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GENETIC SELECTION OF POULTRY BASED ON DIGESTIVE CAPACITY – IMPACT ON GUT MICROBIOTA

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Introduction

The genetic selection of animals has always searched to improve the growth performance of animals and feed efficiency, and this trait will remain the main objectives of genetic selection. Feed efficiency can be improved by selecting for growth, feed conversion ratio (FCR) or feed intake, which are heritable (Pym, 1990). As digestive efficiency is one of the components of feed efficiency, its improvement contributes to the global aim of optimization of feed efficiency, and therefore can be used for genetic selection (Mignon-Grasteau et al., 2004). This capacity to digest feed is all the more important now that more and more unconventional feedstuffs with variable or low digestibility values will be used for animal feeding instead of conventional feedstuffs such as wheat, maize and soya to decrease competition for crop vegetal resources between man and animal. Moreover in Europe, the use of local feedstuffs is needed to decrease the dependence on soya as a protein source, as it mainly comes from importation. Although nutritional means such as feed additives (enzymes, phytobiotics . . .) can be used to adapt animals to these new feedstuffs, genetic selection can also be used to improve adaptation of animals (Dockès et al., 2011). However, the genetic selection performed up until now on feed efficiency has led to chickens with non optimal digestive efficiency, as shown by their lower apparent metabolisable energy corrected for nitrogen retention (AMEn) compared to slow growth rate lines (Carré et al., 2008). Indeed, high growth rate commercial chickens, Ross PM3 broilers, exhibited a 2-9% lower AMEn than a chicken line with median growth rate but selected on AMEn.

Moreover, nowadays, sustainability of poultry breeding needs to include in

addition to economic objectives, environmental and sociological objectives. Regarding preservation of the environment, an increased digestive efficiency means less animal wastes and manure. Regarding social demands, an increase 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 bacteria. Indeed, it has been shown that the undigested compounds are involved in dysbacteriosis (Klis and Lensing, 2007), or the occurrence of necrotic enteritis due to certain *Clostridium perfringens* strains (Timbermont et al., 2011), or coccidiosis (Crévieu-Gabriel and Naciri, 2001). Moreover, the undigested dietary compounds increase the quantity of fermentation substrates in the litter and the frequency of pododermatitis (Shepherd and Fairchild, 2010).

To answer to these needs, at the Poultry Research Unit of INRA, a divergent selection program began in 2002 using AMEn as the criterion for digestive efficiency (Mignon-Grateau et al., 2004). Contrarily to diets based on maize and soyabean meal, a diet containing a high level of wheat (336 g/kg) allowed a rather high difference in metabolisable energy between chicken lines (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 (Choct et al., 1999; Carré et al., 2002). These problems of wheat digestion largely depend on wheat samples, those with high viscosity and hardness resulting in lowest digestibilities (Carré et al., 2002). The program of divergent selection was thus performed by using a high concentration (525 g/kg) of high viscosity wheat due to its richness in arabinoxylan and a medium hardness value (Rialto cultivar, Carré et al., 2002, Mignon-Grasteau et al., 2004). Moreover, a high fat concentration with high vegetal oil added (60-80 g/kg) instead of 10-20 g/kg as usually done for such median growing lines, was used to increase differences between birds, due to the difficulty of young birds to digest lipids. The two lines were named D+ (high digester) and D- (low digester) and have been selected on AMEn during 8 generations, and reproduced without selection for 2 additional generations. Heritability of AMEn estimated on the first, second and eighth generations was high (0.30 to 0.38, Mignon-Grasteau et al., 2004; de Verdal et al., 2011b).

These divergent lines now represent a unique model to study the physiological limiting factors of digestion. The interest in using such genetic lines, instead of alternative approaches such as knockout animals or polymorphism genotyping, lies in the fact that a hierarchy in genetic determinants can be proposed. Estimation of the genetic parameters makes it possible to establish how selection impacts on the phenotype, as the morphology of the gut organs, and offers the opportunity to anticipate any undesirable effects of AMEn selection, before its introduction in selection schemes. Genetic correlations can also be used as a tool to propose

selection criteria related to the objective of selection but easier to measure than the trait itself. Finally, a quantitative trait locus analysis is currently undertaken on a classical F2 cross between these 2 lines which, combined with the phenotypic differences observed between D+ and D- birds, will help to propose a list of candidate genes for selection.

This selection has been performed on AMEn used as the criteria for digestive efficiency. Animal digestive capacity corresponds to its capacity to extract compounds from the diet by hydrolyzing macromolecules to absorbable molecules and absorb them, with its enzymatic / absorbent system, and also with the help or competition of its digestive microbiota. Several recent studies showed the essential role of host microbiota, particularly digestive microbiota in the physiology of the host, not only for digestive tract function, but also for the whole host physiology. Moreover as the host and its digestive commensal microbiota co-evolve after their first contact, a high individual variability in digestive microbiota is observed (Zhu et al., 2002; Gabriel et al, 2007; Torok et al, 2008). Thus several studies have shown that digestive microbiota depends on animal genetics. For example, in humans, microflora are much more similar between homozygous twins than between unrelated persons or heterozygous twins (Van de Merwe et al., 1983; Stewart and Chadwick, 2005; Zoetendal et al., 2004; Dicksved et al., 2008). Differences in digestive microbiota according to animal genetics has also been observed in other mammals (Toivanen et al., 2001; Gulati et al., 2012), and also in birds such as the chicken (Lumpkins et al., 2010). It can be expected that genetic selection on a trait that has a direct effect on the biotope of digestive microbiota, that is digestive area, has an impact on the microbiota. However, due to the numerous effects of digestive microbiota on the host, the relationship between host and microbiota is probably not only a change of microbiota due to a change in its biotope, but also the establishment of a new balance between the host, its capacity to hydrolyze dietary compounds and its microbiota (Hooper et al., 2002). We thus analyzed digestive microbiota of D+ and D- lines, that have been divergently selected based on AMEn, to understand the relationships between the microbiota and digestive efficiency.

In this chapter, after a review of current knowledge on digestive microbiota in chicken and its effect on the host, the phenotypic characteristics of the D+ and D- lines known at the present time will be presented. These characteristics concern their digestive values as well as the consequences on their zootechnical performances, and digestive tract. In the last part of this chapter, data obtained recently on the digestive microbiota will be presented, as well as further work needed to increase knowledge on relationships between digestive microbiota and digestive capacity of chicken.

Digestive microbiota in poultry and effects on its host

HISTORY OF STUDIES OF THE MICROBIOTA

Several studies on digestive microbiota in poultry were performed between 1970 and 1980 both on conventional and germ-free birds, to study the effect of presence or absence of microbiota. Moreover, other studies were performed by using high doses of antibiotics or probiotics, showing the effects of modifications of digestive microbiota . With the announcement of the ban on antimicrobial growth promoters in animal feed toward the end of the XX century, a new development of studies appeared that investigated the digestive microbiota of rearing animal such as poultry, and mainly focused on means to control it. Thus several reviews have been performed on digestive microbiota description and its role that we will sum up subsequently (Fuller, 1984; Furuse and Okumura, 1994; Mead, 1997; Vispo and Karasov, 1997; Apajalahti et al., 2004; Gabriel et al., 2006; Rehman et al., 2007; Dibner et al., 2008).

Until recently, one of the main problems with studying microbiota, was the availability of appropriate methods to explore it. Indeed a large majority of species cannot be easily cultivated (70-90%). Thanks to the development of new independent approaches to culture in microbial ecology (particularly for environmental microbiota and for digestive microbiota in human due to implication in health), new tools became available and have been used to study digestive microbiota of livestock.

The most commonly used methods at the present time are qualitative methods such as fingerprint techniques such as gradient gel electrophoresis with denaturing compounds or temperature, or terminal restriction fragment length polymorphism analysis, or capillary electrophoresis single strand conformation polymorphism, and quantitative methods, such as fluorescent *in situ* hybridization or quantitative PCR (Inglis et al., 2012).

