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PhD thesis

STUDY OF THE CHICKEN PHYSIOLOGICAL PROFILE IN DIFFERENT GUT SITES AND UPON DIFFERENT ENVIRONMENTAL FACTORS

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I want to thank my family, my friends, my supervisors and my colleagues
in having attended me during this important experience

Abstract

Physiological aspects of chicken intestinal tracts are key targets of study due to their important repercussion on productive yield and animal health. Characterization of chicken gut, including spatial variations in microbial community, gut-associated immunity and relation with diseases has highlighted the importance of gut homeostasis and its sensitivity to external factors like stressors or dietary strategies. In this context, a survey on molecular mechanisms and differential gene expression along gut sites may deepen knowledge on functional aspects and provide hints for actions for maintaining gut homeostasis and testing various experimental factors.

This thesis aimed to analyze at molecular level chicken gut physiological profile and possible functional differences between tracts, to highlight potential biomarkers linked to factors influencing gut status. In this view, microarray analysis has been chosen to realize a wide exploration of chicken gut in its gene landscape.

Trial 1: in an exploratory perspective, aim of the study was to evidence the differential tissue gene expression of jejunum and of cecum of chickens at 42 days of age. Genes and enriched gene clusters for biological functions were differentially represented in the two tissues. In jejunum, expected genes and functions related to nutrient metabolism emerged, with interesting correlations to mammals and new key aspects like sulfur transport-related genes and immune pathways tuning. In cecum, gene clusters emerged, as already seen in humans, such as cell turnover related-genes, and genes linked to sulfation for gut barrier maintenance. Results indicated the different functions of the two tissues and revealed key aspects for possible new in-depth investigations in chickens.

Trial 2: to apply former trial hints to possible in-field conditions affecting poultry gut and health, the study aimed to investigate effects of a GOS prebiotic *in ovo* injected in counteract the detrimental effect of the heat-stress in growing chickens. Energetic metabolism-related gene sets were enriched in GOS, mainly in jejunum, and lipid metabolism-related gene sets in GOS might have contributed in gut barrier maintenance with less immune system activation, mainly in cecum. No differences in blood parameters were seen. Only few butyrate-related bacteria were increased by GOS, while heat stress confirmed its effect on microbial imbalance. Without considering thermal treatment, GOS showed to induce a long-term effect on transcriptomic profile of jejunum and cecum. Instead, GOS had no additional efficacy in counteracting heat stress compared to control group.

In conclusion, the microarray utilization was confirmed to differentially highlight functional characteristics between gut tracts in chickens, with possibility to identify useful gene biomarkers. Different functions along gut tracts were highlighted also in the second study with the *in ovo* injection of GOS, with positive effects on gut transcriptome. When applied to the experimental model of heat stress, chosen as stress model due to its important role in production diseases, especially in the

Mediterranean area, the intestine seems to response in a homogeneous way along the different tracts, without considering the *in ovo* treatment. The concomitant analysis of microbiota in cecum offered the possibility to correlate changes in microbial community with functional genes, to identify new potential biomarkers of gut homeostasis in different conditions.

Abstract

La caratterizzazione fisiologica dei diversi tratti intestinali del pollo è un importante oggetto di studio viste le sue ripercussioni sulle prestazioni produttive e sulla salute degli animali. Tale caratterizzazione, dalla variazione nella composizione microbica lungo i tratti intestinali, alla risposta immunitaria fino al coinvolgimento dell'intestino in diverse patologie, ha evidenziato l'importanza dell'omeostasi intestinale in relazione a fattori esterni, quali stress o strategie nutrizionali. In tale contesto, un'analisi sui meccanismi molecolari e di espressione genica nei diversi tratti intestinali potrebbe rivelare nuovi aspetti funzionali utili al mantenimento dell'omeostasi e per testare diversi fattori sperimentali.

Questa tesi ha analizzato a livello molecolare il profilo fisiologico intestinale nel pollo considerando le possibili differenze tra i tratti e con lo scopo di evidenziare possibili marcatori collegati a fattori che possono influenzare lo stato dell'intestino. A tal fine, l'analisi tramite microarray è stata scelta per effettuare un'approfondita esplorazione a livello genico nel pollo.

Studio 1: a scopo esplorativo, si è cercato di evidenziare possibili differenze a livello di espressione genica e relativa funzione biologica tra digiuno e cieco in polli di 42 giorni di età. Nel digiuno si sono evidenziati dei gene set arricchiti nell'espressione di geni relativi al metabolismo dei nutrienti, riscontrati anche i mammiferi, così come relativi all'assorbimento dello zolfo e all'attivazione del sistema immunitario. Nel cieco sono emersi alcuni gene set riscontrati anche nell'uomo, inclusi geni relativi al turnover cellulare e all'utilizzo dello zolfo nel mantenimento della barriera intestinale. Le differenziazioni emerse fra i due tessuti possono rappresentare punti di partenza e spunti per ulteriori studi nel pollo.

Studio 2: volendo applicare le potenzialità del primo studio, in questa prova si è analizzato l'effetto di un prebiotico (galacto-oligosaccaride, GOS), iniettato *in ovo*, in polli successivamente allevati in condizioni da stress da caldo, uno dei maggiori problemi nell'area mediterranea. L'iniezione *in ovo* ha avuto un effetto a lungo termine del GOS sul trascrittoma intestinale, evidenziando gene set arricchiti relativi al metabolismo energetico soprattutto nel digiuno, così come gene set relativi al metabolismo lipidico. Questi ultimi, insieme con gene set impoveriti relativi all'attivazione immunitaria, soprattutto nel cieco, potrebbero aver contribuito al mantenimento della barriera intestinale, anche senza evidenti differenze nei parametri ematici di infiammazione. Il prebiotico, nel cieco, sembra aver stimolato alcuni batteri coinvolti nel metabolismo dell'acido butirrico. Il prebiotico non è stato efficace nel contrastare lo stress da caldo.

In conclusione, il microarray ha evidenziato differenze funzionali tra i tratti intestinali nel pollo, sottolineandole anche nella prova di somministrazione *in ovo* del GOS, i cui effetti hanno influenzato positivamente lo stato intestinale in termini di trascrittoma. Lo stress da caldo in generale sembra aver

avuto un effetto costante rispetto ai parametri considerati, a prescindere dal trattamento in *ovo*, se si esclude il dato relativo al microbiota. La concomitante analisi di questo nel cieco ha permesso di formulare ipotesi su possibili correlazioni geniche funzionali, con la possibilità di identificare potenziali marcatori dello stato intestinale in diverse condizioni.

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Introduction

Introduction

1. Poultry industry and production

In the last decades poultry industry has faced an incredible growth thanks to the industry advances in genetics, feeding and disease control, so much so that poultry products (meat and eggs) have become pivotal in human nutrition as one of the most important sources for protein of animal origin worldwide. The economic interest of poultry industry experienced several changes along about the latter sixty years. The most important advantage for poultry industries was the progress on the genetic selection that allow in producing animals that yielded products of highest value, increases the body weight (BW), feed efficiency and carcass composition. The genetic selection led to a production cost lowering and allowed to obtain chicken products, defined as some of the least expensive protein sources in the world (Hammerstedt, 1999; Petracci and Cavani, 2012). From the initial demand for whole carcasses, about forty years ago market demand started moving towards cut-up portions, leading to a bird selection more focused on the carcass part yields (breast and legs). After, in the last two decades, market demand shifted more towards processed products and breast meat with a consequent adaptation by the poultry industry, which moved to a selection of heavier animals with high breast development and weight (Petracci et al., 2015). The nutritional profile (such as the high protein and low-fat content), sensory properties and flexibility for processing, combined with competitive prices compared to other meats have constantly increased the market demand from the middle of the last century until today. Poultry companies faced this increment increasing both the number of reared animals as well as its growth rate (GR) and feed efficiency. Together with the genetic enhancement, the efficiency of the vertical integration of the poultry supply chain has contributed in the modern poultry industry development. As further consequence of the growing poultry products consumption, and also considering the concept of poultry meat as "functional food" (due to its content in conjugated linoleic acid, polyunsaturated fatty acids, vitamins and antioxidants), higher standards of quality to improve meat sensory characteristics and functional properties are required (Havenstein et al., 2003a; b; Petracci and Cavani, 2012; Petracci et al., 2013a). Currently, the genetic progress has brought to have birds weighting around 3 kg of BW that can grow and become ready for the market in about one-half of the time compared to sixty years ago and having a breast yield of 3-4% higher than ten years ago (Havenstein et al., 2003a; b; Aviagen, 2014, 2017) (Table 1). In fact, most of the broiler selection has been directed towards the muscle mass increase, especially focused on breast muscle size, mainly due to the hypertrophy in muscle fibers that has been connected to the less blood supply and possible oxygen deficiency observed in new broiler lines.

Insufficient oxygen supply can also cause intestinal metabolic disorders, such as malabsorption syndrome, as well as higher susceptibility to infections (Scheele, 1997). Furthermore, a concomitant decrease in heart muscle size has been also observed: this condition obviously explains the less cardiac capacity observed in modern broiler lines and has been connected to the cardiovascular problems and relative disease susceptibility (Schmidt et al., 2009; Petracci et al., 2015). These problems are highly accentuated in broilers than in laying hen because of different genetic selection that, in meat-type chicken, has been addressed toward a higher feed consumption and very rapid development of muscle. As an example, laying hen (selected mostly for egg production) stop eating when their metabolism needs are met, while broilers (selected for meat production) continue in eating until their gut is not completely full. (Buzala and Janicki, 2016).

Table 1- Differences in body weight (BW) and breast yield (% of BW) in male chicken hybrids from 1957 up to 2017

Year	Hybrid	Age (d)	Body Weight (g)	Breast Yield (% of BW)
1957[1,2]	Athens Canadian randombred	42-43	591	11.5
2001[1,2]	Ross 308	42-43	2903	19.5
2014[3]	Ross 308	42	3023	22.5
2017[4]	Ross 308	42	3103	23.1

[1]Havenstein et al. (2003a); [2]Havenstein et al. (2003b); [3]Aviagen (2014); [4]Aviagen (2017)

1.1 Fast growing selection and related myopathies

Together with the strictly targeted selection for faster growing and heavier birds, it has been observed an increase in pectoralis major muscle abnormalities (i.e. deep pectoral disease, principal growth-associated myopathy), stress-induced myopathies and higher stress and disease susceptibility (Petracci and Cavani, 2012; Petracci et al., 2015). As reported by Sandercock et al., (2006), the high metabolic rate typical of these new heavier broilers hybrids may lead to metabolic disorders due to imbalances between energy and metabolites supply, resulting in a homeostatic dysregulation and cellular and tissue damage. However, it is not yet clear if only genetic selection is the main cause of these problems: in fact, as reported by Bailey et al. (2015), it seems that non genetic-environmental factors play an equally important or greater role in triggering myopathies.

In any case, it is well understanding that these problems have a high impact on product quality, economic aspect and animal health.

Compared to slower-growing birds, fast-growing hybrids show a modified skeletal muscle structure, with higher number of muscle fibers, fiber hypertrophy and abnormal fibers, which have been associated with the onset of inflammatory status and necrosis (Petracci et al., 2013b). In fact, Mazzoni

et al. (2015) observed pectoralis major muscle myodegeneration accompanied by necrotic fibers, fibrosis and inflammatory infiltration as indicators of chronic inflammation, along with structural and chemical composition abnormalities in heavy broilers reared in intensive systems. Indeed, the higher lipid and lower protein content as possible effect of myodegeneration can compromise meat quality (Mazzoni et al., 2015). The main growth-associated myopathy, deep pectoral disease (DPM), also named Oregon disease or green muscle disease, is a "degenerative" myopathy having as triggering factor ischemia and that has spread rapidly and exclusively in modern selected broiler strains characterized by a high breast yield. It is defined as an ischemic necrosis following a muscle suffering caused by the blood vessel occlusion due to too much pressure exerted by the muscle itself. Consecutively, necrosis is replaced by fibro-adipose tissue with negative effect on meat composition and value (Petracci and Cavani, 2012; Kuttappan et al., 2016).

The other very well-known breast abnormality associated with the intensive selection is the PSE-like meat (pale, soft and exudative, being similar in the aspect to what observed in PSE pork meat). While in pigs have been found the genetic single mutation responsible for this abnormality, in poultry both genetic selection and environmental factors have been considered as main triggering aspects, having an increased *post mortem* acidification as common point (Petracci and Cavani, 2012). This muscle acidification is can occur differently depending on the two possible triggering factors: genetic role (i)Genetic: the higher metabolic rate in breast of fast-growing broilers favors the achievement of extreme acid conditions, in which too much glycogen content is degraded to lactic acid, reaching a too low pH *post mortem* (below 6) close to the isoelectric point of myofibrillar proteins; (ii) Environment: stress conditions affect and increase the muscle acidification rate through release of calcium ions that stimulate enzyme activity, with denaturation of sarcoplasmic proteins and loss of membrane integrity. In both cases, meat results in pale color, soft consistency and poor water holding capacity (Petracci and Cavani, 2012; Petracci et al., 2015).

Recently, other two muscle myopathies have been described, even if their pathogenesis is poorly known: white striping (WS) and wooden breast (WB), sharing some main histological characteristics. WS and WB are both characterized by multifocal degeneration and atrophic fibers, loss of cross striations and vacuolar degeneration. Then, in WS there is tissue regeneration characterized by adipocyte infiltration and fibrosis, resulting in white striations parallel to the muscle fiber direction (Russo et al., 2015). Fibrosis regeneration has been also observed in WB, where accumulation of interstitial loose connective or collagen-rich connective tissue are present, with a final meat hardness typical of this muscle abnormality; in WB it has also been observed white striping presence, with a final product resulting hard and with pale areas. WB is furthermore characterized by interstitial inflammatory cell infiltration, which can be also present in WS (Sihvo et al., 2014; Soglia et al.,

2015). Thus, in WS and WB myopathies an important negative nutritional value change occurs, with a decrease in protein content and increase in collagen and fat contents, which negatively affect the meat quality and its economic value (Petracci et al., 2015; Russo et al., 2015).

Since the incidence of myopathies DPM, WS and WB is higher in heavy birds with high breast yield (Bailey et al., 2015), the hypothesis that genetic direction towards a faster GR may favour these alterations is understandable. However, Russo et al. (2015) reported no direct genetic effect, even if they found that BW and average daily gain (ADG) are both predisposing variables influencing WS pathology and that WS is correlated to DPM, sharing ADG as risk factor (Russo et al., 2015). Hence, it is plausible that high GR plays a role as indirect effect of the genetic drive. Both growth and stress-related myopathies may also occur as consequence of stress conditions, which affect cellular balance, stimulating protease and lipase activity (Soglia et al., 2015) and other plasmatic enzymes, such as creatine kinase (CK): as reported by Sandercock et al. (2009), release of CK in circulation indicates alteration in muscle membrane permeability (due to alteration in fiber membrane integrity by protease activity), so CK could be considered as marker for tissue damage. Furthermore, these authors also observed a strong correlation between CK plasma concentration and BW in heavy broilers and higher ion muscle concentration compared to laying hens and traditional chickens at the same age, indicating a possible onset of muscle degeneration (Sandercock et al., 2009).

2. Gastro-intestinal tracts

2.1 Digestive tract characteristics

Digestive tract of poultry is generally similar to that of the other vertebrate species, except some peculiarities and the faster transit of food. From a structural point of view, bird gastro-intestinal tract (GIT) consists of beak, esophagus, crop, proventriculus (glandular stomach), gizzard (muscular stomach), small intestine (duodenum, jejunum and ileum), paired isolated ceca, colon-rectum and cloaca (Figure 1). After a first stay in crop, a feed storage organ where fermentation by lactic acid bacteria (LAB) occurs, food is then digested and mechanically grinded in proventriculus and gizzard, respectively, which act as stomachs. Gizzard is recognized as "teeth" of poultry GIT where feed breakdown occurs, and thanks to its low pH, gizzard acts also as microbial barrier (Oakley et al., 2014b; Stanley et al., 2014). Compared to mammals, bird digestive tract is shorter, consequently implying a limited retention time of digesta, reason why it needs to be extremely efficient (Klasing, 1999; Rodrigues and Choct, 2018). After swallow, food moisturization and grounding occur not in mouth, differently from mammals: after crop fermentation, feed continues towards proventriculus and gizzard, where digestion comes with gastric juices. Then, nutrients are mainly digested and absorbed in small intestine, with a short retention time in duodenum, which, anatomically, forms a loop around pancreas from which receives digestive enzymes. After that digestion goes on up to the end of jejunum, recognized as the major site of absorption of small intestine. As last small intestine segment, ileum has the role for nutrients, water and mineral absorption, even if a part of nutrient digestion can also occurs (Svihus, 2014). Like in mammals, small intestine is characterized by a single epithelial cell layer (absorptive, goblet and entero-endocrine cells) that lines villi and crypts, overlaid by a mucus layer, representing the interface between gut and microbiome and formed by mucins secreted by goblet cells. Intestinal villi, defined as protrusions of lamina propria into gut lumen to amplify the absorptive area, are the functional units of small intestine and change along the different segments, becoming shorter and smaller in ileum, where minor digestive functions are required compared to the proximal part. However, since chickens do not have much of a large intestine, ileum represents the last useful tract to adsorb nutrients. Going on with the large intestine, another main distinctive feature in birds are the two ceca, blind-ended bags located at the junction between ileum and colon, usually long and well developed and characterized by a meshwork of long interdigitating villi, of which the majority filters, as a sieve, fluids and small particles coming from ileum. Presence of this villus meshwork is an avian peculiarity (Clench, 1999). Avian ceca have key role in microbial carbohydrate degradation and fermentation, microbial vitamin and amino acid (AA) synthesis, nitrogen compounds degradation, urea recycling into AA and water absorption and balance (Clench,

1999; Klasing, 1999; Yamauchi, 2002; Oakley et al., 2014b; Svihus, 2014). Cecum is the most diverse gut section, characterized by the longest feed retention time compared to the upper parts and by the highest short chain fatty acid (SCFA) concentration that are absorbed by the host. In fact, cecum represents the principal site where complex nutrients such as cellulose and other non-starch polysaccharides are fermented, with high fermentation rate of facultative anaerobes and strictly anaerobic bacteria (Józefiak et al., 2011; Borda-Molina et al., 2018). Large intestine ends with a very short rectum, extending between the ileo-cecal junction and the cloaca. For histological structure, avian rectum is very similar to small intestine, but with shorter villi and richer in lymphoid follicles (Klasing, 1999).

Another avian noteworthy peculiarity in is that digesta moves in both peristaltic and anti-peristaltic ways along the GIT. Except the ordinary progression and transport of feed from duodenum to colon, thanks to intestinal caudal peristaltic contractions, in poultry also a reverse peristalsis (cranial) occurs, to improve feed retention time and digestion. Anti-peristaltic movements and reflux happen at three levels: (i) from gizzard to proventriculus due to gizzard contractions (for a greater feed exposure to proventriculus enzymes); (ii) from jejunum and duodenum back to stomachs (to enhance digestion in fasting); (iii) from cloaca to ceca as physiological continuous process (Klasing, 1999; Rodrigues and Choct, 2018).

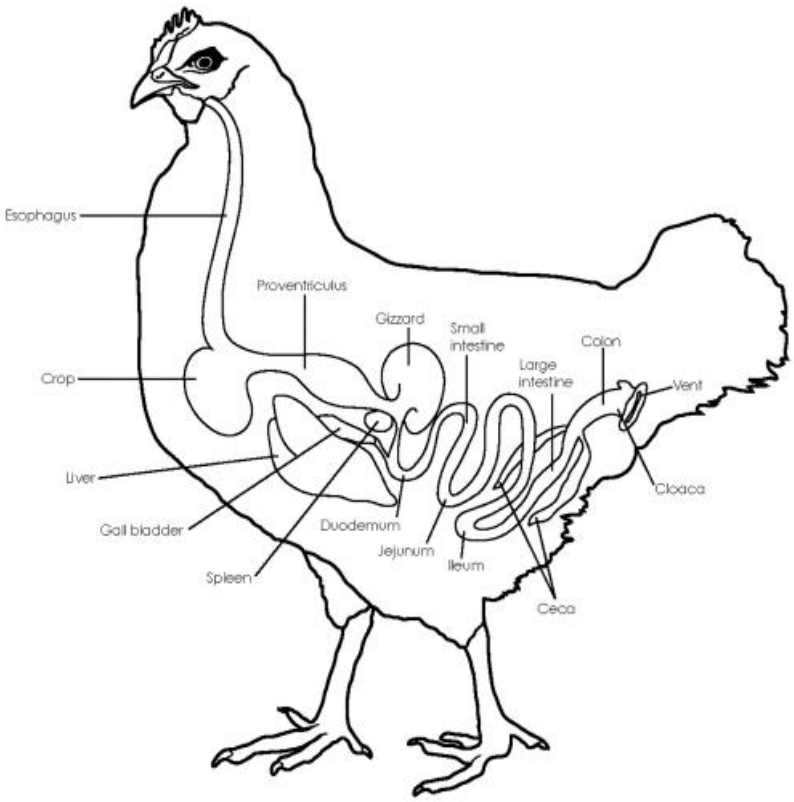


Figure 1. Chicken gastro-intestinal tracts (Clavijo et al., 2018)

2.1.1 Post-hatching digestive tract development

Already during incubation, and specifically in the late embryonic development, small intestine weight grows with a greater rate than chick body: at day 15 of incubation villi start in growing and shaping and between day 17 and 20 of incubation three different stage of villus development and maturity can be visualized, from elongated pear-shaped villi, to shorter ones and to nascent villi. Then, from this period up to the hatch, small intestine to body weight ratio increases from 1 to 3.5% and a minimal enzyme activity is developed (Uni et al., 2003). During incubation, yolk provides the embryo much of nutrition through circulation while, close to hatch and after, yolk is delivered to the GIT, contributing in small intestine development up to 48 hours post-hatching (Yegani and Korver, 2008). It is in this period that chick faces up the transition from the use of lipid-rich yolk as nutrient source to carbohydrate and protein-rich feed (Uni et al., 2003). So, the proportional growth of intestine is higher than that of BW at hatching because chicks, after a first uptake of yolk nutrients, rapidly need to develop an efficient nutrient uptake capacity supported by a proper gut maturation. During the early post-hatch period, distribution of nutrients follows a rigorous partitioning between "demand" of the tissues as largely users of energy and proteins, and "supply" of the tissues. The intestine is the primary nutrient supply organ and small intestinal epithelium determines growth potential of chicks (Hu and Guo, 2008). The sooner gut achieves its full functional capacity, sooner the chicks can exploit the diet for an efficient physiological development, including a complete achievement of immune competence , important for disease resistance (Uni et al., 1995; Lilburn and Loeffler, 2015). Intake of exogenous feed accompanies a fast development of digestive organs, with great and critical importance of timing and form of the diet and its nutrients available to chick on gut development (Yegani and Korver, 2008).Indeed, immediately after hatching, intestinal absorption rate is higher for fatty acids (mostly for unsaturated fatty acids), followed mainly by glucose, and, in few days, absorption rate for AA and carbohydrates highly increases (Cardeal et al., 2015). Early access to feed has been recognized as fundamental factor affecting intestinal function also at later stage of in life, since feeding delayed has been seen to slow down gut development (Geyra et al., 2001; Yegani and Korver, 2008). Compared to mammals, the faster gut development in chicks is reflected in the increasing number of enterocytes during first few days after hatching (Uni et al., 2003). While at hatching enterocytes are immature like in mammals post-farrowing, appearing small, round-shaped and without the typical polarized brush-border, they acquired polarity in only 24 hours and, in duodenum and jejunum, epithelial surface increases rapidly through cell hypertrophy (Geyra et al., 2001). In particular, jejunum seems to account for the greatest increase in absorption post hatching, developing a greater absorptive area with higher and denser villi already after 72 hours compared to

duodenum which, in turn, expands denser villi than ileum (Uni et al., 1999). In fact, differently from the preceding segments, chicken ileum enterocytes seem to be morphologically mature already at hatching, and in this segment, hypertrophy is limited and slow (Geyra et al., 2001). Next to these morphological changes, also digestive and absorptive capacity increase, with a greater functional maturation appearing first in duodenum than in the other distal segments. This maturation for digestive activity occurs during enterocyte migration from crypt to villus with increase in expression of gut nutrient transporters and pancreatic and brush-border enzymes, like disaccharidases, aminopeptidases and alkaline phosphatase, this latter recognized as enterocyte maturation marker (Uni et al., 1998). The presence of proliferative enterocytes not only at crypt level but also along the villus has been reported in all segments in chicks (while in mature bird, like in mammals, cell renewal is guaranteed only by proliferating crypt stem cells that migrate up to villus tip) (Geyra et al., 2001). After, cell proliferation undergoes a subsequent gradual decrease with age, with a slowest decline in jejunum, meaning that, here, villus mitosis is more important for growth compared to the other segments. Regarding crypts development, not-well defined crypts at hatching become distinct in 24 hours, increasing in number and size to provide enterocytes for villus growth and increase cell renewal rate (Geyra et al., 2001). Villus volume and crypt depth increase, result complete in duodenum around days 6 and 7 and after they become less considerable, while in jejunum and ileum this increase continues up to day 14 (Uni et al., 1998, 1999).

Next to the development of digestive functions and structures, contemporary development of gut-associated lymphoid tissue (GALT) happens: GALT is a set of lymphoid structures connected to intestine and representing immune system at gut level. Its development in chicks occurs during the late embryogenesis (Yegani and Korver, 2008). As further component of innate host response, gut mucus layer is formed by goblet cells that release mucins: goblet cells start their development in the late embryonic and immediate post-hatch period, firstly containing only acidic mucins and after hatch producing also neutral mucins (Smirnov et al., 2006).

2.1.2 Intestinal tract of fast-growing hybrids

Selection for GR in modern chickens has been positive correlated to feed intake and feed efficiency, and changes in production-related traits seem to have implied changes at intestinal morphological level and function (Yamauchi, 2002). Since the growing period of broiler has been drastically reduced to reach a market weight in half time compared broiler predecessors, post-hatch period has become proportionally an important phase of the total bird growing period and life. In heavy birds gut increases much more its development rate at hatching compared to non-selected birds, since selection

for metabolic characteristics led to a necessary early development of energy-supplying organs, first of all the digestive tract, which has adapted to the rapid GR of heavy broiler chickens in order to provide the proper nourishment (Scheele, 1997; Geyra et al., 2001; Lilburn and Loeffler, 2015). In fact, compared to non-selected light types chickens, which have a lower GR, modern fast-growing heavy broiler lines show higher crypt depth and villus surface at earlier age, meaning a more extensive absorptive surface area and higher intestinal function. Moreover, along with more matured and activated epithelial cells already at hatching and faster enterocyte migration rate, higher absorption of starch and nitrogen has been reported (Uni et al., 1995; Yamauchi, 2002). Despite broiler digestive tract must face a very high rate feed consumption, nutrient absorption and metabolism necessary for the rapid growth that characterizes these new genetic lines, small intestine length results shorter compared to the predecessor broiler strains, with higher villus height in jejunum and ileum (Lumpkins et al., 2010; Svihus, 2014). Mott and colleagues (2008) observed a decrease in expression of some nutrient transporter-related genes in small intestine of chicks selected for high BW, assuming that a higher digestive efficiency might require less nutrient absorption maximization compared to low BW chicks (Mott et al., 2008). Further, in fast-growing broilers it has been also seen that ileum has a role in starch digestion and absorption: in fact, ileum seems to be able to cope an increase need for digestive capacity through increasing its villus surface, like in case of jejunal dysfunction (Svihus, 2014). More, increase in jejunal villus height observed in fast-growing broiler upon short-term fasting has been pointed up as inverse response compared to other genetic lines (such as laying hens) where, instead, fasting usually induces decrease of jejunal villus height. This divergence may mean a greater capacity of this high selected lines in contrasting fasting period by preparing gut for a successive nutrient uptake maximization. This is also supported by the fact that in these birds, villi slough very often, indicating a high presence of proliferative cells and cell turnover in the small intestine (Thompson and Applegate, 2006). The diversified genetic selection for different lines affected not only growth performance but also digestive tract, as it is for broilers and laying hens. Compared to broilers, laying hens have a large and more muscular gizzard and longer intestine in relation to body weight, as well as longer digesta retention time in crop and gizzard and higher pancreas growth. These characteristics stand for a different digestive behavior, meaning an increasing in feed breakdown and digestibility in laying hens compared to broilers. This is already notable at hatching, where activity of disaccharidases is higher in broiler than laying hen chicks, later this enzymatic activity is completely reversed between the two lines (Buzala and Janicki, 2016).

2.2 Intestinal microbial community

Gastro-intestinal microbiota represents one of the denser ecosystems, showing complex and high microbial diversity (with bacteria as predominant microorganisms) and harboring from 10^7 to 10^{11} bacteria per gram of gut content in poultry, where each gut tract counts as a separate section (Stanley et al., 2012).

Methods of study of gut microbial profile have changed over time: classical microbiological culture techniques are unable in identifying most of the bacteria, since most of them are unculturable. New modern techniques culture-independent have been developed making possible to deepen the knowledge of intestinal microbial community. In the early 2000s, community-fingerprinting techniques have been introduced to provide bacterial profiles by assaying genomic DNA with PCR amplification, most of them based on 16S rRNA, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), temporal temperature gradient gel electrophoresis (TTGE) and others (Stanley et al., 2014; Shaufi et al., 2015). More recently, the advent of next generation sequencing (NGS)-based methods, from 16S rRNA amplicon sequencing to metagenomic analyses, has revolutionized the research approach in studying diversity and function of gut microbiota. 16S rRNA targeted amplicon sequencing technique allowed a more in-depth analysis to characterize the complex microbial composition through amplification and sequencing of the 16S small subunit ribosomal genes of bacteria, in particular the hypervariable regions (Borda-Molina et al., 2018). Processing of raw sequences from 16S rRNA genes allows to produce clusters of almost identical sequences, referred to operational taxonomic units (OTUs) providing taxonomic information (Choi et al., 2015). Metagenomics is the highest throughput NGS approach with high scale analysis and a large volume of sequence data that can provide more insights into gut microbial communities through targeted functional gene amplification or through directly sequencing of whole genomes with unprecedent depth and coverage. It allows to explore in depth metabolic pathways correlated to different ecological functions in the gut (Choi et al., 2015; Shaufi et al., 2015).

2.2.1 Spatial bacterial composition along the gastro-intestinal tract of adult chickens

Also due to high costs of the metagenomic approach, most of actual knowledge on bacteria diversity and functionality along chickens GIT is attributed to 16S rRNA targeted amplicon sequencing-based studies (Table 2). Microorganisms of GIT can be located in the lumen, under the mucus layer or can adhere to the mucosa, forming a cell layer. These strictly mucosa-associated bacteria play a pivotal role in host-microbiota interaction (Gabriel et al., 2006). Generally, in adult and healthy chickens,

except for the wide spectrum of microbial fluctuations occurring during the short and rapid growth, microbiota population has reached a steady balance in its composition, which differs between gastro-intestinal compartments according to gut section functions. The most abundant bacteria in adult chickens' intestine are gram positive (gram +) and the five most common phyla are Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Cyanobacteria, with Firmicutes as the most representative phylum (Lu et al., 2003; Xiao et al., 2017). These main phyla account for more than 90% of all sequences, among which *Lactobacillus, Clostridium, Ruminococcus,* and *Bacteroides* are the most relevant genera, next to a wide proportion of bacteria belonging to unclassified species or genera (Rubio, 2018). At crop level, where starch breakdown and lactate fermentation occur, bacterial community (composed by both associated-mucosa bacteria and bacteria in digesta) is highly dominated by *Lactobacillus* genus, with a concentration of 10^9 colony forming units (CFU) /g content and including species such as *L. salivarius, L. fermentum, L. reuteri* and *L. acidophilus* (Rehman et al., 2007; Ranjitkar et al., 2016) The non-secretory and squamous crop epithelium allows lactobacilli adhesion and biofilm formation, making a hardly accessible environment for other bacteria, also due to the high lactic acid concentrations (pH ~5). From here, lactobacilli diffuse to proventriculus, a thick-walled stomach where they again dominate, and to the remainder of the gut to establish their dominance in the small intestine. In the gizzard there is an increase in bacterial diversity, always dominated by species of *Lactobacillus* genus, but a decrease in bacteria number and activity, due to the very low pH (~3). Actinobacteria and Proteobacteria phyla are present in minor proportion (Ranjitkar et al., 2016).

As for the crop, duodenum and jejunum are dominated by lactobacilli, even if minor species such as *Enterococcus* spp. can be found. Furthermore, Proteobacteria and Actinobacteria phyla are present along with Bacteroidetes, even if in less extent (Stanley et al., 2012; Awad et al., 2016; Xiao et al., 2017). Due to the high transit rate, reflux movements from jejunum, low pH and bile salt dilution, bacterial density is particularly low in duodenum, where the rapid feed passage rate allows only a small proportion of carbohydrate fermentation (Rehman et al., 2007). Due to the rapid transit of digesta and to high concentration of gastric acid, microbiota in jejunum is limited to acid-tolerant bacteria, mostly *Lactobacillus* species (Lan et al., 2004). In ileum, most of microbial population is again made up by *Lactobacillus* genus, followed by *Clostridiaceae* family, with *Clostridium* as main genus represented; then, except unclassified bacteria, a less proportion of *Streptococcus* spp. and *Enterococcus* spp. have been detected and also coliforms can be present (Lu et al., 2003; Ranjitkar et al., 2016; Xiao et al., 2017). In contrast to these studies, a recent study on microbial dynamics along chickens' gut tracts highlighted a prevalence of *Clostridiaceae* and a very low percentage of *Lactobacillus spp.* in ileum (Shaufi et al., 2015). The other most present phyla in ileum are

Proteobacteria and Bacteroidetes (Shaufi et al., 2015; Xiao et al., 2017). Compared to previous tracts, Actinobacteria phylum has been found at very low level (Xiao et al., 2017). As main group dominating small intestine, the important role of *Lactobacillus* spp. is strictly linked to the nutrient metabolism and it has been hypothesized that lactobacilli might be also involved in nutrient absorption (Xiao et al., 2017). At small intestinal level, the absorption of bacterial fermentation products occurs, such as lactic and volatile fatty acids, and the host can use them as energy source. In addition, small intestinal bacteria population degrade and process different substances such as mucus produced by goblet cells and sloughed epithelial cells (Lu et al., 2003). Moreover, at ileal level bile salts coming from duodenum and jejunum are deconjugated by microbiota, then they are in part reabsorbed and in part delivered to ceca (Volf et al., 2017). While small intestinal microbial community has the main role in supporting gut in digestion and nutrient absorption, cecal microbiota has in fermentation task its primary function (Awad et al., 2016).

The most populated and complex microbial community resides in ceca, with a density around 10^{11} (bacteria per g/content) (Lu et al., 2003). In small intestine, digesta retention time takes only 2.5 hours, but in cecum can last 12-20 hours to favor longer digestion, fermentation and absorption. At the entrance of each cecum, long intertwining villi act as a sieve to leave out coarse particles, reason why ceca content is mainly liquid, with soluble particles (Sergeant et al., 2014). Compared to other gut sections, in cecum microbial population forms a separate cluster and, while in the precedent tracts *Lactobacillaceae* represented most of Firmicutes, here this phylum is characterized by the predominance of obligate anaerobic bacteria, with facultative anaerobes in less proportion and a very variable composition. The higher microbial diversity of ceca compared to the upper gut is supported by higher organic acid concentration and variation (Rehman et al., 2007; Ranjitkar et al., 2016; Xiao et al., 2017). Bacteria belonging to Clostridia class are reported to be dominant by most of studies on ceca microbial community profiling using 16S rRNA gene sequencing (Lu et al., 2003; Gong et al., 2007; Danzeisen et al., 2011; Shaufi et al., 2015; Ranjitkar et al., 2016). Inside this class, most reported abundant families are *Ruminococcaceae, Lachnospiraceae* (Danzeisen et al., 2011; Ranjitkar et al., 2016) and *Clostridiaceae* (Lu et al., 2003; Ranjitkar et al., 2016). A smaller proportion of *Lactobacillus* species (Lu et al., 2003; Gong et al., 2007; Ranjitkar et al., 2016), *E. coli* and other coliforms (Gong et al., 2007) are present. The second main phylum in cecum is that of Bacteroidetes (Lu et al., 2003; Shaufi et al., 2015; Ranjitkar et al., 2016), even if in a recent study of Xiao and colleagues (2017) Bacteroidetes resulted to be the main dominant phylum in cecum (Xiao et al., 2017), while Awad and colleagues reported Tenericutes as second most abundant phylum in cecum and found Bacteroidetes at very low level (Awad et al., 2016). According to these studies, also a recent study on cecal microbiota composition by whole DNA shotgun metagenomic sequencing in

adult broiler chickens reported *Clostridia* as most abundant class (70.5%) within Firmicutes (with *Ruminococcaceae* as most abundant family, 29.53%), followed by *Bacilli* class (20.7%) (De Cesare et al., 2017).

Beside known bacterial genera, cecal microbiota in chicken have been estimated in consisting of over 600 species from more than 100 genera with most of them still unknown and unclassified (Stanley et al., 2014).

Nevertheless, since many factors contribute to differences in gut microbial composition such as diet, environment and rearing conditions, it is not ever easy to compare with different studies. Regarding Actinobacteria, even if *Bifidobacterium* genus is known as often representing culturable anaerobe of this minor phylum in chickens, it seems to be reported as relatively low and rare in GIT in terms of Actinobacteria-related abundance from 16S rRNA gene amplicon sequences (Clavijo and Flórez, 2018), but also in cecal microbiota from a study based on TTGE identification (Zhu et al., 2002). Cecal microbiota has the main task to digest and ferment cellulose, starch and resistant polysaccharides, producing the highest SCFA concentration than elsewhere in the gut: after fermentation, protonate forms of SCFAs (like acetate, propionate and butyrate) can pass through the cecal epithelium and catabolized by the host, contributing in host nutrition. Furthermore, SCFAs improve mineral absorption, decrease ceca pH inhibiting some pathogens and, mainly butyrate, can be used as energy source from epithelial cells (Józefiak et al., 2011; Oakley et al., 2014b; Sergeant et al., 2014). Digestion of complex nutrients such as non-starch polysaccharides (NSP) occurs thanks to many microbial hydrolytic enzymes like those of butyrate-producing bacteria such as some species of *Clostridium*, *Ruminococcus*, *Eubacterium*, *Fusobacterium*, *Roseburia* and *Faecalibacterium* (Gong et al., 2002; Rinttilä and Apajalahti, 2013). In fact, metagenomic studies revealed thousands of different genes encoding NSP-degrading enzymes including sequences for glucanases (acting on oligosaccharides), xylanases and endoglucanases domains (xylan-degrading enzymes) in chicken cecum (Sergeant et al., 2014; Borda-Molina et al., 2018). Furthermore, SCFA production seems to be promoted also by the presence of hydrogen-consuming bacteria that uptake hydrogenases so avoiding hydrogen accumulation, which could inhibit fermentations (Sergeant et al., 2014). Also bacterial groups belonging to Bacteroidetes, such as *Bacteroides*, are involved in breaking down polysaccharides and, more, in anti-inflammatory cytokine production, another important cecum-related function (Xiao et al., 2017). The importance of butyrate-producing bacteria and resistant carbohydrate degraders has been also associated with good chickens performance (Stanley et al., 2014). Beside the carbohydrate fermentation, cecal bacteria take part in nitrogenous metabolism, with SCFA and ammonia production from dietary and urinary (e.g. uric acid) nitrogenous sources. Ammonia can be so integrated into glutamate, useful for bacterial protein and glucose synthesis

(Gabriel et al., 2006). Another important function of some cecal bacteria regards also fatty acid transformation: bacteria like some *Roseburia* species can form conjugated linoleic acid from linoleic acid, with control on fat metabolism (Danzeisen et al., 2011).

