiota. Moreover faecal digestibility allow maintaining animal alive for further measurements, and to measure digestibility on a period of one or several days.

However due to hind gut fermentation (mainly in caeca, but also potentially in the rectum), as well as use of undigested compounds by digestive microbiota in post ileal segments and production of bacterial biomass (mainly protein and lipid), estimated to represent 20% of DM in feces, ileal digestibility is a more accurate measurement of nutrient availability from the point of view of animal. Indeed, it allows separating two different phenomena: digestion until end of small intestine due mainly to direct host factors, and post-ileal digestion mainly due to digestive microbiota. In chicken, although this hindgut digestion is lower developed than in mammal as pig, ileal digestibility shows different value than faecal digestibility (Dublecz *et al.*, 2006; Ravindran *et al.*, 1999).

However one need to take into account that digestive microbiota is implied in ileal digestion as we will see later, and that digestive microbiota in the caeca produced nutrients that can be absorbed by the host, as carbohydrates and amino acids (Moreto et Planas 1989) although the quantitative importance of these phenomena is not known, and supposed to be of very lower amplitude than post-ileal modifications.

### **1.1.2. Digestive microbiota in chicken**

The microbiota of the gastrointestinal tract is a complex community of many different species of microorganisms, mainly bacteria. Thus, in the following text, when we used the term microbiota, it refers to bacteria.

Until recently, one of the main problems with studying microbiota, was the availability of appropriate methods as a large majority of species cannot be easily cultivated (70-90%). Thanks to the development of new independent approaches to culture, molecular methods based on 16 S ribosomal DNA, new tools are available. Among these methods, qualitative

methods have been developed such as fingerprint techniques (DGGE, TGGE, TTGE, t-RFLP, ARISA ...). Quantitative methods as fluorescent in situ hybridization (FISH), quantitative PCR and low throughput clone analysis have also been used. These methods allow obtaining a more precise and complete image of the microbial diversity than cultures. More recently, high throughput sequencing of variable regions of 16S rDNA (pyrosequencing belonging to Next Generation Sequencing, NGS) has been developed, and begin to be used for poultry digestive microbiota. It allows again more deep insight in digestive microbiota. In the following part, we will see what is known in chickens.

It is generally admitted that digestive microbiota of animals depends on its development after the first contact between early-colonizing bacteria and digestive tract, and later, depends on the conditions meet by the bacteria in the digestive tract along the bird live. These conditions are within bacteria cross talk and the interactions of bacteria with the digestive tract environment. This latest is composed of host-microbial interaction and digestive biotope composition. This biotope may depend on animal environmental factor as diet (feedstuff, technological treatment, feed additives) and rearing environment of which stress, and host factors, as host genetics and animal physiological state (age, health status). All these conditions lead to a strong selective pressure allowing only microbial populations that are able of establishing a mutualistic relation with the host to be maintained in this gut ecosystem, and to cooperate with other bacteria in microbial food networks, where fermentation end products from one organism can act as a substrate for another, a phenomenon known as “cross-feeding” (El Aidy *et al.*, 2013). As the digestive microbiota and the host co-evolved after their first contact, they are considered as a supraorganism with numerous cross-talk between microbial and host cells (Lederberg, 2000). They seem to be in a mutualistic relationship when the equilibrium is reached. Moreover stochastic phenomena are implied as in whatever microbial ecosystem. It is generally thought that constant community structure is likely to provide continuity of microbial metabolic activities for the host and give rise to stable microbe-dependent phenotypic traits. On the contrary, an unsteady digestive microbiota is thought to have negative consequence on animals.

In chicken, as reviewed recently, implantation and localization of digestive microbiota is specific to these birds and their rearing conditions (Apajalahti *et al.*, 2004; Dibner *et al.*, 2008; Gabriel *et al.*, 2006, 2012; Yeoman *et al.*, 2012). Before hatch, digestive tract may not be sterile, but the content in bacteria seems to be very low. After hatch, further development of digestive microbiota of birds depends on the environment of the eggs. In modern commercial

chicken hatcheries, eggs are fumigated by formol to remove bacterial contamination, thus limiting the bacterial source to incubator and hatcher environment, which are also disinfected, and maintained in surpressure with filtered air. Next chickens are manipulated for sexing and vaccination. In the boxes for transportation, bacteria from outside and from other chickens can be sampled by individuals thanks to retrograde movement of the cloaca and rectum (Bar-Shira and Friedman 2005; Clench, 1999). When chicks arrive in the farm, new bacterial sources are people handling, first feed and water, and litter. Thus, in one day old chickens, bacterial load has been measured to be  $10^8$  and  $10^{10}$  bacteria per g of content in ileal and caecal content respectively, and in three days old chickens, a stable quantity is reached,  $10^9$  and  $10^{11}$  respectively (Apajalahti *et al.*, 2004).

In the chicken, the major sites of bacterial localization are the crop and the caeca, and to a lesser extent the small intestine. For example Guardia *et al.*, (2011) determined that the total bacterial load was  $5.5 \times 10^{11}$ ,  $5.3 \times 10^{10}$  and  $7.4 \times 10^{12}$  copies of 16S rDNA/g of fresh samples in the crop, the terminal ileum and the caeca respectively.

The crop is considered as the inoculum of the following digestive tract, with the dominant group being *Lactobacillus*, although not the unique genera (Gong *et al.*, 2007; Guardia *et al.*, 2011; Peinado *et al.*, 2013).

After a fall in the total amount in the proventriculus and the gizzard, due to their low pH, biotope conditions at the beginning of the small intestine are not in favor of developing microbiota with high oxygen pressure, high concentration in antimicrobial compounds such as digestive enzymes and bile salts. In the following small intestine, the environment becomes more favorable to bacterial growth thanks to lower oxygen pressure, and lower digestive enzyme and bile acids concentrations. Due to these changes along the small intestine, microbiota evolved between the upper part (duodenum-jejunum) to the lower part of the small intestine, with *Lactobacillus* being always the dominant bacteria.

However, it can be noted that due to reverse peristaltic contractions in chicken from the cloaca to the gizzard, the digestive biotope is subjected to fast modification that may be not in favor of bacteria development (Sacranie *et al.*, 2012).

In the lower digestive tract, the caeca, the biotope is the most favorable for bacterial growth. Indeed, oxygen pressure is lower and this biotope is relatively stable, due to the low renewal of the digestive content (Gabriel *et al.*, 2012). Thus, it is the major site of bacterial fermentation in chicken, and it has been evaluated that 50% of the biomass would be of bacterial origin (Clench, 1999). It may contribute to energy extracted from the feed by the

host-microbiota association, thanks to reabsorption of bacterial metabolites. Contrarily to the crop and the small intestine, the bacterial composition of the caeca is more diverse, and the major phylum are Firmicutes and Bacteroidetes, mainly genera *Clostridium* (Guardia *et al.*, 2011; Lu *et al.*, 2003; Stanley *et al.*, 2013a). Apajalahti *et al.*, (2004) found 640 different species, and more recent studies using 16S rRNA pyrosequencing showed as much as 783 operational taxonomic units or OTU (Danzeisen *et al.*, 2011; Moore *et al.*, 2011; Nordentoft *et al.*, 2011). It was also observed that chicken caecal microbiota was markedly different from faecal microbiota, and display more diversity (Lei *et al.*, 2012)

This digestive microbiota can be located in the lumen, in the mucus layer(s) or at the mucosal surface. The luminal microbiota depends on available nutrients, transit rate and the presence of antibacterial compounds. It is the main studied microbiota. The mucosal microbiota in the crop, has been described as adhesive to the mucosa developing several cell layers. Mucosal microbiota, in the small intestine of chicken, would be adherent to the epithelial cells, or localized in the single mucus layer. In the caeca, digestive microbiota as been described to form a 200 cells deep layer, or may be localized in the upper layer of mucus near the intestinal content, and not in the lower layer of mucus. This mucosal microbiota depends on available substrates coming from the mucosa and molecules coming from the digestive content diffusing into the protein matrix of mucus. It also depends on bacteria adhesins, specific adhesive sites on the mucus or mucosa, on mucus or cell renewal rates, on antibacterial substances contributing to the innate immune system of the digestive tract. As these bacteria are in narrow contact with the host, it may be supposed that they have a more direct effect on the host. In the chicken, this mucosal microbiota, is different from the luminal microbiota, but has been relatively little studied compared to the studies in digestive contents. Moreover, in the digestive tract, distribution of microbiota is not homogenous due to different biotopes present in the digestive tract, and there are niches with specific microbiota (Pédrón *et al.*, 2012).