QUANTITY AND DIVERSITY OF THE MICROBIOTA OF CHICKEN ALONG THE GASTROINTESTINAL TRACT

Briefly, at hatch, the digestive tract of the chicken contains a relatively low number of bacteria, although not sterile as shown by detectable bacteria in embryo from 16 d, with about 100 colony forming units (CFU) / g in caeca and yolk sac in embryo at 18 d, and 10^6 CFU / g and 10^4 CFU / g in caeca and yolk sac respectively 3 h after hatch (Binek et al., 2000; Kizerwetter et al., 2008; Pedroso, 2009; de

Oliveira et al., 2010). Further development of digestive microbiota depends on the environment of the eggs at hatching, and rearing environment following hatch.

To be able to grow in a given part of the digestive tract, microorganisms need substrates and have to multiply at a sufficiently fast rate to compensate for elimination by several mechanisms such as antimicrobial substances and predators (e.g. other bacteria and bacteriophage), digestive transit, and cell and mucus renewal. This explains why microbiota can vary to a large extent among the different parts of the gastrointestinal tract.

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 5.5×10^{11}, 5.3×10^{10} and 7.4×10^{12} copies of 16S rDNA/g[1] 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* (Fuller, 1984; Gong et al., 2007; Guardia et al., 2011). The proventriculus and the gizzard, due to their low pH lead to a fall in bacterial population. In the duodenum, conditions are not favorable to bacterial development for several reasons: high oxygen pressure due to villi movement leading to exchange of oxygen between blood vessels and digestive content, high concentration in antimicrobial compounds such as digestive enzymes and bile salts, reflux movement from jejunum to gizzard leading to a fast modification of the biotope. 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, the latter being reabsorbed by the host and degraded by microbiota. Due to these changes along the small intestine, microbiota evolved between the upper part (duodenum-jejunum) to the lower part of the small intestine (Torok et al., 2008). However, the dominant bacteria are *Lactobacillus* (Fuller, 1984; Lu et al., 2003; Guardia et al., 2011).

In the caeca, the biotope is the most favorable for bacterial growth for several reasons. Firstly, oxygen pressure is lower due to lack of villi and reduced mobility limiting the exchange with the intestinal wall and the thick mucus layers. Secondly, this biotope is relatively stable, due to the low renewal of the digestive content. Indeed, the continuous entrance of substrates are mixed with already present digesta and the caeca are emptied only 1 to 2 times per day (Clench, 1999). The substrates are composed of fine particles of digesta and urinary compounds rich in uric acid backflowed from the cloaca, that can be used by several bacterial

1 Expression of results: copies of ADNr16S due to the reference used for the assay, knowing that the number of copies of ADNr16S varies greatly according to bacteria species (Lee et al., 2009; Rastogi et al., 2009). It varies from 1 to 15, with for the most important bacterial groups in digestive tract of chicken : 4 to 6 for *Lactobacillus*, 3 to 15 (average 9) for *Clostridium*, 5 to 7 for *Bacteroides*, 7 for *E.coli*.

species (Mead, 1997). Thus in these digestive contents, it has been evaluated that 50% of the biomass would be of bacterial origin (Clench, 1999). In contrast to the crop and the small intestine, the bacterial composition of the caeca is more diverse, and the major group is *Clostridium* (Lu et al, 2003; Guardia et al., 2011; Moore et al, 2011).

This digestive microbiota can be located in the lumen or at the mucosal surface or in the mucus layer(s). The luminal microbiota depends on available nutrients, transit rate and the presence of antibacterial compounds. It can be considered that this microbiota acts mainly as an aid for starch hydrolysis in the crop (Szylit et al., 1980; Champs et al., 1981)). In the small intestine, bacteria are considered as competitors of the host, due to their high metabolic potential with high hydrolystic activity In the caeca, digestive microbiota allows the production of short chain fatty acids (SCFA) from undigested compounds. The mucosal microbiota in the crop, has been described as adhesive to the mucosa developing several cell layers (Fuller 1984). Mucosal microbiota, in the small intestine of chicken, would be adherent to the epithelial cells (Yamauchi et al, 1990; Pearson et al., 1992), or localized in the single mucus layer (Johansson et al., 2011). In the lower part of the digestive tract, in the caeca, digestive microbiota have been described to form a 200 cells deep layer (Fuller, 1984), or may be localized in the upper layer of mucus near the intestinal content, and not in the lower layer of mucus (Johansson et al., 2011). This mucosal microbiota depends on available substrates coming from the mucosa such as desquamated cells or mucins, and molecules coming from the digestive contents diffusing into the protein matrix of mucus. It also depends on bacteria adhesins (Juge et al., 2012), specific adhesive sites on the mucus or mucosa, on mucus or cell renewal rates, on antibacterial substances such as secretory antibodies or peptides as defensins, or expression of innate immune receptors, contributing to the innate immune system of the digestive tract (Salzman et al., 2010; van Dijk et al., 2008; Hooper and Macpherson, 2010; Crhanova et al., 2011). These bacteria are particularly important from a physiological point of view, due to their narrow contact with the host and their function in the control of pathogens, modulation of digestive mucosal immunity, and their effect on digestive epithelium cells. However, in the chicken, this mucosal microbiota, different from the luminal microbiota, from the first works of Fuller (1984) has been relatively little studied compared to the studies on digestive contents (Zhu et al 2002; Collado and Sanz, 2007; Gong et al., 2007; Guardia et al., 2009; Moore et al., 2011; Malmuthuge et al., 2012).

The bacterial community present in the digestive tract shows a high phylogenetic complexity, particularly in the caeca. Apajalahti et al. (2004) found 640 different species, and more recent studies using 16S rRNA metagenomic pyrosequencing showed as much as 783 operational taxonomic units or OTU (Danzeisen et al., 2011; Moore et al., 2011; Nordentoft et al., 2011).

This bacterial community can be modified by several factors such as the first inoculum (Gulati et al., 2012), dietary components (Mathlouti et al., 2002; Apajalahti et al., 2004) or diet structure (Williams et al., 2008; Amerah et al., 2009) or feed additives such as antibiotic growth promotors (Danzeisen et al., 2011) or alternatives to antibiotic growth promotors such as organic acids, prebiotic, probiotic, vegetal compounds, enzymes, clay, charcoal ... (Gabriel et al., 2006; Yang and Choct, 2009; Huyghebaert et al., 2011; Bedford and Cowieson, 2012), or by stress (Suzuki et al., 1989), the nervous system (Lyte et al., 2010), rearing environment (Putskam et al., 2005; Gong et al., 2008; Guardia et al., 2011) and host genetic as indicated previously.

The phylogenetic complexity of digestive microbiota, and its high number of genes as shown by recent analyses of functional gene content using pyrosequencing in chicken (Qu et al., 2008; Danzeisen et al., 2011), derives from the intermediate of numerous metabolites and/or the direct action of bacteria on numerous physiological processes on the host, that can be beneficial or deleterious.

EFFECTS OF THE DIGESTIVE MICROBIOTA ON THE HOST

Digestive microbiota can be considered as an organ in the digestive tract that uses nutrients and metabolites, recognizes and synthesizes neuroendocrine hormones, interfaces with the nervous system that innervates the gastrointestinal tract, and as digestive epithelium products cell biomass (Lyte, 2010). As indicated previously, the digestive microbiota and the host have co-evolved after their first contact, and are considered as a supraorganism with numerous cross-talk between microbial and host cells (Lederberg, 2000). They seem to be more than mutually tolerant, and to be in a mutualistic relationship when equilibrium is reached.

Thus, the digestive microbiota has an effect on its live environment, the digestive tract. It contributes to the development, morphology and functionality of the digestive tract (Coates 1980, Furuse and Okumura 1994; Bäcked et al., 2005), stimulates mucin production and uses them as substrate, and it may modify intestinal transit.