Table 2- Main bacterial composition in young chicken gastro-intestinal tract (GIT) in different 16S rRNA targeted amplicon sequencing-based studies.

Chicken GIT	Age (d)	Main phyla and dominant bacterial groups (% relative abundance from 16S rRNA gene amplicon sequences)	Sampling type	References
Crop	36d	- **Firmicutes** (~98%) → *Lactobacillus* spp. (dominant, ~94%) - **Actinobacteria** (<1%)	Gut content	Ranjitkar et al., 2016
Gizzard	36d	- **Firmicutes** (~87%)→ *Lactobacillus* spp. (dominant, 58%), *Planococcaceae* (9.5%), *Staphylococcus* spp. (4.8%), *Ruminococcaceae* (4.7%), *Clostridiaceae* (3.3%) - **Actinobacteria** (9.5%) - **Proteobacteria** (2.4%)	Gut content	Ranjitkar et al., 2016
Duodenum	42d	- **Firmicutes** (>60%)→ *Lactobacillus* spp. (dominant, >35%) - **Proteobacteria** (>20%) - **Actinobacteria** (~10%) - **Bacteroidetes** (< 10%)	Gut content	Xiao et al., 2017
Jejunum	25d	- **Firmicutes** → *Lactobacillus* spp. (dominant, >99%)	Gut mucosa	Stanley et al., 2012
	28d	- **Firmicutes** (54.85% mucosa-associated + 84.56% content) - **Proteobacteria** (28.94% mucosa-associated + 13.05% content) - **Actinobacteria** (~1%)	- Gut content - Gut mucosa	Awad et al., 2016
	42d	- **Firmicutes** (>60%)→ *Lactobacillus* spp. (dominant, >35%) -**Proteobacteria** (>10%) - **Actinobacteria** (> 10%) - **Bacteroidetes** (< 10%)	Gut content	Xiao et al., 2017
Ileum	49d	- **Firmicutes** → *Lactobacillaceae* (dominant, ~70%), *Clostridiaceae* (11%), *Streptococcus* spp. (6.5%) and *Enterococcus* spp. (6.5%) - **Bacteroidetes** → *Bacteroides* spp. (1%)	Gut content	Lu et al., 2003
	42d	- **Firmicutes** (85%) →*Clostridiaceae* (dominant, 83%: *Clostridium* spp. as most represented), *Lactobacillus* spp. (<4%) - **Proteobacteria** (5%)	Gut content	Shaufi et al., 2015
	36d	- **Firmicutes** (97%) → *Lactobacillus* spp. (dominant, 68%), *Clostridiaceae* (~20%,	Gut content	Ranjitkar et al., 2016

		Clostridium spp. as most represented), *Streptococcus* spp. (5%) - **Actinobacteria** (1.7%)		
	42d	- **Firmicutes** (>60%)→ *Lactobacillus* spp. (dominant, >35%) - **Actinobacteria** (> 10%) - **Proteobacteria** (< 10%) - **Bacteroidetes** (< 10%)	Gut content	Xiao et al., 2017
Cecum	49d	- **Firmicutes** → *Clostridia* (dominant, ~65%: *Clostridium* spp. and *Ruminococcus* spp. as most represented), *Fusobacterium* spp. (14%), *Lactobacillus* spp. (8%) - **Bacteroidetes** → *Bacteroides* spp. (5%) - **Proteobacteria** (2.8%)	Gut content	Lu et al., 2003
	35d	- **Firmicutes** → *Clostridia* (dominant, 40%: *Ruminococcaceae* as most represented, with *Faecalibacterium prausnitzii* 14% and *Ruminococcus* spp. 6%), *E. coli* (11%), *Lactobacilli* (7%)	Gut mucosa	Gong et al., 2007
	35d	- **Firmicutes** (~80%) → *Clostridia* (dominant, ~65%: *Lachnospiraceae* and *Ruminococcaceae* as most represented)	Gut content	Danzeisen et al., 2011
	42d	- **Firmicutes** (49%) → *Clostridia* (dominant, ~45%) - **Bacteroidetes** (21%) → *Bacteroidia* (dominant, 20%: *Alistipes* spp. and *Bacteroides* spp. as most represented)	Gut content	Shaufi et al., 2015
	28d	- **Firmicutes** (81.50% mucosa-associated + 70.86% content) → *Clostridia* (dominant) - **Tenericutes** (13.38% mucosa-associated + 22.61% content) - **Proteobacteria** (3.73% mucosa-associated + 22.61% content)	- Gut content - Gut mucosa	Awad et al., 2016
	36d	- **Firmicutes** (~75%) → *Clostridia* (dominant: *Ruminococcaceae* 36%, *Lachnospiraceae* 22.7%, *Clostridiaceae* 4.8%), *Lactobacillus* spp. (3.3%) - **Bacteroidetes** → *Alistipes* spp. (20%)	Gut content	Ranjitkar et al., 2016
	42d	- **Bacteroidetes** (>50%)→ *Bacteroides* spp. (dominant, ~40%) - **Firmicutes** (~40%) - **Proteobacteria** (<10%)	Gut content	Xiao et al., 2017

2.2.2 Factors affecting gut microbiota

Even if gut microbial community is quite defined in adult chickens, different factors can influence its composition. From a genetic point of view, intensive selection for high feed efficiency may have contributed in some distinguished characteristics between chickens. In laying hens differing for feed

efficiency has been observed higher biodiversity and different composition in cecal microbiota of better feed efficient hens, with higher abundance of *Lactobacillus* species. Higher *Lactobacillus* spp. abundance has been found also in duodenum of hens with better feed efficiency, even if in this tract, differently from cecum, a lower microbial diversity has been also reported (Yan et al., 2017). Stanley and colleagues (2012) found *Bacteroides* genus-related sequences differentially abundant in cecum of better feed efficient broilers, while *Clostridia* resulted to be more present in low feed efficient animals, considering a possible connection between higher feed efficiency and higher *Bacteroides* presence, maybe linked to undigestible carbohydrate degrading and propionate producing capacity (Stanley et al., 2012). However, it has also been reported that, while Firmicutes increase nutrient absorption, Bacteroidetes may decrease it (Choi et al., 2015). Different rearing systems also affect microbial composition. Indeed, it has been reported differences in cecal microbiota between commercial broiler chicken and free-range chicken grown in semi-wild conditions, with Firmicutes as dominant phylum in the former while in the latter Bacteroidetes increased reaching the same level of Firmicutes (Mancabelli et al., 2016). Being the main important site for NSP fermentation, cecum and its microbiota resulted also to be affected more than upper gut tracts both by dietary water-soluble NSP and by NSP-degrading enzyme enriched diets: the stimulation of butyrate-producing and cellulose and starch-degrading bacteria as well as SCFA production has been correlated with better performance (Gabriel et al., 2006; Józefiak et al., 2011; Stanley et al., 2014). Lastly, metabolic potential of cecal microbiota changes in response to antibiotics, with repercussion on butyrate-producing bacteria such as *Roseburia* spp. (Danzeisen et al., 2011)

2.2.3 Temporal microbial community development in growing chicken

Differentiation in microbial community along gut sections follows gut maturation, with general temporal shift and fluctuations in microbiota within 42 days of age. The microbial transient succession is high in chicks, then a more stable state corresponding to skeletal growth period seems to occur,with again fluctuations after 28 days of age and then stabilization (Lu et al., 2003; Shaufi et al., 2015). As the GIT grows, changes in bacterial community occur. Once reached a steady state development and stable environmental conditions, microbial community stabilizes and decreases in diversity (Lumpkins et al., 2010). Firmicutes phylum results predominant at all ages, with genus-relative abundance decreasing as chicken age increases due to the increase in heterogeneity and diversity of microbiota. In chicks post-hatched, lactobacilli appear as earlier colonizers along all GIT and after, throughout growth, they stay stable in upper gut while are mostly replaced in ceca. Indeed, in crop, *Lactobacillaceae* family is dominant and stable at all ages like in gizzard where

Lactobacillaceae are dominant and Actinobacteria phylum increases as chicken age increases (Ranjitkar et al., 2016). In small intestine, bacterial community establishment take less time than in cecum, where protracted variations in microbiota occur. Around first days post-hatching, small intestine harbors streptococci, enterococci and coliforms (with *E. coli* as most abundant inside Firmicutes phylum), but they undergo a decline and are replaced by lactobacilli, whose proportion increases with age becoming predominant already in 2-week older chickens. In cecum, a longer transient microbiota occurs during chicks development, with more presence of facultative anaerobes in chicks few days post-hatching, then replaced by anaerobes (Rehman et al., 2007; Awad et al., 2016; De Cesare et al., 2017). Indeed, during first days of life in the healthy chicks ceca, predominant bacterial class within Firmicutes seems to be that of Bacilli, followed by Clostridia class, while within Proteobacteria, *Enterobacteriaceae* family-members result as predominant (De Cesare et al., 2017). *Enterococcaceae* (e.g. *Enterococcus* spp.) and *Lactobacillaceae* (e.g. *L. salivarius, L. acidophilus* and *L. fermentum*) appear in highest proportion around 3-4 days of age, but then show a decline (Lu et al., 2003; Józefiak et al., 2011). At 7 days of age, Oakley et al. (2014a) reported Clostridiales order as dominant in cecum, and particularly *Faecalibacterium* genus as dominant at day 21 (Oakley et al., 2014a). Between day 15 and 22, a development of a more mature microbiota seems to occur especially in ileum and ceca, with a striking increase in relative abundance of *Lactobacillus* spp. and Clostridia class in the former and a decrease in LAB and increase in Bacteroidetes in the latter. Particularly, in cecum, lactic acid produced by LAB act as substrate for butyrate-producing bacteria development and indeed, it results lower in older chickens (Ranjitkar et al., 2016). Cecal SCFA concentration changes according to chicks growth, with higher acetate concentration during first days after hatching, while butyrate and propionate increase after (Józefiak et al., 2011). Around 40 days of age, cecal bacteria are in a steady state with total anaerobes (such as *Clostridium* spp. and *Bifidobacterium* spp.), enterococci, *Bacteroidia* and a less proportion of lactobacilli (Rehman et al., 2007). Inside Clostridia class, Oakley et al. (2014a) reported *Faecalibacterium* genus as dominant along with an increase in *Roseburia* genus at day 42 of age, both butyrate-producing bacteria (Oakley et al., 2014a). So, early cecum colonization from facultative anaerobes is a transitory period, where aerobes consume oxygen and its shortage modifies and drives cecum environment towards more reducing conditions, becoming favorable for the successive strictly anaerobic growth (Awad et al., 2016).

As mentioned before, different factors may influence microbiota composition and characteristics. Targeted selection for fast-growing birds seem to affect gut bacterial dynamics in new chick lines. Indeed, during the early age (first week post-hatching), in ileum of new broiler lines bacterial communities clustered with those of historical line, probably due to a still in progress development of intestine together with a not yet established microbiota (unstable environmental condition of GIT

environment), but along with GIT maturation, a clear separation of a more stable microbiota resulted between modern broilers and older lines (Lumpkins et al., 2010). More recently, differences in microbiota between post-hatched chicks of a modern or a heritage lineage have been highlighted. Modern lineage chicks showed a more diverse and differentiated microbiota and the authors hypothesized that genetic selection created a new habitat in GIT in order to obtain a line of chickens with high growth performance, so maybe selecting for a potentially more nutritionally efficient microbiota compared to heritage lines (Pedroso et al., 2016).

2.2.4 . Functions of gut microbial population

As already described before, the first role of microbiota certainly regards nutrient exchange with host's gut (mutualism). On one hand, bacteria provide SCFAs, ammonia, AAs and vitamins, on the other, host also provides nutrients, such as those from dietary compounds but also mucins. In fact, presence of some mucin-degrading bacteria in gut has been associated with intestinal health. Moreover, gut digestive physiology is affected by microbiota since its development post-hatch and throughout animal life. Microbiota can influence digestive enzyme activities and furthermore, bacteria-derived SCFAs feed enterocytes. It has been observed that germ-free chicken have smaller small intestine and ceca and thinner gut walls compared to conventional ones (Clavijo and Flórez, 2018). Those host digestive and nutritional functions improved by and linked to commensal colonization are due to modulation of gut gene expression. Differences in bacterial composition induce different gene activation. Not only is different gene activation linked to nutrition, but it also regards immune functions (Schiffrin and Blum, 2002). In fact, other important roles of microbiota concern pathogens exclusion and modulation of immune system, contributing in gut health maintenance (Rodrigues and Choct, 2018).

Among main pathogen bacteria that can colonize poultry GIT, the most common are *E. coli* (usually at low abundance in gut throughout chicken life), species of genus *Salmonella* (as sporadic and transient bacteria in chicken intestine) and *Clostridium* (with *Cl. perfringens* as principal cause of necrotic enteritis). These pathogens impair gut health status and can produce toxins and induce diarrhea (Gabriel et al., 2006; Oakley et al., 2014b; Choi et al., 2015). Also *Campylobacter* can be found in chickens, but is usually non-pathogenic for avian species while it is for humans (Oakley et al., 2014b). Nevertheless, it has been reported that infection by *Campylobacter jejuni* increases *Clostridium* spp. presence, probably because *Campylobacter jejuni* acts as hydrogen sink and induces higher mucus production, both stimulating factors for *Clostridium* growth. So, higher abundance in both *Campylobacter jejuni* and *Clostridium spp.* may result in higher endotoxin production with

increase of gut permeability and consequent possible pathogen colonization and gut diseases (Awad et al., 2016). As mentioned, commensal gut bacteria have a protective role against pathogen colonization. Indeed, over mucosal surface, the dense and complex layer formed by microbial communities in healthy birds acts by blocking passage and adhesion of enteric pathogens (Pan and Yu, 2014). Furthermore, this competitive exclusion to limit pathogen invasion is also expressed through production of bacteriocins and antimicrobial metabolites such as lactic acid and other SCFAs, (Gabriel et al., 2006; Rinttilä and Apajalahti, 2013), along with competing for essential nutrients and epithelial binding sites and through gut immune response modulation (Gabriel et al., 2006; Burkholder et al., 2008). It has been reported that *Salmonella* colonization in ceca of chicks after hatching can be limited by treatment with cecal microbiota from healthy adults and that lactobacilli counteract coliform growth, demonstrating the importance of gut beneficial bacteria establishment in order to prevent or reduce pathogen invasion (Gabriel et al., 2006; Choi et al., 2015). In support of this, Schokker et al. (2015) compared two broiler lines with same GR but differing for bacterial infection susceptibility and observed that the more resistant broiler line had higher counts for enterococci and lactobacilli and lower *Escherichia spp.* counts (Schokker et al., 2015).

2.3 Gut homeostatis and determinant variables

Well-being is closely connected to gut health, based on complex functions and mechanisms that ensure a steady and regular intestinal homeostasis. These mechanisms include intestinal structure and mucosal integrity, efficiency of the immune system and relationship between host and gut commensal microorganisms.

Luminal commensal bacteria and pathogens are separated from internal environment by the intestinal barrier, composed by both physical, chemical, immunological and microbiological components (Yegani and Korver, 2008). Mucosal strategies of defense together with control of inflammatory reactivity are prerequisites for gut protection and mucosal integrity preservation (Schiffrin and Blum, 2002).

2.3.1 Gut barrier structure

- *Mucus layer*

With the task of protecting gut epithelium, mucus layer is a component of gut mucosal innate immune system, being the first line of defense against pathogen invasion of intestine. Between its functional properties, there are those of trapping bacteria and providing colonization site and nutrients for commensals. Released in mucus layer, antimicrobial peptides produced by Paneth cells (such as

defensins) and IgA immunoglobulins (Igs) contribute in gut barrier maintenance, slowing diffusing into the mucus. Mucin glycoproteins, as major mucus components, are produced by goblet cells and classified as membrane-bound (transmembrane mucins covering goblet cell apical surface forming the glycocalyx) or secreted (gel-forming) mucins, and are densely decorated with complex carbohydrates (Pan and Yu, 2014; Pelaseyed et al., 2014; Broom, 2018). Mucus thickness increases as moving towards the lower intestine. While, in physiological conditions, small intestinal mucus is not or weakly attached to the epithelium, forming a diffusion barrier with peptides with high antimicrobial activity (which create an antibacterial gradient), in lower intestine (most prominent in colon) microorganisms stay in an outer loose mucus layer, clearly separated from an inner denser and epithelium-adherent layer free of bacteria (Pelaseyed et al., 2014; Johansson and Hansson, 2016). The inner mucus layer is further converted by proteases to the expanded outer loose layer, which lubricate feces and where mucin glycans allows mucus-associated bacteria to make niches. In fact, mucin domain surfaces present glycans that interact with bacterial adhesins and supply energy, since some bacteria have enzymes to degrade mucin glycans: different carbohydrate chains determining mucin functional properties can influence also microbiota composition. While the inner adherent mucus layer requires proteases to be dissolved, the loose layer, even if penetrable, protects small intestine from bacteria invasion because of the continue flow towards the low intestine, which contrasts on its own bacteria mobility (Johansson and Hansson, 2016; Broom, 2018). Mucus aspects such as mucin differentiation (neutral, sialo- and sulfo-mucins), goblet cell density and mucus thickness are key factors playing in gut barrier mechanism. Among mucin types, acidic ones seem to more protect gut against bacteria translocation (Broom, 2018).

- *Epithelium*

Placed under the glycosylated mucin-rich layer, a single layer of epithelial cells bordering the intestinal lumen provides physical and biochemical obstructions to microbes thanks to brush border of actin-rich microvillar extensions, intercellular tight junctions (TJs) and antimicrobial peptides production (Artis, 2008). The structure of gut epithelium is based on a monolayer of columnar epithelial cells constituted of four major cell types: goblet cells (producing mucins), Paneth cells (producing antimicrobial peptides), endocrine cells and enterocytes, most abundant cell type responsible for final digestion and nutrient absorption. All these cells derive from stem cells localized in the crypts of small intestine. These cells maintain the intestinal tract integrity by creating a strength barrier, held steady by TJ protein complexes. Regulatory molecules concurring in gut barrier forming and in paracellular permeability regulation (Kurashima et al., 2013; Broom, 2018). Paracellular way represents the major path for passing gut epithelium and getting to submucosa. In healthy conditions, spaces between epithelial cells are well sealed by the apical junctional complex, composed of the TJs

and subjacent adherents junctions with a support of a peri-junctional ring of actin and myosin. Adherents junctions allow close bonds between cells and support the assembly of the TJs, a complex of transmembrane proteins (e.g. claudin and occludin family), peripheral membrane proteins (e.g. zonula occludens like ZO1) and regulatory molecules. All these proteins concur to "fuse" the plasma membranes of adjacent cells, reducing the paracellular passage of solutes (Turner, 2009). Intact and selective permeable TJ barrier is also necessary for transcellular pathway, which depends on the trans-epithelial concentration gradient. This type of transport (e.g. Na^+-nutrient co-transport), in turn, could enhance paracellular pathway by modifying the TJs, through a signal transduction of kinases that involves myosin light chain kinase (MLCK) activation too (Turner, 2009). Dispersed throughout intestinal epithelium there are also intra-epithelial lymphocytes (IELs), between major players of immune response and represented by a large cell pool of a mixture of different T cells. Their connection with enterocytes is important for their immune function (Brisbin et al., 2008; Pelaseyed et al., 2014). IELs are the primary gut immune effector cells and have a pivotal role in eliciting protective immunity against enteric pathogens: These cells can recognize pathogens through expression of innate immune receptors and can release antimicrobial compounds and secrete hormones, cytokines and chemokines to activate the adaptive immune response (Lee et al., 2010; Chen et al., 2015). An increase in IEL count in all intestinal tracts means a possible onset of inflammation (Ashraf et al., 2013).

- *Lamina propria and GALT*

Located under the epithelium, lamina propria and gut-associated lymphoid tissue (GALT), with relative immune cells, represent sub-epithelial immune components that intervene when mucus and epithelial cell lining fail in gut defense. Immune cells include antigen-presenting dendritic cells (DCs), macrophages, lymphocytes (T and B cells) and granulocytes (Broom, 2018).

With the role in gut enteric infection protection, homeostasis, maintenance and inflammation control at submucosal level, immune cells of innate immune system mainly lie in peripheral circulation and in lamina propria, a thin vascular layer under the epithelium (Pan and Yu, 2014). DCs have the role to sample and present antigens to T-cells for action or tolerance response, while macrophages screen intercellular spaces and act through phagocytosis without triggering inflammatory response. B cells and specifically plasma cells also play an important part since they produce and release in the mucus layer IgA (major Ig class in mucosal tissues), which are then transported into the lumen through the enterocytes and bind to bacteria, blocking their crossing through the epithelium (Hooper et al., 2012; Pelaseyed et al., 2014). Release of IgA from B cells depends from stimulation of both T cell-dependent and independent pathways and take role in mucosal immunity through interaction and control of microbial population which, in turn, exerts a modulatory activity on IgA levels (Broom,

2018). DCs can stretch their dendrites through the TJs, directly getting to the intestinal lumen and sampling antigens: these cells have a large variety of pattern-recognition receptors (PRRs) able to recognize members of microbiota or pathogens, such as extracellular Toll-like receptors (TLRs), glycoproteins recognizing specific bacterial molecules (such as peptidoglycans, lipoproteins, lipopolysaccharides etc.). This mechanism allows immune cells of innate response to kill any bacteria passing the epithelium and to discriminate between commensals and pathogens and, in case of commensals, to do not trigger inflammation (Schiffrin and Blum, 2002; Artis, 2008; Brisbin et al., 2008). In fact, immune cells of lamina propria are specialized in managing pro-inflammatory response and in switching it off at epithelium level (Schiffrin and Blum, 2002). TLRs are also expressed by enterocytes at epithelial level, demonstrating the active role of epithelial cells in innate defense: trough TLRs, enterocytes can influence and activate DCs and macrophages, as well as lymphocytes, by processing and presenting antigens (Artis, 2008; Kogut, 2013). Innate immune signaling molecules owned by enterocytes are linked through signaling pathways to one of major immune response regulators, nuclear factor NFκB (Pelaseyed et al., 2014). Furthermore, also goblet cells have an immunological role. They can take up luminal antigens and directly deliver them to DCs, through a way called goblet cell-associated antigen passage that probably exploits endocytosis or vesicle transport. So, even if a part of innate immune response, goblet cells seem to play a role also in the adaptive immune system activation. This particular secondary task of goblet cells seems to be activated by microbiota (Johansson and Hansson, 2016). Sampling of luminal materials by DCs and the passage of small bacterial products seem to be regulated also by TJs. In a healthy epithelial layer, DCs release retinoic acid and anti-inflammatory cytokines such as transforming growth factor β (TGFβ) and interleukins (e.g. IL-10), which mediate IgA production and regulatory T cell differentiation, aimed in maintaining a homeostatic balance. The loss of barrier integrity can lead to pro-inflammatory cytokine production, that can allow to diseases development. DCs, in fact, can also trigger T helper 1 and 2 differentiation, with release of tumor necrosis factor (TNF) and interferon γ (IFN-γ) (Turner, 2009; Chen et al., 2015). Main role in adaptive immune response is played by lymphocytes (T and B cells, involved in cell-mediated and antibody-mediated response, respectively) which, in avian, are organized in lymphoid structures or disseminated in lamina propria and epithelium as lymphocyte aggregates (follicles) or scattered cells (Pan and Yu, 2014). Together, the organized lymphoid structures and the disseminated lymphoid follicles and single immune cells form GALT (Kamada et al., 2013). Bursa of Fabricius is the primary lymphoid organ, while secondary lymphoid complexes are Peyer's patches (in small intestine), Meckel diverticulum and cecal tonsils (Brisbin et al., 2008; Casteleyn et al., 2010). Peyer's patches are covered by a single layer of follicle-associated epithelium made up by enterocyte-like cells, goblet cells and M cells (Pelaseyed et al.,

2014). Cecal tonsils, formed by clusters of aggregated lymphoid tissue, are located at the cecum-rectum junction and are very similar to mammalian Peyer's patches, suggesting their role in antigen sampling. Chickens Peyer's patches, similar but less numerous than mammalian ones and disseminated along the small intestine, mainly in jejunum, consist of lymphoid follicles with T and B lymphocytes. Precisely, inside lymphoid follicles, B cells stay in a network formed by DCs, while the inter-follicular areas are full of T cells (Brisbin et al., 2008). While B cells play role in antibody-mediated response through Ig releasing, cell-mediated immune response is regulated by T cells: particularly, some T lymphocytes are key players in homeostasis maintenance, such as regulatory CD4+ T cells. These adaptive immune components need molecular adaptation to specific bacterial molecules for recognition (Schiffrin and Blum, 2002).

2.3.2 Relationship between host immune system and bacteria in gut health

The cohabitation between host and microbial community of intestinal lumen is a sensitive balance, whose variation can lead to various pathological conditions. Beneficial bacteria participate in gut immune homeostasis through physiological stimulation and modulation of both innate and acquired immune response (Clavijo and Flórez, 2018). Considering innate immune response, the continuous exposure to microbial challenges promotes and maintains lively the barrier function, supporting and increasing production of antimicrobial peptides and IgA concentration in the mucus layer and promoting epithelial turnover. Furthermore, butyrate produced by fermentative bacteria is used by goblet cells to produce mucins. Luminal microbiota also strengthens gut barrier and epithelium integrity by stimulating pathogen recognition systems such as TLRs. When injured, gut epithelium activates healing processes that involve some PRRs to promote tissue restoration: during these processes, activation of TLRs by microbiota seems to occur, along with increase of anti-inflammatory cytokines to try in restoring homeostasis (Chen et al., 2015). Furthermore, as mediators of host and microbiota connection, TLRs seem to have important role also in mucus production (Kurashima et al., 2013; Johansson and Hansson, 2016). In particular, gut bacteria in conventionally reared chickens seem to stimulate sialic acid mucins, while higher sulfate mucin concentration has been observed in birds with lower bacterial load (Clavijo and Flórez, 2018). From an acquired immune response point of view, microbiota regulates intestinal lymphocyte recruitment and cytokine release (Gabriel et al., 2006; Clavijo and Flórez, 2018). Stimulation of gut immune system, in turn, regulates microbiota composition. In example, B cells stimulated by microbiota release IgA which, in turn, regulate microbial composition and function (Kamada et al., 2013) and prevent commensal microbiota from enhancing an overexpressed immune response against commensal themselves (Haghighi et al., 2006).

Commensals do not lead to a strong epithelial defensive response, but primarily they maintain an immune-modulation status in the host. Two mechanisms of control are recognized: bacteria-bacteria interaction (such as competitive exclusion) and bacteria-host interaction (development of gut and regulation of gut functions) (Schiffrin and Blum, 2002). So, bacterial community exerts a dual function in the gut, stimulating mucosal defense but meanwhile maintaining immune reaction. This balance of immunotolerance is regulated through mechanisms involving gene activation, such as those related to regulatory cytokines at IELs level (Schiffrin and Blum, 2002). In fact, microbiota modulates IELs activity through inhibition of signaling events linked to expression of pro-inflammatory cytokines and chemokines. Similarly, metabolites produced by commensal bacteria from nutrients can also downregulate inflammatory pathways (e.g. butyric acid) and elicit regulatory cytokines (Artis, 2008). This balance between pro and anti-inflammatory cells and cytokines is also influenced by the ability of commensals in T cell subsets shaping (e.g. through stimulation of serum amyloid A, SAA, that induces T cell differentiation) to maintain gut homeostasis (Hooper et al., 2012; Kamada et al., 2013).

In chickens, GALT reaches its functional maturity by week 2 post-hatch and its correct development requires the presence of microbiota. In post-hatched chicks, colonization of gut by commensal bacteria generally leads to a mild inflammation, with macrophage and granulocyte infiltration in lamina propria (Pan and Yu, 2014). It has been seen that, at birth, GALT contains functional immature lymphocytes B and T which get their whole functionality during the first 2 weeks of age (Sato et al., 2009). Furthermore, in post-hatched chicks Ig-producing cells are few and they increase their number in response to gut microbial colonization, probably thanks to bacterial lipopolysaccharide mitogenic effect on B cells (Rubio, 2018). In this sense, it has been showed the capacity of some selected LAB to stimulate gene expression of gut TLR in post-hatching chicks, with effects on immune system (Sato et al., 2009). When contact between gut epithelium and bacteria occurs, or when epithelium is partially invaded by bacteria, microbial membrane components (such as lipopolysaccharides, on the outer membrane of gram -) act as potent triggering signals, activating B cells and stimulating production of Igs. This stimulation has been seen to be impossible without bacterial presence. In fact, Igs gene expression lacks in cecum of germ-free chickens, consistent with absence of B lymphocytes, supporting the statement that presence of microbiota induces antibody production (Volf et al., 2017). Given the importance of chick immunocompetence at hatching to face environmental antigens, early microbial colonization covers an important role (Rubio, 2018). Furthermore, Igs expression in cecum develops along with the gradual microbiota establishment during growing, and induction of expression of genes for enzymes involved in sulfur metabolism-related processes has been reported in cecum of conventional chickens compared to germ-free ones, assuming an important role of

microbiota in sulfonate groups providing for mucin enrichment, used to form a strong protective mucus layer (Volf et al., 2017).

Then, beside the capacity of beneficial bacteria in excluding and competing with pathogens, considering that influence on gene expression patterns could be strictly related to gut health, a direct or indirect manipulation of gut microbiota really may represent a key factor to counteract or prevent gut disorders and diseases (Schiffrin and Blum, 2002).

3. Feeding strategies to improve poultry health

Antibiotics have been largely used in animal production for decades. Since their discovery in the 1920s, antibiotics had a pivotal role in progress and growth of animal industries (Gadde et al., 2017). Next to those used therapeutically, most of the antibiotic use was practiced for prophylactic aims since the 1950s. The use of in-feed antibiotics at sub-therapeutic level (as AGP) rose with the intensification of livestock production to maintain gut ecosystem balance, to improve average daily weight gain and feed efficiency, as well as to reduce mortality (Huyghebaert et al., 2011; Lin et al., 2013; Gadde et al., 2017). Net effect of AGP on poultry industry account for about 3-5% of increase of growth and feed efficiency (Gadde et al., 2017). Animal performance benefits come from AGP main effect in shifting in gut microbiota, which result in a total lower microbial load with less energy consumption and a balanced microbial population probably less capable of eliciting an inflammation response. This effect seem to increase energy harvest from nutrients and help the animal in showing all its genetic potential (Huyghebaert et al., 2011; Gadde et al., 2017). Hence, growth promoting by AGP is mediated by an enhanced nutrient availability because less used by microbiota and because of the improved nutrient absorption through a thinner gut wall (Brisbin et al., 2008; Huyghebaert et al., 2011), together with a higher control of gastrointestinal tract infections, with a general final reduced maintenance cost of the gut (Lin et al., 2013). A quite recent study on effects of medicated or non-medicated diets on shift in chickens ileal microbiota showed a particular influence of AGP on Firmicutes division, with elimination of *Lactobacillus* species compared to their high proportion in non-medicated fed birds (Lin et al., 2013). Among indirect effects of AGP, it has been suggested that reduction in *Lactobacillus* spp. enhances lipid metabolism. In fact, bile salt deconjugation by *Lactobacillus* species through bile salt hydrolases (BSH) enzymes leads to a lessen lipid digestion and absorption, while decrease in these bacteria may mean more energy available for growth (Begley et al., 2006).

On the other hand, the routinely and extensive use of antibiotics, mainly as AGP, during the last 50 years has been associated with the development and increasing of drug-resistant bacteria, a real threat and serious problem for animals and public health, since the altered microbiota stimulated by antibiotics is transferrable to other hosts with consequent risk of transference of antibiotic resistance genes from livestock to human microbiota (Sugiharto, 2016; Gadde et al., 2017). This increasing problem has led to the total ban of antibiotic growth promoters (AGP) in the European Union in 2006 (Regulation EC n° 1831/2003) (Danzeisen et al., 2011; Stanley et al., 2014; Ducatelle et al., 2018). After the ban for AGP as feed additives in animal feed, gut health problems related to gut dysbiosis, barrier leakage and intestinal inflammation have increased and become big issues in poultry

(Ranjitkar et al., 2016; Ducatelle et al., 2018). These problems had implemented the search for alternatives to improve growth performance, animal health and to avoid diseases such as NE, gut dysbiosis and immune system dysregulation (Borda-Molina et al., 2018).

3.1 Feed additives to prevent the use of antibiotics

3.1.1 Exogenous enzymes

It is well recognized enzyme effect in promoting feed efficiency and growth in poultry. Among enzyme feed additive classes, those commonly used include phytases, carbohydrases (like xylanase, amylase etc.) and proteases. Most of these dietary exogenous enzymes act on non-digestible and antinutritional factors of animal feeds such as phytic acid, NSP and endosperm cell-wall carbohydrates that could have negative impact on poultry production, improving dietary nutrients availability (Ferket et al., 2005; Gadde et al., 2017).

NSP (including pectins, oligosaccharides, β-glucans) could exert anti-nutritive effects in poultry, increasing bulk and viscosity of digesta and mucus layer thickening, hindering the activity of digestive enzymes. Furthermore, in NSP-rich diets, the risk is an exceeding fermentation at small intestine level and bacteria overloaded, with less activity of the hindgut microbiota and greater competition with the host for nutrients. Supplementation of diets with enzymes can help in containing ileal fermentation and volatile fatty acid amount, with more fermentable material available for cecal microbiota, where fermentation products are readily absorbed by epithelial wall (Huyghebaert et al., 2011). In example, endoxylanases release nutrients that become available for endogenous enzymes and inhibit excess of fermentative bacterial load in small intestine by limiting nutritive substrate trough an increased digesta passage rate and digestion (Ferket et al., 2005).

NSP enzyme-related improved performance is related to an increase in digestibility and absorption of nutrients otherwise not digested by host enzymes, to an inactivation of anti-nutritional factors and to an increase in non-digested nutrient solubility and cecal fermentation. Furthermore, exogenous enzymes make an indirect effect on microbiota composition, which may be affected by the released short-chain oligosaccharides from NSP with potential prebiotic effects, with further influence on performance (Gadde et al., 2017). Enzymes like xylanase and β-glucanases can stimulate LAB growth, which adhere to gut epithelium and compete with pathogens for binding sites (Borda-Molina et al., 2018). Also morphology of small intestine seems to be affected by some enzymes, how it has been observed in jejunum of broiler fed a xylanase, where crypt depth was reduced and associated with an increase in chicken growth (Yang et al., 2008) and in chicken jejunum with dietary lysozyme, where increase in villus length led to higher surface area and absorption (Abdel-Latif et al., 2017).

Lysozyme seems also to be able to implement immune and oxidant status in small intestine by increasing mRNA of related genes (Abdel-Latif et al., 2017).
Often, endogenous enzymes are mixed and used together to maximize their effect by making a combination with multiple anti-nutritive substance targets (Sugiharto, 2016). However, benefits of feed enzymes can vary due to differences in diet composition, enzyme type and source and animal genetic (Gadde et al., 2017).

3.1.2 Organic acids

Organic acids are largely found in nature as constituents of plants and animal tissues. Furthermore, as already said, they also derived from microbial fermentations of carbohydrates as it occurs in poultry ceca and they are positive correlated with growth and performance. Organic acids can be monocarboxylic acids (e.g. formic, lactic, acetic, propionic and butyric acid) or carboxylic acids with hydroxyl group and can be added to animal feeds as individual ones, as salt form or as blends (Huyghebaert et al., 2011; Gadde et al., 2017). General in-feed organic acid main mechanisms of action lay on their capacity in decreasing pH and buffering effect of feed at crop, proventriculus and gizzard level and on their ability in passing from dissociated to undissociated form (depending on pH) to explicate the antimicrobial effect. In fact, organic acids can pass bacterial cell membrane and, once inside, dissociate and lower pH, so that pH-sensitive bacteria dead (such as some pathogens), but no acid-resistant bacteria (such as *Lactobacillus* spp.), which instead, increase (Huyghebaert et al., 2011). Reduction in potential pathogenic bacterial species may be associated with improved gut structural architecture: the trophic effect has been seen in duodenum and jejunum of broiler chickens fed butyric or fumaric or lactic acid, where, regardless of organic acid type used, the dietary supplementation increase villus height, maybe due to a reduced bacterial load, less inflammation and mucosal challenge with consequent increase in villus function (Adil et al., 2010). More, organic acids favor mineral absorption and can be used for epithelial cell nourishment (such as SCFAs) (Gadde et al., 2017); lastly, they also promote digestion by increasing pancreatic secretions (Adil et al., 2010).
There are evidences on benefits on gut health of organic acids, particularly on bacteriostatic effects of fatty acids like butyrate (Rinttilä and Apajalahti, 2013), which has been seen to strengthen mucosal barrier by stimulating antimicrobial peptides release in mucus and tight junction protein expression (Huyghebaert et al., 2011). Nevertheless, it is worth remembering that concentration of in-feed organic acids is reducing going towards the hindgut, due to absorption and metabolism in the upper gut (Adil et al., 2010), except when incapsulated. In terms of performance, even some positive effects

were reported (Adil et al., 2010), not ever coherent effects on growth exist, maybe due to different types and levels of organic acids used and diet ingredients (Gadde et al., 2017).

3.1.3 Phytogenics

Phytogenics or phytobiotics additives are a wide range of natural bioactive compounds deriving and extracting from various plant sources (principle classified in herbs and spices) with antimicrobial activity and immune enhancement as major properties and with polyphenols and flavonoids as main bioactive components and potent antioxidants (Sugiharto, 2016; Gadde et al., 2017). Mechanisms of action of these additives are not fully elucidated and their effectiveness remains controversial, but in general they act by stimulation cell membrane disruption in pathogens, stimulation growth and settlement of beneficial bacteria (lactobacilli and bifidobacteria) and as immunomodulators of immune response (such as higher cytokine expression and cell proliferation) (Sugiharto, 2016; Gadde et al., 2017). It has been recently observed the capacity of some plant extracts in mitigating detrimental effects of *Salmonella enteriditis* infection in cecum of chickens through selectively enhancing abundance of several beneficial bacterial genera such as *Faecalibacterium* and *Lactobacillus,* while, when plant extracts were provided without gut infection, no effects on microbial diversity were recorded (Varmuzova et al., 2015). Another important effect is reduction of oxidative stress and increase of antioxidant response in tissues, increase in pancreatic enzyme production and stimulation of growth of gut cells (Gadde et al., 2017). Naturally, given the wide variety of plant sources, variation in composition due to biological factors, storage and different type of processes (Huyghebaert et al., 2011), it is obvious that a very broad range of variables exists and efficacy depends on type, dose, process applied to the compounds together with all external variables such as diet, genetic etc.

Between processed phytogenic compounds, essential oils and oleoresins stand out (Gadde et al., 2017). Essential oils are volatile compounds isolated from plants. Different studies on effect and efficacy of many different essential oils from aromatic plants have been conducted and their antioxidant activity, along with influence on microbiota composition have been reported: as examples, ginger root essential oil supplementation showed an increase in LAB counts in chickens jejunum (Tekelì et al., 2004) and an improved antioxidant status of broiler chickens under heat stress, with decrease of serum malondialdehyde, one of major aldehyde products of lipid peroxidation in stress situations (Habibi et al., 2014). A decrease in CFU of *E. coli* and other enterobacteria was also observed in digesta of ileo-colon of chickens fed ginger and garlic essential oils (Dieumou et al., 2009). In contrast with precedent studies, where no improvement of performance by dietary

phytogenic substances was observed in chickens reared until 42 days of age (Tekelì et al., 2004; Dieumou et al., 2009), Habibi and colleagues reported an improvement in body weight in chickens fed ginger root powder in the first growing period (at 22 d), but not after (Habibi et al., 2014). Still differently, Kamboh and colleagues (2013) instead reported improved in weekly performance of broilers during the finisher period of growth (22-42 d) with dietary supplementation of two plant-derived flavonoids; moreover, they noticed an ameliorating action of these antioxidants in chickens under persistent seasonal heat stress, through downregulation of some stress biomarkers in blood and muscle (Kamboh et al., 2013).