In recent years evidence has accumulated to support the idea that the community structure of the gut microbiota is a major contributor to the phenotype of the animal host, due to its narrow contact with the host. Digestive microbiota can be considered as an organ in the digestive tract that uses nutrients and products metabolites, recognizes and synthesizes neuroendocrine hormones, may interface with the nervous system that innervates the gastrointestinal tract or via neuropeptides (Holzer *et al.*, 2012; Lyte, 2010). Digestive microbiota may act on several host functions as digestive physiology via its products as short

chain fatty acids (SCFA) that have regularly functions via neural and humoral pathways (Al-Lahham *et al.*, 2010; Mroz *et al.*, 2006; Reilly *et al.*, 1995; Tappenden et McBurney, 1998) or steroid compounds (Groh *et al.*, 1993). Moreover, interactions between the microbiota and the host are being considered pivotal in the early programming of gut function that may be mediated by epigenetic mechanisms with consequence throughout life (Lalles, 2012).

It may be assumed that both quantitatively rich microbiota, as caecal content, and poorer, as small intestinal content or mucosal microbiota, may have effects, that may be due to their high metabolic activity or more targeted action respectively. Moreover, due to the distance away effect of microbiota, via neurohormonal effect or via their metabolites or constituents, or even themselves, that pass through the digestive epithelium, and carried out in blood, it is not necessarily in proximity of the highest load of bacteria in the lower part of the digestive tract that the highest effects are reached. Thus, Larsson *et al.*(2012) observed by comparing germ-free and conventionally raised mice, a higher effect on the gene expression of the small intestine than in the colon.

The question can be raised if modifiable microbiota as in the upper digestive tract of chicken, due to reverse peristaltic contractions, can have a stable effect on the host, contrarily to high stable microbiote in caecal contents.

Among the various effects of microbiota on the host physiology, digestive microbiota has effect on the digestive area. It contributes to the development, morphology and functionality of the digestive tract. These effects may have consequences on animal digestion of nutrient as we will detail later. The commensal microbiota is also implicated in digestive health. It contributes to the protection against harmful microorganisms (barrier effect) and stimulates immune system, leading the host digestive tract in a physiological inflammatory states corresponding to homeostasis. Digestive microbiota contributes to detoxification of some compounds, but also to production of toxic substances.

The digestive microbiota can also influence extra-digestive physiology of the host. Indeed, in mammals, effects have been observed on the animal metabolism as fattening, or on the central nervous system with effects on behavior. In chicken, the presence of microbiota has been shown to increase total protein synthesis of 6-8%, and energy requirement (Furuse and Okumura, 1994; Muramatsu *et al.*, 1987). Digestive microbiota may also contribute to mineral and vitamins nutrition. Moreover the bacterial activity has consequences on health, and thus animal welfare, such as conjunctivitis and respiratory problems due to irritant compounds products released by bacterial fermentation in the litter material. These



fermentations also have consequences on contact dermatitis or pathogen development in the litter. All these effects have consequences on animal production, as well as on growth performance and product quality.

## **1.2. Digestive efficiency and gut microbiota composition relationship**

In mammals, with a high bacterial load in the colon, it is generally admitted that digestive microbiota is implicated in digestive physiology and digestion (March, 1979). We will see in the following part, that in birds as chickens, several data are accumulated to show link between digestive microbiota and digestion.

### **1.2.1. Observation in chicken**

#### **1.2.1.1. Parallel change in digestive efficiency and digestive microbiota**

Several studies have been performed between 1970 and 1985 by using germ-free chickens compared to conventional animals to study the effect on DE. Thus, for lipids, in young chicken of 3 weeks, microbiota led to a decrease of 2 points of apparent faecal digestibility for vegetal oil and 10 points for animal fat (Boyd and Edwards, 1967, Kussaibati *et al.*, 1982b). Digestibility of saturated fatty acids as palmitic and stearic acids are highly decreased whereas this of unsaturated fatty acids as oleic and linoleic acids is not modified by microbiota (Boyd and Edwards, 1967). However the change in faecal digestibility may be due in part to endogenous lipids and bacterial biomass. For protein digestibility, effect of microbiota may depend on sensitivity to hydrolysis of proteins, bacteria being able to hydrolyze some resistant proteins for enzyme host (Kussaibati *et al.*, 1982a; Salter 1973; Salter and Fulford, 1974). Due to the effect of digestive microbiota on nutrient digestibility, it can have an effect on metabolisable energy, positive or negative, (Furuse and Okumura, 1994; Kussaibati *et al.*, 1982b).

In a study comparing conventional chickens to chickens with limited microflora obtained by rearing birds in sterilized conditions, Maisonnier *et al.*, (2003) also observed a significant decrease of faecal lipid digestibility. This negative effect of digestive microbiota on lipid digestibility and the apparent metabolizable energy value of diet, corrected to zero nitrogen retention (AMEn) was also observed in a study by using high dose of antibiotics (Garcia *et al.*, 2007).

Following the ban of the use of antibiotic growth promoters (AGP) in feed, several studies have been performed to explore alternative feed management strategies as feed additives or cereal form, to improve digestive health of chickens, particularly to control digestive microbiota (Alloui *et al.*, 2013; Gabriel *et al.*, 2013). Moreover, consequences of other factors as type of cereals or rearing conditions have been studied. Among these works, some of them also studied if the DE is also modified. Indeed some of these additives can be used as first objective to modify digestive microbiota, but they may also have on DE.

In these studies following both digestive microbiota and DE, some showed that the two are modified, but some of them observed that only one of these two markers is modified, with no consequence on the other marker. Thus, the majority of studies reporting effects of AGP or their alternatives, studying ileal digestibility and digestive microbiota, observed positive effect on digestibility. However, one must keep in mind that all of the studies performed on alternatives to AGP are not necessarily publicly available, thus this representation may be a biased image of the reality. Indeed, although to our knowledge, no study reports negative effect of alternatives to AFC on ileal digestibility, this may happen. Indeed, the additives may have a nutrient cost for the host at the digestive level if they lead to increase bacterial load in the digestive tract that have nutrient requirement for their growth, as the objective of these alternatives is not necessarily to reduce the digestive bacterial population but to enhance the gut health function via the increase of beneficial microorganism, competitive exclusion of pathogens and stimulation of the immune system.

Thus, use of virginiamycin was observed by Zulkifli *et al.*, (2012) to lead to increase ileal protein digestibility and increase of *Lactobacillus* and *E. coli* counts in ileal content. Introduction of enzymes in diets has been shown in several studies to lead to increase ileal digestibility and change in digestive microbiota count or their products of fermentation mainly in caeca, but also in ileum (Baurhoo *et al.*, 2011b; Bhuiyan *et al.*, 2010 ; Choct *et al.*, 1999; Cowieson et Masey O'Neill 2013; Nian *et al.*, 2011b). By the use of herbal product or essential oils, increase ileal digestibility has been reported with modifications of ileal microbiota (Amerah *et al.*, 2011; Zulkifli *et al.*, 2012). With insoluble fiber, Amerah *et al.*, (2009) observed an increase in ileal starch digestibility and a difference in ileal microbiota observed by fingerprint. Use of mannanoligosaccharide in a wheat diet was observed to increase ileal digestibility and led to change in microbiota of the small intestinal mucosa and caecal content (Yang *et al.*, 2008a).

The type of cereal grains has been shown to lead to change in ileal digestibility as well as change in digestive microbiota in ileal and caecal content, but also in gizzard content and mucosa of the small intestine (Baurhoo *et al.*, 2011a; Rodriguez *et al.*, 2012; Shakouri *et al.*, 2009; Yang *et al.*, 2008a). Technological treatments of cereals have been shown to improve ileal digestibility and change microbial digestive contents (Amerah *et al.*, 2011; Bhuiyan *et al.*, 2010).