These effects have consequences for animal digestion of carbohydrates, lipids, and proteins. Whereas Szylit et al. (1980) and Champs et al. (1981) showed that the microbiota is involved in starch digestion by bacterial hydrolysis in the crop, it acts mainly as competitor of the host in the small intestine. However, microbiota may have a positive effect by releasing nutrients that may be absorbed by the host in the small intestine or caeca, the latter also being able to absorb carbohydrates and amino acids (Moreto and Planas 1989), although the extent and benefit for the host of this activity is not known. Moreover fermentation of undigested compounds, mainly in the caeca, and bacterial metabolism of uric acid, allows

producing SCFA, that can be absorbed by the digestive epithelium (Mead, 1997). It may represent 3-4% of energy supply or even 3 times more, but more studies are needed to determine the true involvement of digestive microbiota in energy supply (Jozefiak et al., 2004). Ammonia may also be absorbed and converted to non essential amino acids, although the biological importance is not known (Vispo and Karasov, 1997). 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, such as palmitic and stearic acids are highly decreased, whereas digestibility of unsaturated fatty acids, such as oleic and linoleic acids is not modified by microbiota (Boyd and Edwards, 1967). This is due to deconjugation of bile salts by some bacterial species, such as *Lactobacillus* (Kim and Lee, 2005). 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 hydrolyse some resistant proteins for enzyme host (Salter 1973; Salter and Fulford, 1974; Kussaibati et al., 1982a). Concerning starch digestibility, no effect of microbiota was observed with maize starch by Kussaibati et al. (1982a). Due to the effect of digestive microbiota on nutrient digestibility, it can have an effect on metabolisable energy, positive or negative, (Kussaibati et al., 1982b; Furuse and Okumura, 1994). Moreover, for diets rich in soluble non-starch polysaccharides, leading to increased viscosity of digestive contents, microbiota is considered to be involved in the negative effect observed on digestion (Bedford and Cowieson, 2012), although according to Maisonnier et al. (2003) it is not the main factor. It can also not be ruled out that if chickens have access to 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.

The commensal microbiota is implicated in digestive health of animals. It contributes to the protection against harmful microorganisms (barrier effect) and stimulates the immune system (Ismail and Hooper, 2005; Sharma et al., 2010), leaving the host in a permanent inflammatory states (Klasing et al., 1991). Digestive microbiota contribute to production of toxic substances and conversely to detoxification of some compounds.

The digestive microbiota can also influence extra-digestive physiology of the host. Indeed, commensal bacteria by their metabolites or constituents, or even themselves, that pass through the digestive epithelium, have effects on the animal metabolism for example fattening (Bäcked et al., 2004; Cani et al., 2007), or on the central nervous system with effects on behavior (Lyte, 2010; Diamond et al., 2011).

These effects of microbiota lead to an increase of protein synthesis in the liver (metabolism and detoxification of bacterial products) and in the intestine (organ

with high turnover) of +25% and + 45% respectively, corresponding to an increase of total protein synthesis of 6-8% (Muramatsu et al., 1987). Energy requirement is also increased by microbiota (Furuse and Okumura, 1994). Digestive microbiota may also contribute to mineral and vitamin nutrition (Gabriel et al., 2006).

Moreover the bacterial activity has consequences on non digestive pathologies, and thus animal welfare, such as conjunctivitis and respiratory problems due to irritant compounds products released by bacterial fermentation in the litter material (Thomke and Elwinger, 1998). These fermentations also have consequences on contact dermatitis (Shepherd and Fairchild, 2010) or pathogen development in the litter. All these effects have consequences on animal production, as well as on growth performance and product quality.

Globally, the digestive microbiota represents a cost for the animal, due to the competition with the host enzymes in the small intestine, and due to the stimulation of the immune system, and thus maintenance metabolic cost. However the digestive microbiota provides a protection against harmful microorganisms by the barrier effect and stimulation of immune system, is involved in starch hydrolysis in the crop, and allows energy recovery from compounds undigested by the host enzymatic system in the caeca. It may allow the animal to be more adaptable to environmental changes, as suggested by changes with environmental factors such as dietary composition or rearing conditions (Vispo and Karasov, 1997), thus improving its robustness. The balance state between the genetics of the host, dietary compounds and the digestive microbiota, has consequences for the host phenotype at the digestive level, and also at the animal scale.

In conclusion, from a nutritional point of view, the optimal microbiota would be one that allows for conversion of non digestible feed compounds and endogenous products (such as mucus and desquamated cells) by the host, to absorbable compounds such as SCFA. From a point of view of global host physiology, optimal microbiota would be one that converts unused digestive compounds by the host to absorbable energetic compounds and also allows for the best beneficial /harmful ratio for optimal host physiological function, as immunity stimulation to protect the host against harmful microorganisms but not too high inflammation, or optimal lipid metabolism.

Experimental model of birds selected on AMEn and consequences for digestive microbiota

The divergent D+ and D- lines allow determination of the limiting factors in digestive efficiency in the conditions of the selection, with a high content of wheat as the cereal source, a cultivar rich in arabinoxylan, and a high content of

lipids, to maximize differences between individuals. The lines were selected on their AMEn at 3 weeks of age. This age was chosen because it represents a key period in gastrointestinal tract development. In order to maintain performances at a common level between lines, body weight was constrained among both lines.

DIGESTIVE CAPACITY AND DIGESTIVE TRACT OF D+ AND D- LINES

D+ and D- birds were characterized for several parameters on a large number of birds, as well as on digestive efficiency parameters, zootechnical parameters and organ size of the digestive tract in the upper and middle part (Mignon-Grasteau et al., 2004; de Verdal et al., 2010b, 2011b). Moreover, several studies were performed to study more deeply some parameters of their digestive physiology.

Due to their mode of selection, D+ birds showed low variability in their digestive capacities as they are near to the upper limit of possible values, whereas on the contrary D- birds showed high variability (Mignon-Grasteau et al., 2010; de Verdal et al., 2011b). This characteristic is also observed for other parameters studied in these two divergent lines as digestive organ size.

The difference between the two lines evolved with successive generations. Therefore, from the first generation of selection, a significant difference in digestive capacity (AMEn, lipid, starch and protein digestibility) was observed, and the differences increased with selection. It was accompanied by differences in anatomy of the digestive tract especially in relative weight of gizzard observed as soon as the first generation (Mignon-Grasteau et al., 2004; Péron et al., 2006), whereas a difference in small intestine relative weight was observed only from the 5th generation (Garcia et al., 2007; de Verdal et al., 2011b).

AMEn and faecal digestibility

Apparent metabolisable energy

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

However, the results are highly diet-dependent. Although 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). Thus D- birds showed higher difficulties to adapt to a wheat diet, compared to D+ birds. Moreover D- chickens showed a limited capacity to digest an easily digestible diet, as shown by their lower AMEn than D+ chickens with a maize diet (-5.2%).

Other feed x genotype interactions have been observed between these lines. For example, a soft wheat cultivar compared to a hard one resulted in an AMEn improvement in D+ birds (+6.1%) but not in D- birds (Péron et al., 2006). A fine particle diet with maize as cereal source resulted in digestion improvement only in the D+ birds (+2.2%), whereas a deterioration was observed in the D- birds (-2.5%) (Rougière et al., 2009).

Heritability of AMEn is also diet-dependent. It has been estimated in different experiments between 0.30 and 0.38 when birds were fed with wheat, and only 0.15 when they were fed with maize (Mignon-Grasteau et al., 2004, 2010a; de Verdal et al., 2011b). The strong positive genetic correlations estimated between these traits recorded in both diet treatments (0.73 to 0.88) however showed that selecting on these traits with wheat diets would improve performance both on wheat and maize diets.

Excreta digestibilities

As D+ birds fed with wheat diets were characterized at the 5th 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). However with a maize diet, at the 6th 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.