3.1.4 Amino acids

Beside main nutritive function of AAs and their requirement for protein synthesis for growth optimization, dietary AA supplementation has been also considered in a perspective of intestinal protection, immunity regulation and microbial maintenance. AAs can regulate different metabolic routes, gene expression and hormone and other important molecule synthesis (Gottardo et al., 2016). In fact, increase in dietary AA level may help in case of malabsorption during intestinal challenge because it is reported that supply of AAs with higher digestibility are beneficial to restore gut when absorptive capacity is impaired and so, consequently, to improve feed efficiency and growth. It seems that higher AA concentration in animal diet may stimulate a better development of intestinal mucosa and that dietary AAs may promote intestinal repair through induction of enzymes needed for mitotic processes and through stimulation of expression of genes for anti-inflammatory response and reparative processes (Bortoluzzi et al., 2018).

Between the different AAs, roles of Threonine (Thr), Arginine (Arg) and Glutamine (Gln) in mucin production, immune function and epithelium proliferation, respectively, are well known. Thr is the third essential AA in poultry after Lysine and Methionine and main component of intestinal mucins, taking role in gut barrier maintenance. In case of gut impairment and consequent higher mucus production, Thr requirement increases. Moreover, Thr is a major component of intestinal IgA and it can also influence some immune parameters such as production of pro-inflammatory cytokines (Bortoluzzi et al., 2018). Arg is used as a precursor of creatine, nitric oxide (cytotoxic mediator of immune cells) and polyamines, which act directly on development or reconstitution of gut epithelium as trophic substances (Gottardo et al., 2016; Bortoluzzi et al., 2018). Together with glutamate, the acidic homologue Gln represents first energy source for small intestinal enterocytes and enhances gut proliferation, furthermore it is considered as essential in inflammatory condition because it supports mucosal repair (Bortoluzzi et al., 2018). Gln, beside its role as nitrogen source for purine and

pyrimidine, has been seen to protect gut epithelium when stressors occur: dietary Gln supply in chickens under heat stress showed to increase villus height in jejunum and ileum, with possible benefits for digestion due to wider gut absorptive area. More, Gln decreased level of TNF-α and increased IL-10, so alleviating intestinal inflammatory response; lastly, Gln improved intestinal barrier and integrity by preventing increase in permeability through upregulation of RNA expression of tight junction protein-related genes (Wu et al., 2018). Broiler chickens fed high dietary amino acid concentrations (Thr, Arg and Gln) and submitted to gut infection challenge have been reported to have improved feed conversion and higher small intestinal recovery and integrity, probably due to an increase in cell proliferation, key factor for gut regeneration (Gottardo et al., 2016).

In a recent study, also dietary Tryptophan (Trp) supplementation was shown to alleviate and counteract negative effects of chronic unpredictable stress in broilers at jejunal mucosal level in terms of intestinal permeability (enhancing tight junction protein abundance), gut immune regulation (enhancing IL-10 RNA gene expression and IgA level), Furthermore, supply of Trp seemed to attenuate increase of serum corticosterone and, as precursor of neurotransmitter serotonin (involved in intestinal motility and secretion), it prevented stress-induced alteration of serotonin signaling (Yue et al., 2017).

Keeping in consideration all these functional role of AAs, it is worth to remember that a correct and adequate level must be defined to do not lead to an AA excess and AA loss.

3.1.5 Probiotics

Probiotics are defined as microbial supplements able to beneficially affect intestinal balance by maintaining healthy indigenous microbiota and counteracting pathogens (Rubio, 2018). Further definition states that *"Probiotics are mono or mixed cultures of live organisms which when administrated in adequate amounts confer a health benefit to the host"* (FAO/WHO, 2001).

Different and variant bacteria (e.g. *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, *Streptococcus* and *Lactococcus* spp.) and yeast (e.g. *Candida* and *Saccharomyces* spp.) have been widely tested and studied as probiotics in poultry (Gadde et al., 2017). Probiotics must reflect some requirements, such as surviving the passage in GIT, being not pathogenic but beneficial for the host, being suitable for manufacturing and delivery, having epithelium adherence-capacity and immune system modulatory effect, improving gut function and being able to colonize host's gut (Baldwin et al., 2018; Clavijo and Flórez, 2018). Beneficial effects of probiotics can be indirectly reflected in an improvement of growth performance.

Recently, positive effects of the dietary supplementation of a *Lactobacillus acidophilus* strain, isolated from GIT of healthy adult chickens, on broilers for the whole rearing period have been reported, with an increase in butyric acid producing bacteria abundance in chicken's cecum content, in relation to changes in metabolic activities (higher abundance of glucosidases). Probiotic supplementation also improved chickens growth performance (BW and FCR), probably by indirect way through improving gut health (De Cesare et al., 2017). Effect of direct fed probiotic on growth performance was also previously observed and linked to a larger amount of LAB in bird gut, with possible indirect effect on performance, since LAB can increase gut available energy by increasing digestibility of carbohydrates and ezyme activities (Nayebpor et al., 2007). Similarly, Mookiah and colleagues (2014) observed improved BW and FCR after feeding multi-strain *Lactobacillus* probiotic in adult broiler chickens, together with an increase in cecal population of lactobacilli and bifidobacterial and a decrease in *E. coli* and aerobe population, which might be linked to the probiotic growth-promoting effect (Mookiah et al., 2014). Differently, no differences in growth performance in young chicks were observed in a study on evaluation of LAB (different *Lactobacillus* species) effects as probiotics (Sato et al., 2009), as well as in a study with probiotic *Clostridium butyricum* (Zhang et al., 2016), since many determinants can affect effectiveness of beneficial bacteria.

In terms of gut and systemic immune system activation, some probiotics have been seen to be able in stimulating immune response and so, indirectly, they might contribute in gut health maintenance. In example, dietary administration of *Saccharomyces* and *Bacillus* probiotics in chicks, from hatching on, has shown to modulate immunity at jejunum and ileum level with increase in both inflammatory and anti-inflammatory cytokines and increase in IgA-producing cells (Rajput et al., 2013). Furthermore, these authors also observed a probiotic capacity in modulation of gut structure, with increment of tight junction RNA gene expression and increase in jejunum villus height and goblet cell number, which have been considered as determinants in the higher BW observed in chicken fed probiotics in this study (Rajput et al., 2013). Beside existing contrasting results on growth-promoting effects of *Bacillus* spp. in chicken (Gadde et al., 2017), modulation of small intestinal morphology (increasing villus height in jejunum and ileum) by direct fed *Bacillus* spp. in young chickens was also reported in a previous study (Lee et al., 2010). These authors also observed a modulation of immune-related parameters through decreasing of acute phase proteins and stimulation of specific IEL subsets, indirectly contributing in gut defense against pathogens in young chicks where immune functions are not still fully completed. A clear change at gene expression level in these IELs was also observed, particularly referred to cytokine transcripts such as IL-2 (involved in cell-mediated immunity regulation), INFγ (regulator of acquired immunity) and other mediators of immune systems (Lee et al., 2010). Also, other probiotics and probiotics mixture have shown to impact immunity, especially

in terms of natural antibodies production. Yurong and colleagues (2005) observed that a probiotic mix (with *Bacillus, Lactobacillus* and *Candida* strains) oral administered to post-hatched chicks favored an increase of intestinal IgA in chicks of one week of age along with an increase in Ig-forming cells in cecal tonsils (Yurong et al., 2005). More, probiotic mixture of *Lactobacillus, Bifidobacterium* and *Streptococcus* strains orally inoculated in neonatal chicks can enhance serum (IgG and IgM) and intestinal (IgA) natural antibodies production against different foreign antigens (Haghighi et al., 2006). The different ability of *Lactobacillus* species in inducing systemic immune response to different antigens was further reported in a study by Brisbin and colleagues (2011), where weekly oral administration of different *Lactobacillus* strains in chicks showed differences in terms of antibodies increase or decrease in response to different antigens (Brisbin et al., 2011).

Stimulation of natural non-specific antibodies and immune cells in the intestine of chickens is linked to the probiotic ability in strengthening Peyer's patches in antigen recognition, like those of probiotics, even if harmless bacteria. Once entered Peyer's patches epithelium, probiotics are presented to lymphoid cells and activate lymphocytes B that become plasma cells: these Ig-forming cells diffuse in lymphoid system and GALT, enter the blood circulation and exit in the intestinal lamina propria where they secrete Ig (Yurong et al., 2005).

First step of infection process of pathogens consists in attachment (or adhesion) key-capacity to intestinal epithelial cells. Counteracting and exclude pathogens from epithelial binding sites is the other important probiotic capacity and is exercised through antimicrobial substances production, competing for nutrients, inhibition of pathogen adherence, immune response enhancing and environmental modulation to create a hostile setting for harmful bacteria (e.g. pH lowering) (Gadde et al., 2017; Baldwin et al., 2018). In example, *Lactobacillus* and *Bifidobacterium* probiotic species can reduce adhesion of pathogens by producing hydrogen peroxide, bacteriocins and organic acids (Clavijo and Flórez, 2018; Rubio, 2018). More, usage of spores of *Bacillus licheniformis* or its direct administration as dietary microbial probiotic in chickens at different doses have shown to be able in preventing necrotic enteritis (and then in counteracting *Cl. perfringens*) in terms of mortality and small intestinal lesions, with an improvement of growth performance too (Knap et al., 2010). A more recent study showed that dietary supplementation of *Clostridium butyricum* improved jejunal mucosa status in chickens challenged with *E. coli* K88, with increase in villus height, crypt depth, cytokine production and digestive enzyme activities (Zhang et al., 2016).

Other reported probiotic effects are lactose intolerance alleviation, blood cholesterol reduction, bile salt hydrolysis capacity (typical of *Lactobacillus* and *Bifidobacterium*, usually used as probiotics): this last effect allows AA releasing from bile salt deconjugation and their utilization as nitrogen, carbon and energy sources from bacteria, furthermore BSH acts as bile detoxifying enzyme for

microbial survival. However, increase in deconjugated bile salt level may be not always safe for the host, since deconjugated bile salts are less reabsorbed and they may damage gut mucosa (Begley et al., 2006).

Anyway, it is important to remind that probiotic effectiveness highly depends on differences in strains of bacteria used and their dietary concentration, as well as on time-point and way of administration. Probiotic administration in early life can help in microbiota composition balance even later, during growing (Clavijo and Flórez, 2018; Rubio, 2018).

3.1.6 Prebiotics

Dietary fiber is a healthy component of the diet, composed by insoluble fiber (cellulose and lignin) and souble fiber (i.e. NSP and polysaccharides) not digestible by enzymes of mammals. Among soluble fiber, carbohydrates with specific peculiarity in stimulating beneficial bacterial species (mostly *Lactobacillus* and *Bifidobacterium* spp.) and indirectly improving host health have risen the concept of "prebiotic", over 20 years ago, build on the "probiotic" concept. During these years the concept of "prebiotic" has changed, passing by the definition of "non-digestible feed ingredients", to "selectively fermented ingredients" with effects on composition and/or activity of gastrointestinal microbiota (Gibson et al., 2017). In 2008, the Food and Agricultural Organization (FAO) updated the definition of prebiotics, defining them as "*non-viable food components that confer a health benefit on the host associated with the modulation of the microbiota*" (Pineiro et al., 2008), so removing the criterion of selective fermentation. Recently, the International Scientific Association for Probiotics and Prebiotics has proposed a new definition of a prebiotics, that is *"a substrate that is selectively utilized by host micro-organisms conferring a health benefi*t", extending the possible utilization of prebiotics not only via fermentation but also in other pathways and amplifying prebiotic effects beyond lactobacilli and bifidobacterial (Gibson et al., 2017).

Prebiotics derive from plants or are synthetized by microorganisms, and are represented by those feed ingredients that can resist to gastric acidity and gastrointestinal digestion and absorption, that can be fermented by certain intestinal bacteria, being source of nutrients for commensals, and that can selectively stimulate growth / activity of those specific beneficial bacteria associated to gut health (Rubio, 2018). In fact, first, presence of prebiotics in the diet can shift poultry gut microbiota to a healthier population, increasing resistance of beneficial bacterial community. Most of commercial prebiotics are substances with low molecular weight and are generally fermented in the low intestine, with production of SCFA and improvement of gut health maintenance (Ferket et al., 2005)

Oligosaccharides are the main components of prebiotic group and can derived from any of the hexose monosaccharides, including glucose, fructose, galactose and mannose (Huyghebaert et al., 2011). Among the most commercially available prebiotics, mannan-oligosaccharides (MOS) are widely well known. They are produced from yeast cell walls of *Saccharomyces cerevisiae* and are known as positive influencers of gut health, in part due to yeast-cell antigen stimulating properties, typical of the mannan chain (Shashidhara and Devegowda, 2003): in fact, MOS can be recognized by receptors of immune system and can boost host immune response (Gadde et al., 2017). Contrarily, Midilli and colleagues (2008) did not observed changes in serum IgG concentration in broilers fed dietary MOS compared to those fed basal diet, hypothesizing that prebiotic doses, as well as animal age and other environmental determinants could impact it (Midilli et al., 2008). No differences in plasma Igs (IgA and IgG) after MOS supplementation in broilers for the whole rearing period were also observed by Kim and colleagues (2011) (Kim et al., 2011a). Similarly, no increase in antibody production in chickens fed MOS was reported in a more recent study, even if increase in leukocytes was observed (Taheri et al., 2014). MOS have also receptor properties for *E. coli* and *Salmonella spp.* fimbriae and can prevent epithelial adhesion of these pathogens, and on the other hand, favor beneficial commensals (Huyghebaert et al., 2011). Dietary MOS provided to layers already showed capacity in modifying cecal microbiota, stimulating growth of obligate anaerobes and decreasing enterobacteria load; furthermore, when these hen cecal content was orally inoculated in post-hatched chicks, it induced the same effects (Fernandez et al., 2002). In support of this, dietary supplementation of MOS favored gut health status by inducing decrease of *E. coli* and *Cl. perfringens* and growth of *Lactobacillus* species and diversity at ileal level in broiler chickens (Kim et al., 2011a), as already seen by Chee and colleagues (2010), who also reported higher bacterial diversity in cecum (Chee et al., 2010). Same observations were reported previously also by Baurhoo and colleagues (2007), who reported an increase in bifidobacteria load in ceca of chickens fed MOS, together with a lower *E. coli* load (Baurhoo et al., 2007). MOS have been also reported to positively affect gut health in terms of intestinal morphology, by increasing jejunal villus height and goblet cell number also in ileum (Baurhoo et al., 2007; Chee et al., 2010), with particular selective increase in acidic and sulfate-acidic goblet cells (Chee et al., 2010), all markers of gut maturation and defense. MOS modulation of intestinal mucin synthesis seems to occur through up-regulation of mucin-related genes (Brennan et al., 2013), with possible specific beneficial bacteria intervention too, since some *Lactobacillus* spp. can up-regulate genic post-transcripts of mucins (Chee et al., 2010). A more in-depth study on MOS molecular mechanisms at jejunal level highlighted that MOS induced changes in the expression of multiple genes involved in immune processes, upregulating those involved in mucosal immunity. More, MOS also upregulated some mitochondrial pathways linked to energy production and

antioxidant response (Xiao et al., 2012). In terms of growth performance, different results are reported. In some cases, it seems that MOS are able to improve growth performance in terms of BW and FCR (Chee et al., 2010; Brennan et al., 2013), or BW and feed consumption (Ashayerizadeh et al., 2009) in broiler chickens, while in others, no beneficial effects on performance traits are reported after MOS administration (Midilli et al., 2008; Kim et al., 2011a; Taheri et al., 2014). Anyway, MOS influence on growth response is age dependent and act better on younger than on older birds (Chee et al., 2010), furthermore doses, type and other external factors play a crucial role in prebiotic effectiveness.

Like MOS, also plant-deriving fructo-oligosaccharides (FOS), have been tested as possible prebiotics in poultry. Effects of supplementation of dietary FOS at different doses in chickens have been evaluated and changes in microbial population in small intestine and cecum content have been observed, with significant increase in *Bifidobacterium* and *Lactobacillus* spp. and decrease in *E. coli* counts. In cecum, FOS also led to an increase of total anaerobes. Furthermore, improved growth performance were reported (Xu et al., 2003). Same effects have been also observed in a more recent study, where FOS showed to affect chicken intestinal bacterial community with increase of beneficial bacteria and decrease of *E. coli* and *Cl. perfringens*, even if no performance improvement was observed (Kim et al., 2011a). Changes in gut microbiota after FOS administration have been also linked to a further observation of a digestive enzyme activity increase (especially of amylase and protease) and of an improved jejunum and ileum morphology, where FOS induced higher villus height and lower crypt depth, along with a higher microvillus height (Xu et al., 2003). Inulin, the longer chain version of FOS, is a heterogeneous blend of fructose polymers extracted from chicory roots and widely recognized as prebiotic (Li et al., 2018), being the only one to date awarded as EU health claim on improving gut function (Gibson et al., 2017). Inulin it is known for enhancing *Bifidobacterium* and *Lactobacillus* abundances in poultry thus improving microbial balance and represents a substrate for SCFA production (Li et al., 2018).

In addition to the above-mentioned oligosaccharides, some studies have been conducted on galacto-oligosaccharides (GOS), polymers of galactose and in high concentration in human milk, as prebiotic candidate in poultry. GOS are commercially produced by β-galactosidase enzymes with trans-galactosylation activity of lactose, come from different fungi, yeast or bacteria, are preferentially utilized by bifidobacteria and have shown to possess significant health benefits and to beneficial affect immune system (Wilson and Whelan, 2017). GOS protective role and improvement of gut barrier function in various aspects have been reported in rats with severe acute pacreatitis. Indeed, GOS-supplemented enteral nutrition induced significant increase in colonic bifidobacteria number, in secretory IgA level in intestinal mucus and in occludin RNA level, indicating an enhancement of

gut barrier (Zhong et al., 2009). In chickens, Jung and colleagues (2008) reported an effect of GOS on broiler fecal microbiota, with preferentially increase of abundance of *Bifidobacterium* species, along with increase in total anaerobes and lactobacilli, even if no improvement in performance traits were recorded (Jung et al., 2008). Same observations have been also reported by Baffoni and colleagues (2012), who recognized GOS as good stimulator of *Bifidobacterium longum* probiotic in vitro and found an increase in bifidobacteria-related genes in fecal samples of young broiler chickens, along with a decrease of fecal load of *Campylobacter* spp. (Baffoni et al., 2012). Given these results, they hypothesized that GOS might be well used in poultry also due the lack of lactase. By this point of view, it is possible that GOS are entirely used by intestinal bacteria, and that the induced increase in bifidobacteria in GOS fed chickens have contributing in the modulation of expression of *Campylobacter* spp. genes involved in epithelial adhesion, so preventing its invasion (Baffoni et al., 2012).

However, prebiotic effects of GOS, like those of the precedent mentioned prebiotics, may be dose dependent, host specific and may be influenced by different environmental determinants.

3.1.7 Synbiotics

Synbiotics combine prebiotic and probiotic effects, such that they act synergistically, and are defined as mixture of probiotics and prebiotics that beneficially affect the host by improving the survival and persistence of living microbial dietary supplements in the GIT, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria (Patterson and Burkholder, 2003). This mixture has been formulated based on the idea that the combination of a prebiotic and a probiotic could improve survival and persistence of the beneficial bacteria (probiotic) in the gut thanks to its specific substrate (prebiotic) available for fermentation (Sugiharto, 2016). Such an example, synbiotic formula including prebiotic GOS and probiotic *Bifidobacterium longum* administered to young broiler chickens showed an increase in fecal bifidobacteria and a significant reductive effect on *Campylobacter jejuni* (Baffoni et al., 2012). A previous study already reported a very similar beneficial effect of a synbiotic (GOS and a *Bifidobacterium lactis*-based probiotic) on bifidobacteria intestinal growth in broiler chickens (Jung et al., 2008). When different doses of a synbiotic, formulated with *Enterococcus faecium* and FOS, was administered to broilers for 42 days (rearing period) changes in cecal content population were observed, first with a reduction in total coliform counts, while an increase in LAB occurred, positively correlated with the probiotic doses inside the symbiotic (Dibaji et al., 2014). Supplementation of diets with a symbiotic showed to significantly improve growth traits (BW, FCR and feed consumption)

and carcass trait quality in broilers (Ashayerizadeh et al., 2009). More, improved growth performance (BW and FCR) were observed when a symbiotic combination of prebiotic isomalto-oligosaccharides and *Lactobacillus* multi-strain probiotic was administered to adult broiler chickens, along with an increase in cecal volatile and non-volatile fatty acid concentration, compared to probiotic and prebiotic alone. However, a synbiotic product effects did not show a 2-fold synergistic action like that given by the sum of the effects of probiotic and prebiotic alone (Mookiah et al., 2014). Contrarily, other trials did not report improvements in growth performance, like in the study of Jung et al. (2008), where in-feed inclusion of symbiotic (GOS and *Bifidobacterium lactis*) did not affect chicken growth traits (Jung et al., 2008).

3.2 Early nutritional supplementations

Early nutritional supplementations have been seen to enhance metabolism, immune system response and GIT microbiome development in chicks (Taha-Abdelaziz et al., 2018). Modern poultry industry is organized so that in the period between hatching and the delivery of neonatal chicks to broiler farms, chicks suffer a delayed feeding of 48-72 hours, with negative consequences on health and growth (Roto et al., 2016). Maternal diet, early chick nutrition or *in ovo* feeding represent new strategies to implement chicks development and help chicks in overcoming the gentle phase of post-hatching. Early life programming through instruments like these nutritional strategies is based on the assumption that development of different disturbs and diseases during life may be driven by environmental experiences had during the embryonic phase or in the critical post-natal period and that feed manipulation at early stages of poultry production could be connected to a healthier intestine later (Rubio, 2018).

3.2.1 Feeding of the hens

Development of the chicken embryo depends on nutrients deposited by the hen in the fertile egg. Particularly in the early post-hatching period, chicks are immune-deficient and so most of their immune defense depends on hen-derived antibodies (Ig), which, in turn, depend in part on nutritional status of the laying hens. Moreover, embryonic and post-hatched chick tissues contain high amounts of polyunsaturated fatty acids (PUFA) and development and maintenance of an efficient antioxidant system is critical. In addition to the standard diet, supplementation of laying hens with other nutrients could lead benefits in the offspring by helping embryo development, stimulating immune response capacity and enhancing antioxidant system (Taha-Abdelaziz et al., 2018).

Given the well-known effect of vitamin E as immune response stimulator and antioxidant factor, its addition to the standard diet in laying hen diet and its transfer to egg yolk and chick tissues has been investigated. A vitamin E supplementation to the standard diet in hens implies increase in its concentration in eggs (Surai, 2000; Rebel et al., 2004), with possible higher antioxidant protection. Next to vitamin E, also selenium represents a functional element in boosting antioxidant activity in post-hatched chicks, as reported by Surai and colleagues (2000), who observed that dietary selenium in hens can increase glutathione concentration in liver of 1-day old chicks, being a cofactor working with glutathione peroxidase and so enhancing antioxidant protection against radicals and metabolites in neonatal birds (Surai, 2000). In terms of immune stimulation, Saunders-Blades and Korver (2015) reported the capacity of a vitamin D metabolite dietary supplementation in hens in increasing % of egg hatchability and in vitro chicks innate immunity, with induction of higher leukocyte bactericidal capacity (Saunders-Blades and Korver, 2015). A general higher amount of dietary vitamins (such as vitamin A, E, C) and minerals (such as zinc, copper and selenium) has been seen to positive influence the % of intestinal infiltrate leukocytes in hens and % of circulating leukocytes in offspring, where also a faster recovery of intestinal state after infection for malabsorption syndrome was recorded, compared to chicks from hens fed basal diet with low nutrients supplementation (Rebel et al., 2004). Effects of maternal diet were furthermore observed with a diet supplemented with vitamins and minerals compared to a basal commercial diet on chicks jejunal gene expression. Both in 3 and 14 days old chicks from hens fed supplemented diet, upregulation of genes involved in epithelial turnover and maturation was found. More, differences in gene expression between 3 and 14 days old chicks were also observed, especially regarding upregulation of genes related to immune development at day 14 and not at day 3 of age, probably meaning an effect of maternal diet on these immune-related genes only on a more mature intestine (Rebel et al., 2006). From the results of this study, supplemented maternal diet seems to be able in influencing gut maturation and gut immune system development differently as chicks intestine grows, so not only at early post-hatching but in a long-term manner that might affect gut health (Rebel et al., 2006).

Given the importance of passive immunity acquired by hens during early post-hatching, Wang and colleagues (2004) investigated effects of different dietary fatty acid ratio of linoleic to linolenic acid on laying hen humoral response and passive immunity in embryos and chicks, in terms of IgG titers: while no effects were observed in serum of hens and embryos, changes in laying hen dietary organic acid ratio changed also IgG levels in serum in chicks post-hatching, highlighting hens diet immunomodulatory effect on chicks immune competence (Wang et al., 2004).

About prebiotics, few studies on their effects on laying hens and offspring have been conducted. As an example, effects of dietary administration of MOS on productive traits and humoral immune

response in laying hens and offspring have been partially investigated. Shashidhara and Devegowda (2003) reported an increase in % of egg hatchability and serum antibody titers in hens fed MOS and in their progeny, so explaining a possible better humoral immune status in chicks, which could be due to MOS directly influence on immunity (through GALT PRRs stimulation) or indirectly, by enhancing intestinal absorption of nutrients, such as minerals (Shashidhara and Devegowda, 2003). Contrarily, in a more recent study on dietary MOS and essential oils in laying hens, no effects on humoral immune response and productive traits have been reported (Bozkurt et al., 2012), thus remarking as already mentioned about additives and dietary supplementations, on which many different variables can affect efficacy.

3.2.2 Post-hatch early feeding

Importance of early feeding resides in avoiding a delay in feeding, with consequent starvation and mobilization of reserves from muscle proteins for the gluconeogenesis and with long-term negative effects on growth (Roto et al., 2016). Post-hatch early feeding positively influence both gut and GALT maturation and development, indirectly affecting growth rate and proportion of breast muscle. Digestion and absorption of AA and carbohydrates in neonatal chicks is limited and the major uptake is for glucose (Cardeal et al., 2015). The effect of early feeding on growth performance is linked to the enhanced metabolic rates, since it highly stimulates digestive enzyme secretion and increases absorption of glucose, AAs and oleic acid by the small intestine (Taha-Abdelaziz et al., 2018). The important role of early nutrition in immune stimulation is explained by the fact that when feed passes GIT, it triggers an antigenic response and, so, earlier it passes through intestine earlier proliferating immune stem cells will meet environmental antigens, amplifying their antibody range (Yegani and Korver, 2008). Early dietary supplementation with vitamin E and selenium seems to enhance innate and adaptive humoral chick immune response probably through cell immune-related modulation of vitamin E, whose absorption is supported by selenium (Singh et al., 2006). Next to the effects of nutrient supplementation on gut immune system, also probiotics play a role in inducing immunocompetence in neonatal chicks and have been proposed as possible immunomodulators post-hatching. Probiotic LAB have been particularly taken into account, and evaluation of their influence on GALT immune function in chicks immediately post-hatched has been investigated. As reported by Sato and colleagues (2009), dietary specific selected LAB (mainly *Lactobacillus* species) enhanced expression of some T cell immune response-related genes (such as IL-2 and IFN-γ) and of TLR-related genes in the foregut of neonatal chicks, with effectiveness during the first 3 days after

hatch. Higher gene expression of TLR may be probably due to the fact that many beneficial effects of lactobacilli are mediated by TLR (Sato et al., 2009).

In terms of gut microbiota, it has been seen that early age feeding of proper bacterial communities highly affect gut colonization and microbiota composition. Newly hatched chicks inoculated with different bacteria inocula originating from adult chicken ceca showed different colonization rates at ileum and cecum level with differences in shaping microbial community, depending on inocula, with a more stable colonization and structure in cecum. Furthermore, the different inocula stimulated different gene expression patterns (Yin et al., 2010). Early feeding provides new dietary antigens which can, in turn, influence microbial colonization and differentiation along the GIT. Early access to feed seems to differentiate microbiota compared to delayed fed chickens. Furthermore, probiotics administration after hatch can help in beneficial gut bacterial establishment which in turn influence an efficient GALT development (Taha-Abdelaziz et al., 2018). In fact, earlier beneficial bacteria enter and colonize GIT, earlier they can adhere to gut mucosa, establish and outline microbiota composition through pH modulation and metabolites. It seems that the first bacterial colonization has the greater impact on the further and following microbiota development and so, indirectly, on gut health (Baldwin et al., 2018). Broiler chicks inoculated immediately post-hatch with a mix of *Lactobacillus* probiotic species got higher body weight at 28 days of age and a lasting effect on development of a different bacterial community was observed at fecal and cecal content level compared to non-treated chickens (Baldwin et al., 2018). In another study, Li and colleagues (2018) evaluated the effect of two prebiotics (inulin and wheat bran) in broiler chicks post-hatching, noting a positive influence on villus height in jejunum and ileum and on cecal microbiota profile, particularly with inulin supplementation that greater stimulated butyrate-producing bacteria (e.g. *Faecalibacterium*), even if no differences were seen on SCFA production, maybe due to less and poor fermentative processes in young chicks (Li et al., 2018).

Besides prebiotics and probiotics, also phytogenic and herbal additives have been studied as nutrients for early feeding given their immunomodulatory properties, antibacterial and antioxidant activities. Starting with dietary herbal products supplementation immediately post-hatch was reported to improve chick performance in terms of BW and feed efficiency and to increase cecal *Lactobacillus* counts and decrease coliforms counts, hypothesizing an specific antibacterial activity of essential oil compounds contained in many herbal additives (Rostami et al., 2015). In a previous study, Kadam and colleagues (2009) reported that early feeding of herbal mix supplementation only during the first 48 hours post-hatching could influence chick live weight already at day 2 of age and further daily weight gain; furthermore, they observed also an increase in blood serum antibody titers against Newcastle Disease vaccination in chicks fed herbal mixture. In this study, phytogenic

supplementation also improved small intestinal morphology in terms of villus growth, explained by a possible mechanism of action of certain herbs in stimulating intestinal epithelium development, enterocyte differentiation and gut function (Kadam et al., 2009).

3.2.3 *In ovo* feeding

As previously said, given the importance of intestinal epithelium in determining the developmental potential of the hatched chicks and since post-hatching represents a critical period in which the intestine has to grow rapidly and start in digesting and absorbing nutrients as soon as possible (Tako et al., 2004), a precocity in these functions may highly and positively affect the following grow period of the chicks. It seems that in *in ovo* feeding can accelerate chick intestine development and avoid the delayed feeding that may occur after hatching, ensuring nutrient availability for growth sustenance (Kadam et al., 2008), and stimulating lymphoid organ, GALT and chick immune defenses development.

Early developmental programming is of increasing interest and represent a very useful application especially in poultry production, where to date, 30-40% of chick life is spent *in ovo* due to the faster growth rate. Application of the *in ovo* administration technique is really feasible because of the readily accessibility of avian embryo and the easily manipulation of *in ovo* environment (Rubio, 2018) by injection of nutrients in one of the five regions recognized as sites for delivery, which are the air cell, the chorio-allantoic membrane, the amniotic fluid, the yolk and the embryo body (Fig.2) (Roto et al., 2016). Injection *in ovo* itself is safe: saline solution (NaCl) injection does not affect embryo and chicks development, as well as BW (Tako et al., 2004) but possible negative effects of inoculation might be due to the volume injected. Usually, 0.1 or 0.2 ml volumes are injected without side effects, similarly to the *in ovo* injection of vaccines (0.1 ml/egg) (Cardeal et al., 2015), which represented first *in ovo* treatments and opened new further investigations (Roto et al., 2016). Also precision in depth of injection has to be taken in account, since an injection of needle too deep may cause trauma to the embryo (Roto et al., 2016).

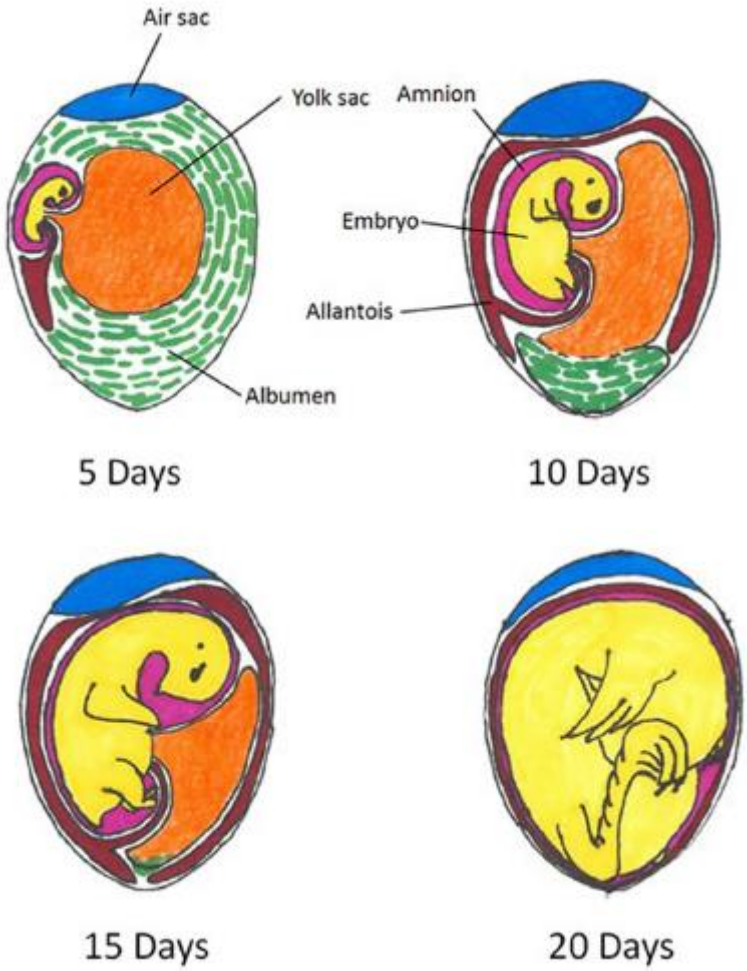


Figure 2 Different compartments of embryonated chicken egg at different ages of incubation (Roto et al., 2016)

Results from first studies on *in ovo* inoculation contributed in identifying effects of timing and of injection sites for nutrient administration, to optimize the technique. Edens et al. (1997) compared the hatchability of broiler embryos submitted to *in ovo* injection in air cell or amniotic with non-injected embryos and found no differences among the groups (Edens et al., 1997). A very early *in ovo* injection was studied at day 7 of embryonic development, in order to evaluate injection sites for nutrients to further improve chick BW at hatch, and relative effects on hatchability. Amniotic fluid, chorio-allantoic membrane, extra-embryonic cavity or yolk sac were chosen as target sites. Hatchability was higher negatively affected by injection in amniotic fluid and chorio-allantoic membrane, embryonic cavities closer to the embryo, probably also for the really early phase of embryo development, so suggesting to consider yolk sac or extra-embryonic coelum as sites for inoculation at this stage (Ohta and Kidd, 2001). Nevertheless, since developing of intestinal absorptive capacities and of immune system occurs later in the embryonic life, nutrient *in ovo* injection has been mostly performed after the first week of age of embryo, such as at day 12 (Pilarski

et al., 2005; Bednarczyk et al., 2011; Maiorano et al., 2012), day 14 (Kadam et al., 2008; Bhanja et al., 2014) or between day 17 and 18 of embryonic development (Tako et al., 2004; Uni et al., 2005; Smirnov et al., 2006). Differently from injection at day 7, no differences in hatchability have been reported with *in ovo* treatment at day 14 of egg incubation compared to untreated eggs, probably due to the less sensitive embryonic age (Cardeal et al., 2015).

- *Main nutrient supplementation*

Majority of *in ovo* studies have focused on main nutrient supplements, such as carbohydrates, AA, vitamins and minerals, necessary for intestinal growing (Roto et al., 2016). In this context, many studies have been conducted on investigation of the effects of *in ovo* treatment with injection in amniotic fluid during the late-term embryonic development. It has been demonstrated that supplying nutrients already during embryonic phase through *in ovo* feeding enhances and accelerates gut development and digestive capacity. Indeed, around day 17 or 18 of incubation (late-term period), inoculation of exogenous nutrients (e.g. carbohydrates) in the amniotic fluid (swallowed by chicks during the last incubation period) can lead to an increase in jejunal villus size and in gut capacity to digest disaccharides (increase of brush border enzyme activities), demonstrating that small intestine is already able in exploiting nutrients (Tako et al., 2004). Furthermore, *in ovo* fed chicks with carbohydrates seem to show a higher BW post-hatch, probably due to a better nutrient digestion and assimilation and a higher intestinal proliferation (Tako et al., 2004; Smirnov et al., 2006), with increasing of jejunum villus surface area (Smirnov et al., 2006). Another study reported how energy supplying (e.g. readily digestible polysaccharides) *in ovo* can support the late-term development of the embryo and the hatching activity, with a higher BW and breast muscle weight at hatching. Insertion of *in ovo* carbohydrates increased liver glycogen reserves, so avoiding gluconeogenesis from muscle AA, demonstrating a correlation between BW and body glycogen level, with a long-term effect on growth of chickens (Uni et al., 2005). Next to this, AA-injected chicks also showed higher BW, together with higher hatchability. In fact, this type of supplementation might stimulate AA utilization and gluconeogenesis from AA, helping the animals in hatching activities (Bakyaraj et al., 2012). *In ovo* administration of Threonine, an important AA during prenatal development, improved chicks BW from d 14 post-hatching onwards (Kadam et al., 2008). It has been seen that *in ovo* AA supplementation like Arginine and Threonine can increase expression of growth-related genes and of mucin-related genes during pre- and post-hatch periods. Such AA are components of mucin backbone and may have enhanced mucin production, whose gene expression is correlated with intestine development (Bhanja et al., 2014).

Next to the positive influence on gut functionality and body weight at hatching, *in ovo* technology seems to represent an effective action to facilitate early life programming of immune system in

embryo (Rubio, 2018). In example, *in ovo* nutrient administration of carbohydrates showed to enhance gut innate defense at mucus layer level, in terms of goblet cell development and gene expression in jejunum. An increased proportion of goblet cells with acidic mucins and a higher expression of mucin mRNA were observed, and these effects might be maintained also in post-hatching days (Smirnov et al., 2006). *In ovo* inoculation can also increase humoral and cell-mediated immunity, as observed with a supplementation of nutrients such as AA, vitamins and fatty acids in chicks (Bakyaraj et al., 2012). Threonine supplementation increased humoral response in young chicks *in ovo* treated (Kadam et al., 2008), while sulfured AA administration has been seen to stimulate and modulate immune gene expression linked to cell-mediated response (Bhanja et al., 2014).