Rearing conditions have also been shown to lead to change in ileal digestibility and digestive microbiota, as delay feeding after hatching and immunological stress (Sarica and Corduk 2013; Yang *et al.*, 2011).

These observations lead to the common idea of beneficial effect of some bacteria as *Bacteroides* and *Lactobacillus Bifidobacteria* and negative effect of *Clostridium* and *E. coli*. However, this is more complex, because all species of a bacteria genera and all strains of a bacteria species have not necessarily the same properties, and their biological effects depend on the digestive biotope (Roy *et al.*, 2008; Yuan *et al.*, 2008). Thus whereas *Lactobacillus* is most of the time associated with beneficial bacteria genera, it was also associated with negative effect on digestibility (Knarreborg, 2002; Ramasamy *et al.*, 2010). Moreover the effect of digestive microbiota on DE depends on localization of this microbiota (digestive segment, localization in content or mucus).

Recently, in our laboratory, Poultry Research Unit of INRA, studies have been performed to search for difference in digestive microbiota between birds having different digestibility estimated by AMEn. These chickens were from a divergent line using AMEn as the criteria for selection (Mignon-Grateau *et al.*, 2004). As explained previously faecal digestibility is a complex phenotypic traits, including animal digestion and microbiota digestion, but this choice was performed in order to maintain animal alive for selection purpose, and in order to study digestion in its globality for research on animal excreta for environment objective. As this selection has been performed with vegetal feedstuffs, it can be expected that difference between ileal and faecal digestibility is small (Ravindran *et al.*, 1999). However, works are in progress in our laboratory to compare these two measurements of digestibility in these animals. Briefly, the selection of these chickens was performed with a diet composed of compounds able to lead to low digestibility, in order to increase difference between individuals. The diet contains a high level of wheat (525 g/kg) of high viscosity due to its richness in soluble non-starch polysaccharides (NSP), arabinoxylan, that may be a substrate

for digestive microbiota, and of medium hard value (Rialto cultivar). Moreover, the fat concentration was high (60-80 g/kg of added vegetal oil) which represent a difficulty for digestion for young chickens. The diet was pelleted. In order to maintain performances at a common level between lines, body weight was constrained among both lines. The lines were selected at 3 weeks of age, as it represents a key period in gastrointestinal tract development. The two lines were named D+ (high digester) and D- (low digester) and have been selected on AMEn during 10 generations. This model has been developed to study the physiological limiting factors of digestion.

To study links between DE and digestive microbiota, we used a F2 cross between the D+ and D- lines with 144 animals. Studied microbiota was those of caecal contents. Links between AMEn and microbiota were observed. High AMEn was associated with low amounts of *E. coli* expressed in absolute values, and also in relative values compared to all other bacterial groups (*Lactobacillus*, *L. salivarius*, *L. crispatus*, *C. coccoides*, *C. leptum*). On the contrary, a low AMEn was associated with high amounts of *E. coli* expressed in absolute, and high amounts of *E. coli* relative to *Clostridium*. These low AMEn are also associated with high amounts of *L. salivarius* expressed in absolute, and a higher proportion of *L. salivarius* compared to *Lactobacillus* groups and *Clostridium* groups (*C. coccoides* and *C. leptum*). Moreover, this F2 cross of D+ and D- lines allows showing that a significant amount of variability of the AMEn can be explained with some components of caecal microbiota. Thus, *L. salivarius* amounts can explain significantly 9% of this variability, with a negative effect. And the bacterial ratio of Log *L. salivarius* to Log *C. leptum* explains a greater part of the variability (13%), with a negative effect.

#### **1.2.1.2. Hypotheses on the mechanisms implied in the relationships between digestive efficiency and gut microbiota**

Changes in digestibility in parallel with change in digestive microbiota can affect different nutrients as protein, lipid, starch and minerals. The mechanisms responsible for the relationship between digestibility and digestive microbiota are not accurately described at the present time. It is needed to determine how animal digestion leads to change in the digestive microbiota and how digestive microbiota leads to change in DE. However hypothesis can be proposed.

DE may impact small intestinal microbiota because digestion leads to undigested particles that can be used as substrates by microorganisms as bacteria. Caecal microbiota may also be

modified by small intestinal digestion as caecal microbiota substrates depend on undigested compounds at the end of the small intestine that can enter in the caeca, with the filter at the caeca entrance.

Digestive microbiota, from the small intestine and caeca, may act on DE, directly or indirectly. In chicken, crop microbiota can be considered as an help for starch hydrolysis in the crop, with amylase coming mainly from *Lactobacillus* species (Champs *et al.*, 1981; Szylit *et al.*, 1980). Microbiota along the following digestive tract may also provide hydrolytic enzymes that may contribute to hydrolysis of polysaccharides as raw starch, or resistant proteins (Kau *et al.*, 2011). Hydrolysis of fiber compounds may also allow release of entrapped molecules and thus enhance nutrient availability. It can also not be ruled out that if chickens have access to a litter it may allow them to practice coprophagy that may allow them to benefit from the bacterial cell composition as proteins or vitamins, although the quantitative importance of this phenomenon is not known, and deserves to be studied. However, in the small intestine, it is generally assumed that digestive microbiota acts mainly as competitor of the host, due to their high metabolic potential with high hydrolysis activity due to their high area links to their very small size. Moreover, microbiota has negative effect of lipid digestion due to deconjugation of bile salts by some bacterial species as *Lactobacillus* as indicated previously (Engberg *et al.*, 2004; Kim and Lee, 2005). Microbiota is also considered to be involved in the negative effect observed in digestion of diet rich in soluble NSP, leading to increase viscosity of digestive content (Bedford and Cowieson, 2012), although according to Maisonnier *et al.*, (2003) it is not the main factor.

However as major site of digestion is jejunum, and small intestinal microbiota mainly concentrated in the ileum, the role of microbiota in digestion may be low. However, in the case of diet difficult to digest, and with more importance of the terminal steps of small intestinal digestion in the ileum, effect of microbiota may be important. Moreover due to retroperistaltis from caeca to ileum and further anterior (Sacranie *et al.*, 2007, 2012), caecal microbiota and its products can influence directly small intestinal microbiota and thus digestive efficiency, although only caecal bacteria able to growth in small intestine can develop in this digestive segment.

Moreover microbiota can modify digestive tract structure and digestive physiology as enzymatic activities or transit as well as positively than negatively. These effects of microbiota may be direct or indirect by mean of bacterial products that can have a systemic effect. These modifications of digestive physiology may have consequence on DE. For example, as proposed by Cowieson and Massey'O'Neill (2013), caecal digestive microbiota

may have an effect on ileal digestion via ileal brake mechanism, via release of peptide YY secreted by the neuroendocrine cells in the distal tract in response to the presence of SCFA. Thus DE may be modulated by change of digestive microbiota. However the latter can be modified by the former and is affected by several other factors, as microbiota is as an organ in links with other animal physiological functions. All this equilibria must be taken into account to proposed control of DE by digestive microbiota.

### **1.2.1.3. No parallel change in digestive efficiency and digestive microbiota**

Some studies shown change in ileal digestibility, but not in digestive microbiota, and on the reverse, no change in ileal digestibility, and change in digestive microbiota.

Thus, in some studies, although AGP as avilamycin, or alternatives to AGP, as enzyme and products of essential oils, were observed to improve ileal digestibility, not effect was observed on digestive microbiota (Cao *et al.*, 2010; Mountzouris *et al.*, 2010; Sarica and Corduk 2013). On the contrary, by using germ-free chicken compared to conventional animal Kussaibati *et al.*, (1982a) observed no change for starch digestibility with maize starch. Moreover, in several studies observing change in microbiota with inclusion of additives (enzyme, vegetal products, prebiotic, probiotic) or modification of cereal structure and type, no change in ileal digestibility was observed (Amerah *et al.*, 2009; Baurhoo *et al.*, 2011a; Bhuiyan *et al.*, 2013; Cao *et al.*, 2010; Nian *et al.*, 2011a; Ouhida *et al.*, 2002; Rodriguez *et al.*, 2012; Yang *et al.*, 2008b).