Heritabilities of faecal digestibility of lipids, starch and proteins for the 8th first generations were 0.25 to 0.29 (Mignon-Grasteau et al., 2010a). Genetic correlations between AMEn and faecal lipid, starch and protein digestibilities were 0.80 to 0.91 (Mignon-Grasteau et al., 2004, 2010a). As for AMEn, heritability 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.

Besides the improved digestive efficiency, selection on AMEn shows a positive effect on total animal wastes, composed of undigested compounds and metabolic wastes. Thus D+ birds showed a 34.9% lower nitrogen and a 19.0% lower phosphorus excretion relative to nitrogen and phosphorus intake (de Verdal et al., 2011c). Moreover the ratio of nitrogen to phosphorus in excreta was lowered

by -20.3% in D+ chickens which implies that losses of nitrogen after excretion have to be more limited in D+ than in D- birds (-15% vs -35%) in order to produce a manure equilibrated for fertilization (Mignon-Grasteau et al., 2010b). Indeed heritability of nitrogen excreted / nitrogen intake, phosphorus excreted / phosphorus intake and nitrogen excreted / phosphorus excreted is moderate, 0.29, 0.22 and 0.18 respectively, and the genetic correlations between AMEn and these 3 traits are highly negative, -0.99, -0.64, and -0.84, respectively.

Bird performance

D+ birds were also characterized at the 8th generation by lower feed intake from 17 to 23 d (-21.5%) than D- birds, improved feed efficiency (+58.0%), higher weight gain (WG) (+13.7%) and higher body weight at 23 d (+14.5%) (de Verdal et al., 2011b). The lower feed intake and lower FCR of D+ birds compared to D- birds was observed from the 2nd generation, -11.4% and -25.6% respectively, whereas no significant effect was observed on WG (Mignon-Grasteau et al., 2004). At 8 weeks, age at which birds reached the market weight, the difference in body weight completely disappeared (de Verdal et al., 2010b).

The higher feed intake of D- birds was explained as an attempt to compensate for their poor feed efficiency and thus the lack of energy obtained from the diet (Carré et al., 2008). Differences between lines for feed efficiency, at the 9th generation, were observed to be significantly different from 7 to 21 d and 21 to 53 d when birds reached the market age, but not from 4 to 7 d (de Verdal et al., 2010b). Differences in feed intake were observed to be significantly different from 4 to 7 d.

Heritability of zootechnical performance showed a moderate to high value at the 8th generation, ranging from 0.21 for FCR, 0.30 for WG and 0.47 for feed intake (De Verdal et al., 2011b), near to those obtained at the 2nd generation, with 0.27-0.32 for FCR, 0.31-0.35 for WG and 0.47 for FI (Mignon-Grasteau et al., 2004). These results are consistent with the high genetic correlations estimated between FCR and AMEn, which ranged between -0.77 and -0.99, and with the low correlations between AMEn and body weight at 3 weeks of age (0.10 to 0.24, Mignon-Grasteau et al., 2004; de Verdal et al., 2011b).

Moreover, it is noteworthy that D+ and D- lines showed different behaviours towards a new environment and diet (Pelhaitre et al., 2012).

Digestive tract anatomy and physiology

Studies on characterization of these lines showed anatomical and physiological differences in all the parts of the digestive system which together have major complementary roles. Analysis of covariance with feed intake as covariate showed

that the quantity of feed passing through the gastrointestinal tract cannot be the only cause of difference between the lines (de Verdal et al., 2011b).

Upper part of the digestive tract: crop and stomach

The crop

Digestion of feed in chicken begins in the crop where diet is humidified and is mixed with some enzymes coming from digestive reflux, feed ingredients and microbiota (Duke, 1986 ; Denbow, 1999). Bacterial amylase coming mainly from *Lactobacillus* species has been implicated in starch hydrolysis (Szylit et al., 1980; Champs et al., 1983).

For the D+ and D- lines, observation of crop showed no difference in relative weight at 3 weeks of age or in pH of its content at 7, 21 or 53 d (de Verdal et al, 2011ab). A trend for a higher retention time of fine particles (+88%) in D+ birds was observed at 9 d of age, but only numerically higher at 29 d (+ 43%) with a maize diet (Rougière et Carré, 2010). Differences may be more important with wheat diet, as shown by higher difference in digestive efficiency. Although the absence of line effect on the relative weight of the crop, heritability estimates of this trait for the animal of the 8th generation at 23 d showed a moderate value (0.21).

The proventriculus-gizzard complex

In birds, the stomach is composed of two parts: the proventriculus or glandular part where hydrochloric acid and pepsinogen are produced; and the gizzard or muscular part, where digesta are ground by muscular contractions. Particles are directed through the pylorus to the small intestine as they reach a critical size of about 0.5-1.5 mm (Ferrando et al., 1987). The pylorus is not a sphincter as it allows reflux from the duodenum to the gizzard. This organ is more developed when diet includes coarse particles as whole cereals (Gabriel et al., 2003; Svihus, 2011). Chemical and enzymatic hydrolysis of proteins occurs in the gizzard thanks to the low pH and pepsin, which is active within a large range of pH in the chicken (Crévieu-Gabriel et al., 1999). It allows hydrolysis of the major dietary proteins as well as of the surrounding protein matrix of starch granules in wheat with high hardness value. The mechanical action of the gizzard allows cell wall breakdown and particle size reduction of dietary components, leading to an increased area for improved contact between enzymes and substrates.

It is in this compartment that the differences between D+ and D- birds are the most striking. The relative weights of the proventriculus and gizzard are higher in D+ birds than D- birds, +21.9% and +34% respectively, at 3 weeks of age at the 8th generation (de Verdal et al., 2011b). However a great variability is observed in the

proventriculus weight of D+ birds due to the presence of enlarged proventriculus in some families of D+ birds (Rougière and Carré, 2010; de Verdal et al., 2011b), as observed with ground diet contrary to whole cereal diet (O'Dell et al., 1959; Gabriel et al., 2003; Taylor and Jones, 2004). This difference is absent at hatch and appear after contact with the feed as soon as 7 d, reach a maximum at 3 weeks of age and disappear at 8 weeks with wheat diet (Péron et al., 2006; Garcia et al., 2007; de Verdal et al., 2010b). When fed with a maize diet, the difference was considerably reduced but persisted from 9 to 63 d (Rougière and Carré, 2010).

Evolution of gizzard weight with selection on AMEn is consistent both with its high heritability (0.53) and its positive genetic correlation with AMEn (0.43; de Verdal et al., 2011b). Proventriculus weight was also positively correlated with AMEn (0.59) and with gizzard weight (0.26-0.81; Rance et al., 2002, de Verdal et al., 2011b), which explains its evolution in these lines, despite its poor heritability (0.09).

At the 8th generation, pH of gizzard content was lower in D+ birds than in D- birds at 3 weeks of age (de Verdal et al., personal communication). 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), however the pepsin activity in digestive content is not known.

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 (N. Rideau, personal communication.). This is the region where are located the interstitial cells of Cajal, the pacemaker of gizzard contraction (Reynhout and Duke, 1999). In D- birds, the isthmus mucosa has a more oval shape, is 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. For D- birds, the lower mean retention time can be explained by a failure in the gizzard relaxation process of these birds during resting periods (no access to diet or light) (Rougière et al., 2012). According to Rougière and Carré (2010), the mean retention time in the proventriculus-gizzard system was a major factor associated with genotype differences between the D+ and D- genetic lines.