- *Probiotics and prebiotics in ovo supplements*

Exposition of chicks to delayed feeding after hatching could negatively impact not only gut development and growth, but also gut microbiota establishment. Recently, the research has pointed out that, like pathogens, also commensal bacteria can pass from hens to eggs and that microbiota colonization may occur during the late stage of embryogenesis, so the idea of the egg and embryo as sterile has been shelved (Pedroso et al., 2016; Roto et al., 2016). Furthermore, bacteria in the air of the hatchery can pass shell pores and get the embryo (Lumpkins et al., 2010). In this context, *in ovo* supplementation can help in an early establishment of a healthy intestinal microbiota that can protect the gut from pathogen invasion and improve gut development and growth performance in chicks. Given the known beneficial effects of probiotics and prebiotics, during the last years researches focused on *in ovo* administration of these dietary supplements and relative possible benefits on chicks have been conducted. Lastly, while in-feed or in-water supplementation depends on amount of feed or water intake, making sure that consumption of bioactive compound can vary across the flock, *in ovo* method unifies the effect of the supplementation by ensuring a fixed dose (Bednarczyk et al., 2016).

It has been seen that the *in ovo* feeding of probiotic bacterial strains or competitive exclusion cultures is effective in protecting gut against pathogenic bacteria and in improving growth performance. Improved protection from challenges with enteric pathogens has been proposed as effect of *in ovo* injection of probiotic, such as *Lactobacillus reuteri*, which showed potential to decrease *Salmonella* loads in post-hatch chicks ceca (Edens et al., 1997), even if others did not find any protection activity against *Salmonella* after *in ovo* injection of probiotics (Yamawaki et al., 2013). More recently, *in ovo* injection of a commercial probiotic competitive exclusion product, derived from healthy adult chickens and chosen for its ability in competitively excluding *Salmonella* spp., has been seen to increase microbial diversity in cecum of newly hatched and 7 days old chicks and to decrease the

abundance of Enterobacteriaceae, representing a possible good strategy for an early establishment of beneficial bacteria and for prevention from pathogens (Pedroso et al., 2016). Additionally, *in ovo* administration of a probiotic mixture showed to increase chicks BW during the first week post-hatch and to modulate immune response by altering the expression of immune-related genes in ileum and cecal tonsils, such as downregulation of pro-inflammatory markers (Pender et al., 2017).

More recent and limited are the researches on *in ovo* injection of prebiotics and synbiotics. Despite *in ovo* technology has been mainly used at day 18 of embryonic phase, for injection of prebiotics and synbiotics has been chosen an earlier time point, day 12 of embryonic development, given the higher effectiveness tested empirically. Furthermore, these products have been tested through insertion in the air cell of the eggs and it has been determined that day 12 of incubation is the optimal time for injection in the air cell, when embryo is still immersed in amniotic fluid (Villaluenga et al., 2004) Indeed, due to high solubility of prebiotics, they are easily transported from the air cell into the bloodstream of the vascularized chorio-allantoic membrane and then to GIT. This capacity represents an advantage compared to probiotics, which instead, due to the bigger size, cannot infiltrate into chorio-allantoic membrane (Slawinska et al., 2016). *In ovo* delivery of specific doses of prebiotics is also important to assure high hatchability and proper microbiota development (Bednarczyk et al., 2016). Some studies have investigated the effects of different doses of injected prebiotics, reporting that low doses (e.g. around 3.5 mg/egg) beneficially impact gut bacteria population (Pilarski et al., 2005), while high doses (e.g. around 7 mg/egg) cause decrease hatchability (Pilarski et al., 2005; Bednarczyk et al., 2016). Promising results from direct administration in eggs of prebiotics were reported by Pilarski et al. (2005), who demonstrated that a single early *in ovo* injection of prebiotic oligosaccharides (e.g. FOS and raffinose family oligosaccharides) at 12 day of embryonic development can lead to a long-term maintenance of high level of bifidobacteria in the chicken gut (Pilarski et al., 2005). This was also previously supposed by Villaluenga et al. (2004), who found a greater number of bifidobacteria in feces after the *in ovo* injection (Villaluenga et al., 2004). In addition to this bacterial stimulation, also enhancements in BW and FCR have been reported (Bednarczyk et al., 2011). Next to the effects on bacterial communities, *in ovo* injection of prebiotics seems to be able in inducing a trophic effect on small intestinal growth and maturation in terms of morphology (jejunum villus height), functionality (enzyme activity) and innate immunity (increase in RNA expression of immune-related genes) (Cheled-Shoval et al., 2011). Improving in villus morphology after hatching was also observed after *in ovo* injection of a synbiotic, along with stimulation of LAB development in small intestine, even if no improved growth performance were recorded (Coskun et al., 2015). These data are in agreement with the results of Maiorano and colleagues (2012), who reported that prebiotic or synbiotic injection did not affect chicks growth

performance, except for FCR (Maiorano et al., 2012). Bogucka and colleagues (2016) also investigated effects of both injected prebiotics or synbiotics on gut morphology in the first days post-hatching and found that their effect is different depending on the small intestinal section: villus height increase was observed only in jejunum, while increase in surface area was reported both in jejunum and duodenum. Contrarily, in ileum they observed a reduction in villus height and area (Bogucka et al., 2016). Similarly, another trial reported that injection of two different synbiotics (*Lactobacillus* species and RFO or inulin) increased absorbing surface, villus height and width in jejunum but also in duodenum in chicks at day 1 of age and a long-term effect on villus morphology was observed at day 42 of rearing in jejunum after injection of synbiotic with inulin. In ileum, these authors found wider villi but deeper crypts in post-hatched chicks injected with synbiotic containing inulin (Sobolewska et al., 2017). Further, *in ovo* injection of synbiotics in the air cell at 12 days was shown to accelerate the development of immune organs, next to an increase in lymphocyte proliferation (Slawinska et al., 2014). Stimulation of GALT development has also been reported with both administration of prebiotic or synbiotic at 12 days of incubation in the air cell, with a modulatory effect on adaptive immune cells, like T and B cells, for a rapid colonization of peripheral lymphoid organs (Madej and Bednarczyk, 2016). A recent study on transcriptomic profile of spleen, cecal tonsils and large intestine of chicks treated by *in ovo* prebiotic (GOS) and synbiotic injection revealed a higher potential of GOS compared to the synbiotic in stimulating host transcriptome, maybe due to the bifidogenic effect of this prebiotic. This prebiotic bifidogenic effect seems to be able in inducing a down-regulation of immune-related genes and pathways, with inhibition of cell-mediated and humoral response. Furthermore, GOS strongly promoted GALT maturation, probably due to a proper stimulation of intestinal microbiota (Slawinska et al., 2016). These authors also attempted another explanation for the more potent impact on GALT of GOS compared to synbiotic, hypothesizing that, when in combination, probiotic could have used GOS to its advantage before it got the chorio-allantoic membrane, impeding its effect (Slawinska et al., 2016).

Overall, compared to dietary inclusion, *in ovo* injection technique seems to allows to administer prebiotics at very low doses, for a precise delivery of the compound at early stage (Bednarczyk et al., 2016), with an efficient action on beneficial bacteria like bifidobacteria and *Lactobacillus* species (Bednarczyk et al., 2011) and an increase in microbial population after hatch and a maintenance of these bacteria throughout the growing period (Pilarski et al., 2005; Pruszynska-Oszmalek et al., 2015). Additionally, the action of prebiotics as stimulators of GALT development (Madej and Bednarczyk, 2016) and of a long-term regulation of immune-related pathways and adaptive immune response (Slawinska et al., 2016) are other important factors that deserve to be more in-depth investigated. The observed growth-promoting action of prebiotic *in ovo* administration in some trials

(Pruszynska-Oszmalek et al., 2015; Bednarczyk et al., 2016), as well as the improvement in slaughter performance (Maiorano et al., 2017) need to be deepen with further studies, since conflicting results have been reported from others (Maiorano et al., 2012; Bogucka et al., 2016).

Discrepancies between the different trial results are to be attributed to diverse factors, such as probiotic or prebiotic composition, dosage, application method, diet, condition of animals and other environmental factors (Pender et al., 2017).

4. Animal health: stress and disease susceptibility

As previously mentioned, a worrying increase in patho-physiological changes and metabolic alterations in new chicken lines have been observed, seriously impacting bird health and welfare (Scheele, 1997).

4.1 Cardiopulmonary diseases

The increase in GR means animals need more oxygen for more energy production that is requested for the higher metabolism, but metabolic dysfunctions linked to inability in ensuring enough energy supply for maintenance have been reported, with subsequent respiratory and cardiopulmonary disorders and mortality (Scheele, 1997; Sandercock et al., 2006). Failure in sufficient oxygen amount to support the high metabolic demand leads to an increase in red blood cells proliferation and so to an increase in blood viscosity with final pulmonary hypertension, edema and ascites: ascites syndrome and often the resulting sudden death syndrome are conditions of heart failure observed and positively correlated with high GR in new-line broilers (Deeb et al., 2002). As just mentioned, ascites and heart failure are related to insufficient consumption and transport of oxygen by the circulatory system: blood low oxygen tension inhibits phosphorylation and induces vasoconstriction which, at pulmonary level, creates a decompensation in blood pressure of systemic circulation, causing edema and accumulation of fluid in the body cavities. Increase in ascites susceptibility has been also correlated with low plasma level of thyroid hormones, which slow heart activity (Scheele, 1997).

4.2 Stress susceptibility and enteric diseases

Higher stress sensibility has been also related to the intense GR selection. As reported by Padgett and Glaser (2003), a disturbance at any level of the stress response can lead to an imbalance in physiological functions, inflammation and higher disease susceptibility, such as enteric diseases. Despite the great variety of stress sources, overall effects in response to stress are driven by two commonly pathways: (i) sympathetic-adrenalmedullary axis (SAM) and hypothalamic-pituitary-adrenocortical axis (HPA); (ii) neuroendocrine pathway and important system for body integration (Post et al., 2003). The release of adrenocorticotropin hormone (ACTH) stimulates the adrenals to secret glucocorticoid hormones (cortisol and corticosterone, also recognized as stress hormones) in blood, which exert a different role depending on the conditions. In fact, glucocorticoids act as positive effectors in physiological stress, such as an acute stress, where these hormones stimulate glucose metabolism to produce energy in order to respond to external stimuli. Conversely, the HPA

overactivation under chronic stressful situations may be harmful and can impair health leading to immune dysregulations by affecting cytokines, chemokines and immune cells (e.g. inhibition of antibody production by B cells), which have receptors for glucocorticoids. Furthermore, glucocorticoids seem to be able in regulating the expression of many immune-related genes (Padgett and Glaser, 2003; Post et al., 2003; Hu and Guo, 2008). Chicken are very sensitive to glucocorticoids and their altering effects have been also seen at gut level, in particular on small intestine with a decrease of absorptive function, linked to the delayed epithelial cell turnover and with loss of absorptive surface area (Hu and Guo, 2008). In particular at jejunum level, where mostly of nutrient digestion and absorption occur, glucocorticoid-induced stress has been reported to induce decrease in villus height and absorption area and reduction of small peptide transporter efficiency and so, absorption (Chang et al., 2015). Many different factors, including diet components, infectious agents and environmental conditions have been linked and defined as predisposing factors for enteric diseases, with negative impact on dynamic balance between mucus layer, epithelial cells, microbiota and immune cells (Yegani and Korver, 2008). Indeed, in addition to being the major site of potential exposure to environmental pathogens, gastrointestinal tract is highly sensible to stressors, which can cause shift in gut microbial population and changes in gut mucosa status (at barrier level) and health (Yegani and Korver, 2008; Oakley et al., 2014b).

Differently from high microbial diversity, intact gut barrier and absence of inflammation that distinguish gut health status, the gut impairment is characterized from dysbiosis by a disrupted microbial composition and reduced microbial diversity, with increase in *Enterobacteriaceae,* often accompanied by mucosal barrier disruption, increase in epithelial permeability and by inflammation status (Kogut, 2013; Ducatelle et al., 2018).

At mucus layer level, defects in mucus organization and structure such as short glycans or stressors affecting strength of adherent mucus layer make this first defense line penetrable to bacteria (Johansson and Hansson, 2016). An overproduction of mucus has been noticed in conditions of microbiota composition changes, which can be related to gut dysbiosis and inflammation (Pastorelli et al., 2013).

Intestinal barrier permeability is recognized as the passage of different molecules through the gut epithelial barrier by non-mediated passive diffusion. Passive diffusion into epithelial cells of many molecules coming from gut lumen is counteracted by plasma-membrane bound efflux pumps, expressed also in poultry intestine. Indeed, defects in these pumps can generate inflammation (Ducatelle et al., 2018). At epithelial level, paracellular permeability and barrier function are regulated by epithelial TJs and their tightness and relative alterations are attributable to many gut diseases and disturbs, including gut inflammation, pathogens and oxidative stress. Reduction in TJ

integrity decreases trans-epithelial tissue resistance and alteration in permeability leads to an increasing number of IELs and macrophages in epithelium and to a higher susceptibility to enteric pathogen invasion, since many pathogens (such as enteropathogenic *E. coli* and *Salmonella* strains, *Cl. perfringens, C. jejuni*) act on TJs to pass the gut barrier (Clayburgh et al., 2004; Pastorelli et al., 2013; Awad et al., 2017). Furthermore, deficiencies in some specific immune functions may trigger pathological processes. In example, defects in TLRs imply lack in pathogen-associated molecular pattern recognition and then loss of physiological immune response, which in turn, avoid a regular microbial clearance. Increase in gut bacterial load conduces easily to pro-inflammatory pathway activation (Pastorelli et al., 2013). The steady healthy state is broken when a disruption in the epithelial barrier happens. The passage of bacteria from gut lumen trough the epithelium provokes a great response of mucosal immune cells that release inflammatory cytokines (Kurashima et al., 2013). Antigen-presenting cells in lamina propria trigger the immune response through T cell activation, that release inflammatory cytokines without a real regulatory system to contain and limit the reaction: IFN-γ and TNF can promote the leaky pathway, a paracellular transport of big solutes, so increasing gut permeability (Clayburgh et al., 2004; Turner, 2009).This is one of first problems of gut inflammatory diseases, because the continuous production of cytokines, also from activated macrophages, can further disrupt barrier resistance with further immune response stimulation, establishing a detrimental cycle (Clayburgh et al., 2004). In fact, activation of intracellular pathways like MLCK pathway from pathogenic or pro-inflammatory stimuli can affect TJ regulation. Indeed, stress responses lead to phosphorylation of myosin light chains by MLCK, inducing contraction and opening of TJs. In example, enteropathogenic *E. coli* acts on cell signaling pathways by directly injecting effector proteins in host cell cytoplasm, *Salmonella* strains release endotoxins that act on barrier permeability, while *Cl. perfringens* has an enterotoxin that uses TJs as receptors to attach. Beside pathogens, external stressors can induce these physiological disturbs at gut level and consequent possible inflammatory diseases (Awad et al., 2017).

So, briefly, pathogenesis of inflammatory diseases in intestine involves disruption of epithelial barrier, access of luminal contents (including pathogens) into the lamina propria and an unmodulated immune response (Clayburgh et al., 2004). If uncontrolled, this immune activation leads to an excessive inflammation with intestinal damage with impairment of digestive functions (Brisbin et al., 2008). Considering the systemic level, possibility of translocation through damaged TJs can lead bacteria to reach the liver and stimulate acute phase inflammatory response. Acute phase proteins released in blood by hepatocytes can be measured in serum. However, systemic response and acute phase protein serum levels highly depend on how much great the epithelial damage is and not always the effect on gut barrier is reflected at serum level (Ducatelle et al., 2018).

In a recent study on chickens with gut barrier failure induced, all small intestinal tracts presented longer crypt depth, and, in duodenum and jejunum, wider villi probably meant a less nutrients absorption area compared to narrow villi present in healthy animals. Furthermore, decrease in occludin mRNA level along with increase in serum endotoxins was reported, as results of gut barrier failure with consequent increase of transport from lumen to blood of intestinal gram negative (gram -) endotoxins. Moreover, inflammation onset was hypothesized since increase in serum acute phase protein α1-acid glycoprotein (α-1-AGP) was observed (Chen et al., 2015). In another study, gut barrier failure and enteritis induced by overcrowding stress and *Salmonella enteritidis* infection showed inflammatory infiltration in gut mucosa and repercussions on plasma markers, with increase of corticosterone level, decrease of IgG and increase in IgM (usually acting as first antibody response to infection) and IgA levels (suggesting an increase of IgA also at mucosal level). Lastly, also performance yield was affected (Gomes et al., 2014).

From an economical point of view, a strong immune response to intestinal antigenic stimulation may negatively affect feed efficiency and performance, because the high energetic cost forces a shift of nutrients from production to satisfy immunity requirements, since organism prioritizes proliferation of immune cells, expression of non-self-antigen receptors and production of cytokines and antibodies (Bortoluzzi et al., 2018). It is obvious that in non-homeostasis immune conditions also metabolic requirement suffers and the consequent imbalance for the lack of stable and physiological energetic distribution between compartments results in signaling pathway changes and metabolic disturbances (Yegani and Korver, 2008; Kogut, 2013). Moreover, malabsorption of nutrient consequently to epithelium dysfunction reduces growth and nutrients remain available as substrate for enteric pathogens growth. Pathogens negatively affect growth through mechanisms such as protein metabolism interference, gut nutrient transporter alteration and villus damage, decrease of digestibility through impairment of digestive enzyme activity (Awad et al., 2017). Since feed intake and absorption efficiency of nutrients strictly depend on gut health status and since economic consequences from gut malfunction are of great impact when it comes to intensively reared systems (where gut functions are taken to the limits), intestinal welfare has become pivotal for animal general health and performance (Ducatelle et al., 2018).

Environmental factors like fasting, overcrowding, pre-slaughter and thermal conditions during rearing are between the main stressful conditions affecting gut health (Burkholder et al., 2008).

4.3 Impact of heat stress on poultry production

Due to the lack of sweat gland, birds generally easily suffer heat and increase in drinking and panting (Yi et al., 2016). During production, poultry can be exposed to stressful environmental challenges such as thermal stress, the main environmental factor influencing poultry production and health (Zhang et al., 2012) and being of great concern, especially in hot regions of the world (Deng et al., 2012). Usually the comfort zone of broilers declines from 32°C (at hatching) to 24°C at 3-4 weeks of age and to around 18-21°C thereafter (Lan et al., 2004; Farag and Alagawany, 2018). Heat stress is defined as the bird response to high temperatures (and humidity), exceeding the comfort zone, where abnormal responses to increase heat dissipation, such as panting and increased respiratory rate, are observed (Lan et al., 2004). Fast-growing broilers are highly susceptible to heat stress due to the lower thermoregulatory capacity compared to the previous genetic lines. The excessive hyperthermia is probably due to an imbalance between heat loss and heat production, like under heat stress conditions. The low heat loss capacity, in part probably influenced by the great body size, results in a high thermoregulatory effort with respiratory breathlessness that requires a high metabolic cost, which in turn increases heat production. In addition, a lowering of feed intake occurs, to avoid a further increment in heat production, difficult to disperse (Sandercock et al., 2006). The elevated body temperature of chickens is the triggering factor of metabolic changes that induce oxidative stress as response to heat exposure. Many biochemical reactions normally increase with temperature and so also production of reactive oxygen species (ROS) increases. Furthermore, loss of body thermoregulation and heat production lead to an increase in plasma thyroid hormones concentration, which also accelerate the basal metabolic rate and oxidative metabolism (Lin et al., 2006). Oxidative stress can then persist due to lack of balance between ROS production and antioxidant systems (Varasteh et al., 2015). Acute or short-term (such as during transportation or pre-slaughter holding) or chronic heat stress (like in summer period) can highly impair poultry production, inducing problems including muscle damage, acid-base disturbances, reduced meat quality (Sandercock et al., 2001) and other physiological dysfunctions such as immune response depression (Azad et al., 2013) and impairment of intestine development and functionality (Svihus, 2014). These problems increase, in turn, infectious disease susceptibility and mortality (Varasteh et al., 2015). Depending on if it is acute or chronic heat stress, it shows different impacts on production and metabolism (Slimen et al., 2016).

4.3.1 Heat stress on meat quality

Acute heat stress has been demonstrated to increase superoxide free radicals like ROS in chicken' skeletal muscle, showing the same gene expression pattern having after oxidative stress exposure (Mujahid et al., 2005). Mitochondrial ROS production via enhanced mitochondrial respiratory chain (Azad et al., 2013) may be the mechanism responsible for the transport stress and heat stress-induced muscle damage and for the changes in muscle and meat quality observed in broilers, along with growth retardation. Indeed, ROS can induce indiscriminately molecular changes at DNA, protein (e.g. mitochondrial protein denaturation) and lipid level (e.g. peroxidation of cell membrane phospholipids, leading to cellular damage) with deleterious consequences (Mujahid et al., 2005; Slimen et al., 2016). In addition, heat stress-induced hormonal changes affect calcium regulation and hypermetabolism in skeletal muscle, with dysregulation of calcium homeostasis and increase in glycolytic metabolism with quick decrease of pH and protein denaturation (Sandercock et al., 2001). This homeostatic dysregulation, derangements of ante-mortem muscle cell metabolism and alterations in sarcolemma integrity (due to disruption of intracellular calcium homeostasis) associated with oxidative damage may have profound implications for meat quality (Sandercock et al., 2006). Hyperthermia-associated myopathies are characterized by altered activities of some known biomarkers like aspartate transaminase, lactate dehydrogenase (LDH) and increased activity of isoenzyme CK. Moreover, heat stress increases expression of heat shock proteins (HSP) in muscle, along with increase in cytoprotective proteins aimed to maintain stress tolerance and survival of stressed cells (Kamboh et al., 2013). A study of Akşit et al. (2006) tested the effects of chronic heat stress during rearing and acute heat stress in pre-slaughter crating on broiler stress parameters and meat quality traits. Heat-stressed birds both during farming and pre-slaughter time evidenced a higher heterophil. Indeed, lymphocyte ratio as sensitive stress indicator, and after slaughter, lower ultimate pH and paler breast meat. This study also indicated that particularly chronic heat stress during farming predisposes birds to produce breast meat with PSE-like characteristics (Akşit et al., 2006). A further impairment of metabolism and consequently of meat quality has been observed under chronic heat stress, with an increased intermuscular fat deposition, protein catabolic rate and denaturation (with more loss of water-holding capacity) and lactic acid concentration (due to the higher LDH activity), which in turn increases more pH decline rate (Zhang et al., 2012). Chronic heat stress highly increases reduction of fatty acid oxidation, which are not available for energy anymore, forcing the animal in becoming more dependent on glucose for energy needs and increasing its absorption at intestinal level (Slimen et al., 2016). Under an economic point of view, heat stress substantially means a significant

reduction in chickens growth performance, probably due to a lower efficiency following to changes in metabolic usage of nutrients (Habibian and Ghazi, 2014).

4.3.2 Heat stress on chickens health

Besides the impact on meat quality, heat stress strongly alters physiological and metabolic response affecting animal health: it leads to endocrine disorders (affecting thyroid function and hormone release), has negative consequences on immune response (leukocyte and lymphocyte immunosuppression) and increase inflammatory cytokines and intestinal dysfunction (Farag and Alagawany, 2018).

Heat stress induces excessive production of free radicals and the concomitant release of glucocorticoids and catecholamines may lead to immunosuppression, because these molecules cause depletion of glutathione and then peroxidation of membrane lipids, also in immune cells. Oxidative stress can result in cell death and, in terms of immune system, in reduction of macrophages and of both circulating and lymphoid organ-associated lymphocytes (Lara and Rostagno, 2013; Taha-Abdelaziz et al., 2018). Specifically, if fast and acute, heat stress can enhance antigen-specific cell-mediated immunity, but if the stimuli is prolonged, its delayed effects can suppress the immune cellular response (Shini et al., 2010). Reduction and impairment in macrophage activity is mediated by the reduction of regulatory cytokines like IFN-γ, which further regulates other important immune processes (Quinteiro-filho et al., 2017). Heat stress is also associated to an increase in pro-inflammatory cytokines (e.g. TNF-α and IL-2): in stressful conditions, the presence of these cytokines induces increase in C-reactive protein (CRP) blood concentration, a protein involved in complement system activation to respond to inflammation and tissue damage (Sohail et al., 2010). Furthermore, the release of glucocorticoids causes fast influx of heterophils in blood stream from bone marrow, increasing their circulating concentration (Kamboh et al., 2013). In terms of humoral response, heat stress leads to a decrease in antibody response. Chickens serum natural antibodies are mostly of the IgM isotype, but also IgG and IgA can be found. Specifically, IgG are the major class of blood circulating antibodies produced in the humoral response to neutralize antigens and activate macrophages and the complement system (Wang et al., 2004), so decrease in this Ig isotype may mean a critical impairment of immune response to pathogens. In broilers immune-stimulated with sheep red blood cells both primary and secondary antibody response has been seen to decrease in birds reared under heat stress conditions, in terms of IgM and IgG (Habibian and Ghazi, 2014). Similarly, chickens under heat stress showed a decrease in their IgA plasmatic level and a more pronounced decrease of this Ig class was observed when heat stress was applied during an infection

from *Salmonella enteritidis*, along with decrease in gene expression of cytokines involved in pro-inflammatory response against this pathogen in cecal tonsils (Quinteiro-filho et al., 2017). These authors also observed that under heat stress, this pathogen, after colonization of crop and cecum, can easily reach and replicate in other organs (e.g. spleen, liver and bone marrow), showing a immunological deficiency in controlling *Salmonella* invasion (Quinteiro-filho et al., 2017). Differently from these previous studies, Attia et al. (2017) instead noted an increase in serum Igs to chronic heat stress exposure in healthy broiler chickens (Attia and Hassan, 2017). These different results may indicate a possible different immune response depending on health status of chickens; anyway, many other factors may impact chickens immunity.

At intestinal level, heat stress alters gut epithelium in terms of morphology, with apparently damage to intestinal mucosa, epithelial desquamation, shortening of villus height and deeper crypt depth (Deng et al., 2012; Wu et al., 2018): high environmental temperature reduces feed intake of birds and so also the amount of energy delivered to GIT, which can suffer atrophy. Moreover, low feed intake reduces absorptive area in gut mucosa and digestive enzyme secretion, with delay in gut mucosa development (Wu et al., 2018). Decrease in villus height and crypt depth ratio it has been observed particularly in jejunum, along with further impairment of energetic metabolism and antioxidant enzyme activity (Yi et al., 2016).

A compromised goblet cell activity and mucus composition are consequences of heat stress, with consequent changes in attachment capabilities of commensals and pathogenic microorganisms (Burkholder et al., 2008). Indeed, it has been observed an impairment of host resistance and increase in susceptibility to pathogen colonization in birds under heat stress, with decrease in gene expression of a TLRs involved in gut barrier maintenance (Quinteiro-filho et al., 2017) together with an increase in HSPs concentration, which can act as epithelial surface receptors for pathogens binding (Burkholder et al., 2008). Supporting this, cyclic heat stress has been seen to induce up-regulation of HSPs and of TJs protein mRNA expression in jejunum and ileum, but not in duodenum and colon, showing a different effect on the intestinal tracts. Furthermore, jejunum and ileum showed also higher concentration of proinflammatory cytokines (Varasteh et al., 2015). Modifications in TJ protein gene expression was furthermore reported by Wu et al. (2018), but in this case they found a downregulation of gene expression after a long period of cyclic heat stress and this change was more evident in jejunum than cecum (Wu et al., 2018). Both results from these studies seem to highlight an important different susceptibility to heat stress between the intestinal tracts, and the differences between the two studies might be linked to the different time of heat stress exposition (acute or chronic), with possible differences and changes in gene expression regulation. Anyway, an abnormal TJ-related gene regulation it is involved in the loss of barrier function after a heat stress condition, since inflammatory

cytokine releasing such as TNF-α act on TJs, increasing the permeability. In addition, protection of gut mucosal epithelium is also damaged by the decrease in IgA, IgG and IgM at intestinal level, as reported by Song and colleagues (2018), who observed this lowering in jejunum of broilers (Song et al., 2018). A further dysfunction of innate immunity seems to influence the gut barrier and health: in fact, an increase in the oxidative response of macrophages at peritoneal level may be involved in the inflammation onset, as reported by Quinteiro-Filho and colleagues (2010), who observed a consequent increase in inflammatory cellular infiltration in jejunum mucosa (Quinteiro-Filho et al., 2010).

High temperature influences intestinal bacterial composition, anyway, in poultry, few studies have been conducted on the effect of heat stress on microbiota, mostly focused on some specific bacteria. The observed alteration in microbial composition with decrease in *Lactobacillus* and *Bifidobacterium* counts along with a compromised gut barrier integrity facilitate the access to intestinal basal layer for pathogens (coliforms, *Salmonella* and *Clostridium*) and concur in gut impairment under heat stress, with possible onset of enteric diseases (Burkholder et al., 2008; Lara and Rostagno, 2013). In support of this, Song and colleagues (2014) demonstrated how heat stress can increase viable counts of coliforms and *Clostridium* in broiler small intestine contents, along with a decrease in *Lactobacillus* and *Bifidobacterium* viable counts (Song et al., 2014). Recently, it has been reported that heat stress increased species richness of the bacterial community in ileum of broilers (Wang et al., 2018). Moreover, at phylum level, heat stress seems to increase abundance of Bacteroidetes, as well as of *Bacteroides* genus, at the expense of Firmicutes, which decrease, along with other phyla (Fusobacteria and Proteobacteria) as observed in fecal content of laying hens under heat stress (Zhu et al., 2019). These authors also reported that other taxa like *Ruminococcaceae* or *Lactobacillus* were decreased under heat stress (Zhu et al., 2019). Furthermore, it has been reported that fast-growing chickens seem to be more susceptible to salmonellosis compared to lower growth ones under heat stress, with different kinds of gut immune response compared to slow growing lines (van Hemert et al., 2006), highlighting a possible difference in stress pathogen colonization resistance between the two lines. Burkholder and colleagues (2008) observed changes in commensal intestinal bacterial population of birds under heat stress towards a favorable attachment of *Salmonella enteritidis* (Burkholder et al., 2008) As another example, a specific increase in colonization by *Clostridium perfringens* is reported, which toxins damage the gut and cause necrotic enteritis. Heat stress represent a predisposing factor for necrotic enteritis in broiler and a factor increasing severity of this lesion (Moore, 2016), which is mediated by the effects of heat stress on microbiota, intestinal mucosa and immunity. Diffuse gut mucosa necrosis, increase in fibrin, villus fusion and shortening and high cell epithelial infiltration have been reported in broilers under cyclic heat stress (Tsiouris et al., 2018),

leading to a decrease in digestion and nutrient absorption and consequent impaired growth performance and, when in acute form, to death (Knap et al., 2010).

4.4 Feeding strategies to counteract heat stress

It has been hypothesized that, next to positive effects on chicks body development, early feeding strategies may influence the responsiveness of chickens to adverse environmental conditions (Taha-Abdelaziz et al., 2018), such as heat stress. Several studies have reported the stress alleviating properties of dietary supplementations with phytoextracts and herbal mixtures in birds exposed to heat stress. These feed additives can alleviate the effect of prolonged heat stress conditions and increase productive performance (Kamboh et al., 2013; Song et al., 2018), provide better resistance to oxidative processes (Habibi et al., 2014; Song et al., 2018) and improved immune-modulatory response (Azad et al., 2013) and gut morphology (Song et al., 2018). Moreover, the utilization of antioxidants like dietary vitamin E or selenium in heat stress conditions showed to be effective in preventing PSE-like meat characteristics in broiler chickens (Hashizawa et al., 2013) and in alleviating detrimental effects on immunological responses by circulating lymphocyte level, humoral antibody response and lymphoid organ weight (Habibian and Ghazi, 2014).

At intestinal level, supplementations of probiotics in chickens under heat stress conditions have shown to enhance gut beneficial bacteria (Lan et al., 2004; Song et al., 2014) and improve intestinal micro-architecture probably in part due to the healthy SCFA profile distinguishing probiotic fermentation, with trophic effects on the intestine (Sohail et al., 2012; Ashraf et al., 2013). Lan et al. (2004) found that a *Lactobacillus* probiotic supplementation enriched *Lactobacillus* diversity in jejunum and cecum and reduced mortality of broiler chickens under heat stress. Furthermore, the restored microbial balance in both tracts and the higher abundance of *Lactobacillus spp.* in jejunum were maintained also after the treatment, throughout the chickens growth (Lan et al., 2004). Similarly, beneficial effects of a dietary probiotic mixture of *Lactobacillus spp. and Bacillus subtilis* were observed in terms of increased lactobacilli and bifidobacterial viable counts in jejunum of chickens under heat stress, along with a decrease in coliforms (Song et al., 2014). Besides, dietary supplementation of a probiotic mixture in chickens kept under chronic heat stress showed to beneficially impact gut morphology by inducing increase in ileal villus width and surface area along with crypt depth compared to birds under heat stress but without supplementation (Sohail et al., 2012). Similarly, dietary inclusion of probiotic *Bacillus licheniformis* showed to re-stabilizing villus height both in ileum and cecum of laying hens under chronic heat stress treatment (Deng et al., 2012). These authors also found that probiotic alleviated heat stress negative influence by up-regulating mucosal

IgA secretion, normally decreasing with a delayed stress, probably meaning an ameliorated immune status of the gut. Along with this probiotic treatment ameliorated gut barrier status by increasing the number of intestinal goblet cells, which usually decrease with chronic stressful stimuli (Deng et al., 2012). Similar to laying hens, also in broiler chickens an increase in goblet cell number by probiotic supplementation in diet was observed by Ashraf et al. (2013), who recorded this effect both in duodenum and jejunum of birds under a chronic heat stress condition (Ashraf et al., 2013). More, these authors also reported that probiotic changed goblet cell differentiation, noting an increase in acidic mucins in jejunum of heat-stressed birds: since the maturation process cycle of goblet cells passes from neutral to acidic mucin composition (Ashraf et al., 2013), it is plausible that probiotics may contribute in goblet cell development with greater epithelial protection. Lastly, the probiotic induced a decrease in IEL count in all small intestinal tracts, usually increasing with stressful situations, indicating a possible indirect effect on pro-inflammatory cytokines (Ashraf et al., 2013).

Since, as alternative to probiotics, prebiotics have been proposed as strong microbiome modulators and as substances that directly improve gut barrier and gut-associated immunity (Zhong et al., 2009; Pruszynska-Oszmalek et al., 2015) and some studies have investigated their effects also in chickens environmentally challenged, such as birds reared with heat stress. It was reported that chickens fed dietary MOS and reared under heat stress had less serum cortisol and CRP concentration and presented an enhanced humoral immune response, with increase in anti-inflammatory cytokine production (Sohail et al., 2010). Moreover, the same prebiotic supplementation also improved BW and FCR under heat stress compared to chickens not supplemented (Sohail et al., 2012). In terms of gut barrier and immune response, Ashraf et al. (2013) observed that dietary MOS feeding enhanced the number and the activity of goblet cells in jejunum of broilers kept under heat stress and decreased infiltration of IELs, probably reducing the inflammatory response. These authors also observed that MOS can prevent shortening of intestinal villi induced by heat stress (Ashraf et al., 2013). Moreover, also dietary GOS showed to be able in protecting gut homeostasis under heat stress by preventing stress response-related changes in chicken jejunum, with decrease of inflammatory cytokine, HSP and TJ protein RNA expression (Varasteh et al., 2015). The administration of GOS has further been seen to reduce stress-induced plasma corticosterone level in mice (Burokas et al., 2017).

Few studies reported also beneficial results of dietary synbiotics against detrimental effects of heat stress in chickens, with decrease in intestinal IEL and increase in goblet cell count (Ashraf et al., 2013) and improved growth performance in terms of BW and feed intake (Mohammed et al., 2018).

5. Molecular markers related to gut health: perspectives and application

In view of the facts, gut health sustenance represents an essential prerequisite to guarantee animal efficiency and to preserve animal health and balance throughout the organism. Under stress stimuli gut mucosa is a target of damage and that inflammation-associated oxidative stress can change intestinal epithelial cell phenotype with consequent changes in gene expression. Investigation of changes in intestinal signaling pathways and gene expression, along with variations in microbiota composition, may help in pointing out the gut status condition and in the research for relative biomarkers (Ducatelle et al., 2018), besides most common acute phase protein markers in blood serum such as 1-AGP, ceruloplasmin, transferrin and SAA, that may rise up to 100 fold during an inflammation (Lee et al., 2010). In fact, molecular markers can indicate a specific status of the tissue (due to the immediate RNA response), differently from other markers which are less specific and not directly related to a specific moment of the tissue status.

Modern molecular approaches can offer the access to the knowledge about the presence and the relative expression of thousands of genes in tissues. Nevertheless, it has been observed in the past (Hoffmann and Valencia, 2003) and also now (Graham, 2018) that researchers tend to concentrate the attention on a limited number of genes and protein products, in general on the basis of diffusion of previous studies.

Microarray technology represents one of the most challenging tools to in-deep study gut gene expression profile by obtaining a wide quantity of different transcripts, and to clarify molecular mechanisms and pathways behind the gut well-being. With this kind of analysis, it becomes possible to identify many novel molecular markers as indices for a physiological or an altered status of intestinal environment.

For human the gene expression in different tissues is currently collected by some databases cured by medical projects, such as GTEX (gtexportal.org) or Bio GPS (biogps.org); differential gene expression between different gut tracts was explored and discussed in mice (from the stomach to the colon) (Goebel et al., 2011), in pigs (among different gastric mucosae) (Colombo et al., 2014). In chicken no such kind of survey was detected on current research data bases. However, the different aspects and characteristics of GIT may be linked to different gene pattern, with still not well-known functions. In addition, the spatial differences in dominant bacteria along the gut, and particularly between small and large intestine (Choi et al., 2014) suggest that these variations may affect the expression of genes in the different gut segments.

The wider view furnished by the microarray analysis can be further applied with a perspective of testing various experimental factors. In example, Trevisi and colleagues (2018) applied the microarray analysis to pig jejunal loops *in vivo* to investigate effects of early association with complex or simple microbiota on intestinal states and on relative immune defense development after challenge with *E. coli* (Trevisi et al., 2018). Moreover, the exploration of gut functional aspects may be interesting to provide hints for action (e.g. dietary strategies) to favor gut balance maintenance, as showed by Fukasawa et al. (2007), who used a microarray analysis to investigate small intestine gene profile of mice fed a prebiotic, identifying some marker genes for gut immunomodulation after prebiotic administration (Fukasawa et al., 2007). With this analysis, these authors identified specific immune response-related genes such as MHC and other antigen-presentation function-related genes (Fukasawa et al., 2007). Similarly, Blavi and colleagues (2018) studied molecular mechanisms at the base of the jejunal response to dietary treatments with different level of limestone inclusion in weaning pigs, finding alteration in genes of mediators of immune response such as IFN-γ, TNF-α, IL-2 and IL-6 (Blavi et al., 2018).

Considering the beneficial effects of prebiotics, the importance in finding gene pathways and markers genes involved in gut behavior and response to prebiotics has been highlighted by some transcriptomic microarray studies on chickens, in order to identify induced molecular mechanisms to improve gut health by dietary supplementation (Ibuki et al., 2010; Xiao et al., 2012; Brennan et al., 2013) or by *in ovo* feeding (Slawinska et al., 2016).

In terms of researches on changes in molecular mechanisms at the base of gut impairment, some studies have investigated potential inflammatory markers in chickens' small intestine by studying gene expression of single genes, but not with a broad-spectrum point of view. In example, changes in gene expression for cytokines IFN-γ, IL-4, IL-13, for TLR-2, as well as for mucin-2 were reported as gut inflammatory markers after a probiotic treatment (Pender et al., 2017). In particular, TJ proteins and mucins represent possible biomarkers of gut barrier failure (Chen et al., 2015; Pender et al., 2017), as well as myosin light chain kinase (MLCK) gene, influencing gut permeability (Pender et al., 2017), while gene expression trends of markers like IL-8 or IL-1β can indicate mucosa wound healing (Chen et al., 2015). Furthermore, also different responses to stress stimuli between the intestinal tracts have been observed in terms of gene expression, but only for few specific marker genes, such as HSP and TJ proteins (Varasteh et al., 2015; Wu et al., 2018).