### **1.2.2. Conclusion on these observed relationships between digestive efficiency and gut microbiota and further works**

With the differents studies presented above, we can see that various results have been observed. These discrepancies may be due to different experimental conditions concerning both animal experiment and sample analyses. Indeed concerning animal studies, experimental conditions can be very different, from animal history before arrival for experiment to rearing conditions and diet compositions. Mode of sampling and sample treatments may differ from one study to another. For digestibility assessment, all the nutrients are not necessarily studied, according to objectives of the works, and thus some nutrient having a change in its digestibility may have been not highlighted. Another difference can come from microbiota

analysis. Targeted approaches as bacterial counts of main bacteria can miss difference contrary to new untargeted approaches.

However parallel modifications of digestive microbiota and DE have been observed. This not necessarily involves a links between these parameters. An additive may act on these two parameters via different mechanisms as vegetal products, which, on one hand, may act on digestive physiology and on the other hand may have an antibacterial activity (Gabriel *et al.*, 2013). However DE may be controlled via digestive microbiota via several mechanisms that need to be studied. For example, as proposed by Cowieson et Massey'O'Neill (2013) by decreasing transit rate with higher products of fermentation (SCFA) as modern broilers may have a too rapid feed passage rate particularly due to lower gizzard activity (Croom *et al.*, 1999).

The modification of microbiota can be performed by environmental factor as diet composition or rearing conditions, and also by genetics as we will see in the following part.

## **2. Genetic control of digestive efficiency and digestive microbiota**

Beside technological treatment of diet and feed additives to improve DE via or independently from digestive microbiota, genetic selection may be an interesting tool. Until now, genetic selection of animals has been focused on the improvement of the performances of animals as growth and feed efficiency. These phenotypes are complex traits. Although DE is one of the components of feed efficiency, the selection on feed efficiency did not improve DE. Indeed, in most studies of genetics, a highly digestible diet was used to enable animals to express their genetic potential for feed efficiency and growth. With such diets, DE of birds is not sollicitated, which explains that this component was not found to vary in most studies. At the opposite, a strong genetic link was observed between feed efficiency and DE when using a challenging diet, more difficult to digest (Carré *et al.*, 2008). It also appears that to improve DE by genetics, it must be directly targeted. However, even if this trait is less complex than feed efficiency as it does not include components as heat production, protein and fat deposition or energy expenditure for activity, it is still a complex trait.

Digestive microbiota, which is also a complex trait, may also be under genetic control.

In the following part, the concept of quantitative genetics will be used. This part of the genetics deals with the way that continuous traits (such as body weight, feed efficiency, DE or bacterial species content) are inherited. This approach includes the estimation of the

heritability ( $h^2$ ) of these traits, i.e. the proportion of phenotypic variation in a population that is attributable to genetic variation among individuals. The level of heritability indicates whether selecting on the trait will be easy (high heritability) or difficult (low heritability). In addition, quantitative genetics aims at identifying QTLs, which are genomic regions which variation are associated with the quantitative variation in a phenotypic trait. Identifying QTLs regions is necessary to do a selection assisted by markers (SAM) which takes into account the genotypic information for selection. It is also the first step to identify genes on which the variability of the trait relies on. QTL can be found at the chromosome level, or at the genome level, the latest one using more strict statistical test than at the chromosome level.

## **2.1. Effect of genetics on digestive efficiency**

### **2.1.1. General observation in monogastric farm animals**

In poultry, contrarily to diets based on maize and soybean meal, a diet containing a high level of wheat (336 g/kg) induced a rather high difference in metabolizable energy between lines of chicken (Pym *et al.*, 1984). Indeed, wheat diets were often observed to result in low digestibilities when compared with maize diets and to lead to high variability between birds (Carré *et al.*, 2002; Hughes and Choct, 1997) suggesting a genetic factor in digestibility. These problems of wheat digestion largely come from high viscosity and hardness, which decreases digestibility and then the value of wheat. These factors vary largely between wheat varieties.

In pig, the digestive loss of nutrients and energy ranges from 15% to 25% of the total intake in usual rearing systems (Le Goff and Noblet, 2001). In addition to variations in diet composition, feed technology and animal development (body weight), animal breed can impact digestibility coefficients of nutrients and energy (Le Gall *et al.*, 2009). Differences in energy digestibility have been observed between lines selected for differences in adiposity (Sundstöl *et al.*, 1979). Comparing Asian breeds with commercial European lines has generally shown that the latter have a lower digestive efficiency than the former, especially when given fibrous feeds (Février *et al.*, 1992; Kemp *et al.*, 1991; Len *et al.*, 2009; Morel *et al.*, 2006). Recently, in a study on 20 pigs, originating from four boars and three to four sows per boar, and fed a diet with high dietary fibre content, Noblet *et al.*, (2013) observed that the apparent faecal digestibility was affected by boar origin, with a difference of 2.2 and 2.6



points between the extremes for energy and nitrogen, respectively. These preliminary results suggest the possibility of selecting growing pigs for an increased digestive efficiency when fed high DF diets. However, these preliminary data deserve a confirmation on a much greater number of pigs to achieve estimations of genetic parameters for digestibility.

In poultry, two attempts to select directly for different digestive efficiencies in broiler chickens have been performed. Zhang *et al.*, (2003, 2005) attempted to select for phytate phosphorous (P) bioavailability in a randomly bred chicken population. Selection for this trait for three generations improved P bioavailability. Although the heritability of the trait was very low ( $h^2=0.05$ ), it was significantly different from zero. The other selection on digestive efficiency was performed in our laboratory as indicated previously (Mignon-Grasteau *et al.*, 2004). This selection, performed within a breed of chicken fed with a diet rich in wheat, concerned faecal digestive efficiency, and has proven the existence of individual variability of digestive efficiency for lipid, protein, starch and energy ( $h^2$  between 0.33 and 0.47).

### **2.1.2. Current knowledge in chicken**

As indicated previously, the divergent lines available in our laboratory at the Poultry Research Unit of INRA, have been divergently selected using AMEn as the criteria for digestive efficiency (Mignon-Grasteau *et al.*, 2004). The two lines were named D+ (high digester) and D- (low digester) and have been selected on AMEn during 10 generations.

By using wheat diet, the AMEn at 3 weeks of age showed a higher value of +13.2% between D+ and D- birds at the 2<sup>nd</sup> generation (Mignon-Grasteau *et al.*, 2004), and +33.5% at the 8<sup>th</sup> generation (de Verdal *et al.*, 2011), showing an increased divergence in this selected trait. At 8 weeks of age, this difference between the lines disappeared at the 2<sup>nd</sup> generation (Carré *et al.*, 2005), whereas it persisted at the 9<sup>th</sup> generation (de Verdal *et al.*, 2010b). Thus the results observed at 3 weeks of age would still hold for the whole production cycle.

As D+ birds fed with wheat diets were characterized at the 5<sup>th</sup> generation by a higher AMEn than D- birds (+36.5%), the D+ birds were characterized by higher faecal digestibilities of lipids, starch, and proteins, with a highest difference observed for lipid (+58.0%), intermediate for starch (+39.3%), and lowest although significant for protein (+13.3%) (Carré *et al.*, 2007).

Heritability of AMEn has been estimated in different experiments on the first 2<sup>th</sup> and 8<sup>th</sup> generations and is between 0.30 and 0.38 when birds were fed with wheat (de Verdal *et al.*, 2011; Mignon-Grasteau *et al.*, 2004, 2010). Heritabilities of faecal digestibility of lipids, starch and proteins for the 8<sup>th</sup> first generations were 0.25 to 0.29 (Mignon-Grasteau *et al.*, 2010).

Feed by genotype interactions were observed. Thus, the results are highly diet-dependent. When birds were fed a corn diet easier to digest, differences between lines were still significant but much lower for AMEn and coefficients of digestive utilization, values in D+ line being 1 to 8 % higher than in D- line (Rougière *et al.*, 2009). Whilst D+ chickens showed a small variation in AMEn between maize and wheat (2.9%), D- chickens displayed a high AMEn variation (10.3%) (Carré *et al.*, 2008). With a maize diet, at the 6<sup>th</sup> generation, differences between lines were lower as well as for AMEn (+6.4%), and for digestibility, the higher difference was observed for protein digestibility (+9.1%), followed by lipid digestibility (+5.6%), and the lowest difference although significant was for starch digestibility (+1.3%) (Rougière *et al.*, 2009). Thus the limiting factors for the D- birds digestibility were dependent on the cereal source. Heritability of AMEn is also diet-dependent. It has been estimated to be only 0.15 when they were fed with maize (Mignon-Grasteau *et al.*, 2010). As for AMEn, heritability of digestibility was much lower when birds were fed with maize (0.04 to 0.09), except for starch that presented equivalent levels of heritability with both diets.