These studies with D+ and D- lines bring new knowledge related to digestive physiology, concerning the role of gizzard. For a long time, the importance of gizzard in the optimal digestion of proteins and lipids in the chicken has been recognized, by showing decrease of digestion in gizzardectomized chicken especially with coarse particles (Fritz et al., 1936). Several studies showed that a greater development of the gizzard is related to improved digestibility (Hetland and Svihus, 2001; Ravindran et al., 2006). In D+ and D- lines, the individual relative weights of the gastric compartment were found to be strongly positively linked to retention times

(Rougière and Carré, 2010). This is in agreement with the observation that a longer gizzard mean retention time (adjusted to a mean body weight) is associated with a heavier gizzard in Leghorn than in broiler chickens (Shires et al., 1987). Moreover, a significant positive relationship was observed in D+ and D- lines between the retention time of fine feed particles in the proventriculus-gizzard system and the digestive efficiency assessed by measured AMEn / calculated AMEn or by faecal digestibility of proteins (Rougière and Carré, 2010). Thus gastric retention time was proposed as a major limiting factor for digestive efficiency in chickens. Furthermore, retention time in the stomach of D+ and D- lines was modified by the introduction of fibre in the diet, but with a different effect according to line: fibre decrease transit time in D+ birds, and increase it in D- birds (Rougière and Carré, 2010). According to these results, it has been hypothesized that the critical size controlling gastric emptying depends on rheologic properties of feed particles of the diet, but also on genetic factors. However retention time seems not to be the only major limiting factor as decreased retention time with fibre in D+ birds led to decreased AMEn and protein digestibility, but in D- birds, the increased retention time with fibre led to increased protein digestibility, but decreased AMEn.

Middle part of the digestive tract: the small intestine

The small intestine is the site of hydrolysis of feed compounds by pancreatic and intestinal enzymes with the help of bile acids for lipids. The intestinal surface area is highly developed in order to increase the contact between its epithelium and components in the digestive tract, and to allow for final hydrolysis and absorption by enterocytes.

The higher feed intake of D- birds contributes to higher intestinal content in these birds (+54%, Garcia et al., 2007). This higher intestinal content may in part be responsible for the higher development of intestinal segment tissue to cope with higher digesta quantities to process. However, statistical analysis that included feed intake as a covariate showed that it is not the only factor responsible for the difference in the digestive tract (de Verdal et al., 2011b). According to Rougière and Carré (2010); the intestine enlargement observed in D- compared to D+ birds was probably an adaptive process trying to counteract the low digestive efficiency in D- birds in the upper part of the digestive tract. It can also be an adaptation of D- birds compensating for their higher sensitivity to the negative effect of viscosity of arabinoxylan on absorption (Garcia et al., 2007). This higher development is facilitated by the availability of space in the rib cage due to lower stomach development in these birds.

The contrast in development between the upper and middle part of the digestive tract is consistent with the moderate negative genetic correlation between gizzard

weight and jejunum density (-0.56) (de Verdal et al., 2011b). This is in agreement with results showing lower development of gizzard and higher development of small intestine with ground wheat instead of whole wheat inclusion (Gabriel et al., 2003), or with a diet with fine instead of coarse particles (Nir et al., 1994). The difference in small intestine between D+ and D- lines appeared later than those of the gizzard, being not observed at hatching and 7d, but detected at 9 d with maize diet, and persisted until 53 and 63 d with wheat or maize diet, although the difference between lines was considerably reduced at the end of the rearing period (de Verdal et al., 2010ab; Rougière and Carré, 2010). This delay in divergence in the upper and lower part of the gastrointestinal tract is consistent with the hypothesis that the small intestine grows in response to the functional efficiency of the gizzard. One can assume that the difference in small intestine size is not due to a delay in development of the digestive tract at the beginning of life, as observed with delay in feed accessibility to young chicken (Noy and Uni, 2010), as the yolk sac relative weight is not significantly different between the two lines at hatch (de Verdal et al., 2010a), and none were empty (de Verdal, personal communication).

This higher relative intestinal weight in D- birds at 3 weeks of age concerns each of the three segments, although less pronounced in duodenum (+15%) than in jejunum (+37%) and ileum (+40%) (de Verdal et al., 2011b). This discrepancy between the 3 segments could be due to the fact that the absorption process predominates in the jejunum and ileum (Denbow, 1999). This higher relative weight is due to increased length (+3%, +6% and +4%, for duodenum, jejunum and ileum respectively) but mainly to increased density (weight to length ratio) (+12%, +30% and + 31%, for duodenum, jejunum and ileum respectively). Heritability estimates of intestinal traits for birds in the 8th generation at 23 d showed high values for weight, length and density of these 3 small intestinal segments (from 0.28 to 0.50) (de Verdal et al., 2011b). High positive genetic correlations were observed between the weight of the duodenum, the jejunum and the ileum (0.62 to 0.88), as observed by Rance et al. (2002). This implies a parallel evolution of the 3 intestinal segments.

The increased density of intestinal segments may be explained in part by changes observed at the microstructure of the intestine. Indeed, D- chickens have higher villi height in the jejunum, greater villus area and crypts size in the three intestinal segments (de Verdal et al., 2010a). Moreover, the tunica muscularis is thicker in the three intestinal segments of D- birds that may be due to higher intestinal content to move along the intestine (de Verdal et al., 2010a).

The higher villi area of D- birds may suggest a higher potential of membrane hydrolysis and absorption in their intestine. Moreover, in the jejunum, they may have a higher efficiency as higher villi may mean more mature enterocytes, as enterocytes mature when moving up the villi. Although increased development

in absorptive surface in the intestine was observed, D- birds are not able to compensate for their lower proventriculus-gizzard functionality, as shown by their lower total tract digestibility of starch, proteins and lipids. This may be due to a lower digestive functionality of epithelium. This may also be due to a lower accessibility of nutrients in the small intestine of D- chickens. This may be in part due to a limitation in absorption capacity, as shown by the pronounced effect of xylanase in a wheat diet on conjugated bile salts in D- birds supplemented with antibiotics, as viscosity primarily acts on absorption (Garcia et al., 2007).

The higher development of crypt size in D- birds, may indicate a more intense cell production, necessary for higher villi development. No difference between lines was observed for the villus height / crypt depth ratio, showing a similar balance between membrane hydrolysis / absorptive potential and cell turnover in the two chicken lines.

More goblet cells per villus were observed in D- birds in jejunum and ileum, which may lead to higher mucin secretion (de Verdal et al., 2010a). This may be due to higher need to protect the epithelium due to heavier digesta content or protection against microorganisms (Forstner and Forstner, 1994; Laboisse et al., 1996; Johansson et al., 2011). Other mechanisms of innate immune system may be stimulated, such as lymphocyte cells, and contribute to higher density of intestinal tissues of D- birds in jejunum and ileum. This higher mucin production may lead to higher endogenous wastes, as these glycoproteins are difficult to hydrolyze, and may thus contribute to lower apparent protein digestibility of the D- birds. Moreover if this increase mucin production leads to a thicker mucus layer, although a higher number of goblet cell does not necessarily lead to a thicker mucus layer, it may restrict nutrient absorption (Iiboshi et al., 1996), contributing to lower digestibility. This may explain the high negative genetic correlation observed between AMEn and small intestinal weight segments, jejunum (-0.67) and ileum (-0.77) (de Verdal et al., 2011b).

All these modifications at the intestinal level might lead to a higher maintenance cost for D- birds as the intestinal epithelium has a high turn-over, and mucin production represents an energy cost, and this may contribute to the lower feed efficiency of the D- birds.

D+ birds fed with a maize diet had a higher relative weight of the pancreas (+15%), which was positively associated with the gizzard weight (Rougière and Carré, 2010). It suggests a common pathway for regulation of growth of the pancreas and gizzard. However, with a wheat diet, difference lower relative weight of pancreas (-16%) was observed in D+ birds (Péron et al., 2007). In the latter study, proteolytic activity in this organ expressed per body weight, was lower in D+ birds when wheat was of low hardness value, but without a difference when wheat was of high hardness value. No data is available in regards to digestive enzymatic activities in intestinal contents.