Objectives of the Thesis

First of all, this thesis wanted to aim at a in-depht exploration of chicken gut profile in terms of transcriptome characterization, to provide and exploit possible new key aspects and potential biomarkers as indicators of gut status. In particular, this thesis wanted to point out the importance of intestine as key target for occurrence of changes which may have repercussions on the whole organism, with possible impact on productive yield and animal health. Moreover, in this sense, the present work wanted to investigate the chicken gut behavior, in terms of transcriptome and microbiota, and its sensitivity to external factors like stressors or dietary strategies considering a possible different response depending on the specific intestinal tracts. A survey on molecular mechanisms and differential gene expression along gut sites may deepen knowledge on functional aspects and provide hints for actions for maintaining gut homeostasis and testing various experimental factors. Furthermore, a concomitant analysis of microbiota was included to see if changes in microbial community might be linked to different functional genes.

Study 1:

Exploring differential transcriptome between jejunum and cecum tissue of broiler

Aim of the study

It is well known how selection for fast-growing broiler chickens has directly or indirectly targeted and differentiated some functional aspects linked to growth performance, like high intestinal specificity. In addition to this, other functional aspects of chicken intestine may have been enhanced, also linked to the spatial microbial community along the GIT. Furthermore, considering the particular and different characteristics distinguishing small and lower intestine in chickens, it might be useful to know more in-depth which molecular mechanisms may be at the base of the differences between tracts, with possible new point of views on the gut role.

In an exploratory perspective, the aim of the present research was to evidence the differential tissue gene expression of jejunum and of cecum of chickens at 42 days of age.

Materials and Methods

The experiment design was approved by the Ethical Committee of the University of Bologna on 03.05.2017(ID363/2017-PR). A total of 150 Ross 308 male chicks were reared at the experimental facility of the University of Bologna within an environmental controlled poultry house and randomly allotted in 6 pens of 6 m^2 each (25 birds/pen). All the chicks were vaccinated against coccidiosis, infectious bronchitis virus, Marek's disease virus, Newcastle and Gumboro disease. Stocking density was defined according to the European legislation in force (European Commission, 2007) to simulate the environmental conditions usually adopted in the intensive production system. Each pen was equipped with 2 circular pan feeders able to guarantee at least 2 cm of front space/bird and 10 nipples, while the floor was covered with chopped straw (2 kg/m2). According to the legislation in force (European Commission, 2007), birds received 23L:1D of artificial light from 0 to 7 d and in the last 3 days before slaughter, whereas a photoperiod of 18L:6D was adopted in the remaining days.

All the birds received the same standard commercial diet composed by three feeding phases: Starter (0-10d), Grower (11-25d) and Finisher (26-42d) (Supplementary Table 1).

At slaughter age (day 42), 24 birds (4 birds/replicate) were selected for a balanced distribution between pens and for body weight homogeneity. Jejunum and cecum mucosae were collected by gently scraping after tissues rinsing in PSB to remove residues of digesta, and immediately frozen in liquid nitrogen and then stored at -80°C. From both tissues, total RNA was extracted using GeneJET RNA Purification Kit (Thermo Scientific) according to manufacturer's instructions and RNA quantity and quality were evaluated using Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA) and agarose gel electrophoresis, respectively. After, RNA integrity was evaluated through Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). Samples out of 5 subjects were discarded since RNA integrity was compromised in one of the two tissues. Thus, the whole transcriptome microarray analysis was then performed on 38 samples of both tissues obtained from 19 subjects, using Affimetrix© GeneChip Chicken Gene 1.0 ST Array and hybridized arrays were scanned on Affimetrix© GeneChip Scanner 3000 7G System (Affymetrix, Santa Clara, California, USA).

Data and Statistical Analysis

Data analysis was carried out on the CEL files using Transcriptomic Analysis Console (TAC) Affymetrix© software (4.0.1.36). Transcripts were considered as differentially expressed transcripts (DET) when showing a >=2-fold change ratio (FCR) and a False Discovery Rate (FDR) < 0.05

between intestinal tracts. Volcano Plot and Hierarchical Clustering of DET were obtained by TAC (Figure 1). Transcripts were annotated primarily based on TAC and then using the gene annotation available for *Gallus gallus* (release 85) in Ensembl (Zerbino et al., 2018), based on sequences of Affymetrix probes. Excluding non-protein-coding RNA's, 12397 genes were recognized. Those genes that were also in the list of DET were defined as differentially expressed genes (DEG). The lists of DEG for cecum and for jejunum were submitted to DAVID (Huang et al., 2009) for functional annotation clustering and summarization option used were in general Functional_Categories, Gene_Ontology, Pathways and Protein_Domains. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapper was used to visualize the pathways more significantly enriched (Kanehisa and Goto, 2000).

Gene set analysis was carried out on using Gene Set Enrichment Analysis (GSEA) software, which performs a gene set analysis, where gene sets are defined as groups of genes with common biological function, chromosomal location or regulation (Subramanian et al., 2005) and was based on C2.CP:KEGG, c5.BP and C5.MP gene set collections (MSigDB, Broadinstitute). Gene sets were considered significantly enriched with False Discovery Rate (q-value) ≤ 0.05. Furthermore, to visualize differences between jejunum and cecum, Enrichment Map (http://baderlab.org/Software/EnrichmentMap20) plugin for Cytoscape 3.2.1 (http://www.cytoscape.org) was used to evidence the links between gene sets, considering node cut off FDR q-value of 0.10. The nodes were joined if the overlap coefficient was ≥ 0.4.

Results

In total, 671 and 681 DET were found in jejunum and cecum; of them, 524 and 608 were defined as DEG.

The lists of the first 20 DETs in jejunum and cecum, ranked for the FCR, are presented in Table 1 and 2 respectively, while the full lists for DETs are reported in Supplementary Table 2 and 3. APOB (apolipoprotein B) and CBS (cystathionine beta synthase) were the genes with the highest FCR in jejunum and cecum respectively. DEGs for jejunum and cecum were then processed in DAVID and the lists of DAVID functional annotations significantly enriched of DEGs in the jejunum and cecum are presented in Table 3 and 4, respectively.

In jejunum, most of the DEG-enriched annotations were related to the integral components of the cell membranes (26% of total DEGs) and to PPAR (peroxisome proliferator-activated receptor) signaling pathway, peroxisome and lipid metabolism. The DEGs of PPAR signaling pathway enriched in jejunum and relative link with lipid metabolism are visualized by the KEGG scheme in Fig.2. It worth

to signal that, beside those genes expected to control the lipid metabolism, most of those involved in gluconeogenesis were upregulated in jejunum (PCK1, phosphoenolpyruvate carboxy-kinase 1, soluble, 19.4 FCR; AQP7, aquaporin 7, 17.5 FCR; GK, glycerol kinase, 2.7 FCR).

In cecum, 69 DEGs (11.3 % of total) were associated to Disulfide bond category, 9 to Cysteine and methionine metabolism, and 42 were in the Glycoprotein category. Other enriched categories were related to cell cycle and extracellular matrix. The link between cysteine and methionine metabolism, sulfate metabolism and disulfide oxidoreductase activity, involving several DEGs, is represented in Fig. 3. The process starts with the catabolism of methionine and cysteine, more stimulated in the cecum by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH), then the DEG signatures involve the "minor" sulfate pathway comprising CBS, CTH, sulphide quinone reductase-like (SQOR), thiosulphate sulphurtransferase (TST) and sulphite oxidase (SUOX). The direction towards transport of sulphate is marked by the high FCR of expression of PAPSS2 (3'-phosphoadenosine 5'-phosphosulfate synthetase) in cecum, joined with the higher expression of sulphotransferases SULT1E1 and SULT1C3. Hydrogen sulfide is generated also from cysteine via 3-mercaptopyruvate with joined oxidation of reduced thioredoxin (TXN) by 3-mercaptopyruvate sulfurtransferase (MPST).

Data of protein-coding gene expression were then analyzed by GSEA that tests the enrichments in predefined sets. Using the KEGG-based list, consisting of 186 gene sets, 14 and 24 gene sets were enriched for jejunum and cecum respectively. The full results of this analysis were then processed to create an Enrichment map where enrichment sets are eventually linked when sharing relevant numbers of genes. Fig. 4 represents the enriched sets for jejunum and cecum according to KEGG list, while the lists of all the gene sets enriched in jejunum and cecum are reported in Supplementary Table 4 and 5, respectively.

Concerning jejunum, some results confirmed the observation based on DEG by DAVID such as those related to peroxisome and PPAR signaling. Nevertheless, several gene set related to the regulation of immunity were also evidenced, particularly concerning IgA production and the tuning of the immune response. An interesting upregulated gene set was RENIN_ANGIOTENSIN_SYSTEM, and also genes related to tryptophan and histidine metabolism were more involved in jejunum, than in cecum. Two larger gene aggregates based on Gene Ontology – Biological Processes and Molecular Functions - were also tested. Besides groups of genes similar to those already mentioned, for jejunum several genes sets related to digestion, absorption and to bile acid metabolic process (for Biological Processes), and exo-enzyme and transporter activity (for molecular functions) were among those more enriched.

In cecum the most enriched gene sets were those related to the cell cycle, but these were also associated to genes sets controlling the turnover of mature cells, firstly those in the HEDGEHOG SIGNALING PATHWAY. There was also the linked ECM_RECEPTOR_INTERACTION and FOCAL_ADHESION, evidenced by the Enriched Map. Other interesting enriched gene sets were VIBRIO_CHOLERAE_INFECTION, PATHOGENIC_ESCHERICHIA_COLI_INFECTION and TASTE_TRANSDUCTION. In cecum, the examination of enriched aggregates based on Gene Ontology (Supplementary Table. 6) evidenced a long list of biological processes in general referable to the cell cycle and it regulation. The observation of the list of upregulated molecular function was useful to better focalize on some functions already evidence in the preceding analysis. Thus, there were LAMININ_BINDING and FIBRONECTIN_BINDING that were informative about EXTRACELLULAR_MATRIX_STRUCTURAL_CONSTITUENT and PROTEIN_DISULFIDE_OXIDOREDUCTASE_ACTIVITY that can be associated to the previously evidenced Disulfide bond category.

Non-coding transcripts that were differentially expressed between the two different mucosae were also found: 4 micro RNA (miRNA) and 2 small RNA (snoRNA) in jejunum and 4 miRNA and 1 snoRNA presented statistically significant expression with FCR≥2.0 (Supplementary Table 7).

Discussion

The small intestine is deputed to digest and absorb nutrients thus it is not surprising that in the comparison with cecum emerged gene sets related to secretory enzymes and transporters. Nevertheless, the detailed check of genes with highest FCR signaled also some genes already known to be typically present in the small intestine, but not often considered for chicken. Between these genes, we found retinol binding protein (RBP2, 151.6 FCR), abundantly expressed in small intestinal epithelial cells and essential for retinol and β-carotene absorption and metabolism: its presence was previously already reported at chick duodenum level and it has been associated in rats to the expression of PPARα gene (Takase et al., 2000), which was also identified in this study as jejunal DEG in PPAR signaling pathway by DAVID. Another identified gene was lactase (LCT, 48.6 FCR), previously identified in chicken (Freund et al., 1997) and whose enzyme activity was investigated both in chick and in adult chicken (Chotisnky et al., 2001). Other genes identified were cubilin (CUBN, intrinsic factor-cobalamin receptor, 44.6 FCR), whose function at intestinal level is linked to the uptake of vitamin B_{12} (Christensen et al., 2013), and beta-carotene 15,15-monooxygenase 1 (BCMO1, 11.3 FCR), key enzyme in vitamin A metabolism in mammals and also characterized in chickens (Lietz et al., 2010).

Furthermore, the study of pathways evidenced other aspects connected with absorption of nutrients. The presence of several DEGs associated to renin–angiotensin system (glutamyl aminopeptidase, ENPEP, 143.7 FCR; angiotensin I converting enzyme 1 and 2, ACE1 and ACE2, 35.5 and 90.2 FCR respectively) evidences that also the chicken presents the enterocyte renin–angiotensin system, as in rat where it was fond in brush border, epithelial cells, lamina propria, muscularis mucosa, submucosal blood vessels and muscularis propria (Wong et al., 2007). Particularly this system was found to control of SGLT1-dependent glucose uptake across intestinal brush border membrane, sodium and water absorption, digestion and absorption of peptides (Garg et al., 2012). Furthermore, of practical relevance is the observation, based on knock-out mice, that an excess of dietary sodium impairs the digestive efficiency via the renin-angiotensin system (Weidemann et al., 2015).

The enrichment of peroxisome and PPAR-related gene sets was evidently related to the absorption and processing of fats by jejunal enterocytes; however, the inspection of these set evidences also the important presence of a local gluconeogenesis, where glucose is produced and used by small intestine itself or released into the portal blood (Mithieux et al., 2005). As already previously reported in rat and human (Rajas et al., 2000; Yanez et al., 2003; Lin et al., 2013), key enzymes and their mRNA for gluconeogenesis have been found in small intestine, such as PCK, one of the two major regulatory genes of gluconeogenesis (Mithieux et al., 2005). In line with previous studies, we found PCK1 as upregulated gene in jejunum compared to cecum. The relevance of the small intestine as endogenous source of glucose and the modulation of this production by the diet in some animals (Kirchner et al., 2005; Mithieux et al., 2005) and by insulin action (Croset et al., 2001) have been reported, but not for poultry. This activity raises particularly when subjects are underfed or given high protein diets, since intestinal gluconeogenesis has been associated with amino acid availability (Sinha et al., 2017), whether this availability comes from diet or from long fasting period with trigger of protein catabolism (Habold et al., 2005). Like dietary protein, also diets rich in dietary fiber induce gut gluconeogenesis gene expression and, as well as for amino acids, propionate deriving from fiber can be used as precursor (Mithieux et al., 2014). The presence of a portal sensing of intestinal gluconeogenesis in other species (Mithieux et al., 2005; Penhoat et al., 2011) suggests that local gluconeogenesis in chicken jejunum could be a relevant site of variability in the interaction between feed characteristics and individual control of feed intake. Finally, the indication that succinate produced by intestinal microbiota activates intestinal gluconeogenesis and, in turn, improves gut homeostasis (De Vadder et al., 2016) can be considered as a potential connection of this function with the presence of typical microbial metabolites in chicken, such as lactic acid produced by locally dominant lactic acid bacteria (Lu et al., 2003).

Sulphur metabolism emerged also as a new key aspect in the comparison between the transcriptomes of the chicken jejunum and cecum tissues. Beside sulfur amino acids, inorganic sulfate is an essential source for several physiological processes. The high jejunal FCR (32.9) of solute carrier family 13 member 1 (SLC13A1, also known as NaSi), recognized as the main apical sodium (Na+)-dependent transporter into the enterocyte (Whittamore and Hatch, 2017), supports that small intestine is the main site of sulfate absorption in chicken. Moreover, in mice, cecum was recognized as a site of active secretion of sulfates, exchanged for chlorides, by solute carrier family 26 member 3 (SLC26A3, also known as DRA) (Whittamore et al., 2013): the same transporter SLC26A3 was upregulated in cecum (8.1 FCR) and this may indicate that also in chicken the cecum is a source of release of sulfates into the lumen. This could have also a relevance because sulfates can be used by local microbiota, while it is known that some toxin derived by pathogen bacteria downregulate SLC26A3 by the raise of intracellular cyclic AMP or GMP (Berni-Canani et al., 2011) and, conversely, beneficial bacteria up-regulate SLC26A3 gene expression in CACO2 cell culture (Kumar et al., 2014) and in pig jejunal loops in vivo (Trevisi et al., 2018). Interestingly, several genes related to sulfur metabolism were upregulated in ceca obtained from chickens conventionally reared or associated with some groups of bacteria, compared to those obtained from germ free chickens (primarily CBS, SULT1C3, SULT1E1, TXN, PAPSS2, TST). In particular, these genes were related to sulfotransferases and enzymes recognized as sulphate donors: in cecum, sulfonate groups are widely used both for sulphate conjugation and mucin sulfation for mucus layer building (Volf et al., 2017). Thus, it may be possible that several pathways and functional associations related to sulfur (such as cysteine and methionine metabolism, disulfide bond and disulfide oxidoreductase activity that we found in cecum) are induced or up-regulated by the presence (germ free cecum vs conventional) or the major concentration or quality of bacteria (cecum vs jejunum) in chicken. An indirect confirmation can result also from some considerations about mucin structure in cecum. A reduction in sulfomucins in goblet cells in the jejunum, ileum, and colon was associated to the reduced availability of circulating serum sulfates in SLC13A3 knockout mice (Dawson et al., 2009). Mucin sulfation is important to provide the structure, the complexity and the protection against microbial penetration of mucins (Deplancke and Gaskins, 2001), that are denser and sulfated in chicken cecum than in jejunum and in chicken than in human (Struwe et al., 2015). In fact, the sulfated structures detected in cecum are about 57% of all O-glycans compared to the 33% in the small intestine (Struwe et al., 2015). The main enzyme responsible of sulfation of mucin glycans, galactose-3-O-sulfotransferase 2 (GAL3ST2), was more expressed in cecum (3.5 FCR). It worth to note that for this gene an extreme variation of individual gene expression was seen here both for cecum (from 3.5 to 9.7 log2 microarray values) and jejunum, and also in a previous set where two different hybrid genetic lines were compared for gene expression in broiler

ilea (Zampiga et al., 2018). Our data cannot allow deciding if this observation is related to a variation related to a genetic polymorphism in GAL3ST2 or it is the result of other determinants. Nevertheless, it should be considered the hypothesis of the individual variation of the sulfation of intestinal mucin glycans. Finally, bacteria can release hydrogen sulfide with their metabolic action. Presumably, the balance between dietary organic and inorganic sulfur, endogenous release and net use by bacteria has never been assessed. However more knowledge on these fluxes could also be relevant for methionine and cysteine use by the chicken and to properly adequate the feeding requirements to the ideal cecum microbiota.

Sulfur is also important for the organization of the extracellular matrix (ECM). Laminin (LAMB1), an ECM structural glycoprotein whose gene was up-regulated in cecum than in jejunum (3.4 FCR), contains nearly 200 disulfide bonds (Fass and Thorpe, 2018). The same was also for a keratan sulfate proteoglycan, lumican (LUM). These genes were among those that enriched the gene sets related to ECM structure and formation. ECM is important as structural support, biochemical or biomechanical cue for cecum cells, and the general observation that pathways related to ECM were enriched for cecum, may indicate that a specific attention on the structures should be paid when the gut barrier is considered to be improved. This can imply a revision of sulphur or sulphur amino acids requirements.

The local metabolism of other amino acids was seen to be affected by the type of intestinal mucosa. In jejunum, the pathways related to histidine and tryptophan emerged principally. However, the inspection of the list of high-ranking genes for these sets (Dopa decarboxylase, aromatic L-amino acid decarboxylase, monoamine oxidase A, monoamine oxidase B, aldehyde dehydrogenase 3 family member A2) refers to functions (oxidation, decarboxylation) that are not specific of the metabolism of these single amino acids, but rather associated to the use of other essential amino acids. Jejunum is an important site of absorption of amino acids. Thus, it can be considered normal that part of them are used locally as a first-pass metabolism, as seen for example also in young pig (Stoll et al., 1998). Conversely, the cecum may depend for local metabolism on nutrients other than essential amino acids and derived from blood, and thus may activate less these pathways.

The enrichment of several pathways related to immunity in jejunum worth a specific attention. In fact, both sets related to activation (T cells, natural killer cells) and to immune depression were present. The first DEG for the set PRIMARY_IMMUNODEFICIENCY was adenosine deaminase (ADA, FCR = 5.67). Adenosine is a purine nucleoside that is typically released extracellularly particularly in case of tissue injuries and can be detected by specific cell surface detectors and modulates almost all functions of innate and acquired immunity (Antonioli et al., 2018). ADA is important because protects the immune system by the adenosine-induced excessive depression with the catalyses of the conversion of adenosine to inosine. In fact, combined immunodeficiency diseases

can be seen in human related to genetically derived impaired ADA function (Bradford et al., 2017). Other DEGs were those related to the presence of T cells (CD3D and CD3E) and particularly to cytotoxic types (CD8A). Taking these data together, it looks like jejunum required in general more tuning of the immune system because more exposed to offenses of bacteria, also may be due to a less defence of passive barrier like in cecum. In support of this, it might be worthwhile to consider again PPAR signaling, enriched in jejunum, from an immunological point of view. In fact, in this study we identified FABP (fatty acid binding proteins) between DEGs in PPAR signaling pathway. FABP are a class of molecules that mediate lipid response and metabolism but are also closely linked to inflammatory processes: they are involved in modulation of lipid-sensitive pathways in cells like macrophages and it has been suggested the importance of FABP presence for gut barrier health (Chen et al., 2015). A decrease in FABP2 mRNA expression was observed in jejunal mucosa with gut barrier failure in broiler chickens (Chen et al., 2015): in our study, FABP2 was identified as differentially expressed gene in jejunum and so, beside to its function in lipid metabolism, it might be involved also in sustaining gut barrier maintenance and defence (more necessary in jejunum that in cecum).
A final consideration should be addressed to the relevant presence of enriched gene sets related to the cell cycling and mitosis in cecum, compared to jejunum. Both tissues are in general having important turnover. However, it is possible that in chicken the cecum undergoes to a more important pressure, including a controlled apoptosis to maintain optimal barrier. This is indicated particularly by the enrichment of Hedgehog signaling pathway that is important to the control of large intestinal homeostasis. Interestingly two of the non-coding transcripts that were also differentially expressed in cecum (gga-mir-196-4 and gga-mir-1732) are the same cluster on chromosome 2 as the two Hox genes, whose differential expression explains principally the effect on the Hedgehog signalling pathway (HOXA9 and HOXA10). A similar cluster is seen also in human (De Kumar and Krumlauf, 2016) and the integration of miRNA into this system reflects the relevance of certain miRNA to control the expression of gene clusters related to the maturation but also to the maintaining of the tissue differentiation in the cecum. Furthermore, it should be considered that the sampled chickens were still growing and thus cecum was still maturing, may be with much intensity than jejunum.

Conclusions

By performing a double exploratory functional analysis on chicken gut transcriptomic profile, this study confirmed some known and expected intestinal functions, such as those related to nutrient digestion and metabolism and to cell turnover and revealed and highlighted new interesting correspondences with mammals not reported before in poultry, such as gluconeogenesis and renin-

angiotensin system in jejunum. Furthermore, some key aspects emerged from analysis of DEG and pathways indicating a different biological characterization between the different gut sites, which diverge in terms of gene expression towards their main biological processes. In fact, in jejunum, new key aspects related to sulfur transport activity specific for this site along with immune pathways tuning emerged; in cecum, a more intense activity for cell turnover and sulfur utilization for structural components suggested a specific activity at epithelial level, maybe involved in gut barrier maintenance. These findings may represent useful hints for further insights on molecular aspects of gut tissues and for possible investigations with various experimental factors.

Table 1. **List of the first 20 differentially expressed transcripts in jejunum of broiler chickens at 42 days of age (n=19) compared to cecum, ranked for the fold change ratio**

Fold Change	P-value	FDR P-value	Gene Symbol	Description
207.3	6.4E-19	1.7E-16	APOB	apolipoprotein B
151.2	1.7E-15	1.7E-13	RBP2	retinol binding protein 2, cellular
143.7	1.5E-22	3.8E-19	ENPEP	glutamyl aminopeptidase (aminopeptidase A)
111.4	5.5E-21	4.0E-18	MEP1A	meprin A, alpha (PABA peptide hydrolase)
98.2	6.0E-21	4.2E-18	SI	sucrase-isomaltase (alpha-glucosidase)
90.2	4.9E-21	3.7E-18	ACE2	angiotensin I converting enzyme 2
88.0	2.2E-22	4.1E-19	SLC6A19	solute carrier family 6 (neutral amino acid transporter), member 19
74.2	9.2E-23	3.4E-19	MGAM	maltase-glucoamylase (alpha-glucosidase)
73.2	9.5E-19	2.3E-16	SLC7A9	solute carrier family 7 (amino acid transporter light chain, bo,+ system), member 9
66.3	1.5E-21	1.6E-18	SLC15A1	oligopeptide transporter, member 1
64.8	1.9E-22	3.8E-19	SLC9A3	(NHE3, cation proton antiporter 3), member 3
58.4	2.9E-15	2.7E-13	ENPP7	ectonucleotide pyrophosphatase/phosphodiesterase 7
57.9	1.7E-20	8.3E-18	CLDN10	claudin 10
55.6	1.4E-22	3.8E-19	MGAT4D	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme B-like
49.6	1.6E-14	1.2E-12	LCT	Lactase
49.2	6.6E-22	8.0E-19	CNOT2	CCR4-NOT transcription complex, subunit 2
46.6	2.3E-19	6.6E-17	TM4SF4	transmembrane 4 L six family member 4
46.3	9.6E-19	2.3E-16	MME	membrane metallo-endopeptidase
45.8	5.1E-22	7.1E-19	MEP1B	meprin A, beta
45.0	3.7E-16	4.3E-14	FABP2	fatty acid binding protein 2, intestinal

Transcripts were considered as differentially expressed transcripts (DET) when showing a >=2-fold change ratio (FCR) and a False Discovery Rate (FDR) < 0.05 between intestinal tracts

- The full list of differentially expressed genes is reported in Supplementary table 2.

Table 2. **List of the first 20 differentially expressed transcripts in cecum of broiler chickens at 42 days of age (n=19) compared to jejunum, ranked for the fold change ratio.**

Fold Change	P-value	FDR P-value	Gene Symbol	Description
244.4	6.17E-21	4.18E-18	CBS	cystathionine-beta-synthase
121.2	1.44E-21	1.55E-18	ENSGALG00000021450	C factor like
78.4	1.19E-23	7.73E-20	MAL	mal, T-cell differentiation protein
40.8	1.42E-19	4.34E-17	AQP8	aquaporin 8
24.5	6.06E-16	6.60E-14	NOXO1	NADPH oxidase organizer 1
22.5	1.76E-14	1.24E-12	CA4	carbonic anhydrase IV
18.0	3.58E-18	7.28E-16	HOXA10	homeobox A10; homeobox protein Hox-A10-like
17.9	3.44E-20	1.40E-17	SLC38A4	solute carrier family 38, member 4 (SNAT4)
15.8	2.11E-15	2.01E-13	SLC26A4	solute carrier family 26 (anion exchanger), member 4
15.1	5.97E-22	7.80E-19	PON2	paraoxonase 2
14.2	4.79E-15	4.14E-13	TFCP2L1	transcription factor CP2-like 1
14.2	2.93E-21	2.55E-18	SELENBP1	selenium binding protein 1; selenium-binding protein 1-A-like
14.0	7.89E-15	6.28E-13	ATP6V0D2	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2
13.2	3.34E-20	1.39E-17	PADI3	peptidyl arginine deiminase, type III
12.9	5.97E-15	4.99E-13	PLET1	Placenta Expressed Transcript 1
12.9	3.03E-14	2.05E-12	GJB2	gap junction protein, beta 2, 26kDa
12.0	8.16E-18	1.57E-15	LY6E	lymphocyte antigen 6 complex, locus E-like
11.7	1.74E-16	2.28E-14	gga-mir-196-4	microRNA 196-4
11.2	8.74E-17	1.25E-14	GSTA4	glutathione S-transferase alpha 4
11.0	2.26E-23	1.03E-19	B4GALNT3	beta-1,4-N-acetyl-galactosaminyltransferase 3

Transcripts were considered as differentially expressed transcripts (DET) when showing a >=2-fold change ratio (FCR) and a False Discovery Rate (FDR) < 0.05 between intestinal tracts

- The full list of differentially expressed genes is reported in Supplementary table 3.

Table 3. **List of DAVID functional annotation significantly enriched of differentially expressed genes in the jejunum of broiler chickens at 42 days of age (n=19), compared to cecum.**

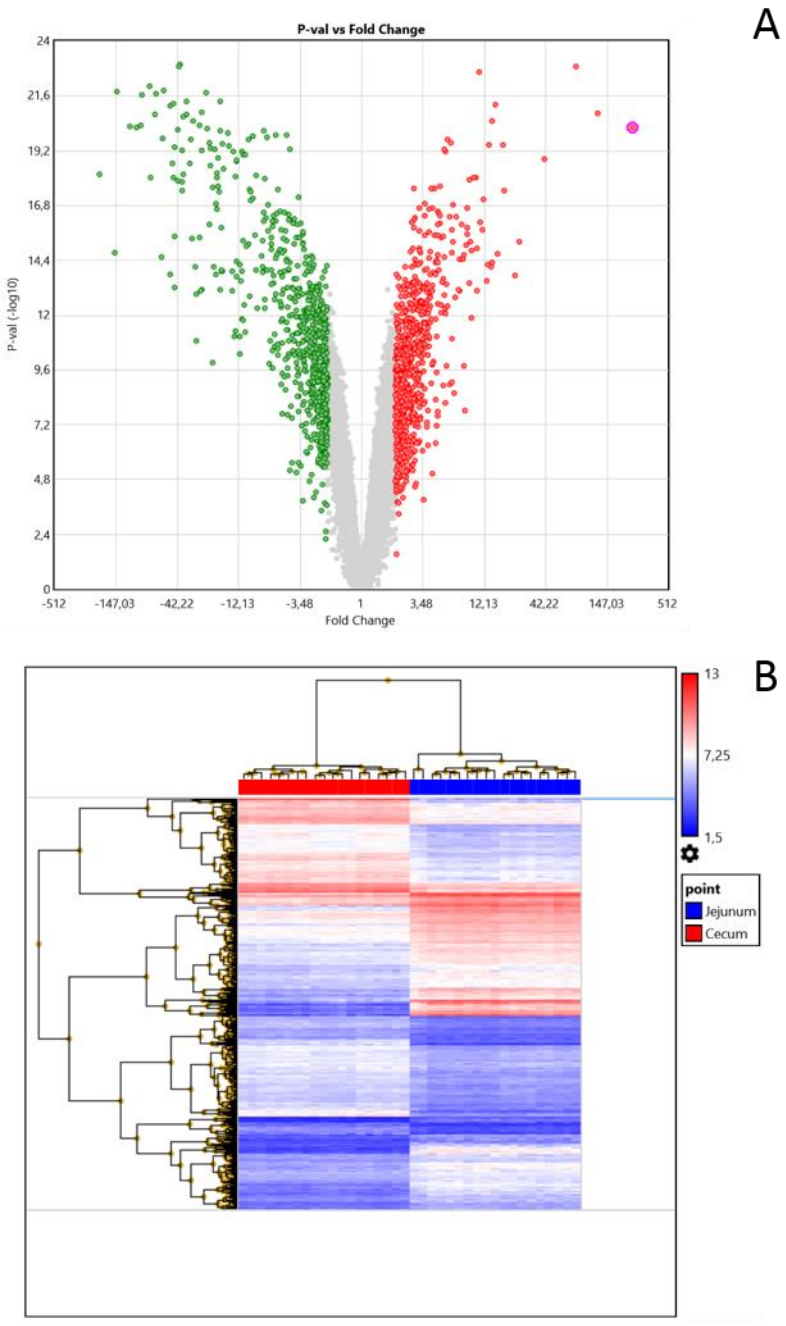
Functional Annotation	Gene Count	%	*P*-Value	Benjamini
PPAR signaling pathway	18	3.4	9E-11	9E-09
Metabolic pathways	78	14.9	3E-08	1E-06
Peroxisome	12	2.3	3E-04	1E-02
Glycerophospholipid metabolism	12	2.3	1E-03	3E-02
Histidine metabolism	6	1.1	1E-03	3E-02
Fatty acid degradation	7	1.3	2E-03	4E-02
Transmembrane helix	173	33.0	1E-11	3E-09
Transmembrane	173	33.0	2E-11	2E-09
Membrane	179	34.2	9E-10	6E-08
Transport	39	7.4	5E-04	2E-02
Cholesterol efflux	7	1.3	2E-05	3E-02
Apical plasma membrane	20	3.8	2E-08	5E-06
Integral component of membrane	138	26.3	3E-07	3E-05
Brush border membrane	8	1.5	1E-05	7E-04
Peroxisome	10	1.9	9E-05	4E-03

Table 4. **List of DAVID functional annotation significantly enriched of differentially expressed genes in the cecum of broiler chickens at 42 days of age (n=19), compared to jejunum.**

Category and functional Annotation	Gene count	%	*P*-Value	Benjamini value
KEGG_PATHWAY				
Cysteine and methionine metabolism	9	1.5	0.0001	0.012
Cell cycle	16	2.6	0.0001	0.009
Focal adhesion	20	3.3	0.0008	0.032
UP_KEYWORDS				
Disulfide bond	69	11.3	0.0000	0.002
Mitosis[1]	12	2.0	0.0000	0.001
Developmental protein	29	4.8	0.0000	0.002
Glycoprotein	42	6.9	0.0000	0.002
ATP-binding	48	7.9	0.0002	0.008
Secreted	35	5.8	0.0002	0.007
Alternative splicing	18	3.0	0.0003	0.008
Cytoskeleton	17	2.8	0.0016	0.030
Phosphoprotein	26	4.3	0.0033	0.053
GOTERM_BP_DIRECT				
Chromosome segregation	10	1.6	0.0000	0.044
GOTERM_CC_DIRECT				
Proteinaceous extracellular matrix[2]	24	3.9	0.0000	0.000
Midbody[3]	12	2.0	0.0001	0.013
Kinesin complex[4]	9	1.5	0.0002	0.012
GOTERM_MF_DIRECT				
Heparin binding	15	2.5	0.0000	0.001
Chemoattractant activity	7	1.2	0.0001	0.022

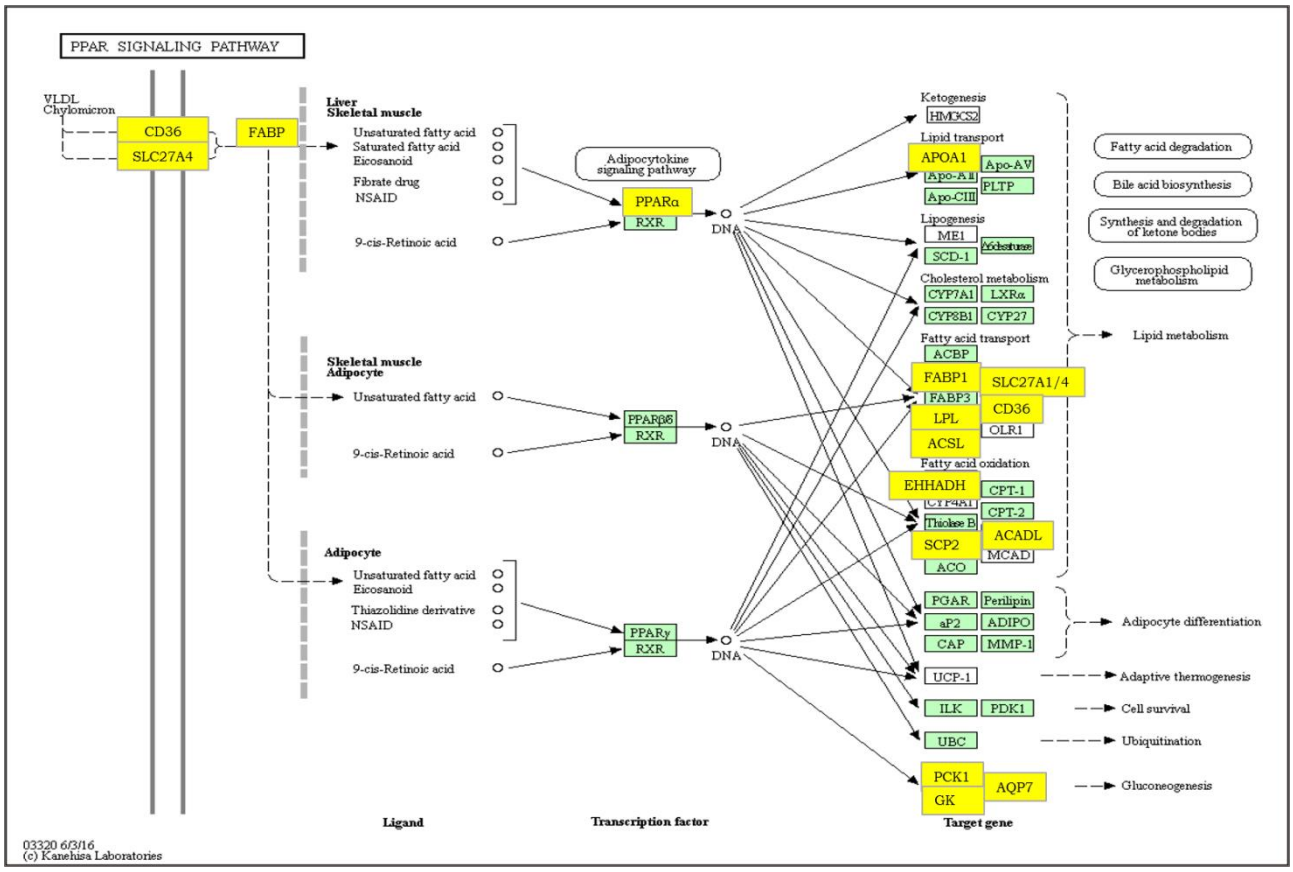
Other categories statistically significant: [1]Cell division, centromere, cell cycle, Nucelotide binding, DNA binding, Chromosome; [2]Extracellular exosome, Extracellular space, Focal adhesion; [3]Spindle microtubule; [4]Kinethocore, Condensed chromosome kinetochore

Figure 3. **Volcano Diagram (A) and Hierarchical Clustering (B) showing the distribution of transcripts differentially expressed in jejunum and cecum of broiler chickens at 42 days of age (n=19 per intestinal tissue).**



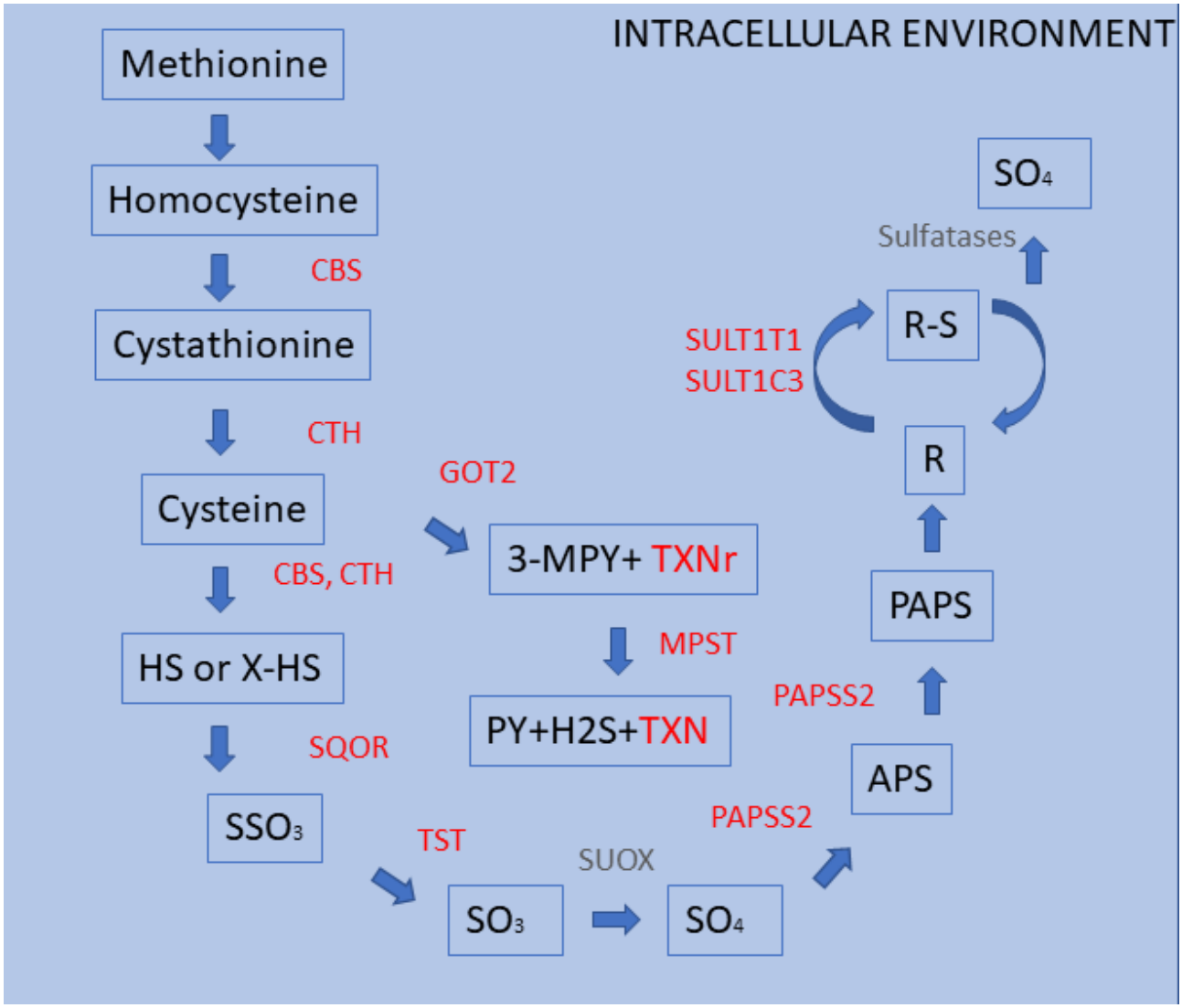
A: Volcano Diagram, jejunum in green, cecum in red. B: Hierarchical Clustering

Figure 2. **PPAR signaling enriched with differentially expressed genes (DEGs) in jejunum of broiler chickens at 42 days of age (n=19), by KEGG**.