These differences in digestive efficiency were associated with anatomical and physiological differences between these two lines in all the parts of the digestive system as previously reviewed in Gabriel *et al.*, (2012). It can be noted that the lower feed intake of D+ birds compared to D- birds (-21.5% at the 8<sup>th</sup> generation), cannot be the only cause of difference between the lines (de Verdal *et al.*, 2011).

Briefly, the most striking differences between D+ and D- birds are observed for the proventriculus-gizzard complex. The relative weights of the proventriculus and gizzard are higher in D+ birds than D- birds, +21.9% and +34.0% respectively, at 3 weeks of age at the 8<sup>th</sup> generation and pH of gizzard content was lower in D+ birds than in D- birds (de Verdal *et al.*, 2011, 2013). Pepsin activity in the proventriculus tissue was observed to be higher in D+ birds when expressed as per animal body weight (Péron *et al.*, 2007). The isthmus area between the proventriculus and the gizzard showed a 4 times larger lumen and a 1.4 larger

total area of this region for D+ than for D- birds (Rideau *et al.*, In press). This is the region where are located the interstitial cells of Cajals, the pacemaker of gizzard contraction (Reynhout and Duke, 1999). In D- birds, the isthmus mucosa has a more oval shape and more twisted, and its muscular part is more developed than in D+ birds. A higher mean retention time was observed in the stomach of D+ than in D- chickens at 9 and 29 d with a maize diet (Rougière and Carré, 2010). This may improve nutrient accessibility in D+ birds by increasing time for grinding and enzymatic activity. This physiological parameter may be the major factor associated with genotype differences between the D+ and D- genetic lines (Rougière and Carré, 2010).

A higher relative intestinal weight was observed in D- birds at 3 weeks of age. It concerns each of the three segments, although less pronounced in duodenum (+15%) than in jejunum (+37%) and ileum (+40%) (de Verdal *et al.*, 2011), mainly due to increased density (weight to length ratio), +12%, +30% and + 31%, for duodenum, jejunum and ileum respectively. This increased density may be explained in part by an increased development of epithelium area and thicker tunica muscularis. Moreover more goblet cells per villus were observed in D- birds in jejunum and ileum (de Verdal *et al.*, 2010a). Digestive contents of D+ and D- lines showed some difference in their composition in terms of pH and bile salts. The pH of the intestinal content is higher for D+ birds in duodenum and ileum at 21 d and in ileum at 53 d (de Verdal *et al.*, 2013, de Verdal, pers.comm.). Intestinal contents of D+ birds show more conjugated bile acids and total bile acids (Garcia *et al.*, 2007).

In the caeca, heavier digestive contents were observed in D+ birds at the 8<sup>th</sup> generation at 3 weeks of age (+80%; H. de Verdal, comm pers.) and a higher relative weight tissue was observed with a maize/soybean diet at 4 weeks of age (+29%; Rougière and Carré, 2010) . Moreover, the transit time was twice as long in D+ birds (Rougière and Carré, 2010). Thus at 3 weeks of age, caecal functions appeared more developed in D+ than in D- birds.

Detection of QTLs controlling DE was undertaken on 820 chickens of the F2 cross between the D- and D+ lines fed the wheat diet used for the selection and leads to the identification of several genomic regions involved in this trait (Tran *et al.*, In press). Nine QTLs were detected. They were mainly found for components of AMEn (mainly digestibility of starch) and only one for AMEn itself. This result is consistent with the results of previous QTL studies devoted to FCR, that found few QTLs for this trait, but rather QTLs on components of FCR, such as feed intake, growth and body composition. Moreover the fact that most QTL were found for digestibility of starch is probably due to the fact that starch has the highest proportion in

dietary content. For DE, two QTL significant at the genome level were present at the same position for digestibility of starch and dry matter, two traits highly genetically correlated. Moreover, chromosome wide QTLs were detected for AMEn, digestibility of starch, dry matter and protein at the same region on other chromosomes. Other chromosome wide QTLs were identified for digestibility of starch and dry matter on three other chromosomes. Moreover, 11 QTL controlling digestive anatomy-related traits were observed. On two chromosomes, co-localization between QTL controlling digestive efficiency and the ratio of intestine length to body weight were observed. However, it is to note that most of these QTLs are only significant at the chromosome level. This is probably linked to the fact that these QTLs are not fixed in the F0 population. Moreover, digestive efficiency traits are probably polygenic in their determinism, as it includes a large number of physiological processes such as digestive secretions, enzymatic hydrolyses, absorption, motility and neurohormonal regulations. Most of the QTL have a low effect in favor of a polygenic control, with the exception of QTLs controlling digestibility of starch on one chromosome. Now, further studies are needed to refine the position of these QTLs, in order to be able to identify the candidate genes underlying these effects. An ongoing study is performed in our laboratory for this (ADIGEN Project AGENAVI 2014-2015). In a further phase, it will be needed to validate these results in commercial populations and breeding environments.

## **2.2. Effect of genetic on digestive microbiota**

### **2.2.1. Observation in animal**

From about seven years according to Web of Science database, an increasing number of studies are now evaluating the contribution of host genetics to the diversity of the microbial community in the digestive tract, and the analysis of host genetics is just beginning to be incorporated into studies to estimate its contribution to the diversity of the gut bacteria.

#### **2.2.1.1. Effect of genetics on digestive microbiota in mammals and model animals**

There is increasing evidence that genetics of the host influences and interacts with gut microbiota in various mammals. Results of different types of studies on the role of the genetics in shaping the composition of the gut microbiota, have recently been gathered in a review by Spor *et al.*, (2011) and more recent studies presented below.

Comparing the faecal microbiota in human twins or between human differing by varying degrees of genetic relatedness, has led to some conflicting results. Whereas some of these studies using fingerprint technique suggest a strong influence of host genotype (Dicksved *et al.*, 2008; Van de Merwe *et al.*, 1983; Stewart *et al.*, 2005; Zoetendal *et al.*, 2001), more recent studies using sequencing did not (Turnbaugh *et al.*, 2010) although these approaches yield support for a role of host genetics. The effects are likely to be small, and detecting them in a healthy population will require a large number of subjects, because of confounding factors due to genetic diversity of human populations and strong environmental effects, primarily diet, but also gene–environment interactions that may overshadow the effect of genetics.

In animals, results were more conclusive. With laboratory mice, comparison of caecal microbiota showed more similar fingerprint between more related animals than with the others (Hufeldt *et al.*, 2010). The analysis of faecal samples from twin calves revealed higher similarity in fingerprint profiles for twins compared to their coresident indicating that the individual microflora might be genetically or epigenetically influenced (Mayer *et al.*, 2012).

In a study comparing the faecal microbiota of humans and 59 other mammalian species by 16S rRNA gene sequencing, Ley *et al.*, (2008) observed that host diet and phylogeny both influence bacterial diversity. In a study of human and four species of great apes, the host phylogeny explained 25% of the variation in the faecal microbial community analyzed by pyrosequencing with a high sampling depth (10 000 sequences per individual) to accurately assess the diversity present in these complex microbial communities (Ochman *et al.*, 2010).

Linnenbrink *et al.*, (2013) studying the caecal microbiota by D-loop sequencing of house mice from eight locations across Western Europe (France, Germany) found a small influence of genetics, assessed by the genetic distance between populations. Thus, for these mice populations, genetics has a weaker influence than environment to explain the diversity in the digestive microbiota. Moreover, the authors observed that the influence of host genetics was limited to the mucosa communities, this environment being more intimately dependent from the host. Thus in caecal mucosa, geography and genetics explained 16% and 6% of the microbiota variation respectively, and in caecal content, geography explained 11% of the microbiota variation, whereas genetics was not significantly implied. However, the small scale of genetic divergence among the populations included in this study may be limited with regard to the potential influence on microbial communities.