Digestive contents of D+ and D- lines showed some differences in their composition in terms of pH and bile salts. pH of the intestinal content showed no difference between lines at 7 d, but was higher for D+ birds in duodenum and ileum at 21 d and in ileum at 53 d (de Verdal, personal communication), which is the opposite to what was observed in the gizzard content, as was observed with whole wheat feeding (Gabriel et al., 2003).

Intestinal contents of D+ birds showed more conjugated bile acids and total bile acids (Garcia et al., 2007). This may be due to higher synthesis or lower degradation by digestive microbiota (Garcia et al., 2007) and probably explains partly the difference in lipid digestibility between the lines. However, supplementing diet with xylanase and antibiotics together from 8 d of age did not suppress the difference in lipid digestibility between lines despite similar bile acid levels. This means that this difference in digestion efficiency between lines could not be explained only in terms of bile salts. Other limiting factors should exist in D- birds that relate to the secretion of digestive enzymes or absorption capacities (Rougière et al., 2010).

No difference in transit time was observed between the lines when fed a maize/soyabean diet (Rougière and Carré, 2010). However, with wheat the results may be different.

Besides the composition in small intestine in terms of pH and bile acids that differed between the two lines, starch, protein and lipid content are probably not the same, as suggested by the differences in faecal digestibility between the two lines, and the different gap between digestibility of starch, proteins and lipids of the two lines with larger difference for lipids, intermediate for starch and lowest for proteins with wheat diet as characterized at the 5th generation (Carré et al., 2007). Thus, undigested compounds of D+ birds are lower and may be relatively more concentrated in protein, and conversely undigested compounds of D- birds are more important and may be far more concentrated in starch. It must however be taken into account that the undigested components measured at the faecal level, as performed until now for the evaluation of digestibility in the D+ and D- lines, are only an approximation of components at the end of the small intestine. Indeed it does not take into account further bacterial metabolic modifications. Indeed bacteria use the undigested components in the lower digestive tract with additional urinary products as substrates leading to bacterial biomass, with a specific composition[2] and bacterial products as SCFA. Despite these microbial metabolic activities, a difference between composition of small intestinal content of the two lines can still be presumed, due to the relative low modification of microbiota in birds compared to mammals, although not negligible, as assessed

2 About 50-70% of crude protein, 8-10% lipids and 20-25% saccharides with structural and exo-polysaccharides and nucleic acids, but with variability according to species

by its relatively low contribution in excreta, estimated to be 11% of dry matter and 25% of protein in chickens (Parsons et al., 1982).

Contrary to these differences in chemical composition of intestinal content, and despite differences in gizzard development in the two lines, distribution of particle size in the digestive contents of the ileum of the two lines after feeding a diet with wheat of high or low hardness values, showed no significant difference (Péron et al., 2007).

Lower part of the digestive tract: Caeca/colon

The lower part of the digestive tract has been less studied in the D+ and D- lines than the upper and middle part, as it is widely accepted that in birds, major digestive processes occur in the upper and middle parts, with the lower part being relatively small compared to that of mammals.

The caeca are two blind sacs at the end of the small intestine. Villi present in the proximal area of caeca act as filters allowing entrance of only liquids and small particles of the digesta. Moreover urine can backflow from the cloaca. Caeca are the site of reabsorption of water and electrolytes, immune cell production, and the major site of bacterial fermentation in chicken and may contribute to energy extracted from the feed by the host-microbiota association, thanks to reabsorption of bacterial metabolites.

Although less studied, some data are available about the caeca of D+ and D- birds. At the 8th generation, at 3 weeks of age, higher digestive contents were observed in the caeca in D+ birds (+80%; H. de Verdal, personal communication) and at 4 weeks of age with maize / soya diet a higher relative tissue weight (+29%; Rougière and Carré, 2010). Moreover higher transit time was observed in D+ birds (x2; Rougière and Carré, 2010). Thus at 3 weeks of age, caecal functions appeared more developed in D+ than in D- birds, which may contribute to higher energy extracted from the diet, and thus higher AMEn.

Moreover, as explained previously for small intestinal composition, one can expect that the differences in composition of caecal contents in terms of starch, proteins and lipids between D+ and D- birds are similar to the difference in composition of excreta, despite modification by bacterial metabolism as explained before, and filtration of digesta at the caecal entrance. Thus contents may be relatively rich in protein for D+ birds and conversely relatively rich in starch for D- birds. Moreover, concerning the caeca, urinary compounds composed mainly of uric acid are present, and may be more concentrated in D+ than D- birds (De Verdal et al., 2011c).

Concerning the colon, also named rectum in birds, it has a short size, lower than caeca, and lower retention time. It may be implied in water and electrolyte

reabsorption as well as SCFA and other bacterial metabolites, in a lower extent than the caeca. This digestive part has been little studied in birds due to its low size, and has not been studied in D+ and D- lines.

All the changes observed in the digestive tract morphology and physiology lead to a change in terms of available substrates and digestive environment such as pH, those parameters being implied in digestive microbiota development.

CONSEQUENCES OF SELECTION ON AMEn ON DIGESTIVE MICROBIOTA

Digestive microbiota of D+ and D- birds

Digestive microbiota of the two divergent chicken lines was studied in birds from the 10th generation, in two digestive segments, the small intestine, more precisely the terminal ileum and the caeca. For these studies, birds were reared on litter for the first days of life, and placed in individual cages after 10 days of life, as in other works on these lines for AMEn determination. The analyses showed clear differences between microbiota of the two lines in both the digestive contents and in the mucosa.

In the ileum contents, no significant difference in total bacteria load per gram of fresh weight was shown with a mean value of 4.28×10^{10} copies of 16S rDNA/g (Konsak et al., 2012). As small intestine content is 50% more important in D- birds (Garcia et al., 2007), the total bacterial biomass in the small intestine is expected to be higher in D- birds. Comparison of bacterial fingerprint, which provides an overview of the major bacteria, showed variability between animals of the same line was slightly higher in D+ birds (Gabriel et al., 2011; Konsak et al., 2011). Moreover, statistical analysis of the fingerprints of the two bird lines showed significant difference between the two lines. More precisely, 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 (Konsak et al., unpublished data). Moreover, quantitative analysis of the main bacterial groups found in the digestive tract of chickens (*Lactobacillus* and *Clostridium* genus, for the main phylum Firmicutes, *E. coli* species for the phylum Proteobacteria, and *Bacteroides* for the phylum Bacteroidetes) of this microbiota, showed difference (Konsak et al., 2012). Thus, D+ chickens showed a higher amount of *C. coccoides* and D- chickens a higher amount of *E. coli*.

In the caeca contents, as in the ileum contents, no significant difference in total bacteria load per gram of fresh weight was shown with mean value of 4.36×10^{11} copies of 16S rDNA/g (Konsak et al., 2012). As caecal content is 80% higher in D+ birds (H. de Verdal, personal communication), the total bacterial biomass in this organ is expected to be higher in D+ birds compared to D- birds, and clearly higher than those of their small intestine, in contrast to D- birds that may have a similar or slightly higher bacterial load in the small intestine than in their caeca. Conversely to the ileum content, variability between microbiota of animals of the same line was lower in D+ birds (Gabriel et al., 2011; Konsak et al., 2011). 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 (Konsak et al., unpublished data). Moreover, quantitative analysis of the main bacterial groups showed in D+ birds more *C. leptum* group, and in D- birds more *Lactobacillus*, and particularly *L. salivarius*, a dominant lactic acid bacteria in the broiler digestive tract (Engberg et al., 2000; Souza et al., 2007; Gong et al., 2007), and more *E. coli* (Konsak et al., 2012).