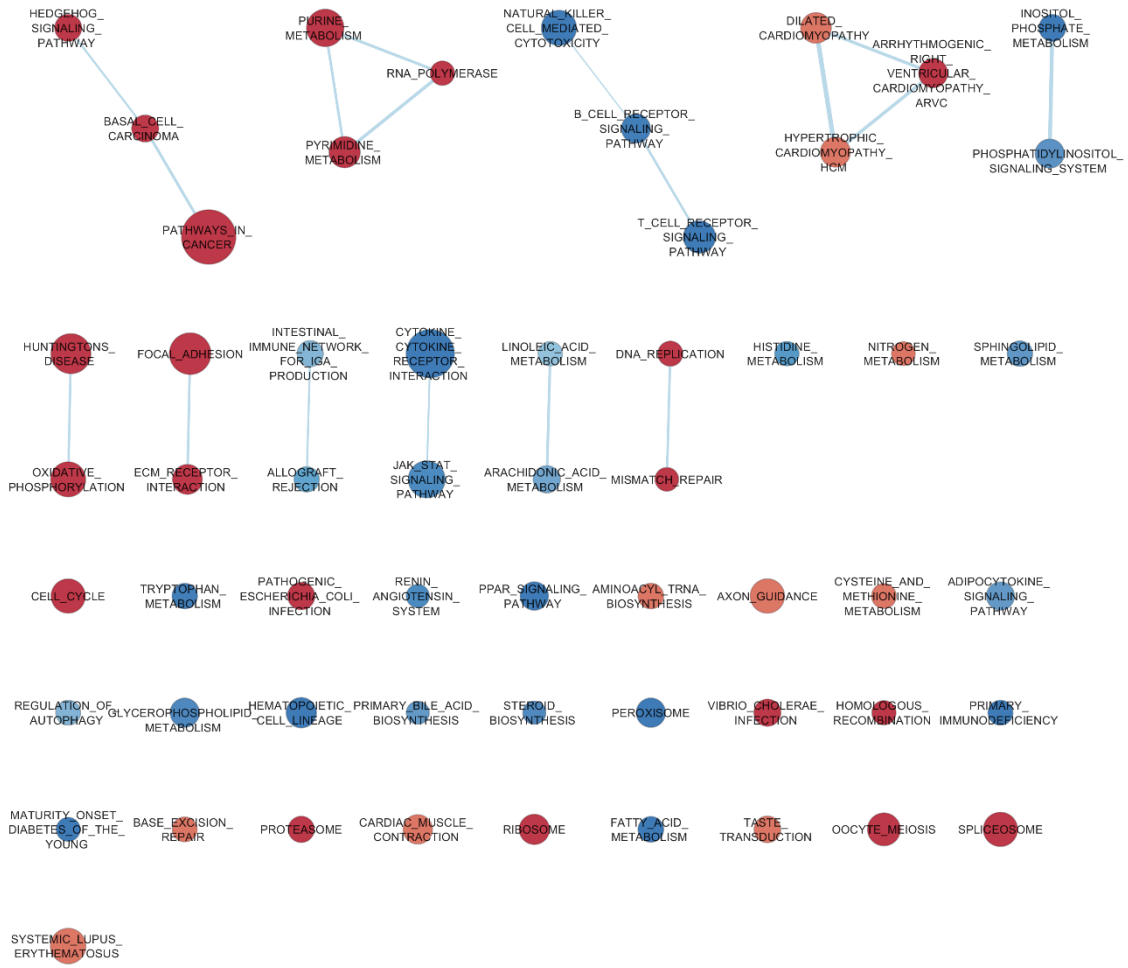
Genes over-expressed in jejunum are shown in yellow. CD36: CD36 molecule; ACADL: acyl-CoA dehydrogenase long chain; ACSL: acyl-CoA synthetase long chain family member (ACSL3, ACSL4, ACSL5); APOA1: apolipoprotein A-1; AQP7: aquaporin 7; EHHADH: Bi-enzyme enoyl-CoA hydratase/ 3-hydroxyacyl CoA dehydrogenase; FABP: fatty acid binding protein (FABP1, FABP2, FABP5, FABP6); GK: glycerol kinase; LPL: lipoprotein lipase; PPARα: peroxisome proliferator-activated receptor alpha; PCK1: phosphoenolpyruvate carboxykinase 1; SCL27A1/4: solute carrier family 27 (fatty acid transporter), member 1, member 4; SCP2: solute carrier protein 2.

Figure 3. **Gene enrichment in the cysteine and methionine catabolism, sulfate metabolism and disulfide oxidoreductase activity in cecum of broiler chickens at 42 days of age (n=19)**



Genes over-expressed in cecum respect to jejunum are shown in red (FDR>2, except was at least = 1.8). The alternative sulfate-generating pathway controlled by cysteine dioxygenase type 1 (CDO1) is not represented, because the gene coding this enzyme was mildly expressed, with values not differing to jejunum mucosa. CBS = cystathionine β-synthase ; CTH = cystathionine γ-lyase; SQOR = sulphide quinone reductase; TST = thiosulphate sulphurtransferase (TST); SUOX = sulphite oxidase (not spotted on chicken Microarrays); APS = 5' adenosine-phosphosulfate; PAPS = 3'-phosphoadenosine 5'-phosphosulfate; PAPSS2 = PAPS synthetase; SULT1E1 and SULT1C3 sulphotransferase, family 1C member 3 and family 1E member 1; GOT2 = Glutamic-Oxaloacetic Transaminase 2; 3-MPY = 3-mercaptopyruvate; MPST = 3-mercaptopyruvate sulfurtransferase; PY = pyruvate; TXN = thioredoxin. Adapted from Dawson et al., 2015.

Figure 4. **Nodes of gene sets enriched in jejunum and cecum of broiler chickens at 42 days of age (n=19 per intestinal tissue) in Enriched Map plugin for Cytoscape 3.2.1, according to KEGG gene list**



Nodes represent gene sets enriched in jejunum (red color) and cecum (blue color). Node size represents the number of genes in each gene set.

Node cut off with FDR q-value of 0.10. The nodes were joined if the overlap coefficient was ≥0.4.

Study 2:

In ovo injection of a galacto-oligosaccharide prebiotic in broiler chickens submitted to heat-stress: impact on cecal microbiota, transcriptomic profile of jejunum and cecum and plasma immune parameters

Aim of the study

Given that *in ovo* technology can be considered as promising solution for bioactive compounds delivery since it ensures a protection of gastro-intestinal tract as early as from the hatching and since it represents a possible useful tool for counteracting stress-related immune suppression in chickens, the possible beneficial effects of prebiotics injected *in ovo* to counteract the negative impact of heat stress deserve to be investigated.

The main aim of this study was to investigate the effect of *in ovo* injection of GOS on the intestinal microbiota, transcriptomic profile and plasma immune parameters of broiler chickens kept under thermoneutral condition or under chronic heat stress condition during the last phase of rearing period.

Materials and Methods

Animals tested, experimental groups and overall sampling

The experiment was carried out based on 2x2 factorial design with GOS *in ovo* injection and chronic heat stress as factors. Fertilized eggs of broiler chickens (Ross 308, 3,000 eggs) were incubated in a commercial hatchery following the procedure commonly used at commercial levels. At day 12 of egg incubation, a single dose (0.2 ml) of 0.9 % physiological saline (0.9% NaCl) (CON) or 0.9 % physiological saline+3.5 mg GOS/egg (GOS) was injected into the air chamber of fertilized eggs of the chicks. The GOS consisted of a formulation of non-digestive mixture of trans-galactooligosaccharides from milk lactose digested with *Bifidobacterium bifidum* (Clasado Biosciences, Jersey, UK). At hatching, chicks were sexed and vaccinated against coccidiosis, Infectious Bronchitis Virus, Marek's disease virus, Newcastle and Gumboro disease. Hatchability was calculated as the number of chicks being hatched and expressed as percentage of fertile eggs with apparently live embryos selected at 12 days of incubation by candling. The dose of 3.5 mg/egg of GOS we used in this study was chosen on the basis of a previous dose optimization trial, which showed that this dose does not reduce the hatchabilty rate compared to a control group injected with physiological saline (Bednarczyk et al., 2016). A total of 300 male chicks/group (i.e., CON and GOS) were transferred to the experimental facility of the University of Bologna within an environmental controlled poultry house. The experiment design was approved by the Ethical Committee of the University of Bologna on 03.05.2017 (ID363/2017-PR). In the poultry house, the 300 chicks belonging to each group were divided in two subgroups (150 chicks/subgroup). Each subgroup was split into 6 replicates (25 birds/replicate) and kept either in thermoneutral (TN, 25°C) condition up to 42 days, when the trial ended, or chronic heat-stress (HS) at 30°C all day long between day 32 and 42, for a total of ten days of heat stress. Overall, these treatments resulted in a total of four experimental groups: control group keeps in thermoneutral condition (CON/TN); control group keeps under heat stress the last 10 days of rearing (CON/HS); GOS *in ovo* group keeps in thermoneutral condition (GOS/TN); GOS *in ovo* group keeps under heat stress the last 10 days of rearing (GOS/HS). All the birds received the same standard commercial diet composed by three feeding phases: Starter (0-10d), Grower (11-25d) and Finisher (26-42d) (Supplementary Table 1).

At day 32, a total of 48 birds were randomly selected from both the CON (n=24 birds) and GOS (n=24 birds) group (4 birds/replicate) and humanely euthanized. The entire GIT of the 48 individual selected birds was dissected out and a small sample (i.e., 0.5 to 2 g) of cecum content was collected into 15ml sterile plastic tubes. The samples collected were immediately frozen in liquid nitrogen and then stored at -80°C until further testing. At the time of slaughter (i.e., day 42), further 48 birds were

randomly selected from both the CON (n=24) and GOS (n=24) group, keep both in thermoneutral condition (i.e., CON/TN and GOS/TN) and under heat stress the last 10 days of rearing (i.e., CON/HS and GOS/HS). The entire GIT of the 48 individual selected birds was dissected out and processed as previously described. Moreover, jejunum and cecum mucosae were collected by gently scraping after tissues rinsing in PSB to remove residues of digesta. Both caecum contents and mucosae were immediately frozen in liquid nitrogen and then stored at -80°C. At the same time point, blood was collected from wing vein from 2 birds/replicate using 6 ml EDTA coated vacutainer tubes (Vacumed K3 EDTA, vacuum system). Immediately after sampling, tubes were centrifuged at 4000 xg for 15 minutes at 4° C. Then plasma was quickly dispensed in vials, snap frozen in liquid nitrogen and stored at -80°C.

DNA, RNA and plasma analysis

The DNA was extracted from each sample of caecum content using a bead-beating procedure (Danzeisen et al., 2011). Briefly, 0.25 g of cecal content were suspended in 1 ml lysis buffer (500 mM NaCl, 50 mM Tris-Cl, pH 8.0, 50 mM EDTA, 4 % SDS) with MagNA Lyser Green Beads (Roche, Milan, Italy) and homogenized on the MagNA Lyser (Roche) for 25 seconds at 6500 rpm. The samples were then heated at 70°C for 15 min, followed by centrifugation to separate the DNA from the bacterial cellular debris. This process was repeated with a second 300 µl aliquot of lysis buffer. The samples were then subjected to 10 M v/v ammonium acetate (Sigma, Milan, Italy) precipitation, followed by isopropanol (Sigma) precipitation and a 70% ethanol (Carlo Erba, Milan, Italy) wash and re-suspended in 100 ul 1X Tris-EDTA (Sigma). The samples were treated with DNase-free RNase (Roche) and incubated overnight at 4°C, before being processed through the QIAmp® DNA Stool Mini Kit (Qiagen, Milan, Italy) according to manufacturer's directions with some modifications. Samples were measured on a BioSpectrometer® (Eppendorf, Milan, Italy) to assess DNA quantity and quality. The libraries were prepared following the Illumina 16S Library preparation protocol, amplifying the variable V3 and V4 regions of the 16S rRNA to obtain a single amplicon of approximately 460 bp. Sequencing was performed in paired-end in the Illumina MiSeq with the MiSeq Reagent kit v2 500 cycles, characterized by a maximum output of 8.5 Gb.

From both jejunum and cecum mucosae issues, total RNA was extracted using GeneJET RNA Purification Kit (Thermo Scientific) according to manufacturer's instructions and RNA quantity and quality were evaluated using Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA) and agarose gel electrophoresis, respectively. After, RNA integrity was evaluated through Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA).

The whole transcriptome microarray analysis was performed using Affimetrix© GeneChip Chicken Gene 1.0 ST Array and hybridized arrays were scanned on Affimetrix© GeneChip Scanner 3000 7G System (Affymetrix, Santa Clara, California, USA).

Plasma immunoglobulin A (IgA), immunoglobulin G (IgG) and serum amyloid A (SAA) concentrations were measured according to the protocol of the commercial chicken-specific IgA (Catalogue number ECH0083), IgG (Catalogue number ECH0031) and SAA (Catalogue number ECH0090) Fn-test ELISA kits (Wuhan Fine Biotech Co., Ltd., Wuhan, China). The samples were examined in duplicate at the dilution 1:1, 1:100000 and 1:4 respectively. The IgA, IgG and SAA concentrations of the samples have been interpolated from the standard curves using software curve expert to 1.3.

Data Analysis

The reads obtained for each sample were analysed by using USearch and the mean values of the relative frequency of abundance of each taxonomic category calculated within each group were compared by using the t test of Tukey-Kramer (i.e., CON vs GOS at 32 days; CON/TN vs GOS/TN at 42 days; CON/HS vs GOS/HS at 42 days). Such test aimed to evaluate differences between abundances of the same group of microorganisms between GOS-injected and CON samples collected under different conditions (i.e., TN and HS). The p-values < 0.05 were considered statistically significant. Moreover, abundances of the 42 days samples were fitted to a multiple linear regression model. The regression was applied to each group at every taxonomic level using two covariates: temperature (T) (i.e., TN vs HS) and treatment (i.e., CON vs GOS). In this analysis, for each taxonomic category, the regression coefficients, relative to the temperature and the treatment, measure the correlation between the taxonomic groups and each covariate after taking into account the other covariate. Furthermore, each coefficient was paired to a p-value that tests the null hypothesis that the coefficient is equal to zero. For each parameter with significant p-value ($\alpha=0.01$), a so called regplot showing how the other two sub-groups are distributed was plotted. For both t-test and multiple regression Python scripts, allowing an automated analysis on multiple samples, were used. Alpha diversity was assessed by using the Shannon index and the beta diversity was quantified by both Bray-Curtis and Unifrac indices.

Transcriptome data analysis was carried out on the CEL files using Transcriptomic Analysis Console (TAC) Affymetrix© software (4.0.1.36). Transcripts were considered as differentially expressed genes (DEG) when showing a $\geq$ 2-Fold Change ($\log_{10}$ 2 ratio) and a False Discovery Rate (FDR) <

0.05 between treatments. Furthermore, an exploratory functional analysis was then carried out using Gene Set Enrichment Analysis (GSEA) software, which performs a gene set analysis, where gene sets are defined as groups of genes with common biological function, chromosomal location or regulation (Subramanian et al., 2005). GSEA analysis was based on C2.CP: KEGG gene set collection (MSigDB, Broadinstitute) and gene sets were considered significantly enriched with FDR (q-value) ≤ 0.05. Finally, to evaluate differences between jejunum and cecum within each treatment (i.e., GOS vs CON and HS vs TN) Enrichment Map (http://baderlab.org/Software/EnrichmentMap20) plugin for Cytoscape 3.2.1 (http://www.cytoscape.org) was used in order to visualize the overlap of gene sets, considering a FDR q-value <0.01. The nodes were joined if the overlap coefficient was ≥0.5.
Results from plasma IgA, IgG and SAA were analyzed by two-way ANOVA with R software (Stats Package) considering environmental condition (TN or HS) and *in ovo* injection (CON or GOS) as factors and considering significative differences with $P \leq 0.05$.

Results

Hatchability did not differ between the *in ovo* treatments, with a high rate in both GOS and CON (~90%).

Cecal microbiota

Most of bacteria colonising the cecum belonged to the phylum Firmicutes, followed by Proteobacteria, Actinobacteria and Tenericutes (Table 1). Applying the t-test, the identified phyla did not show significative differences between the CON and GOS group at both 32 and 42 days (Supplementary Table 2). Taking into account the effect of both covariates in the multiple regression analysis (i.e., *in ovo* treatment and thermal treatment), the mean relative frequency of abundance of Firmicutes decreased in both GOS and HS treatments (Fig. 1 A), while the thermal treatment alone (HS) decreased the mean relative frequency of abundance of Actinobacteria compared to TN treatment, independently by the *in ovo* treatment (Fig. 1B). Instead, Proteobacteria mean relative frequency of abundance was increased by GOS compared to CON *in ovo* treatment, independently by the thermal one (Fig. 1C). The Firmicutes identified in the cecum were mainly represented by Clostridia, Bacilli and Erysipelotrichia at both sampling time (Table 2). The identified classes did not show significative differences between *in ovo* treatments (i.e., CON vs GOS) at both 32 and 42 days with t-test (Supplementary Table 3). However, it is interesting to underline the protective effect of GOS in comparison to the CON group against Gammaproteobacteria, including many foodborne pathogens as *Salmonella enterica* and *Escherichia coli*, in the HS group (0.8 vs 1.2%), although this

difference was not significantly different (Supplementary Table 3). Taking into account both the impact of *in ovo* treatment and thermal treatment with the multiple regression analysis, they affected the mean relative frequency of abundance of Clostridia, increased in saline group (CON) under HS (Fig. 2 A), Bacilli, decreased in both GOS and HS treatment (Fig. 2 B), and Erysipelotrichia, increased in both GOS and HS treatments (Fig. 2 C).

The most representative orders identified in the cecum of birds at both sampling times were Clostridiales, Lactobacillales and Erysipelotrichales (Table 3). The identified orders did not show significative differences between *in ovo* treatments at both 32 and 42 days with t-test (Supplementary Table 4). The only exception was represented by Bacillales significantly higher in the CON than GOS group in the HS group (1.0 vs 0.4%) (p=0.0009). Considering the multiple regression analysis, the interaction between the factors affected the mean relative frequency of abundance of Clostridiales, increased in both CON and HS treatments (Fig. 3 A) and of Erysipelotrichales, increased in both GOS and HS treatments (Fig. 3 B).

The most representative families identified in the cecum of birds at both sampling times were Ruminococcaceae, Lachnospiraceae, Erysipelotrichaceae, Lactobacillaceae, Eubacteriaceae and Streptococcaceae (Table 4). From t-test, the identified families did not show significative differences between *in ovo* treatments at both 32 and 42 days (Supplementary Table 5). The only exception was represented by Enterococcaceae, significantly higher in the CON group than the GOS group in the TN group (0.2 vs 0.1%) (p=0.0457). Considering both the impact of *in ovo* GOS treatment and HS treatment in the multiple regression analysis, they increased the mean relative frequency of abundance of Erysipelotrichaceae (Fig. 4 A). Furthermore, the thermal treatment affected the mean relative frequency of abundance of Lachnospiraceae, Peptostreptococcaceae (increased under HS condition, compared to TN) and Bifidobacteriaceae (decreased by HS treatment, compared to TN) independently by the *in ovo* treatment (Fig. 4 B, C, D) while GOS treatment increased the mean relative frequency of abundance of Ruminococcaceae compared to CON, independently by the thermal treatment (Fig. 4 E).

The most represented genera in the cecum of birds belonging to the tested groups were *Faecalibacterium*, *Lactobacillus*, *Clostridium IV*, *Lachnospiraceae_incertae_sedis*, *Eubacterium*, *Streptococcus* and *Blautia* (Table 5). The identified genera did not show significant differences between CON and GOS group at 32 and 42 days with t-test (Supplementary Table 6). The only exception was represented by *Enterococcus* significantly higher in the cecum of birds belonging to the CON group in comparison to GOS in the TN group (i.e., 0.2 vs 0.1%) (p=0.0457). Taking into account the impact of both covariates in the multiple regression analysis, both GOS and HS treatments decreased the mean relative frequency of abundance of *Faecalibacterium* (Fig. 5 A) and *Blautia* (Fig.

5 B). Instead, GOS treatment increased the mean relative frequency of abundance of *Butyricicoccus* compared to CON treatment, independently by the thermal treatment (Fig. 5 C).

Faecalibacterium prausnitzii was the most abundant species in all groups at both 32 and 42 days, followed by *Eubacterium hallii* and *Eubacterium desmolans* (Table 6). The identified species did not show significative differences between CON and GOS groups at 32 and 42 days, with the t-test (Supplementary Table 7). The only exception was represented by *Blautia glucerasea* significantly higher in the cecum of birds treated with GOS in comparison to the CON group in the HS group (i.e., 0.8 vs 0.1%) (p=0.0334). Considering both the impact of *in ovo* treatment and thermal treatment in the multiple regression analysis, both GOS and HS decreased the mean relative frequency of abundance of *Faecalibacterium prausnitzii* at 42 days (Fig. 6).

Overall, both the alpha diversity assessed by using the Shannon index and the beta diversity quantified by both Bray-Curtis and Unifrac indices did not show significative differences within and between tested groups (Fig. 1 and Fig. 2 supplementary).

Transcriptomic profile

No interaction between environmental conditions (i.e., TN and HS) and *in ovo* treatments (CON vs GOS) was detected in jejunum and cecum mucosa of broiler chickens at 42 days of age for differentially expressed genes (DEG). No single DEG was detected for GOS vs CON treatment in either tissue. Conversely, HS vs TN up- and down-regulated 12 and 13 genes in jejunum and 2 and 9 genes in cecum, respectively. Regarding the gene set analysis, in jejunum mucosa, 11 significantly enriched gene sets were observed in GOS group, mainly linked to energetic metabolism and oxidation (PEROXISOME, SPHINGOLIPID METABOLISM, CYTOCHROME P450 METABOLISM, PENTOSE-PHOSPHATE PATHWAY, FATTY ACID METABOLISM; FDR $\leq$ 0.008), while in CON group 13 enriched gene sets were detected, including CELL CYCLE, DNA REPLICATION AND RIBOSOME (FDR $\leq$ 0.002) as first three gene sets (Table 7a and 7b). In cecum mucosa, 11 enriched gene sets were observed in CON group, most of them grouped and linked to immune cell response (T CELL RECEPTOR SIGNALING PATHWAY; B CELL RECEPTOR SIGNALING PATHWAY; CHEMOKINE SIGNALING PATHWAY; NATURAL KILLER CELL MEDIATED CYTOTOXICITY; FDR $\leq$ 0.028); only 1 gene set was enriched in GOS group for cecum (Table 8a and 8b).

For HS, in jejunum, GSEA analysis showed enrichment in 4 gene sets, including METABOLISM CYTOCHROME P450 and METABOLISM OF XENOBIOTICS BY CYTOCHROME P450 (FDR $\leq$ 0.001), while 14 enriched gene sets were found in TN group, among which OXIDATIVE PHOSPHORYLATION as first gene set and immune-related gene sets (INTESTINAL IMMUNE

NETWORK FOR IGA PRODUCTION, CHEMOKINE SIGNALING PATHWAY, B CELL RECEPTOR SIGNALING PATHWAY; FDR ≤ 0.016) were observed (Table 9a and 9b). In cecum mucosa, only 7 enriched gene sets were found in HS group while 27 gene sets resulted enriched in TN group, including gene sets linked to energetic metabolism (SPHINGOLIPID METABOLISM, STARCH AND SUCROSE METABOLISM, OXIDATIVE PHOSPHORYLATION; FDR = 0.000) and immune response (NATURAL KILLER CELL MEDIATED CYTOTOXICITY, B CELL RECEPTOR SIGNALING PATHWAY, T CELL RECEPTOR SIGNALING PATHWAY; FDR ≤ 0.001) (Table 10a and 10b). Considering both tissues, under HS a less gene up-regulation occurred, while in TN conditions much more genes up-regulated were observed.

Fig 7 shows Enriched Map for gene sets enriched in cecum and jejunum of broiler *in ovo* injected with GOS or CON: considering the prebiotic treatment, gene sets resulted enriched in a quite homogeneous way in the two tissues. Enriched gene sets for immune cell signaling pathways were found as up-regulated in CON group, while gene sets such as PENTOSE-PHOSPHATE PATHWAYS, FATTY ACID METABOLISM AND PEROXISOME were generally up-regulated in GOS group, confirming the GSEA results where, with GOS, a general up-regulation of gene sets related to energy metabolism was reported while in CON gene sets related to immunity were underlined. Fig. 8 shows Enriched Map for gene sets enriched in cecum and jejunum mucosa of broilers *in ovo* injected and submitted to TN or HS condition. Considering the heat stress treatment, again gene sets resulted enriched in homogeneous way in the two tissues and most of gene sets were enriched in TN group, such as OXIDATIVE PHOSPHORYLATION, STARCH AND SUCROSE METABOLISM together with amino acid metabolism and immune response-related gene sets.

Plasma IgG, IgA, SAA

No interaction between thermal conditions and *in ovo* treatments was detected for serum IgG, IgA and SAA levels. No differences were seen in serum immune parameters between thermal treatments and between *in ovo* injection treatments (Table 11).

Discussion

Cecal microbiota

Few studies have been conducted on the effect of *in ovo* prebiotics administration on chicken's microbiota because previous researches mainly focused on the effects of the *in ovo* delivery of prebiotics on gut and GALT development. Additionally, few studies exist on the effect of heat stress on chicken's microbial ecosystem, despite the important negative incidence of this stressor on poultry physiology.

According to previous studies (Shaufi et al., 2015; Ranjitkar et al., 2016; Awad et al., 2017), the phylum of Firmicutes is the most common and represented in chicken cecum, with Clostridia class and Clostridiales order as the predominant ones (Oakley et al., 2014a). The second main represented class in cecum of adult chickens found in this study was that of Bacilli. This trend in terms of taxonomic resolution was maintained in adult chickens and, considering t-test analysis for differences in abundance between GOS and CON, these taxa were not affected both at 32 and 42 days of age. The same was seen also at order taxonomic level, except for Bacillales that decreased in GOS group compared to CON, under HS condition. The maintenance of this equilibrium with or without GOS supplementation is however probably due to the high taxonomic level, in line with what is already known in poultry, where a matured healthy cecum presents for the most part anaerobic bacteria (Xiao et al., 2017). In particular, our findings correspond to the results reported from De Cesare et al. (2017), with Clostridia as the most abundant class in adult broiler cecum within Firmicutes, followed by Bacilli class (De Cesare et al., 2017). At family level, *Ruminococcaceae* was the most abundant one in chicken cecum, as also reported by others (Gong et al., 2007; Ranjitkar et al., 2016; De Cesare et al., 2017), followed by *Lachnospiraceae* family, as already seen previously (Danzeisen et al., 2011; Ranjitkar et al., 2016). Even if no effect of GOS treatment alone has been seen on the major families, a higher abundance of *Enterococcaceae* has been seen in CON compared to GOS group in TN conditions, as well as the same trend has been seen for one of its major genera, *Enterococcus*, indicating a probable direct effect of GOS on this genera. *Faecalibacterium* was the major recognized genus in chicken cecum in the present work, as previously reported (Oakley et al., 2014a) and its species *Faecalibacterium prausnitzii* was the most abundant one in all groups and it has been already previously reported to be one of the largest groups in chicken cecum (Gong et al., 2007). Even if not changes from GOS treatment have been seen on *Faecalibacterium*, an increase in *Blautia glucerasea* abundance has been observed in GOS group, in HS condition. *Blautia* spp. are known to be butyric acid-producing bacteria and a recent study reported an increase in some *Blautia* spp. in rats fed prebiotics, specifically FOS. Between *Blautia* spp. these authors detected *Blautia glucerasea* within different prebiotic treatments (cellulose, raffinose and FOS) (Bai et al., 2016), so it may be possible that, in the present study, it has been stimulated also by GOS.

When the multiple linear regression analysis was applied at day 42, some trends and changes in taxa resulted to be affected by *in ovo* treatment, thermal treatment or by interaction of both.

Proteobacteria were increased in GOS group, contrarily to what was reported by Burokas et al. (2017), who observed that mice fed GOS had lower Proteobacteria in cecum (Burokas et al., 2017), even if bacterial differences inside the phylum depending on animals may influence or interact with prebiotic. At family level, multiple linear regression analysis reported an effect of GOS treatment on

Ruminococcaceae, which increased. To date, few studies specifically focused on GOS effects on cecal microbiota so not many information are available, anyway, a recent study on effect of dietary GOS on cecum microbiota in mice reported an increase in *Ruminococcaceae* (Burokas et al., 2017). Since it is known that *Ruminococcaceae* family includes many species of butyrate-producing bacteria (like *Ruminococcus* spp.) (Rinttilä and Apajalahti, 2013), which allow digestion of nutrients like prebiotics in cecum, this might suggest why this family increased in GOS group, even if we found no significative results at genus level. However, also contrasting results have been observed by Corrigan and colleagues (2018), who reported a decrease in *Ruminococcaceae* after dietary supplementation of a yeast- mannan fraction, even they observed an increase in some other butyrate metabolism-related bacteria (Corrigan et al., 2018). It is therefore possible that some other factors may influence this family trend in different dietary treatments. At genus level, GOS treatment induced an increase in *Butyricicoccus*, a particular genus found firstly in chicken cecum and characterized by a high activity in butyrate production (Eeckhaut et al., 2008), so highlighting GOS capacity in stimulating butyrate-producing bacteria.

Considering the thermal treatment as covariate in the regression analysis, HS induced decrease in Actinobacteria phylum abundance. Differently, Zhu and colleagues (2019) observed an increase in Actinobacteria abundance in fecal content of laying hens under HS (Zhu et al., 2019), but the high variability in this phylum composition, which includes both gut commensals but also pathogens, may cause different responses. In our case, the phylum decrease could be linked to the decrease of one of its major families, *Bifidobacteriaceae*, lower under HS compared to TN treatment, confirming the negative impact of HS on bifidobacteria (Burkholder et al., 2008; Lara and Rostagno, 2013; Song et al., 2014). These results are in line with results of Burokas et al. (2017), who reported a decrease in Actinobacteria in stressed mouse cecum, along with decrease in *Bifidobacteriaceae* (Burokas et al., 2017). At family level, HS induced an increase in *Lachnospiraceae* and *Peptostreptococcaceae* compared to TN treatment. Regarding *Lachnospiraceae*, a decrease in their relative abundance has been recently found in fecal content of laying hens under HS (Zhu et al., 2019), even if a recent study of an experimental model of water immersion restraint stress on mice revealed that *Lachnospiraceae* can also increase when a stress source is applied, given their relation with immune response (Li et al., 2017). Since HS negatively impacts microbial ecosystem by changing abundance balance of its taxa, it may be possible an increase in these families, also because it has been seen how HS can increase richness of the gut bacteria (Wang et al., 2018). A decrease in bacterial richness in cecal microbiota of chickens treated with antibiotics has specifically decreased *Peptostreptococcaceae* abundance (Costa et al., 2017), meaning a possible direct correlation between richness and abundance of this taxa.

Considering the interaction between the two factors, Firmicutes abundance was affected under HS (compared to TN group) depending on the *in ovo* treatment, with a decrease in GOS compared to CON group under HS. It has been recently reported a decrease in abundance of Firmicutes phylum under HS in feces of laying hens fed a standard diet, together with feed intake decrease (Zhu et al., 2019). Also in our trial the heat stress-related feed intake decrease was observed (data not shown), but the effect of HS on Firmicutes abundance decrease was observed only when coupled to GOS treatment, so multiple factors may have induced the imbalance between taxa. A possible explanation for these different results might be related to the HS type, which was much higher in layers (cyclic, from 25 to 34°C, for 46 days) (Zhu et al., 2019) than in broilers of our study (30°C constantly for 10 days). Considering this, it might be possible that in our study HS alone did not affect or slightly affected Firmicutes abundance, without a significant change, but along with GOS the effect was more evident since they decreased in GOS-HS group and not in CON-HS group. The changes we found at transcriptomic level induced by GOS (such as on immune-related gene sets, as further reported) might be maybe linked to changes in microbiota community and the association of GOS with HS treatment might have enhanced some of these changes.. An effect of interaction between the two factors was further seen at class level, where *in ovo* CON group seemed to have reacted to HS with an increase in Clostridia abundance, further confirmed from the observed increase in Clostridiales order abundance in CON-HS groups. HS effects on Clostridiales order have been recently reported in ileum of broilers, but with a reverse effect compared to our study (Wang et al., 2018). In fact, these authors found a decrease of Clostridiales under HS (Wang et al., 2018), but differences between tracts (small intestine and cecum) might be involved, along with differences related to other external factors, so further studies may improve our knowledge on HS effects at this taxa level. The mean relative frequency of abundance of Bacilli class was affected by the interaction between the two factors, with a decrease in GOS group in response to HS. This might indicate a possible effect of GOS specifically in HS condition, previously seen also in t-test analysis, where Bacillales order decreased in birds treated with GOS compared to CON under HS. An effect of treatment interaction on Erysipelotrichales was observed, with increase of this order abundance in GOS and HS group compared to CON and TN group and the same result was found at family level, with increase of *Erysipelotrichaceae*. The importance of this family in relation to inflammatory disorders in GIT has been reported, even if with contrasting results. In fact, abundance of *Erysipelotrichaceae* has been seen to increase in colorectal cancer but both decrease or increase in different models of inflammatory bowel diseases (IBD), probably due to differences in gut microbiota and immune response between human and animal models (Kaakoush, 2015). Furthermore, in a study on the effect of dietary GOS on mouse cecum microbiota, the authors found *Erysipelotrichaceae* as one of family significantly

decreased in prebiotic group compared to CON (Burokas et al., 2017), which seems in contrast with our results. However, the increase in *Erysipelotrichaceae* abundance in the present study in GOS group when the animals are under HS is a result of the interaction between treatments. Considering the transcriptomic analysis, we found immunity-related gene sets downregulated both in GOS and HS group so, it may be possible a connection between this trend in immune response in both GOS and HS with the change in *Erysipelotrichaceae* abundance. At genus level, *Faecalibacterium* and *Blautia* genera have been affected by both the treatments, decreasing in GOS group and in HS condition compared to TN condition, and the same effect was seen for the species *Faecalibacterium prausnitzii*. Particularly, *Faecalibacterium* is known for its anti-inflammatory activity and it decreases in intestinal inflammatory conditions, as observed in ileum of broilers (Wang et al., 2018), similarly to the results reported for cecum in this study. Similar changes have been reported for *Blautia* genus, since a decreasing trend of its abundance in fecal microbiota of laying hens under HS has been observed (Zhu et al., 2019). These are all butyrate-producing bacteria and their beneficial effects at intestinal level are known (Banerjee et al., 2018) and, given the differences in their abundance compared to TN condition, it is possible that the bifidogenic effect of GOS did not work in stress condition, maybe due to a less metabolization of the prebiotic, which instead was able in stimulating beneficial bacteria in TN condition.

Transcriptome

In both tissues GOS group compared to CON resulted in gut transcriptome impoverished of gene sets related to immunity, even if more highlighted in gene sets top list of cecum CON than jejunum. In fact, in cecum, PHOSPHATIDYLINOSITOL SIGNALING SYSTEM gene set was enriched in CON and not in GOS: inside this gene set, the gene PIK3C2B (gene belonging to PI3K phosphatidylinositol-phosphate 3 kinase family, involved in signaling pathway for cell proliferation, migration etc.) was one of the first in the gene list upregulated in cecum CON group. Expression of PI3K seems to be positively correlated with toll-like receptors (TLRs) genes expression which, in turn, is up-regulated by cytokines in inflammatory conditions, as reported by Gao and colleagues (2018) (Gao et al., 2018). Phosphorylation of PI3K and MAPK (mitogen-activated protein kinase family), induced by TLRs signaling, leads to NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation, which acts as transcription factor for immune response and as stimulator for pro-inflammatory cytokines production (Gao et al., 2018). In fact, MAP2K2 (MAPK family) was the second and third gene we found respectively in the other up-regulated immune-related gene sets in cecum CON top list, T CELL RECEPTOR AND B CELL RECEPTOR SIGNALING PATHWAY. These gene sets were found upregulated also in jejunum CON compared to GOS. This means a

possible correlation and close role with PIK3C2B (in PHOSPHATIDYLINOSITOL SIGNALING SYSTEM gene set) in immune system stimulation. In support of this, CD8 T cell marker gene was found as third and first gene again listed in T CELL RECEPTOR AND B CELL RECEPTOR SIGNALING PATHWAY gene sets, respectively. CD8 T cells can secrete different cytokines, have pro-inflammatory function and affect B cell response too (Mosmann et al., 1997). In cecum of CON group, other gene sets related to immunity and cytokines were found: CHEMOKINE SIGNALING PATHWAY and NATURAL KILLER CELL MEDIATED CYOTOTOXICITY. Generally, release of cytokines by immune cells is negatively correlated with the cytochrome p450 (CYP) drug/xenobiotic metabolic capacity, meaning that a decreased metabolic activity could be associated to inflammatory status (Christensen and Hermann, 2012). In fact, in a recent study, it has been reported that inflammation induces downregulation of hepatic and extrahepatic CYP enzymes drug metabolism (Stavropoulou et al., 2018). DRUG METABOLISM CYTOCHROME P450 and METABOLISM OF XENOBIOTICS BY CYTOCHROME P450 gene sets were found enriched in GOS group, specifically in jejunum. Even if CYP enzymes are more present in liver, small intestinal mucosa is the most important extrahepatic site of biotransformation in human, where CYP enzymes have a role in metabolic processes (Bezirtzoglou, 2012; Christensen and Hermann, 2012). These results indicate a gene and function similarity between human and chicken at small intestinal level. Since intestinal metabolic processes mainly occur in the small intestine, it might be possible that CYP gene sets were poorly enriched in cecum. In both enriched CYP metabolism-related gene sets, CYP2D6 (xenobiotic detoxifying CYP enzyme) and UGT2A1 (xenobiotic/endobiotic compound-metabolizing enzyme of UDP glucuronosyltransferase 2 family) genes were found among the first genes of the list. A similar UDP glucuronosyltransferase, UGT1A1 (drug-metabolizing enzyme involved in gut epithelial barrier maintenance) was studied by Gao and colleagues (2018): they observed a decrease of gut UGT1A1 protein concentration in rats with colitis, confirming the negative correlation between metabolic capacity and inflammation. After, they also reported a decrease of UGT1A1 gene expression in a condition of gut dysbiosis induced by Gram negative bacteria both in normal and colitis rats, showing a possible relevant role of microbiota in xenobiotic metabolizing enzymes expression regulation (Gao et al., 2018). Enriched gene set for RETINOL METABOLISM was also found in GOS group, mainly in jejunum, but also highlighted in both tissues by Enriched Map, and similarly to CYP metabolism-related gene set, first genes of the list in jejunum GOS were related to xenobiotic metabolism (UGT2A1 and CYP3A7). Considering these observations, microbial population might have developed differently in CON and GOS in both tissues, leading to a different immune stimulation. It might be possible that a different microbiota had led to a higher immune defenses recruitment in CON group compared to GOS where, particularly in jejunum, the enriched

gene set of CYP metabolic enzymes might be related to a better gut function, since CYP intestinal role also concerns endogenous metabolism, such as that for fatty acids (Bezirtzoglou, 2012) and a good epithelial barrier with less expenditure in immune system stimulation and consequent more energy saving.