Genes of the host affecting digestive microbiota have also been observed in animal or human. For example, in pig, Meijerink *et al.*, (2000) found that the adhesion of F18 fimbriated *E. coli* to intestinal mucosa and subsequent susceptibility to swine edema disease was controlled by fucosyl-transferase 1 gene, implied in H antigen production on red blood cells. In human, a mutation of the gene encoding for the protein pyrin, a regulator of innate immunity, was shown to be linked to significant changes in bacterial community structure of feces studied by sequencing of 16S rDNA clones libraries and FISH (Khachatryan *et al.*, 2008). In the pathogenesis of inflammatory bowel diseases, two genes implied in mucosal immunity were significantly associated with shifts in microbial compositions obtained by sequencing clones libraries from intestinal tissues (Frank *et al.*, 2011).

Candidate gene approaches, in which one gene is deleted or added to a model host organism, show that a single host gene can have a tremendous effect on the diversity and population structure of the gut microbiota (Spor *et al.*, 2011). Most of the genes shown to have an impact on the composition of the gut microbiome are components of the immune system, and a few others have roles in metabolism. Genes implied in immunity affecting digestive microbiota are for example genes coding for defensins, cytokines, IgA, HLA of the major histocompatibility complex, receptor or signaling molecule implied in innate immune system. Thus, several studies in monogenic models (knockout mice) have demonstrated the role of innate immune response in altering the composition of mouse gut microbiota and disease susceptibility. For example, deficiency in Toll-like receptor 5 (TLR5) alters the abundance of microbiota at species level leading to features characteristic of metabolic syndrome (Vijay-Kumar *et al.*, 2010). Mice deficient in a regulatory factor implied in immune functions had greater instability in the composition of the faecal microbiota, both daily and over 5-day intervals, than control mice (Thompson *et al.*, 2010).

Regarding genes implied in metabolism, a few host genes have been studied for their impact on the gut microbiota. One is the gene coding apolipoprotein AI, another example is the leptin-encoding gene, OB (also known as LEP) (Spor *et al.*, 2011). More recently, Buhnik-Rosenblau *et al.*, (2012) observed that the deletion of iron metabolism genes in the mouse host affects the composition of its faecal bacteria observed with fingerprint by t-RFLP and sequencing. It may be due to change in luminal iron content of the gut which is one of the important elements essential for bacterial growth. Gene involved in the regulation of developmental processes, have also been observed to have consequences on digestive

microbiota as deficiency in T-bet (Tbx2), that promotes a colitogenic microbial population and ulcerative colitis (Garrett *et al.*, 2007).

However, some of these studies have shown a much weaker effect of genetics than a change in diet on the microbial composition (Hildebrandt *et al.*, 2009; Zhang *et al.*, 2010).

Studies have been performed with genetically inbred mouse lines that provide a resource for high resolution analysis of complex traits. The interest in using such genetic lines, instead of approaches such as polymorphism genotyping or knockout animals, lies in the fact that a hierarchy in genetic determinants can be proposed.

With eight inbred mouse lines, Kovacs *et al.*, (2011) concluded that the faecal microbiota was substantially different in different genetic backgrounds by using DNA fingerprinting methods based on length variability regions within the bacterial ribosomal RNA operon. Nevertheless, the small sample size, and the pooling of samples, which is known to result in underrepresentation of rare taxa, may have hindered the assessment of the full magnitude of the genotype effect (Manter *et al.*, 2010).

By studying the intestinal microbiota by pyrosequencing of 10 genetically distinct inbred mouse strains, Campbell *et al.*, (2012) studied the effects of host genetics and environmental on caecal microbiota. They found significant correlations between the mouse strains and their gut microbiota, reflected by distinct bacterial communities. Common environment had a reduced, although detectable effect, and the microbiota response to this factor varied by strain. They identified discriminative and strain-specific bacterial phylotypes. Cohabitation of different strains of mice revealed an interaction between host genetics and environmental factors in shaping gut bacterial consortia, as bacterial communities became more similar under common environments but remained strain specific.

However, in these studies with inbred mouse lines, the maternal effect was confounded with genotype effects. To disentangle between maternal and genotype effects, different methods have been used as cross-fostering (swapping offspring between two mothers after birth), uterine transplants of embryos of one genotype into a dam of another genotype, and inoculation of one microbiota into a set of germ-free mice. Thus, in a study involving reciprocal transplantations of digestive microbiota between germ-free zebra fish and mice, it has been observed that after transplantation, the bacterial lineages found in each recipient animal resembled the donor microbiota, but the relative abundance of each taxonomic group was altered to be more similar to the typical microbiota of the recipient (Rawls *et al.*, 2006). It

shows that selection pressure in each host acts to influence community structure during and after colonization.

Now, quantitative genetics (QTL mapping) is emerging as a highly promising approach that can be used to better understand the overall architecture of host genetics influence on the microbiota, and to uncover potential candidate gene controlling microbial diversity in the gut (Spor *et al.*, 2011). These studies are performed on large number of animals, from divergently selected lines, that can be intercrossed between 2 generations (producing a F2 cross) or during more than 6 generations (production of an advanced intercross lines, AIL). The F2 crosses have been widely used to detect the presence of QTL. As continuous intercrosses used to produce AIL result in animals with many recombinations in the genome, AIL allow a better mapping resolution (Darvasi et Soller, 1995).

A recent study using QTL mapping method detected genome-wide linkages with the relative abundance of several taxa (Benson *et al.*, 2010). The faecal microbiota of AIL population of mice selected for wheel-running behavior was analyzed with pyrosequencing on 645 mice. The authors identified 13 significant QTLs and 5 suggestive QTLs for which host genetic variation is significantly linked with relative abundances of 26 of the 64 conserved taxonomic groups (defined as core microbiota) that varied quantitatively across most animals in the population. In several instances, one QTL was associated with more than one taxon, indicating that host genetic composition can influence population structure. These QTL regions contain genes implied in immunity.

More recently, McKnite *et al.*, (2012) used this genetic mapping approach with recombinant inbred strain of mice in combination with gene expression within the gastrointestinal tract. This population resulted from the combination of 2 mice strains and displays important differences in susceptibility to obesity and other morphologic, immunologic, behavioral and metabolic traits. The faecal microbiota studied by next-generation sequencing revealed important quantitative differences in microbial composition among these strains. These differences in gut microbial composition are influenced by host-genetics, which is complex and involves many loci. Linkage analysis defined QTLs restricted to a particular taxon, branch or that influenced the variation of taxa across phyla. Study of gene expression within the gastrointestinal tract and analysis of the sequence of the parental genomes in the QTL regions uncovered candidate genes with potential to alter gut immunological profiles and impact the balance between gut microbial communities.



With the exponential increase in the number of SNP (eg. 6K 5 years ago, 600K today in chicken), the interval mapping approach used for QTL detection studies evolved toward association studies. This approach is called Genome-wide association studies (GWAS). The aim is to relate genome-wide variation in genetic markers to variation in digestive microbiota. GWAS studies identify SNPs and other variants in DNA which are associated with components of digestive microbiota. Such studies in large populations will enable the detection of genes for which variation is related to complex phenotypes that can be triggered by differences in the digestive microbiota. However these studies cannot conclude on the causal effect of a given gene.

This systemic genetics approach was used by Parks *et al.*, (2013) with more than 100 inbred strains of mice to assess gene-by-diet interactions common to obesity, allowing an improved mapping resolution. They observed a strong relationship between genotype and caecal microbiota composition analysed by pyrosequencing. Indeed a strong effect of genetics was observed on the composition and plasticity of the gut microbiota in response to altered dietary composition, after feeding a high-fat and high sucrose diet instead of a control diet. A genome region which contains three amylase genes may contribute to gut microbiota composition.

In pigs, the SUS FLORA project (2011-2014 Genomic ANR; C. Rogel-Gaillard) being developed at INRA has created Large White cohorts allowing to uncover the contribution of genetics of the host to the microbiota composition via GWAS (Estellé *et al.*, personal communication). In fact, the intestinal microbiota has been associated to porcine immune and health traits (Lepage *et al.*, 2012) that are themselves under genetic control (Flori *et al.*, 2011).