In the ileal mucosa, no difference in total bacteria load per tissue between lines was observed in the distal part of ileum with 2.49×10^{9} copies of 16S rDNA/segment (Konsak et al., 2012). However, the concentration in the mucus layer may be different. Indeed, higher digestive content and higher tissue weight implied higher mucosa area. Moreover, a higher number of goblet cells in villi in D- birds may lead to a higher mucus layer thickness. Thus a lower bacterial concentration in mucus matrix may be assumed. Quantitative analysis of the main bacterial groups of the digestive tract of chicken, showed in D- birds, more *L. salivarius* (Konsak et al., 2012).

In the caecal mucosa, no difference in total bacteria load per tissue between lines was observed with 7.66×10^{9} copies of 16S rDNA/segment (Konsak et al., 2012). However, as this tissue is more developed in D+ birds, mucosal area is higher. Moreover, as explained previously, bacterial load is expected to be higher, which may lead to higher thickness of the mucus layer(s) to protect the epithelium. In consequence, the bacterial density in the mucus layer may be lower in D+ birds. 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 (Gabriel et al., 2011; Konsak et al., 2011; Konsak et al., unpublished data). In this mucosa, quantitative analysis of the main bacterial groups, showed more total *Lactobacillus*, as well as *L. salivarius* and *L. crispatus* in D- birds (Konsak et al., 2012).

To go further in the characterization of the digestive microbiota, by quantification of the main bacterial groups, a study was performed on a F2 cross between D+ and D- lines and on a high number of birds (144 animals) with high range of AMEn (from 7.6 to 16.1 MJ/kg) (Gabriel et al., unpublished data). The study was focused on one of the more discriminant biotope observed previously,

caecal content (Gabriel et al., 2011). Significant relationships were observed between a faecal nutrient component, starch content and the concentrations of caecal bacteria, *Lactobacillus* and ratio between *Lactobacillus* and *Clostridium*. A higher starch content is thus associated with a higher development of *Lactobacillus*, especially *L. crispatus* and *L. salivarius*, and with higher ratios of *L. crispatus* to *C. leptum* and of *L. salivarius* to *C. leptum*, and conversely with a low ratio of *C. leptum* to *Lactobacillus*. Significant heritability estimates were observed for bacterial numbers, or higher for bacterial ratios. 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. These results obtained on F2 cross of divergent genetic lines confirmed previous studies in mammals as well as in chickens that proposed that digestive microbiota may be dependent on host genetics. For future studies, it would also be interesting to evaluate the importance of genetics of the host on microbiota development under different diets, as other studies showed that chicken microbiota development was affected by the diet.

Are the differences of microbiota only the consequence of digestive biotope modification?

The differences in digestive microbiota between the two lines of chickens selected on digestive capacity may be partly due to the consequence of change of biotopes of the digestive segments due to differences in digestive physiology and intestinal contents.

As indicated previously the inoculum of middle and lower parts of the digestive tract is the microbiota of the crop. At present, no statistical difference between the two lines was observed at the level of this organ, except a trend to a higher retention time in D+ birds at 9 d but not at 29 days with maize diet (Rougière and Carré, 2010). The microbiota of this organ has not been studied until now.

Going down to the following segments of the digestive tract, microbiota undergoes the acidic pH of the stomach. As mentioned previously, in D+ birds, the contents have a lower pH and a higher retention time, leading to more deleterious conditions for bacterial survival in these birds. After this chemical selection of bacteria, leading to high reduction of their number, some of them persist due to development of acid survival systems (Jensen et al., 2012; Hong et al., 2012a; Ramos-Morales, 2012).

In the small intestinal contents, conditions of bacterial growth became more appropriate, especially towards the distal part. Microbiota development depends on

environmental conditions, digestive transit, presence of inhibitors, growth factors, predators, and available substrates (from exogenous and endogenous origins).

As stated previously, these environmental conditions are not the same in the small intestine of D+ and D- birds. In D+ birds, pH was higher than in D-birds, which may affect bacterial balance. In D- birds, if the higher quantity of goblet cells in villi leads to a thicker mucus layer, it may have a negative effect on oxygen diffusion from blood vessels (Saldena et al., 2000) leading to lower oxygen concentration in digestive content, and thus on bacterial balance according to their sensitivity to oxygen. Substances such as bile acids can inhibit bacterial growth. In D+ birds, these acids contents are higher, which gives them higher power to control microbiota growth in the small intestine.

Apart from the environmental conditions in the small intestine that differ between the two lines, available substrates are probably not the same. Firstly, due to the difference in digestibility between the two lines, substrates are present in higher amounts in D- birds. Secondly, as the differences in digestibility efficiency between lines are larger for lipids, intermediate for starch and lowest for proteins as characterized at the 5th generation by Carré et al. (2007), relative quantities of these compounds in the small intestine are probably different as explained previously. According to the concentration of these substrates, some bacteria groups are more adapted, according to their enzymatic equipment. Thus, as the intestinal content of D+ birds may be well balanced with relatively high concentration in proteins, this may explain the higher content in *Clostridium* able to use various substrates according to species and strains (Fonty and Chaucheyras-Durand, 2008). On the contrary, as the intestinal content of D- birds may be more concentrated in starch, which is favorable to *Lactobacillus,* and may explain the higher load of *L. crispatus*.

In addition to changes in proteins, saccharides and lipids composition of small intestinal content, the origin of these compounds and thus their susceptibility to hydrolysis may vary. They are composed of undigested dietary components and components produced by the host. The former depend on diet composition, and are composed of undigested dietary proteins, starch and lipids, and dietary fibre mainly coming from plant polysaccharides such as arabinoxylan of wheat. Components produced by the host are mucus, mainly mucin (glycoprotein with low sensitivity to hydrolysis, Carlstedt et al., 1993), desquamated cells from the digestive tract and dead bacterial cells. This second source of substrates represents an important source of proteins. It depends on mucus production and rate of cell turnover in the digestive tract (animal and microbial cells). In the case of D- birds, this part may represent a higher amount than in D+ birds as suggested by the higher number of goblet cells in villi of these birds and their higher crypt depth.

In the caeca, as for intestinal content, D+ birds may have a relative well balanced composition although richer in proteins, and may have higher content of uric acid, whereas D- birds may have a relative high starch concentration. In

the D+ chickens that are subjected to lower fermentation in the small intestine compared to D- birds, one can suppose that undigested digesta are composed essentially of components undigestible by the host enzymes such as non-starch polysaccharides and endogenous proteins, and may favour fermentation by certain bacteria, which could explain the higher development of caeca in D+ birds. As for the ileum, the caecal content composition may explain the preferential development of *Clostridium* in D+ birds and *Lactobacillus* in D- birds.

For the bacteria of the mucosa, present in the mucus layer(s), no difference in total load was observed between the two lines, as well as in the small intestine and in the caeca. However, as explained previously, a lower concentration in mucus matrix of the small intestine of D- birds, and of the caeca of D+ birds may be assumed. These differences may be due to modification of the composition of the major component of mucus, mucin. And yet, these glycoproteins represent binding sites for bacteria. Moreover mucin is a substrate for bacteria, as well as desquamated cells and molecules able to pass through the mucous gel. Quantity of desquamated cells may be changed if cell turn-over is affected by selection as suggested by higher crypt depth in the small intestine of D- birds. The reachable molecules in the mucus may be modified due to changes in animal digestion as well as bacterial fermentations in the digestive lumen. This may explain the higher development of *Lactobacillus* in the digestive mucosa of D- birds compared to D+ birds. Moreover changes in digestive physiology may lead to changes in physicochemical parameters, such as pH or redox potential, in the mucin matrix. It can also not be ruled out that AMEn selection may have led to selection on antimicrobial peptides as defensins, as the genetic of the host is implied in the level of gene expression of these peptides (Hong et al., 2012b). However, we do not have such information about the D+ and D- lines.

We thus saw that the differences between the D+ and D- lines in digestive efficiency, anatomy and histology of the digestive tract compartments may explain some of the differences in composition of their microbiota. Reciprocally, the digestive microbiota, as indicated at the beginning of this paper, has numerous effects on the digestive tract, and also on the whole physiology of its host and can thus influence digestive and feed efficiency of the host.