Furthermore, the enriched gene set for PENTOSE-PHOSPHATE PATHWAY found in GOS group, in jejunum firstly but further highlighted in both tissues by Enriched Map, might mean higher energetic metabolism, where genes for enzymes involved in glucose metabolism such as FBP1 (fructose 1,6 biphosphatase 1) and PFKL (phosphofructokinase) and for enzymes involved in ribose metabolism (RBKS, ribokinase) were in the top of the list of gene set in jejunum GOS. The hypothesis of a higher energetic metabolism in GOS may be also linked to PEROXISOME enriched gene set, the first gene set in jejunum GOS list, but again highlighted in both tissues by Enriched Map. Peroxisomes are pivotal to several lipid-metabolizing pathways (Morvay et al., 2017): in fact, inside the gene set in jejunum, at the top of the list there was ACOX2 gene (acylCoA oxidase 2, involved in branched fatty acid degradation), along with EHHADH gene (encoding for a beta oxidation pathway enzyme), both key enzymes for beta oxidation also found in mammal small intestine (Morvay et al., 2017), and CAT gene (catalase, H_2O_2 detoxifying enzyme and peroxisomal marker). PEROXISOME gene set was more enriched in jejunum than cecum, as reported also by Morvay and colleagues (2017) which found peroxisomes being mainly present in small than in large intestine of mice, as well as a higher expression of CAT and ACOX2 genes, due to higher involvement of small intestine in lipid uptake (Morvay et al., 2017). Then, peroxisome involvement in metabolic oxidation processes, in turn, can be linked to the enrichment of FATTY ACID METABOLISM gene set in jejunum, where again EHHADH was one of genes at the top of the list, along with ALDH3A2 (aldehyde dehydrogenase, detoxifying aldehydes from lipid peroxidation) and ACOX1. In support to this hypothesis, we found also PPARs SIGNALING PATHWAY gene set in jejunum: this pathway regulates peroxisomal beta oxidation of fatty acids (and the gene ACOX2 found in PEROXISOME gene set was in this list too) along with retinoid receptors. PPARs are also upstream regulators of UDP glucuronosyltransferases (involved in xenobiotics metabolism) such as UGT1A1, as reported by Gao and colleagues (2018), which also hypothesized that microbiota might regulate these receptors in the gut (Gao et al., 2018). SPHINGOLIPID METABOLISM gene set resulted also enriched in GOS, in jejunum firstly but evidenced in both tissues in Enriched Map. At the top of the list of this gene set in jejunum GOS, genes GLA (alfa-galactosidase, hydrolytic enzyme for galactose and glucose production), NEU3 (sialidase3, glycohydrolytic enzyme removing sialic acid from glycol-lipids/proteins) and ENPP7 (coding for a protein probably involved in gut mucosa protection) were found. Beneficial bacteria like Bacteroides can produce sphingolipids and these molecules are

involved in bacterial-host interaction in immune system modulation and in gut homeostasis maintenance, acting as signal molecules (Heaver et al., 2018): it might be possible that in GOS group, a different microbiota (compared to CON group) influenced lipidic metabolic pathways and higher sphingolipid metabolism might have contributed in gut function and barrier maintenance. As final hypothesis, a less activated immune system along with a high energetic metabolism might explain GOS higher growth (data not shown) compared to CON group.

From GSEA analysis, only one gene set resulted enriched in cecum in GOS group, that is ECM RECEPTOR INTERACTION. Considering ECM role in structural supporting gut mucosa, it is possible that GOS has led to an improvement in barrier maintenance in cecum. This hypothesis may be supported also by the gene sets found in Enriched Map. In fact, CELL CYCLE and DNA REPLICATION gene sets resulted enriched in cecum when compared to jejunum, suggesting more cell turnover, maybe involved in gut barrier maintenance. Results from microbiota analysis revealed a possible bifidogenic effect of GOS on butyric-producing bacteria, and butyrate is used as main energetic sources in cecum (Sergeant et al., 2014).

As expected, HS strongly affected animal response. Many gene sets resulted impoverished in HS group compared to TN, such as those related to OXIDATIVE PHOSPHORYLATION, amino acid metabolism-related gene sets (VALINE, LEUCINE AND ISOLEUCINE DEGRADATION gene set) and immune response-related gene sets (T CELL AND B CELL RECEPTOR SIGNALING PATHWAY; NATURAL KILLER CELL MEDIATED CYTOTOXICITY). As already well reported, HS leads to mitochondrial damage (being mitochondria main responsible of reactive oxygen species -ROS- production, in case of oxidative stress), and this damage leads to inactivation of the respiratory chain with downregulation of cellular energy production due to alteration of oxidative phosphorylation pathway (Slimen et al., 2016). This may explain the impoverished gene set found in HS group of this study. Furthermore, downregulation of cellular energetic metabolism might be linked also to another impoverished gene set in HS group compared to TN, regarding STARCH AND SUCROSE METABOLISM, where AGL (amylo-alpha 1,6-glucosidase, 4-alpha glucanotransferase, a glycogen degradation enzyme) and PYGB (glycogen phosphorylase) genes were upregulated in TN. Chronic HS seems to negatively affect also protein metabolism (Slimen et al., 2016). In fact, we found amino acid metabolism-related gene set impoverished in HS compared to TN group, with the gene ACAD8 (acylCoA dehydrogenase family member 8) at the top of the list. ACAD8 encodes for a dehydrogenase involved in branched-chain amino acids (BCAAs) metabolism: this enzyme is a mitochondrial enzyme and it might be that its functionality is affected by the oxidative stress damage at mitochondrial level. In fact, it seems to be especially affected by chronic heat stress, along with general decrease of protein breakdown (Slimen et al., 2016). Our observations on the impoverishment

of immune response-related gene sets found in HS group are in line with what is already reported on poultry, where HS induces a general immunosuppression (Lara and Rostagno, 2013), compared to TN group. In TN group for example, gene PIK3R2 (a lipid kinase of the PI3K family, involved in phosphatidylinositol phosphorylation in growth signaling pathway and in activation of NF-κB complex inducing immune response) was found upregulated in all three gene sets (T CELL AND B CELL RECEPTOR SIGNALING PATHWAY; NATURAL KILLER CELL MEDIATED CYTOTOXICITY).

These results might be directly due to HS downregulation linked to cortisol effect, but they might also be a consequent of feed intake decrease (data not shown), with a general adjustment of the cell metabolic activity. In fact, as reported by Slimen et al. (2016), growth and health are not priorities in the metabolism of heat-stressed animals, due to the lower metabolic rates (Slimen et al., 2016).

Furthermore, the homogeneous response for enriched gene sets in cecum and jejunum under the thermal treatment, as showed by the Enriched Map, lead to the consideration that, despite possible variations of the local microbiota, under stress stimuli different intestinal tracts seem to be affected in a similar way at gene level.

Plasma IgG, IgA, SAA

Regarding results on serum immune parameters IgG, IgA and SAA (Table 5), no differences were found between environmental and *in ovo* treatments.

IgG are the major class of blood circulating antibodies produced in the humoral response to neutralize antigens and activate macrophages and the complement system (Wang et al., 2004). Some previous studies reported IgG decrease as marker of heat stress-induced immunosuppression (Bartlett and Smith, 2003; Park et al., 2013). However, these authors observed IgG suppression following extreme heat stress conditions (over 33°C), so probably the heat stress induced in our study did not stimulate IgG level in the same way.

About IgA concentrations, it may be possible that, since this Ig class is found primarily at intestinal level, only a strong impairment of gut barrier may provoke high IgA serum level (due to damaged mucosal layer and to exposition of antigens), which probably did not happen in our case. However, in contrast with previous results, a more recent trial reported that IgG and IgA significantly increased in broiler chickens under chronic heat stress (Attia and Hassan, 2017). Since Ig belong to serum non-specific molecules released in different contexts of immune responses and in response to different inflammatory processes, many factors may affect their trend in serum. Our results regarding serum IgG level in GOS or CON group are in line with results reported by Midilli and colleagues (2008) where dietary prebiotic (0.2%) supplementation in broiler chicken did not affect IgG serum level at

d 42 (Midilli et al., 2008). Conversely, in a study on turkeys was reported that IgG values were higher in the group fed dietary mannooligosaccharide (0.5%, MOS) than in CON (Cetin et al., 2005). About IgA, as we observed in this study, also Kim and colleagues (2011) did not find differences in plasma IgA levels in broiler fed 0.5% dietary prebiotic fructo-oligosaccharides (FOS) or MOS at d 21 (Kim et al., 2011b). In contrast, Rezaei and colleagues (2015) found increased blood IgA levels in broiler fed 1% palm kernel oligosaccharide prebiotic at d 36 (Rezaei et al., 2015). Possible explanations on different results may refer to the doses and to the different delivery ways of prebiotics (orally or *in ovo*).

Regarding SAA levels, our results contrast with those found by Hartog and colleagues (2015), where a dietary prebiotic (1.5% prebiotic multifiber mixture) significantly reduced SAA serum levels in mice in induced colitis (Hartog et al., 2015). Other previous studies reported significant increases in serum SAA levels in chicks infected with bronchitis virus (Nazifi et al., 2011; Asasi et al., 2013) and in chicken vaccinated against infectious bronchitis virus and New Castle disease (Kaab et al., 2018), where a strong stimulation of immunity against infections happens. Even if SAA is a phase-acute protein proposed to be a general marker of stress, it might be possible that SAA concentration in serum changes depending on the inflammation type and stress level, so being related to the severity of stimulation or damage.

Conclusions

In this study the GOS prebiotic affected the intestinal transcription of genes with different impact related to the specific functions of each intestinal tract. A general favorable effect of GOS prebiotic may be recognized due to the enrichment of energetic metabolism-related gene sets, mainly in jejunum. In addition, the enrichment of lipidic metabolism-related gene sets in GOS group might have contributed in gut function and barrier maintenance, which might also be linked to a less immune system activation, mainly at cecum level. GOS treatment alone did not affect generally cecum microbiota, with few exceptions presenting the possible capacity in butyrate-producing bacteria stimulation. Considering HS, the experimental model was effective in stressing the animals, according to previous studies, with impairment of gut functions in terms of energy and immunity along with changes in bacteria abundance both at phylum and family taxonomic level, including decrease in potentially beneficial bacteria. In case of effects of interaction between the two factors, in terms of microbiota, GOS seems to provide a different response, maybe depending on the animal physiological status, even this response was not observed in the transcriptomic results. Generally, our results show that the additional efficacy of GOS on transcriptome and microbiota in the case of heat

stress was scarce. Nevertheless, without considering the different environmental conditions, the positive impact of GOS on transcriptome data concurs to sustain the ability of the *in ovo* injection technique to induce positive responses with a long-term effect and confirms the *in ovo* feeding strategy as a promising tool to modify the early program development of the chick gut.

Table 1. **Mean relative frequency of abundance (mean ± standard deviation) of the phyla identified in the chicken tested groups: GOS group (0.2 ml of 0.9% physiological saline + 3.5 mg prebiotic (GOS)/egg) and CON (0.2 ml of 0.9% physiological saline) at 32 days of age, CON or GOS under thermoneutral condition (TN, 25°C), CON or GOS under heat-stress (HS, 30°C constantly) at 42 days of age (n=24 per *in ovo* treatment).**

Phylum	CON[1]	GOS[1]	CON/TN	CON/HS	GOS/TN	GOS/HS
	32 days of age		42 days of age			
Firmicutes	96.3 ± 1.9	96.8 ± 1.2	96.0 ± 1.3	94.9 ± 2.8	96.2 ± 0.9	95.9 ± 1.6
Unassigned	2.3 ± 1.8	1.9 ± 0.9	2.4 ± 1.3	3.0 ± 1.6	2.2 ± 0.7	2.4 ± 0.9
Actinobacteria	0.2 ± 0.1	0.3 ± 0.4	0.5 ± 0.4	0.5 ± 0.6	0.3 ± 0.3	0.3 ± 0.4
Proteobacteria	0.9 ± 0.9	0.9 ± 0.8	0.9 ± 0.9	1.5 ± 2.0	1.0 ± 0.8	1.1 ± 1.3
Tenericutes	0.3 ± 0.7	0.1 ± 0.3	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.3	0.4 ± 0.6

[1] *In ovo* treatments

Table 2. **Mean relative frequency of abundance (mean ± standard deviation) of the classes identified in the chicken tested groups: GOS group (0.2 ml of 0.9% physiological saline + 3.5 mg prebiotic (GOS)/egg) and CON (0.2 ml of 0.9% physiological saline) at 32 days of age, CON or GOS under thermoneutral condition (TN, 25°C), CON or GOS under heat-stress (HS, 30°C constantly) at 42 days of age (n=24 per *in ovo* treatment).**

Class	CON[1]	GOS[1]	CON/TN	CON/HS	GOS/TN	GOS/HS
	32 days of age		42 days of age			
Clostridia	79.8 ± 4.6	80.4 ± 5.6	76.6 ± 5.3	79.0 ± 4.5	77.1 ± 4.9	79.8 ± 4.0
Unassigned	6.5 ± 3.1	6.0 ± 2.8	7.6 ± 2.4	7.6 ± 3.1	7.0 ± 1.5	7.6 ± 1.8
Bacilli	10.4 ± 4.5	10.4 ± 5.7	12.1 ± 6.1	9.0 ± 4.7	11.8 ± 4.4	8.6 ± 3.5
Actinobacteria	0.2 ± 0.1	0.3 ± 0.4	0.5 ± 0.4	0.5 ± 0.6	0.3 ± 0.3	0.3 ± 0.4
Erysipelotrichia	1.9 ± 1.0	1.8 ± 0.7	2.2 ± 1.0	2.2 ± 1.1	2.6 ± 1.0	2.3 ± 0.9
Gammaproteobacteria	0.6 ± 0.5	0.7 ± 0.8	0.7 ± 0.8	**1.2 ± 1.9**	0.7 ± 0.8	**0.8 ± 1.3**
Mollicutes	0.3 ± 0.7	0.1 ± 0.3	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.3	0.4 ± 0.6
Deltaproteobacteria	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1

*Values in bold refer to mean relative frequency of abundance significative different between groups

[1] *In ovo* treatments

Table 3. **Mean relative frequency of abundance (mean ± standard deviation) of the orders identified in the chicken tested groups: GOS group (0.2 ml of 0.9% physiological saline + 3.5 mg prebiotic (GOS)/egg) and CON (0.2 ml of 0.9% physiological saline) at 32 days of age, CON or GOS under thermoneutral condition (TN, 25°C), CON or GOS under heat-stress (HS, 30°C constantly) at 42 days of age (n=24 per *in ovo* treatment).**

Order	CON[1]	GOS[1]	CON/TN	CON/HS	GOS/TN	GOS/HS
	32 days of age		42 days of age			
Clostridiales	79.7 ± 4.6	80.4 ± 5.6	76.4 ± 5.3	78.9 ± 4.5	76.8 ± 4.8	79.6 ± 3.9
(Unassigned)	6.6 ± 3.1	6.1 ± 2.8	7.7 ± 2.3	7.8 ± 3.2	7.2 ± 1.3	7.8 ± 1.7
Lactobacillales	9.8 ± 4.6	9.8 ± 5.8	11.6 ± 6.0	8.0 ± 4.7	11.0 ± 4.4	8.2 ± 3.7
Erysipelotrichales	1.9 ± 1.0	1.8 ± 0.7	2.2 ± 1.0	2.2 ± 1.1	2.6 ± 1.0	2.3 ± 0.9
Bacillales	0.6 ± 0.7	0.6 ± 0.6	0.5 ± 0.3	**1.0 ± 0.4**	0.9 ± 0.6	**0.4 ± 0.3**
Coriobacteriales	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Enterobacteriales	0.6 ± 0.5	0.7 ± 0.8	0.7 ± 0.8	**1.2 ± 1.9**	0.7 ± 0.8	**0.8 ± 1.3**
Anaeroplasmatales	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.3	0.4 ± 0.6
Bifidobacteriales	0.3 ± 0.7	0.1 ± 0.3	0.2 ± 0.4	0.3 ± 0.6	0.2 ± 0.3	0.1 ± 0.3

*Values in bold refer to mean relative frequency of abundance significative different between groups

[1] *In ovo* treatments

Table 4. **Mean relative frequency of abundance (mean ± standard deviation) of the families identified in the chicken tested groups: GOS group (0.2 ml of 0.9% physiological saline + 3.5 mg prebiotic (GOS)/egg) and CON (0.2 ml of 0.9% physiological saline) at 32 days of age, CON or GOS under thermoneutral condition (TN, 25°C), CON or GOS under heat-stress (HS, 30°C constantly) at 42 days of age (n=24 per *in ovo* treatment).**

Family	CON[1]	GOS[1]	CON/TN	CON/HS	GOS/TN	GOS/HS
	32 days of age		42 days of age			
(Unassigned)	22.7 ± 6.3	22.1 ± 5.6	24.6 ± 5.6	28.9 ± 7.4	27.0 ± 5.1	26.6 ± 5.4
Ruminococcaceae	39.9 ± 3.4	37.6 ± 5.1	34.3 ± 4.7	34.0 ± 5.9	32.8 ± 6.0	35.7 ± 7.3
Lachnospiraceae	22.4 ± 4.5	25.2 ± 5.0	23.6 ± 3.8	22.4 ± 5.4	22.8 ± 3.1	23.0 ± 5.8
Erysipelotrichaceae	1.9 ± 1.0	1.8 ± 0.7	2.2 ± 1.0	2.2 ± 1.1	2.6 ± 1.0	2.3 ± 0.9
Lactobacillaceae	8.7 ± 4.4	8.7 ± 5.6	9.2 ± 5.2	6.5 ± 5.1	10.1 ± 4.2	7.1 ± 3.7
Coriobacteriaceae	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Eubacteriaceae	1.6 ± 1.2	1.9 ± 1.8	1.8 ± 1.0	2.1 ± 1.0	1.9 ± 0.7	1.9 ± 1.0
Peptostreptococcaceae	0.1 ± 0.2	0.2 ± 0.2	0.3 ± 0.3	0.3 ± 0.3	0.2 ± 0.2	0.4 ± 0.6
Enterococcaceae	0.0 ± 0.1	0.1 ± 0.1	**0.2 ± 0.2**	0.1 ± 0.2	**0.1 ± 0.1**	0.3 ± 0.4
Enterobacteriaceae	0.6 ± 0.5	0.7 ± 0.8	0.7 ± 0.8	1.2 ± 1.9	0.7 ± 0.8	0.8 ± 1.3
Streptococcaceae	1.1 ± 1.4	1.0 ± 0.9	2.3 ± 3.3	1.4 ± 1.5	0.7 ± 0.8	0.9 ± 0.9
Bifidobacteriaceae	0.0 ± 0.1	0.1 ± 0.4	0.2 ± 0.4	0.3 ± 0.6	0.2 ± 0.3	0.1 ± 0.3

*Values in bold refer to mean relative frequency of abundance significative different between groups

[1] *In ovo* treatments

Table 5. **Mean relative frequency of abundance (mean ± standard deviation) of the genera identified in the chicken tested groups: GOS group (0.2 ml of 0.9% physiological saline + 3.5 mg prebiotic (GOS)/egg) and CON (0.2 ml of 0.9% physiological saline) at 32 days of age, CON or GOS under thermoneutral condition (TN, 25°C), CON or GOS under heat-stress (HS, 30°C constantly) at 42 days of age (n=24 per *in ovo* treatment).**

Genus	CON[1]	GOS[1]	CON/TN	CON/HS	GOS/TN	GOS/HS
	32 days of age		42 days of age			
(Unassigned)	59.5 ± 5.1	61.5 ± 5.5	59.8 ± 6.1	65.1 ± 8.1	63.5 ± 4.9	64.1 ± 5.3
Clostridium_IV	2.3 ± 0.8	2.3 ± 1.0	2.7 ± 1.0	2.0 ± 0.7	2.1 ± 0.9	2.3 ± 0.9
Clostridium_XlVb	0.8 ± 0.4	0.7 ± 0.3	1.1 ± 0.8	1.2 ± 1.1	1.2 ± 0.5	0.9 ± 0.8
Lactobacillus	8.7 ± 4.4	8.7 ± 5.6	9.2 ± 5.2	6.5 ± 5.1	10.1 ± 4.2	7.1 ± 3.7
Blautia	1.1 ± 0.8	1.4 ± 1.0	1.2 ± 0.4	0.9 ± 0.9	1.1 ± 0.9	1.3 ± 0.9
Faecalibacterium	17.9 ± 4.1	15.4 ± 5.3	14.4 ± 4.5	12.5 ± 7.6	12.0 ± 6.0	13.7 ± 7.5
Ruminococcus	0.7 ± 0.6	0.4 ± 0.3	0.4 ± 0.3	0.8 ± 0.8	0.4 ± 0.2	0.4 ± 0.3
Butyricicoccus	0.8 ± 0.7	0.9 ± 0.7	1.0 ± 0.8	0.8 ± 0.6	1.1 ± 0.7	1.0 ± 1.0
Oscillibacter	0.7 ± 0.5	0.8 ± 0.7	0.5 ± 0.2	0.8 ± 0.9	0.4 ± 0.2	0.5 ± 0.2
Clostridium_XVIII	0.6 ± 0.7	0.6 ± 0.5	0.9 ± 0.6	1.0 ± 0.9	1.0 ± 0.7	1.0 ± 0.5
Clostridium_XlVa	0.8 ± 0.5	0.8 ± 0.5	0.4 ± 0.4	0.4 ± 0.4	0.3 ± 0.2	0.4 ± 0.4
Lachnospiracea_incertae_se dis	1.3 ± 1.1	1.4 ± 1.0	1.3 ± 0.7	1.3 ± 1.0	1.2 ± 0.8	1.3 ± 1.5
Anaerostipes	0.3 ± 0.3	0.3 ± 0.3	0.4 ± 0.3	0.4 ± 0.3	0.4 ± 0.4	0.4 ± 0.3
Eubacterium	1.6 ± 1.2	1.9 ± 1.8	1.8 ± 1.0	2.1 ± 1.0	1.9 ± 0.7	1.9 ± 1.0
Enterococcus	0.0 ± 0.1	0.1 ± 0.1	**0.2 ± 0.2**	**0.1 ± 0.2**	0.1 ± 0.1	0.3 ± 0.4
Streptococcus	1.1 ± 1.4	1.0 ± 0.9	2.3 ± 3.3	1.4 ± 1.5	0.7 ± 0.8	0.9 ± 0.9
Pseudoflavonifractor	0.0 ± 0.1	0.0 ± 0.1	0.5 ± 0.4	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.3
Bifidobacterium	0.0 ± 0.1	0.1 ± 0.4	0.2 ± 0.4	0.3 ± 0.6	0.2 ± 0.3	0.1 ± 0.3

*Values in bold refer to mean relative frequency of abundance significative different between groups

[1] *In ovo* treatments

Table 6. **Mean relative frequency of abundance (mean ± standard deviation) of the species identified in the chicken tested groups: GOS group (0.2 ml of 0.9% physiological saline + 3.5 mg prebiotic (GOS)/egg) and CON (0.2 ml of 0.9% physiological saline) at 32 days of age, CON or GOS under thermoneutral condition (TN, 25°C), CON or GOS under heat-stress (HS, 30°C constantly) at 42 days of age (n=24 per *in ovo* treatment).**

Species	CON[1]	GOS[1]	CON/TN	CON/HS	GOS/TN	GOS/HS
	32 days of age		42 days of age			
(Unassigned)	72.6 ± 4.1	74.2 ± 5.5	74.5 ± 5.2	78.3 ± 7.6	78.0 ± 6.7	76.1 ± 8.1
Clostridium_lactatifermentans	0.8 ± 0.4	0.7 ± 0.3	0.9 ± 0.5	1.0 ± 0.9	0.8 ± 0.4	0.5 ± 0.2
Faecalibacterium_prausnitzii	17.9 ± 4.1	15.4 ± 5.3	14.4 ± 4.5	12.5 ± 7.6	12.0 ± 6.0	13.7 ± 4.5
Butyricicoccus_pullicaecorum	0.8 ± 0.7	0.9 ± 0.7	1.0 ± 0.8	0.8 ± 0.6	1.1 ± 0.7	1.0 ± 1.0
Eubacterium_coprostanoligenes	0.2 ± 0.3	0.7 ± 0.7	0.4 ± 0.6	0.3 ± 0.5	0.2 ± 0.2	0.2 ± 0.5
Eubacterium_hallii	1.3 ± 1.1	1.4 ± 1.0	1.3 ± 0.7	1.2 ± 1.0	1.2 ± 0.8	1.3 ± 1.5
Blautia_glucerasea	0.5 ± 0.8	0.6 ± 1.0	0.4 ± 0.3	**0.1 ± 0.2**	0.5 ± 0.8	**0.8 ± 1.0**
Pseudoflavonifractor_capillosus	0.0 ± 0.1	0.0 ± 0.1	0.5 ± 0.4	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.3
Eubacterium_desmolans	1.4 ± 1.0	1.3 ± 1.3	1.4 ± 0.8	1.8 ± 0.8	1.7 ± 0.6	1.7 ± 1.1

*Values in bold refer to mean relative frequency of abundance significative different between groups

[1] *In ovo* treatments

Table 7a. **Enriched gene sets found in jejunum mucosa of broiler chickens (42 days of age) of GOS group (0.2 ml of 0.9% physiological saline + 3.5 mg prebiotic (GOS)/egg) vs CON (0.2 ml of 0.9% physiological saline), after GSEA analysis considering FDR q value ≤ 0.05 (n=24 per *in ovo* treatment).**

GENE SET - JEJUNUM GOS	FDR q value
PEROXISOME	0.000
SPHINGOLIPID METABOLISM	0.000
HISTIDINE METABOLISM	0.001
DRUG METABOLISM CYTOCHROME P450	0.007
METABOLISM OF XENOBIOTICS BY CYTOCHROME P450	0.006
DRUG METABOLISM OTHER ENZYMES	0.006
PENTOSE PHOSPHATE PATHWAY	0.006
FATTY ACID METABOLISM	0.008
RETINOL METABOLISM	0.008
STARCH AND SUCROSE METABOLISM	0.011
PPAR SIGNALING PATHWAY	0.013

Table 7b. **Enriched gene sets found in jejunum mucosa of broiler chickens (42 days of age) of CON group (0.2 ml of 0.9% physiological saline) vs GOS group (0.2 ml of 0.9% physiological saline + 3.5 mg prebiotic (GOS)/egg), after GSEA analysis considering FDR q value ≤ 0.05 (n=24 per *in ovo* treatment).**

GENE SET- JEJUNUM CON	FDR q value
CELL CYCLE	0.000
DNA REPLICATION	0.000
RIBOSOME	0.002
OOCYTE MEIOSIS	0.006
SYSTEMIC LUPUS ERYTHEMATOSUS	0.014
SPLICEOSOME	0.016
PROGESTERONE MEDIATED OOCYTE MATURATION	0.016
B CELL RECEPTOR SGNALING PATHWAY	0.020
MISMATCH REPAIR	0.024
HOMOLOGOUS RECOMBINATION	0.030
NUCLEOTIDE EXCISION REPAIR	0.034
T CELL RECEPTOR SIGNALING PATHWAY	0.033
PROTEASOME	0.050

Table 8a. **Enriched gene sets found in cecum mucosa of broiler chickens (42 days of age) of GOS group (0.2 ml of 9% physiological saline + 3.5 mg prebiotic (GOS)/egg) vs CON group (0.2 ml of 0.9% physiological saline), after GSEA analysis considering FDR q value ≤ 0.05 (n=24 per *in ovo* treatment).**

GENE SET – CECUM GOS	FDR q value
ECM RECEPTOR INTERACTION	0.008

Table 8b. **Enriched gene sets found in cecum mucosa of broiler chickens (42 days of age) of CON group (0.2 ml of 0.9% physiological saline) vs GOS group (0.2 ml of 9% physiological saline + 3.5 mg prebiotic (GOS)/egg), after GSEA analysis considering FDR q value ≤ 0.05 (n=24 per *in ovo* treatment).**

GENE SET - CECUM CON	FDR q value
PHOSPHATIDYLINOSITOL SIGNALING SYSTEM	0.000
T CELL RECEPTOR SIGNALING PATHWAY	0.000
B CELL RECEPTOR SIGNALING PATHWAY	0.009
GLIOMA	0.015
FC GAMMA R MEDIATED PHAGOCYTOSIS	0.016
RIG I LIKE RECEPTOR SIGNALING PATHWAY	0.017
CHEMOKINE SIGNALING PATHWAY	0.015
ENDOCYTOSIS	0.018
NATURAL KILLER CELL MEDIATED CYTOTOXICITY	0.028
UIBQUITIN MEDIATED PROTEOLYSIS	0.035
INOSITOL PHOSPHATE METABOLISM	0.034

Table 9a. **Enriched gene sets found in jejunum mucosa of broiler chickens (42 days of age) of HS group (HS- heat stress, 30°C for 24h/d from 32 to 42 d) vs TN group (TN- thermoneutral, 25°C), after GSEA analysis considering FDR q value ≤ 0.05 (n=24 per thermal treatment).**

GENE SET – JEJUNUM HS	FDR q value
DRUG METABOLISM CYTOCHROME P450	0.001
METABOLISM OF XENOBIOTICS BY CYTOCHROME P450	0.001
RETINOL METABOLISM	0.004
BASAL TRANSCRIPTION FACTORS	0.034

Table 9b. **Enriched gene sets found in jejunum mucosa of broiler chickens (42 days of age) of TN group (TN- thermoneutral, 25°C) vs HS group (HS- heat stress, 30°C for 24h/d from 32 to 42 d), after GSEA analysis considering FDR q value ≤ 0.05 (n=24 per thermal treatment).**

GENE SET – JEJUNUM TN	FDR q value
OXIDATIVE PHOSPHORYLATION	0.000
PARKINSONS DISEASE	0.000
ALZHEIMERS DISEASES	0.000
PROTEASOME	0.011
INTESTINAL IMMUNE NETWORK FOR IGA PRODUCTION	0.010
CHEMOKINE SIGNALING PATHWAY	0.008
CELL ADHESION MOLECULES CAMS	0.015
B CELL RECEPTOR SIGNALING PATHWAY	0.016
HUNTINGTONS DISEASE	0.021
FC EPSILON RI SIGNALING PATHWAY	0.020
AMINO SUGAR AND NUCLEOTIDE SUGAR METABOLISM	0.030
PROTEIN EXPORT	0.040
OLFACTORY TRANSDUCTION	0.044
N GLYCAN BYOSINTHESIS	0.046

Table 10a. **Main gene sets found in cecum mucosa of broiler chickens (42 days of age) of HS group (HS- heat stress, 30°C for 24h/d from 32 to 42 d) vs TN group (TN- thermoneutral, 25°C), after GSEA analysis considering FDR q value ≤ 0.05 (n=24 per thermal treatment).**

GENE SET – CECUM HS	FDR q value
ECM RECEPTOR INTERACTION	0.001
DNA REPLICATION	0.029
SPLICEOSOME	0.062
RNA POLYMERASE	0.047
COMPLEMENT AND COAGULATION CASCADES	0.042
BASAL CELL CARCINOMA	0.035
SYSTEMIC LUPUS ERYTHEMATOSUS	0.042

Table 10b. **Main gene sets found incecum mucosa of broiler chickens (42 days of age) of TN group (TN- thermoneutral, 25°C) vs HS group (HS- heat stress, 30°C for 24h/d from 32 to 42 d), after GSEA analysis considering FDR q value ≤ 0.05 (n=24 per thermal treatment).**

GENE SET – CECUM TN	FDR q value
PEROXISOME	0.000
NATURAL KILLER CELL MEDIATED CYTOTOXICITY	0.000
SPHINGOLIPID METABOLISM	0.000
STARCH AND SUCROSE METABOLISM	0.000
PROPANOATE METABOLISM	0.000
OXIDATIVE PHOSPHORYLATION	0.000
B CELL RECEPTOR SIGNALING PATHWAY	0.000
LYSOSOME	0.000
T CELL RECEPTOR SIGNALING PATHWAY	0.001
PARKINSONS DISEASE	0.005
EPITHELIAL CELL SIGNALING IN HELICOBACTER PYLORI INFECTION	0.008
VALINE LEUCINE AND ISOLEUCINE DEGRADATION	0.008
ALDOSTERONE REGULATED SODIUM REABSORPTION	0.011
ALZHEIMERS DISEASE	0.011
UBIQUITIN MEDIATED PROTEOLYSIS	0.023
CHEMOKINE SIGNALING PATHWAY	0.022
INSULIN SIGNALING PATHWAY	0.022
FC EPSILON RI SIGNALING PATHWAY	0.025
ENDOCYTOSIS	0.031
VIBRIO CHOLERAE INFECTION	0.036
TOLL-LIKE RECEPTOR SIGNALING PATHWAY	0.037
CITRATE CYCLE TCA CYCLE	0.038
FC GAMMA R MEDIATED PHAGOCYTOSIS	0.039
PPAR SIGNALING PATHWAY	0.039
FATTY ACID METABOLISM	0.040
BUTANOATE METABOLISM	0.042
GALACTOSE METABOLISM	0.046

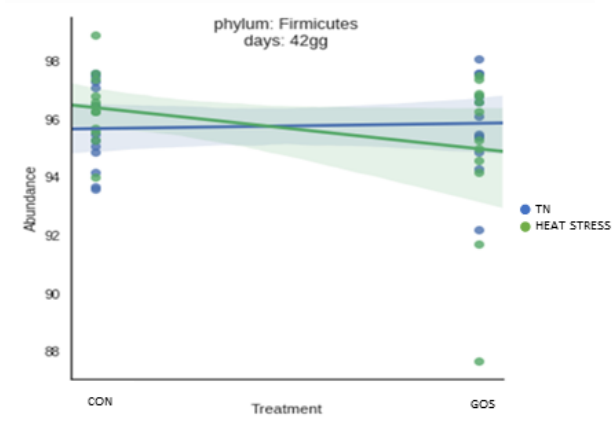
Table 11. **Plasma immune parameters of broiler chickens at 42 days of age with *in ovo* (CON group, 0.2 ml of 0.9% physiological saline, vs GOS group, 0.2 ml of 9% physiological saline + 3.5 mg prebiotic GOS/egg, n= 24 per treatment) and thermal (TN- thermoneutral, 25°C, vs heat stress-HS, 30°C for 24h/d from 32 to 42 d, n= 24 per treatment) treatments as factors**

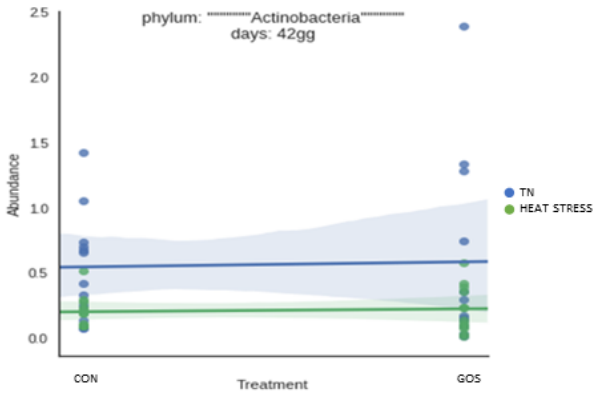
	IN OVO TREATMENT			THERMAL TREATMENT			*P* value		
	GOS	CON	SEM	HS	TN	SEM	*In ovo* Tr.	Environmental Tr.	Interaction
IgG mg/ml	4.43	4.82	0.50	4.61	4.63	0.49	0.58	0.97	0.30
IgA ng/ml	25.01	34.45	6.43	30.50	28.40	6.94	0.36	0.87	0.57
SAA ng/ml	4.16	4.04	0.50	4.24	3.95	0.49	0.88	0.70	0.65

Immunoglobulin G (IgG); Immunoglobulin A (IgA); serum amyloid A (SAA)

Differences are considered significant with *P* value < 0.05. Values are reported as mean ± standard error-

Figure 1. **Regplot for detected phyla which mean relative frequency of abundance resulted significantly affected (*p*-value α=0.01), in the multiple linear regression analysis by Python scripts where the regression coefficients, relative to the temperature (TN- thermoneutral, 25°C, vs heat stress, 30°C for 24h/d from 32 to 42 d) and to the *in ovo* treatment (CON group, 0.2 ml of 0.9% physiological saline, vs GOS group, 0.2 ml of 9% physiological saline + 3.5 mg prebiotic GOS/egg), measured the correlation between each taxonomic group and each covariate taking in account the other covariate.**