#### **2.2.1.2. Effect of genetics on digestive microbiota in birds**

Advantage to work on chicken is that, as in mice used as human model (Gootenberg and Turnbaugh, 2011), all the environmental conditions can be controlled. Moreover, there is no influence of maternal environment and the maternal effect is limited to the eggs formation, and it allows working directly on the target animal species, avoiding the difference of microbiota between the model and the target species as between mice and human (Ley *et al.*, 2005).

In chicken, first studies with AGP showed that their effects on growth was dependent on the animal strain suggesting that genetics may have an effect on digestive microbiota (Nordskog et Johnson, 1953).

By comparing the molecular fingerprint of digestive microbiota of ileal content of 3 genetic lines of chicken feeding the same diet, Lumpkins *et al.*, (2010) observed difference between two modern genetic lines and an historic strain with a much slower rate of development than the 2 other lines. In duck, by comparing the digestive microbiota analyzed by pyrosequencing of two genotypes of ducks (Pekin and Muscovy), fed *ad libitum* or overfed, Vasai *et al.*, (2014) observed that digestive microbiota of caecal content is more affected by genetics than by overfeeding, whereas digestive microbiota of ileal content is affected by overfeeding.

As for laboratory mice, used as human model, several well characterized divergent genetic lines of chickens have been developed as research tools for agronomic research. It allows using a QTL detection approach that allows measuring the heritability of the digestive microbiota as explained for mice previously.

As it can be expected that a genetic selection on DE, that has a direct effect on the biotope of digestive microbiota has an impact on the microbiota, in our laboratory, we used our divergent lines D+ and D- to study effect of genetics on digestive microbiota in chicken. Digestive microbiota of the two divergent chicken lines was studied in birds from the 10<sup>th</sup> generation, in the terminal ileum and the caeca. For these studies, birds were reared on litter the first 10 days of life to be in contact with the environmental microbiota from other birds, and then reared in individual cages for AMEn determination. The analyses showed clear differences between microbiota of the two lines in both the digestive contents and in the mucosa (Gabriel *et al.*, 2011, 2012; Konsak *et al.*, 2011, 2012).

In the ileum contents, comparison of bacterial fingerprint of the two bird lines showed significant difference between them. Identification of specific bacteria showed a higher amount of a strain of a long segmented filamentous organism in D+ birds, belonging to cluster I of *Clostridium*, and a strain of *L. crispatus* in D- birds. Moreover, quantitative analysis by qPCR of the main bacterial groups found in the digestive tract of chickens showed in D+ chickens a higher amount of *C. coccoides* and a higher amount of *E. coli* in D- chickens. In the ileal mucosa, more *L. salivarius* per segment were observed in D- birds.

In the caeca contents, in D+ birds, total bacterial biomass is higher than those of their small intestine, contrarily to D- birds that may have a similar or slightly higher bacterial load in the small intestine than in their caeca. Moreover, a high difference between the fingerprints of the two bird lines was observed, and a higher relative amount of an *E. coli* strain was found in D- birds. Moreover, in D+ birds, higher amounts of *C. leptum* group were observed, and in D- birds more *Lactobacillus*, and particularly *L. salivarius*, a dominant lactic acid bacteria in broiler digestive tract and more *E. coli*. In the caecal mucosa, as in the caecal content, a high difference between fingerprints of the two bird lines was observed, and a higher relative amount of an *E. coli* strain in D+ birds, and a *L. salivarius* strain in D- birds. In this mucosa, in D- birds, more *Lactobacillus* per segment, as well as *L. salivarius* and *L. crispatus* were observed. Thus contrarily to the observation of Linnenbrink *et al.*, (2013) in mice, digestive microbiota was modified both in content and in mucosa of caeca.

Using a high number of F2 birds (144 animals) of these divergently selected line, D+ and D- with high range of AMEn (from 7.6 to 16.1 MJ/kg), and on one of the more discriminant biotope observed previously, caecal content, genetic parameters were determined. Significant heritability estimates were observed for bacterial numbers, or higher for bacterial ratios (Gabriel *et al.*, 2012). Thus heritability ranged between 0.11 and 0.14 for the genus *Lactobacillus*, and more precisely with *L. salivarius*. Higher heritability estimates were obtained ( $h^2$  close to 0.20) for the ratios of *L. salivarius* to *C. leptum* and of *C. leptum* to *C. coccoides*. The highest heritability was estimated for the ratio of *C. coccoides* to *Lactobacillus* ( $h^2=0.34$ ). These estimates imply that the development of microbiota is partly controlled by the genetics of the host.

Moreover, 15 QTLs regions controlling variations of caecal microbiota composition have been identified in our F2 cross. These regions are under analysis to detect candidate genes. Moreover, gene expression within the gastrointestinal tract, in parallel to the determination of major bacteria groups, will allow refining these candidate genes (ADIGEN Project, AGENAVI 2014-2015). It may contribute to explain the observed link between host genetics and its digestive microbiota.

A new study is also in progress on AIL population of D+D- lines, generation 7, with 216 birds (ongoing project: INRA Project GISA Galimide 2014-2015). It will allow refining the relationship between microbiota composition, innate immune system parameters and digestive efficiency (Calenge *et al.*, 2013). In this study, digestive microbiota will be studied by pyrosequencing allowing a more precise description of digestive microbiota.

Recently, Zhao *et al.*, (2013) used two divergent chicken lines to study the genetic effect on digestive microbiota. These two lines of chickens were selected during 54 generations for high (HW) or low (LW) 56-day body weight, and maintained at the same location and reared on the same diets. The chickens were reared in individual cages to prevent exchange between birds and fed a corn-soybean diet. Birds were sampled at 8 months. The digestive microbiota that was targeted was faecal microbiota, and the method used to study it was next generation sequencing (NGS), with high sequencing depth (about 20 000 sequences reads per sample, between 7 600 and 32 900). They showed that quantitative genetic background of the host influences gut microbiomes in chickens, as they observed difference in microbiota at the genera and species level: 29 species were affected by genotype. However they did not obtain significant heritability, although some show high value maybe because of low number of birds used in this study (56 animals, with 12-15 animals per group, 2 sexes, 2 strains).

### **2.2.2. Assessment of present knowledge and hypotheses on this relationship**

Published studies in mammals, fish and birds, show an effect of host genetics on digestive microbiota that may explain a part of individual variability in the case of animal reared in very similar environment, as poultry. Host genotype may exert an effect on the composition of the microbiota by a selective pressure imposed within the gut habitat through secretions as bile acids and other antimicrobial compounds as defensins, control of digestion, gut motility or modification of epithelial cell surfaces. This selective pressure is not the same according to each digestive compartment as different physiochemical properties exist, and depend on host genotype and gene expression in these compartments.

At the present day, studies in mammals have highlighted the implication of genes implied mainly in immunity and metabolism to control digestive microbiota. However, there is bias in the genes that could be found by these studies, which mostly relied on animal models used in these studies, focused on human health diseases as metabolic, immunity or neuropsychiatric disease. However, it is interesting to note that in our QTL detection study, several QTLs for digestive efficiency have been identified on chromosome 16, which role in immunity of chicken is well-known. Other sets of genes, than those of immunity and metabolism, may be implied in the control of digestive microbiota, as the genes implied in the characteristic of the digestive biotopes, as suggested by results of Parks *et al.*, (2013) who found a genome region which contains three amylase genes that may contribute to gut microbiota composition. Genes

implied in digestive physiology will influence the composition of the undigested compounds of the diet in the digestive tract, and then in turn, substrates than can be used by microbiota. Thus genes implied in the digestion steps as hydrolysis and absorption, or digestive transit via neurohormonal regulation, may control microbiota. In poultry, researches ongoing in our laboratory are precisely designed to determine the relationship between microbiota and digestibility, which increase the chance to find such genes.

This effect of host genetics on digestive microbiota is probably the results of adaptation during host speciation and the evolution of pluricellular eukaryotes to their surrounding prokaryotes to evolve to a mutualistic relationship between the host and the colonizers as proposed by Benson *et al.*, (2010). This evolution may imply complex sets of loci, that may have been implied during stepwise change of host and its microbiota. This could explain the evolution of highly specialized digestive organs (e.g. crop and ceca in poultry) that harness microbes for fermentation. By exerting this selective pressure, host genetics control would control microbiota within the gut ecosystem to promote beneficial microbes. This hypothesis is compatible with the suggestion that the adaptive immune system has specifically evolved in vertebrates to regulate and maintain beneficial microbial communities (McFall-Ngai, 2007).