Effect of digestive microbiota of the D+ D- lines on the host

The contribution of digestive microbiota in the difference between D+ and D- lines was shown by the different effect of a high dose of antibiotic from 8 d of age in the diet of these chickens, with higher effects in D- than D+ birds on AMEn and growth performance (Garcia et al., 2007). The higher bacterial load in the small intestine of D- birds may be responsible for the lower pH of small intestinal contents and may lead to fermentation of diet compounds instead of

host digestion causing a diversion of nutrients to the bacteria to the detriment of the host. Thus an inverse relationship between small intestinal bacterial density and growth efficiency has been shown (Apajalahti et al., 2004). On the contrary, in the D+ birds, high fermentative activity in the caeca of undigestible compounds by the host, may lead to higher energy extracted from the diet.

The bacteria may be responsible for the higher epithelium development of the small intestine of D- birds as certain strains have an effect on epithelial cells. It may also be an adaptation of the host to compete with bacteria for the use of dietary compounds. However, the microbiota may increase the integrity of the epithelium through upregulation of cross-bridging proteins (Hooper et *al.*, 2001), and thus decrease intestinal absorption as in birds, paracellular absorption is an important way of nutrient absorption (Caviedes-Vidal et al., 2007). Moreover, the effect of microbiota on intestine could also pass through a stimulation of the intestinal immune system, which is the most important immune system of the body. As presented before higher level of bacteria in the small intestine of D- birds compared to D+ birds may be responsible in part to the higher goblets cells in villi of the D- chickens, as they can stimulate mucin production (Sakata and Setoyam 1995), contributing to innate intestinal immune system. This increased bacterial load may also lead to inflammation and increase expression of antimicrobial peptides as defensins (Menendez and Brett Finlay, 2007). Thus digestive microbiota may contribute to the higher relative weight and density of the small intestine of D- birds.

This high development of bacteria may lead to higher amounts of harmful products that need to be detoxified and may contribute to higher liver relative weight in D- birds (+2.6%; de Verdal et al., 2011b). Moreover the low level of bile acids in the small intestine of D- birds may be in part due to their deconjugation by bacteria such as *Lactobacillus* (Maisonnier et al., 2003; Kim and Lee, 2005), which are present in large quantity in these birds. Consistently with this hypothesis, the beneficial effects of high dose of antibiotics on conjugated bile acids content is higher in D- than in D+ line (+109% and +36% respectively), and consequently improved lipid digestibility and AMEn more in D- birds (+35% and +14%) than in D+ birds (+5.7% and +2.6%, Garcia et al., 2007). Indeed although *Lactobacillus* genus is more often seen as beneficial bacteria, some species and strains can also have negative effects (Guban et al., 2006). Our results obtained on D+ and D- lines, are in agreement with results obtained by Moore et al (2011) with commercial broiler chickens of high and low AMEn (12 birds of the quarter higher and 12 birds quarter lower of a group of animals; +3.5%) showing more *Lactobacillus* in jejunum mucosa of low AMEn birds. This deconjugation of bile acids by microbiota may also contribute to the higher liver weight of D- birds due to extra synthesis of these compounds. Moreover it cannot be excluded that the basal endotoxemia due to commensal bacteria leads to modification of hepathic metabolism with consequence on bile acid synthesis (Beno et al., 2003; Cani et

al, 2007). All these extra-syntheses in D- birds may contribute to a lower feed efficiency.

Conversely, even if *E. coli* is often seen as deleterious bacteria, which may be the case for the detected strain in the caeca content of D- birds, the one detected in D+ birds caecal mucosa may have beneficial effects as some *E. coli* strains are used as probiotics (Zschüttig et al., 2012).

In the same manner as for *E. coli*, some species and strains of *Clostridium*, more frequent in D+ bird digestive tract, can have positive effects, despite this bacterial group often being associated with negative effects. This is for example the case of the long segmented filamentous bacteria present in a higher amount in D+ bird ileum content. This bacteria belongs to cluster I of *Clostridium* and is a common inhabitant of intestinal mucosa in mammals and birds such as the chicken (Snel et al., 1995) and was also found in jejunum contents of chickens (Lu et al., 2003). It is implicated in the development of the intestinal immune system, intestinal motility, and the development of intestinal epithelial cells. *C. coccoides* and *C. leptum* are also in higher number in the digestive tract of D+ birds. These bacteria are considered beneficial as they produce SCFA, such as butyrate contributing to the maintenance of intestinal health (Scheppach, 1994; Eeckhaut et al, 2011). In the chicken, the *C. leptum* group is mainly represented by bacteria *Faecalibacterium* that has several beneficial effects on intestinal tract health (Bjerrum et al., 2006, Lund et al., 2010). By their fermentative products such as SCFA they may contribute to energy from the diet for the host. Thus, by using the F2 cross between D+ and D- lines, links between AMEn and caecal digestive 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*). The observation of associations between caecal microbiota and AMEn with the F2 cross between D+ and D- lines corroborates the results of Torok et al. (2011), obtained with birds of a commercial line, showing correlations between fingerprint of digestive microbiota of ileum and caeca contents and AMEn. These results obtained with D+ and D- lines also confirm results obtained by Moore et al. (2011) by using high and low FCR broiler commercial chickens showing more *Clostridium* in caecal contents of high AMEn birds. Moreover, this F2 cross of D+ and D- lines shows that a significant amount of variability in AMEn can be explained by some components of caecal microbiota. Thus, *L. salivarius* number can explain significantly 9% of this variability, with a negative effect. Similarly the bacterial ratio of Log *L. salivarius* to Log *C. leptum* explains a greater part of the variability (13%), with a negative effect.

It cannot be excluded that the microbiota of D+ is responsible for a decrease in viscosity of the small intestinal contents as some bacterial strains of the digestive tract of the chicken can hydrolyze non-starch polysaccharides (Mead, 1997; Beckmann et al, 2006). However at the present day, no data on intestinal viscosity of D+ and D- lines are available.

Besides the effect of different microbiota of D+ and D- lines on the digestive tract, it may be responsible for effects out of the digestive tract. It can thus be hypothesized that the different behaviour of D+ and D- lines (Pelhaitre et al., 2012) may in part be explained by their different digestive microbiota due to the effect of microbiota on behaviour (Lyte, 2010; Diamond et al., 2011).

Results on digestive microbiota of D+ and D- lines showed that, more than the absolute quantity of bacteria of each group, it is the equilibrium between the different bacterial groups that plays a role in digestive efficiency. Indeed, ratios of different bacteria groups affected more AMEn than quantity of each group, and the ratios were also more controlled by the host, as shown by their higher heritability. This can be explained by the fact that the effect of microbiota is not due to a group of bacteria, but to their interactions. Indeed, as indicated previously in this paper, digestive microbiota is a complex equilibrium between numerous species and even bacterial strains. More precisely, it is the combination of their activity that lead to the global effect of microbiota. Indeed, it is not the presence of the bacterial species that is important for the effect of microbiota, but the activities of all of these bacteria in this complex ecosystem.

Conclusion

The higher digestive efficiency of D+ birds compared to D- birds, fed with a wheat-based diet, appears to be linked to their digestive physiology, while D- birds are limited in their capacity, but also to their high adaptability to specific components of wheat, as shown by the diet-dependent differences between lines.

In D+ birds, the higher development of the proventriculus-gizzard complex leading to higher retention time and thus lower pH, leads to higher digestion in this upper part of the digestive tract, and has been proposed as the major factor responsible for the higher digestive efficiency of D+ birds. This high digestibility in the upper part of the digestive tract may lead to easily digestible compounds in the small intestine, that may be quickly hydrolyzed by bird digestive enzymes and absorbed, leading to a low amount of available substrates for digestive microbiota, with a composition relatively equilibrated although high in protein,