### **3. Conclusion and further knowledge needed**

Digestive efficiency is a major goal in poultry production for several reasons, economic, environmental and sociological. As shown by the works performed in our laboratory, DE is under genetic control. Moreover, DE depends on digestive microbiota composition. This later one and its stability appear also to depend on genetics, via or not via DE, whose results lead to microbial substrates. Thus DE, host genetics and digestive microbiota are linked together.

Further studies are needed to understand these genetic controls and these interactions. It is made easier with the development of high throughput method to study digestive microbiota. Work in progress in our laboratory will contribute to improve knowledge concerning genetic control of DE, with finer delimitation of QTLs. Moreover, if identified gene candidates are confirmed in our further work, after being validated in commercial populations and breeding environment, they can be included in selection schemes, in selection assisted by markers.

Concerning genetic control of digestive microbiota, analysis of identified QTLs region will allow detecting candidate genes. Moreover parallel study of gene expression within the

gastrointestinal tract will allow refining these candidate genes, and may contribute to explain the observed link between host genetics and its digestive microbiota. Assessing the causal role of host genetic variation in gut microbiota composition and dynamics will enable an understanding of the mechanisms of colonization, and the relation to DE. Correlations of QTL and host gene expression to bacterial diversity will likely shed more light on potential physiological roles of digestive bacteria. Understanding the mechanisms of community selection and genetic influences on community structure will have many implications for attempts to alter this community structures in order to increase DE. Moreover, future isolation and physiological studies of bacterial taxa that were discriminatory among the divergent lines selected for DE may improve our understanding of the role of these bacteria.

However, one question to answer when studying digestive microbiota, genetics and host phenotype as DE, is which digestive microbiota has the highest influence on the host : the one with highest concentration or whose with highest contact with the host ? The one of content or mucosa ? The one of small intestine or caeca, and also crop as inoculum of the following digestive tract ? And can feces (intestinal or caecal) be used to represent these microbiota in order to collect samples without killing birds to follow microbiota evolution on the same animal or to work on farm ?

Moreover, several parameters need to be taken into account from sampling conditions, choice of analysis of individual or pool samples, and number of samples per treatment that need to be high due to high animal variability to have a faithful representation of the reality. The different steps of sample processing need also to be adequately chosen to obtain the image the more representative of the reality. These different steps include sample storage conditions, bacterial DNA extraction adapted to the specificity of digestive samples (types of potential bacteria and digestive matrix), PCR conditions, pyrosequencing parameters as choice of sequencing depth, bioinformatics analysis of generated data, and statistical analysis of these data, as all of these steps have impact on the final results. These different steps need standardization and compromises are needed to be practical.

Although genetics appear to have an important part in the control of digestive microbiota, numerous environmental factors are also implied. Thus, environment of first days of life may be crucial in the development of digestive microbiota as proposed by Stanley *et al.*, (2013a). First bacteria in contact with the bird are at hatcher, and further in post-hatch environment during vaccination, boxes for transportation. During this fasting and other stress that birds

undergo (temperature changes, vibrations), digestive microbiota depends on animal endogenous products as mucus, intestinal cells and yolk sac content. The consequences of these post-hatching conditions on digestive microbiota will be studied in our laboratory (ongoing project: INRA Project GISA Whelp 2014-2016; L. Guilloteau and C. Leterrier). At arrival on the farm, bacteria come from the diet, water, litter, and other birds. On farm, digestive microbiota can be impacted by rearing environment as animal density (Guardia *et al.*, 2011) and diet composition as explained previously. The sum of the small differences in each step of microbial colonization may be responsible in part to the variability between individual birds. Maternal effect, although potential not so important than in mammals, may be implied as proposed by Torok *et al.*, (2011). Indeed, maternal nutrition has been shown to alter chick gut development (Rebel *et al.*, 2006), which has been linked indirectly to microbiota development and biological variation between birds (Lumpkins *et al.*, 2010). Moreover, in microbial ecology, it is known that a same initial biocenose can evolve to different states. The objective will be to perform a hierarchy in all these factors, genetic and environmental, in order to manipulate microbiota to improve DE.

Another essential point is that not only the composition but also the functional capacity of the digestive microbiota is highly important regarding the host physiology as DE and performances. Indeed, different bacterial strains of the same species can have profound differences in the interaction with their host. However current phylogenetic characterization of digestive microbiota by pyrosequencing does not allow to the determination of bacteria strain because of information in databases that depend on current knowledge, yet a high proportion of bacteria in complex system as in the digestive tract are not known, as the exploration of these systems are in progress. That's why recently was developed metagenomic approach in order to highlighted all the potential functions of digestive microbiota in human (Qin *et al.*, 2010; Turnbaugh *et al.*, 2009) and also in chicken (Yeoman *et al.*, 2012). However the detection of genes in a metagenomic library does not necessarily means that they are functionally important, as microbiota effect on the host depends on bacterial metabolism that depends to their environment (Roy *et al.*, 2008). As bacteria fulfil their effect on the host by the intermediate of excreted products as protein or metabolites and/or their direct action, to gain better insight into the activity and functionality of the intestinal microbiota, its products can be studied as RNA, proteins and metabolites (Blottière *et al.*, 2013). Metatranscriptomics remains a challenging step due to the difficulty of obtaining intact RNA. Bacterial proteins and metabolites can be studied by targeted approach. Thus specific bacterial enzymes have

been studied. Particularly, the metabolites which represent the actual end products of metabolism are of high significance for the links with host physiology. Large amounts and various compounds are produced by microbiota. Specific metabolites can be studied as those from carbohydrates as it is the main substrate for microbial fermentation. As the main products of carbohydrate metabolism are SCFA, they have been the most studied. As bacteria fermentations switch to protein fermentation when carbohydrate source are depleted, products of protein fermentation have also been studied. Fermentation of bile acids has also been studied due to the various modifications by microbial fermentation and consequences on the host. However, these latest years, metaproteomics and metabolomics are rapidly developing and applied to digestive samples. They allow following all the proteins and metabolites respectively. By following these approaches, novel proteins and metabolites, and mechanisms involved in host physiology as DE, can be identified. Metaproteomic has been used to study digestive microbiota in human (Kolmeder *et al.*, 2012) and in chicken (Tang *et al.*, 2014). Metabolomic has also been applied in human (Nicholson *et al.*, 2012) and in chicken (Bailey, 2010; Gabriel *et al.*, unpublished), and we will study these metabolites in our ongoing project on AIL population of D+D- lines (INRA Project GISA Galmide 2014-2015). To move forward in this area, knowledge about the exact microbiota-derived metabolites that can induce host responses need to be increased.

Digestive microbiota has a complex position in the relation to the host. It can be view as a phenotype trait as whatever other phenotypes, being the results of genetic and environmental factors, but it can also be viewed as an environmental factor that contribute with genetic components to multifactorial phenotype traits of animals as DE. To study these interactions, it appears that an holistic approach is needed which will be possible thanks to the development of the different OMIC methods, and the use of divergent line of animals (Blottière *et al.*, 2013). The main challenge is the integration of complex data in order to identify meaningful relationships. This knowledge of host-gut microbe interrelationships will rapidly increase and allow orientating digestive microbiota to the targeted phenotype.

However, it must be noted that this search in optimal digestive microbiota to improve DE, must take into accounts other effects on animal phenotype, and that there are no trade-off with other important phenotypic traits as immunity, as we will studied in our ongoing project (INRA Project GISA Galmide 2014-2015).



To conclude, knowledge of microbiota composition is important to understand in which digestive biotopes these populations lead to which products having an effect on the host physiology. Deciphering these microbial signatures and their metabolites that govern short and long-term equilibrium, as well as imbalances in host-microbial relationships, may provide novel physiological markers of the targeted phenotype traits, as DE.

Thus, increase in knowledge on the complex dynamic of poultry digestive tract ecosystem is needed in the future, in order to improve phenotypic traits as DE.

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