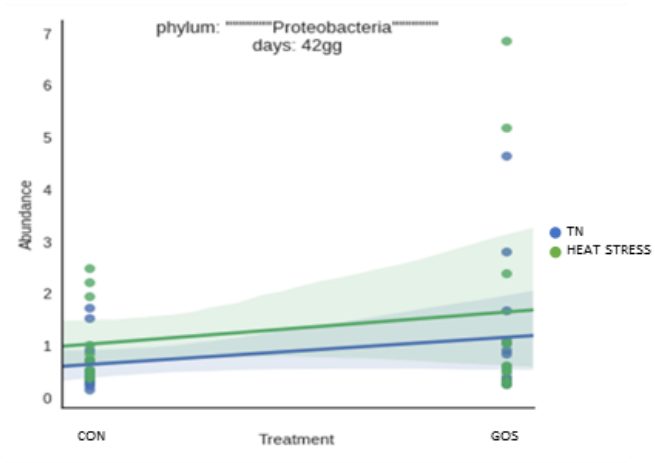




hyla

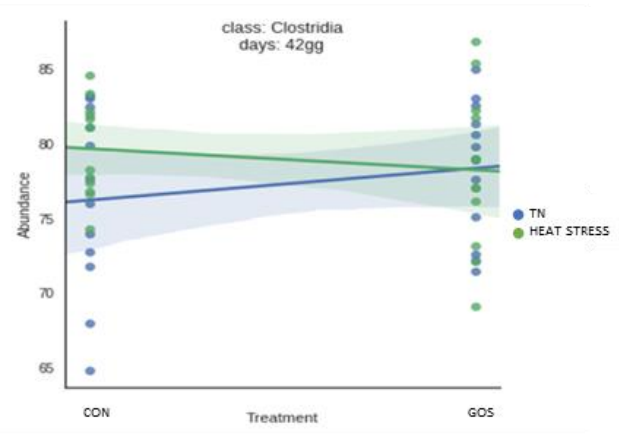
A

B

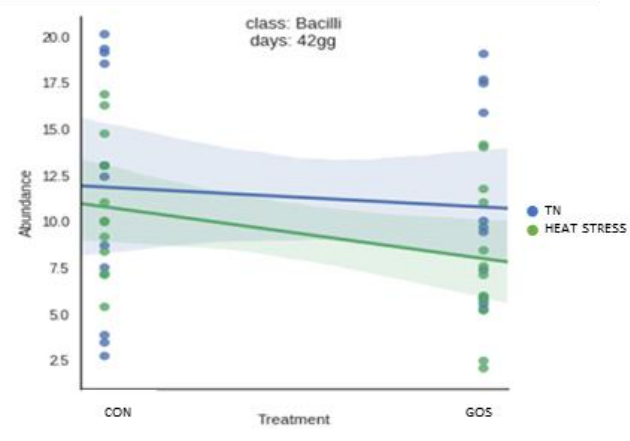


C

Figure 2. **Regplot for detected classes which mean relative frequency of abundance resulted significantly affected (*p*-value α=0.01), in the multiple linear regression analysis by Python scripts where the regression coefficients, relative to the temperature (TN- thermoneutral, 25°C, vs heat stress, 30°C for 24h/d from 32 to 42 d) and to the *in ovo* treatment (CON group, 0.2 ml of 0.9% physiological saline, vs GOS group, 0.2 ml of 9% physiological saline + 3.5 mg prebiotic GOS/egg), measured the correlation between each taxonomic group and each covariate taking in account the other covariate.**



A



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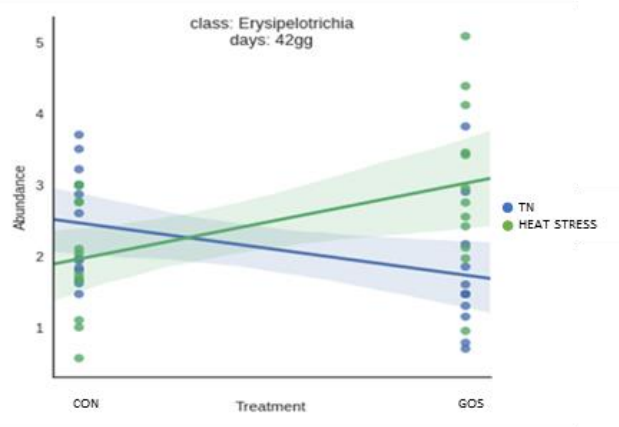
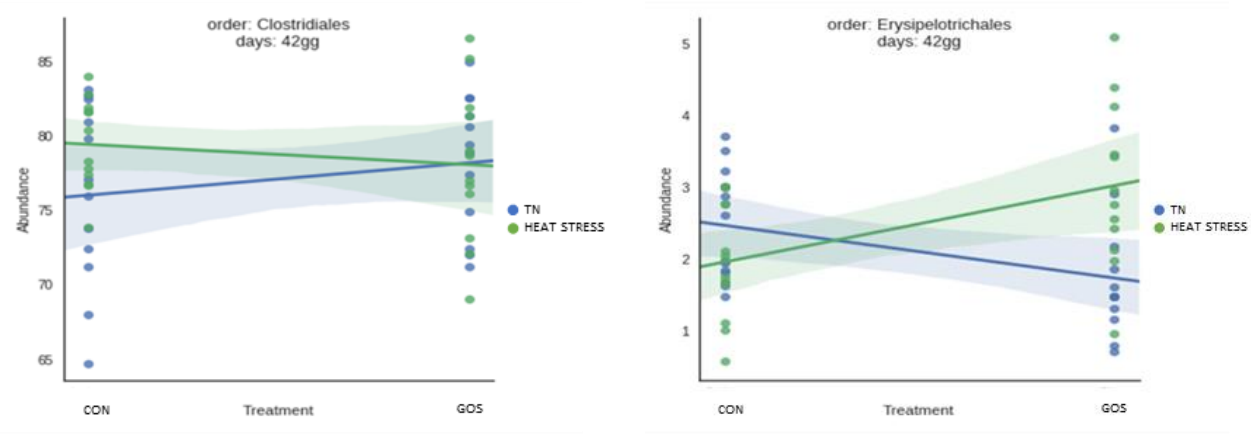


C

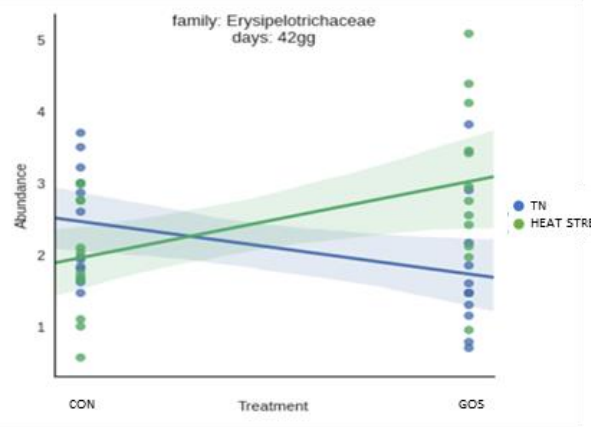
D

Figure 3. **Regplot for detected orders which mean relative frequency of abundance resulted significantly affected (*p*-value α=0.01), in the multiple linear regression analysis by Python scripts where the regression coefficients, relative to the temperature (TN- thermoneutral, 25°C, vs heat stress, 30°C for 24h/d from 32 to 42 d) and to the *in ovo* treatment (CON group, 0.2 ml of 0.9% physiological saline, vs GOS group, 0.2 ml of 9% physiological saline + 3.5 mg prebiotic GOS/egg), measured the correlation between each taxonomic group and each covariate taking in account the other covariate.**



A B

Figure 4. **Regplot for detected families which mean relative frequency of abundance resulted significantly affected (*p*-value α=0.01), in the multiple linear regression analysis by Python scripts where the regression coefficients, relative to the temperature (TN- thermoneutral, 25°C, vs heat stress, 30°C for 24h/d from 32 to 42 d) and to the *in ovo* treatment (CON group, 0.2 ml of 0.9% physiological saline, vs GOS group, 0.2 ml of 9% physiological saline + 3.5 mg prebiotic GOS/egg), measured the correlation between each taxonomic group and each covariate taking in account the other covariate.**



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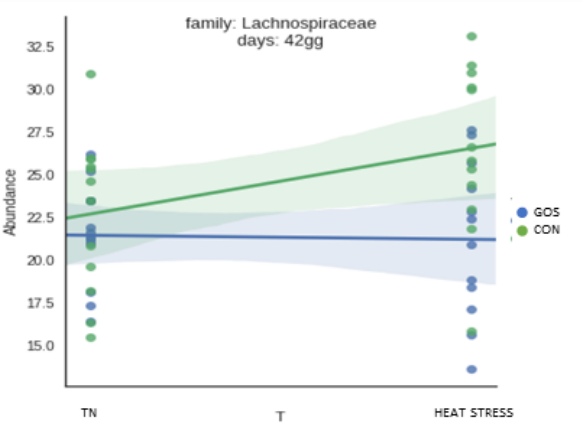

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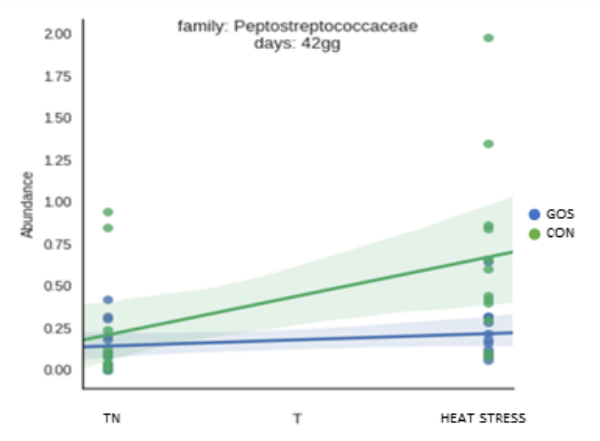

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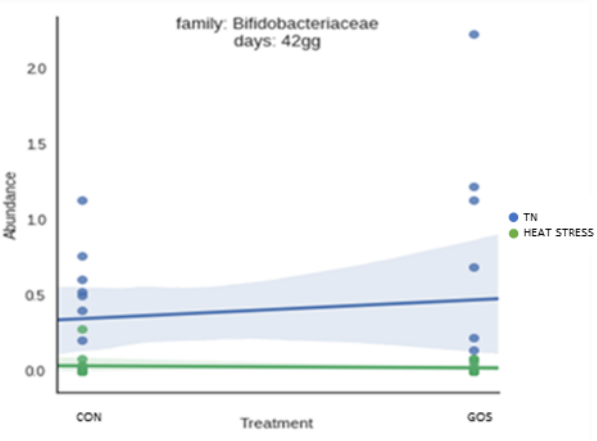

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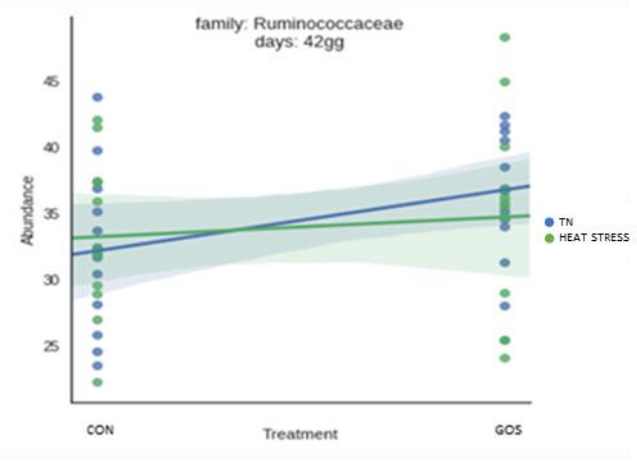
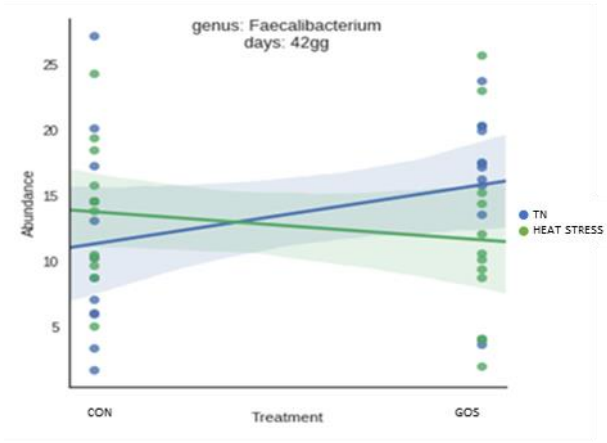

family: Ruminococcaceae
days: 42gg
Abundance
45
40
35
30
25
CON
Treatment
GOS
TN
HEAT STRESS

E F

Figure 5. **Regplot for detected genera which mean relative frequency of abundance resulted significantly affected (*p*-value α=0.01), in the multiple linear regression analysis by Python scripts where the regression coefficients, relative to the temperature (TN- thermoneutral, 25°C, vs heat stress, 30°C for 24h/d from 32 to 42 d) and to the *in ovo* treatment (CON group, 0.2 ml of 0.9% physiological saline, vs GOS group, 0.2 ml of 9% physiological saline + 3.5 mg prebiotic GOS/egg), measured the correlation between each taxonomic group and each covariate taking in account the other covariate.**



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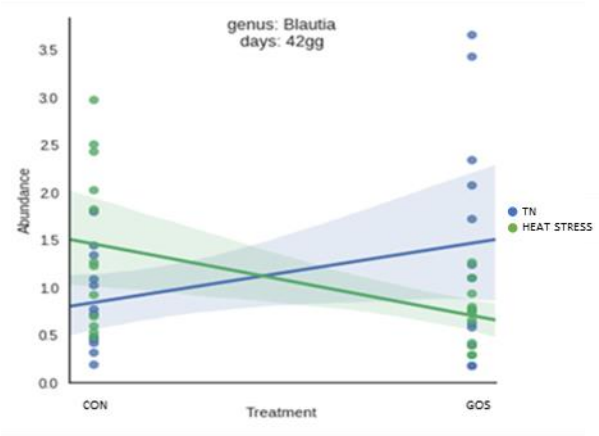

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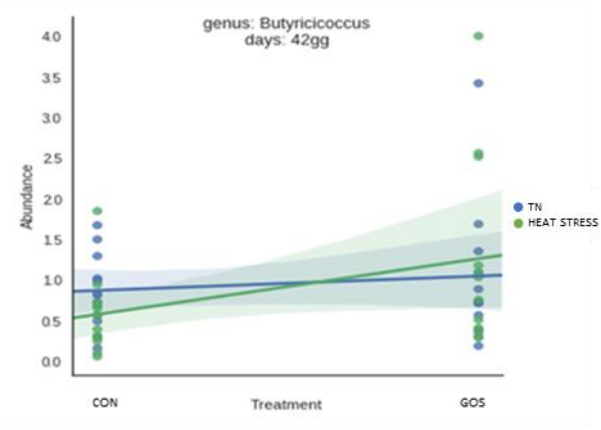


C

D

Figure 6. **Regplot for detected species which mean relative frequency of abundance resulted significantly affected (*p*-value α=0.01), in the multiple linear regression analysis by Python scripts where the regression coefficients, relative to the temperature (TN- thermoneutral, 25°C, vs heat stress, 30°C for 24h/d from 32 to 42 d) and to the *in ovo* treatment (CON group, 0.2 ml of 0.9% physiological saline, vs GOS group, 0.2 ml of 9% physiological saline + 3.5 mg prebiotic GOS/egg), measured the correlation between each taxonomic group and each covariate taking in account the other covariate.**

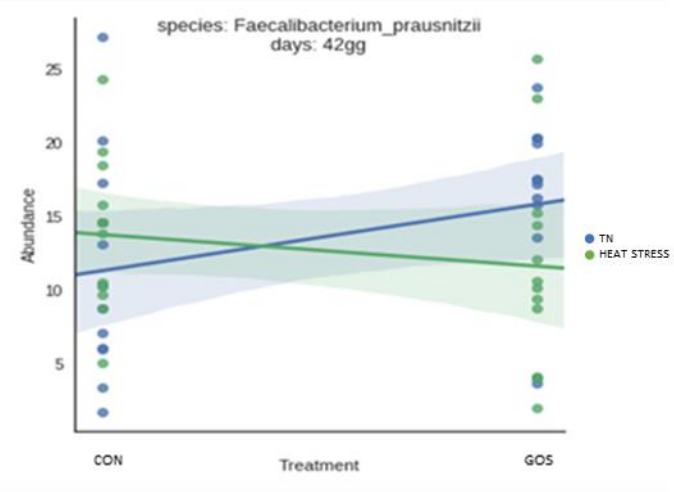
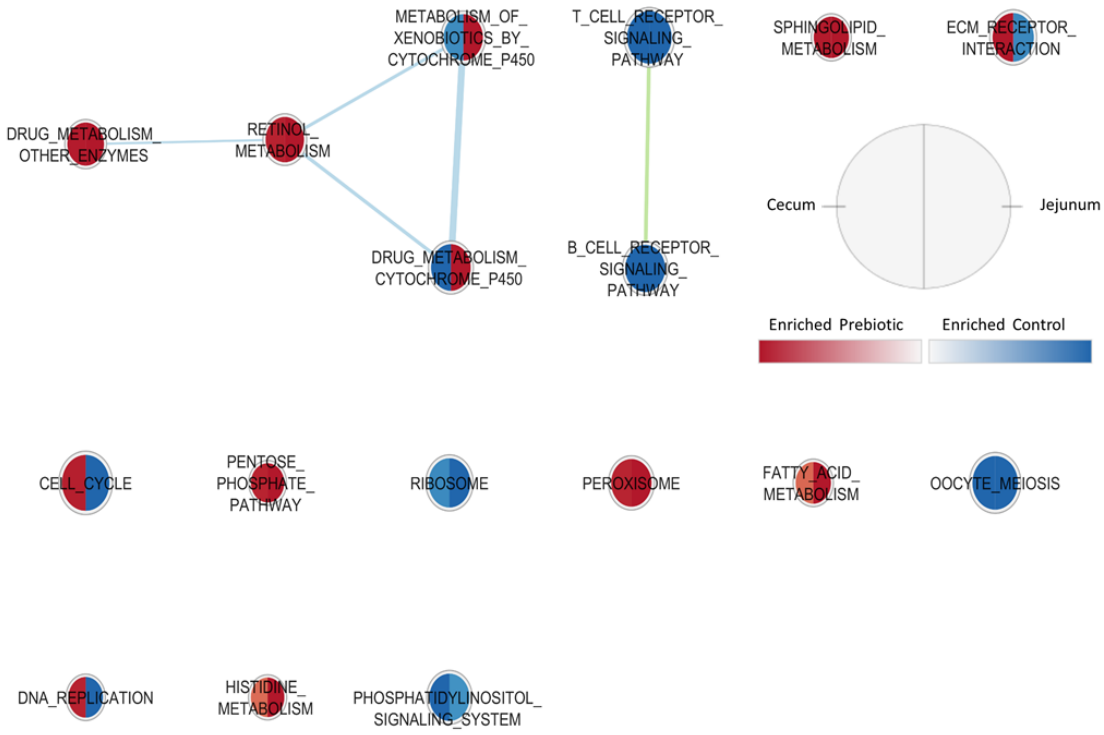


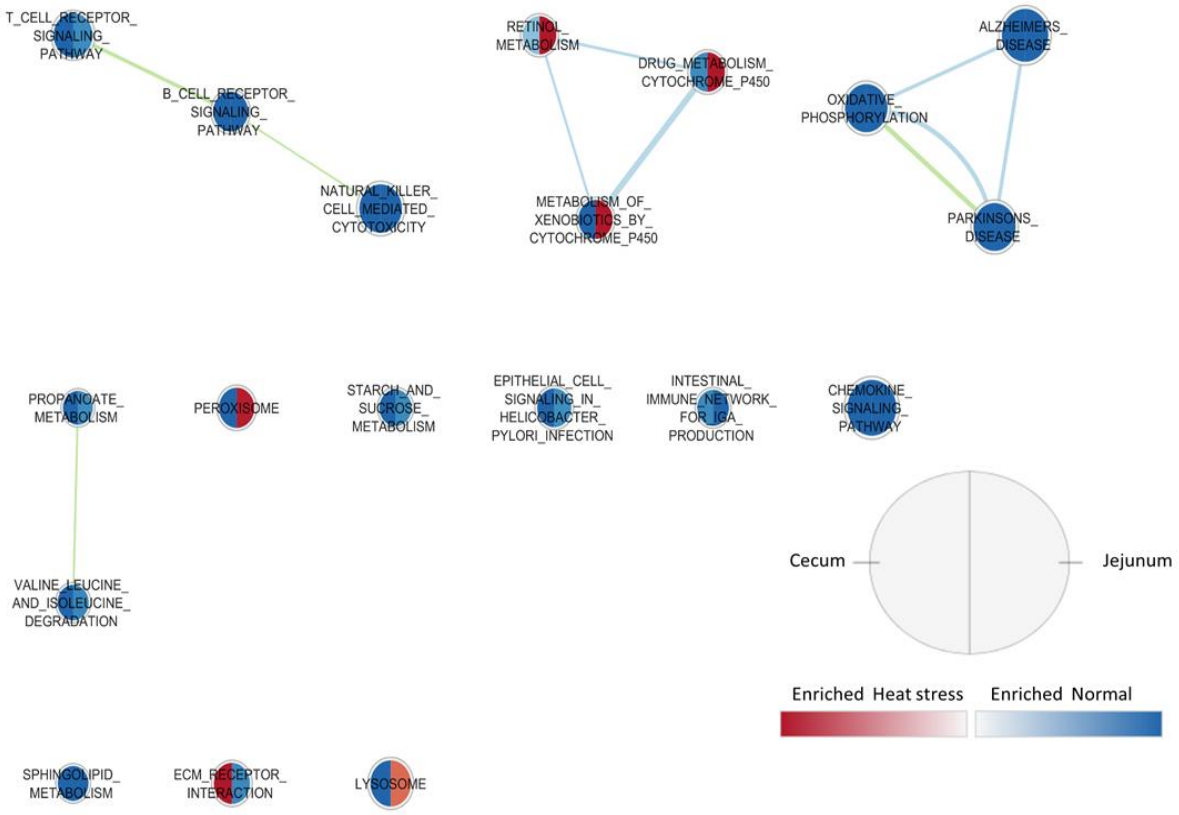
Figure 7. **Enriched Map showing enriched gene sets in cecum or jejunum of broiler chickens (42 days of age) *in ovo* injected with a single dose of physiological saline (0.2 ml of 0.9% NaCl), control (CON), or 0.2 ml of 0.9 % physiological saline+3.5 mg galacto-oligosaccharide prebiotic/egg, prebiotic (GOS) (n=24 per treatment).**



Nodes represent gene sets. Each gene set enrichment is represented for both cecum (left side of the area) and jejunum (right side of the area) tissue, enriched or in prebiotic group (red color) or in control group (blue color). Node size represents the number of genes in each gene set.

Node cut off with FDR q-value <0.01. The nodes were joined if the overlap coefficient was ≥0.5.

Figure 8. **Enriched Map showing enriched gene sets in cecum or jejunum of broilers (42 days of age) *in ovo* injected, in thermoneutral (normal) (TN, 25°C) and heat stress (HS, 30°C constantly) groups (n=24 per treatment).**



Nodes represent gene sets. Each gene set enrichment is represented for both cecum (left side of the area) and jejunum (right side of the area) tissue, enriched or in heat stress group (red color) or in normal (thermoneutral) group (blue color). Node size represents the number of genes in each gene set.

Node cut off with FDR q-value <0.01. The nodes were joined if the overlap coefficient was ≥0.5.

General Discussion

This thesis wanted to focus on the transcriptomic profile of the chicken intestine from a point of view of the intestine as central point and key target for occurrence of changes which have repercussions on the whole organism.

It has been reported the importance of the intestinal homeostasis and consequences of its dysfunction on both animal and human health, also considering the gut role in many diseases, such as metabolic disorders but also stress-related diseases. Furthermore, negative repercussions of the intestinal diseases have been seen in animal production, and particularly in intensive reared animals, like fast-growing chickens.

In this thesis, the application of microarray as modern molecular approach for an in-depth and wide investigation of molecular patterns in chicken intestine revealed possible new interesting points of view on the functional characterization of the gut, in terms of gene expression, differentiated by tract. In fact, in the present work, the analysis of transcriptomic profile by using microarray has been seen to be a valid and useful tool in underlining possible differences between chicken gut tracts in terms of basic biological functions of jejunum and cecum, but also in identifying potential gene markers in the two tissues. Specifically, the sulfur metabolism-related genes CBS (first of the DEG list in cecum compared to jejunum), SULT1C3, SULT1E1, PAPSS2, TST and TXN wereoverexpressed in cecum and may represent possible useful biomarkers of gut status. In fact, these genes have been seen to be possible indicators of a specific cecal bacterial activity (they are down-expressed in germ free chicken cecum) and of a cecal functional maturity (they are involved in cecal mucus layer sulfation). Another interesting possible biomarker gene may be PCK1, we found overexpressed in jejunum compared to cecum, evidencing for the first time in poultry jejunum relevance in gluconeogenesis activity. In this sense, PCK1 expression might be exploited as indicator of local gluconeogenesis trend in chicken small intestine, since its variability is strictly linked to interaction between feed characteristics and individual control of feed intake.

Exploratory analysis for differential gene expression along different gastro-intestinal tracts might be useful not only for poultry application, but also to further increase the knowledge on functions of genes usually studied individually and to enrich the already existing microarray studies on animal models and human. In fact, with this work we have highlighted some interesting gene correlations to mammals and, more specific, to humans.

With this perspective, the application of these hints in the second trial was performed by testing some experimental factors, to further explore possible in-field environmental conditions affecting poultry gut status and to highlight potential biomarkers linked to these factors. In this work, heat stress was

chosen as model of stress given its importance as disease triggering factor, since it represents the main environmental problem inducing poultry production losses, especially in the Mediterranean area, and impairing gut health status. Based on recent literature, in-feed prebiotics, and more specifically GOS, have been chosen as dietary strategy able to promote bifidobacteria gut tract colonization and to support the gut homeostasis in poultry under stress conditions. In particular, dietary GOS alleviated heat stress-related immune response and improved gut barrier at small intestinal level. Moreover, in the recent years the early feeding was recognized as an important tool to improve chicken development and gut health. Thus, we selected the *in ovo* prebiotic injection at day 12 of fetus development, as candidate strategy to deliver a promising GOS formulation, based on non-digestive mixture of trans-galactooligosaccharides obtained from milk lactose digested with *Bifidobacterium bifidum*, in broiler reared in thermoneutral condition or heat-stressed. The experimental model was effective in stressing the animals as evidenced by the animal performance, by the transcriptome of both jejunum and cecum tissues and by the cecal microbiome profile. In general, the *in ovo* injection of the GOS prebiotic affected the transcription of genes related to nutrient metabolism and immune response with different impact related to the specific functions of each intestinal tract and the presence of a few butyric producing bacteria in the cecum content. As far as transcriptome is concerned, these results confirm the observation of the study 1, according to which is important to consider the peculiarity of specific functions of each intestinal tract in researches involving the response of the gut to external factors.

GOS mainly did not protect against the heat stress, as evidenced by the absence of interaction between environmental treatment and dietary treatment on the differentially expressed gene presence in the intestinal tissues and on the blood parameters. Conversely, GOS supplementation interacted with the thermal treatment for the cecum microbiota, with a moderate reduction of abundance of Firmicutes, but mainly related to a reduction of Clostridia and Bacilli, as well as with changes in few lower taxa. In this context, our results show that the additional efficacy of GOS on transcriptome and microbiota in the case of heat stress was scarce. Nevertheless, without considering the different environmental conditions, the positive impact of GOS on transcriptome data concurs to sustain the ability of the *in ovo* injection technique to induce positive responses in the long-term period. It confirms additionally the effectiveness of GOS prebiotic on intestinal tracts at molecular level, providing also the opportunity of making new insights and expanding the already known contribution of GOS on the small intestine and at the cecum level too. Particularly the concomitant analysis of microbiota in cecum showed the probability of a possible correlation between changes in microbial community with functional genes and provided new insights on the possible different responses that GOS

prebiotic may give in terms of microbiota in different conditions, offering possible hints for the identification of new biomarkers of gut homeostasis too in future researches.

As a global consideration, the single *in ovo* injection of a mixture of trans-galactooligosaccharides at day 12 of embryo development as promising early feeding strategy supports previous trials confirming its association with relevant indicators of a proper gut development and homeostasis. Concerning the use of heat stress in the last rearing phase as a model to reproduce the in-field conditions in Mediterranean areas, our results show that the important impact of this treatment is clearly evidenced by the use of the microarray method, to attest gut status and to identify genes for diversified molecular functions depending on gut tract in chickens. However, such a relevant effect does not help to give evidence to other treatments, as it was evidenced by the lack of interaction with GOS treatment. In other terms, the results could be due to the inability of the GOS to counteract the effect of heat stress applied in this study that widely impaired the gut homeostasis of the chickens.

General Conclusion

This thesis underlined the importance of in-depth studying the physiological profile of a tissue, like chicken intestinal tract, highly susceptible to external factors, and how the application of *in ovo* technique as early feeding strategy is long-term effective in influencing transcriptomic profile along the gut tracts. This consequently demonstrates that the microarray technique is a proper tool in detecting molecular mechanisms at the base of biological and functional properties of the different intestinal tracts compared to previous studies where researchers had mainly concentrated on single and limited number of genes. Moreover, the identification of possible functional genes as biomarkers for monitoring gut health allows having a precise intestinal status indication at a specific time point, evidencing the use and the research of possible new prospective potential biomarkers of chicken gut homeostasis in different conditions may be useful and represent a favorable suggestion for future researches.

Lastly, characterization of the intestinal transcriptomic profile should not disregard the analysis of microbiota, including its spatial variations along the gut tracts, given its important role in gut balance maintenance or disruption.

The results of this thesis may contribute to give useful starting points for new further investigations on key molecular aspects of the intestine as possible target of different experimental factors and confirm the *in ovo* feeding strategy as a promising tool to modify the early program development of the chick gut. Finally, other formulations of prebiotics should be tested to properly protect the animals against the heat stress.

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Study 1 – Supplementary Tables

Supplementary Table 1. **Composition of the commercial diets.**

Item	STARTER (0-10 d)	GROWER (11-25 d)	FINISHER (26-42 d)
Corn	42.17	34.96	12.73
White Corn	0.00	0.00	15.00
Wheat	10.00	20.00	25.01
Sorghum	0.00	0.00	5.00
Soybean Meal	23.11	20.63	17.60
Expanded Soybean	10.00	10.00	13.00
Sunflower	3.00	3.00	3.00
Corn Gluten	4.00	3.00	0.00
Soybean Oil	3.08	4.43	5.48
Dicalcium phosphate	1.52	1.20	0.57
Calcium carbonate	0.91	0.65	0.52
Sodium bicarbonate	0.15	0.10	0.15
Salt	0.27	0.27	0.25
Choline chloride	0.10	0.10	0.10
Lysine sulphate	0.59	0.55	0.46
Dl-Methionine	0.27	0.29	0.30
Threonine	0.15	0.14	0.14
Enzyme - Roxazyme G2G	0.08	0.08	0.08
Phytase 0.1%	0.10	0.10	0.10
Vitamin - Mineral Premix [1]	0.50	0.50	0.50
Calculated chemical composition:			
Dry Matter,%	88.57	88.65	88.64
Crude Protein,%	22.70	21.49	19.74
Lipid,%	7.06	8.24	9.74
Crude Fibre,%	3.08	3.04	3.07
Ash,%	5.85	5.17	4.49
Lysine,%	1.38	1.29	1.21
Methionine,%	0.67	0.62	0.59
Methionine + Cysteine,%	1.03	0.97	0.91
Calcium,%	0.91	0.80	0.59
Phosphate,%	0.63	0.57	0.46
Metabolizable Energy (Kcal/Kg)	3.076	3.168	3.264

[1] Provided the following per kg of diet: vitamin A (retinyl acetate), 13,000 IU; vitamin D3 (cholecalciferol), 4,000 IU; vitamin E (DL-α_tocopheryl acetate), 80 IU; vitamin K (menadione sodium bisulfite), 3 mg; riboflavin, 6.0 mg; pantothenic acid, 6.0 mg; niacin, 20 mg; pyridoxine, 2 mg; folic acid, 0.5 mg; biotin, 0.10 mg; thiamine, 2.5 mg; vitamin B12 20 μg; Mn, 100 mg; Zn, 85 mg; Fe, 30 mg; Cu, 10 mg; I, 1.5 mg; Se, 0.2 mg; ethoxyquin, 100 mg.

Supplementary Table 2. **Upregulated assigned transcripts in jejunum mucosa of broiler chickens at 42 days of (n=19) , compared to cecum.**

Fold Change	P-value	FDR P-value	Gene Symbol	Description
207.3	6.39E-19	1.700E-16	APOB	Apolipoprotein B x chilomicroni e LDL
151.2	1.73E-15	1.690E-13	RBP2	retinol binding protein 2, cellular
143.7	1.52E-22	3.810E-19	ENPEP	glutamyl aminopeptidase (aminopeptidase A)
111.4	5.45E-21	3.990E-18	MEP1A	meprin A, alpha (PABA peptide hydrolase)
98.2	6.01E-21	4.180E-18	SI	sucrase-isomaltase (alpha-glucosidase)
90.2	4.89E-21	3.730E-18	ACE2	angiotensin I converting enzyme 2
88.0	2.24E-22	4.100E-19	SLC6A19	solute carrier family 6 (neutral amino acid transporter), member 19
74.2	9.15E-23	3.350E-19	MGAM	maltase-glucoamylase (alpha-glucosidase)
73.2	9.46E-19	2.300E-16	SLC7A9	solute carrier family 7 (amino acid transporter light chain, bo,+ system), member 9
66.3	1.53E-21	1.550E-18	SLC15A1	(oligopeptide transporter), member 1
64.8	1.87E-22	3.810E-19	SLC9A3	(NHE3, cation proton antiporter 3), member 3
58.4	2.93E-15	2.680E-13	ENPP7	ectonucleotide pyrophosphatase/phosphodiesterase 7
57.9	1.69E-20	8.340E-18	CLDN10	claudin 10
55.6	1.38E-22	3.810E-19	MGAT4D	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme B-like
49.6	1.61E-14	1.150E-12	LCT	Lactase
49.2	6.58E-22	8.040E-19	CNOT2	CCR4-NOT transcription complex, subunit 2
46.6	2.31E-19	6.600E-17	TM4SF4	transmembrane 4 L six family member 4
46.3	9.57E-19	2.300E-16	MME	membrane metallo-endopeptidase
45.9	5.06E-22	7.120E-19	MEP1B	meprin A, beta
45.0	3.66E-16	4.330E-14	FABP2	fatty acid binding protein 2, intestinal
44.9	4.05E-20	1.580E-17	MALRD1	MAM And LDL Receptor Class A Domain Containing 1
44.6	6.22E-14	3.800E-12	CUBN	cubilin (intrinsic factor-cobalamin receptor)
42.3	1.27E-18	2.940E-16	PLA2G2E	phospholipase A2, group IIE
40.7	1.27E-23	7.730E-20	ABCG5	ATP-binding cassette, sub-family G (WHITE), member 5
40.5	1.01E-23	7.730E-20	PDZK1	PDZ domain containing 1
38.8	3.33E-18	6.850E-16	CYP2K1L	cytochrome P450 2K1-like
38.4	1.47E-18	3.360E-16	LEAP2	liver expressed antimicrobial peptide 2
37.9	1.97E-21	1.810E-18	ABCG8	ATP-binding cassette, sub-family G member 8
37.3	6.36E-19	1.700E-16	CPO	carboxypeptidase O
35.5	1.61E-21	1.550E-18	ACE	angiotensin I converting enzyme
35.0	4.12E-22	6.290E-19	CLIC5	chloride intracellular channel 5
32.9	2.11E-19	6.130E-17	SLC13A1	solute carrier family 13 (sodium/sulfate symporter), member 1
32.0	1.88E-20	8.640E-18	SLC3A1	solute carrier family 3 (amino acid transporter heavy chain), member 1
31.5	4.13E-16	4.700E-14	APOA1	apolipoprotein A-I
30.6	3.13E-21	2.610E-18	GCG	Glucagon
29.5	7.67E-15	6.160E-13	AGMO	alkylglycerol monooxygenase
29.0	1.19E-13	6.450E-12	FABP6	fatty acid binding protein 6
27.3	4.05E-16	4.640E-14	CYP2C23b	cytochrome P450 2H1
26.9	4.77E-21	3.730E-18	MTTP	microsomal triglyceride transfer protein

26.6	8.24E-14	4.670E-12	SLC2A2	solute carrier family 2 (facilitated glucose transporter), member 2
26.1	7.66E-14	4.420E-12	SLC26A9	solute carrier family 26 (anion exchanger), member 9
23.6	1.67E-22	3.810E-19	GATA5	GATA binding protein 5
23.2	6.39E-21	4.180E-18	FRMPD2	FERM and PDZ domain containing 2
22.1	1.09E-16	1.480E-14	TMEM86A	transmembrane protein 86 A
21.9	4.63E-20	1.770E-17	ST3GAL5	ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 5
21.6	2.77E-19	7.800E-17	FUT9	fucosyltransferase 9 (alpha (1,3) fucosyltransferase)
20.9	1.23E-10	2.720E-09	TMPRSS15	transmembrane protease, serine 15
20.6	2.53E-18	5.590E-16	ALDOB	aldolase B, fructose-bisphosphate
20.2	7.71E-15	6.170E-13	ACOT12	acyl-CoA thioesterase 12
19.8	5.21E-20	1.950E-17	CYP2D6	cytochrome P450, family 2, subfamily D, polypeptide 6
19.4	1.33E-17	2.480E-15	PCK1	phosphoenolpyruvate carboxykinase 1 (soluble)
19.2	1.62E-14	1.160E-12	ASAH2	N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2
19.1	8.97E-19	2.230E-16	SLC2A5	solute carrier family 2 (facilitated glucose/fructose transporter), member 5
19.0	6.05E-19	1.650E-16	SLC1A1	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1
18.9	2.23E-17	3.890E-15	TTLL2	tubulin tyrosine ligase-like family member 2
18.7	1.34E-19	4.140E-17	PLEKHS1	pleckstrin homology domain containing, family S member 1
18.5	2.13E-18	4.800E-16	MFI2	antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5
18.0	3.70E-18	7.380E-16	HKDC1	hexokinase domain containing 1
17.7	8.24E-21	4.870E-18	SLC5A1	solute carrier family 5 (sodium/glucose cotransporter), member 1
17.7	4.06E-22	6.290E-19	HNF4B	hepatocyte nuclear factor 4 beta
17.6	7.58E-14	4.420E-12	SCT	Secretin
17.5	9.93E-15	7.740E-13	AQP7	aquaporin 7
17.4	1.15E-14	8.640E-13	IYD	iodotyrosine deiodinase
16.9	1.12E-14	8.430E-13	GATA4	GATA binding protein 4
16.6	3.93E-19	1.090E-16	ALPP	alkaline phosphatase, placental
15.1	1.03E-20	5.880E-18	ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2
13.9	8.20E-14	4.660E-12	APOA4	apolipoprotein A4
13.6	6.46E-20	2.190E-17	NTS	Neurotensin
13.3	8.08E-12	2.510E-10	SLC5A11	solute carrier family 5 (sodium/inositol cotransporter), member 11,
12.9	4.70E-12	1.590E-10	CALB1	calbindin 1, 28kDa
12.8	4.14E-15	3.620E-13	ANO5	anoctamin 5
12.6	2.00E-14	1.390E-12	CYP4V2	cytochrome P450, family 4, subfamily V, polypeptide 2
12.0	5.13E-11	1.260E-09	ETNPPL	ethanolamine-phosphate phospho-lyase
11.9	6.28E-15	5.200E-13	GIP	gastric inhibitory polypeptide
11.9	8.32E-12	2.570E-10	GLUL	glutamate-ammonia ligase
11.6	3.06E-14	2.060E-12	TMEM252	transmembrane protein 252
11.5	1.21E-14	8.880E-13	GIMD1	GIMAP family P-loop NTPase domain containing 1
11.5	6.73E-20	2.200E-17	SLC22A13L	solute carrier family 22 member 13-like
11.3	1.53E-12	5.830E-11	BCMO1	beta-carotene 15,15-monooxygenase 1
11.2	8.94E-20	2.840E-17	SLC5A9	solute carrier family 5 (sodium/sugar cotransporter), member 9

11.2	5.58E-16	6.240E-14	CYP3A7	cytochrome P450 A 37
11.1	1.72E-12	6.480E-11	SLC34A2	solute carrier family 34 (type II sodium/phosphate contransporter), member 2
10.8	3.66E-13	1.710E-11	SUSD2	sushi domain containing 2
10.8	5.52E-12	1.820E-10	SLC5A12	solute carrier family 5 (sodium/monocarboxylate cotransporter), member 12
10.6	9.00E-20	2.840E-17	GDA	guanine deaminase
9.9	7.50E-16	7.990E-14	REEP6	receptor accessory protein 6
9.9	1.87E-20	8.640E-18	GPD1L2	glycerol-3-phosphate dehydrogenase 1-like 2
9.8	2.98E-18	6.190E-16	AKR1D1	aldo-keto reductase family 1, member D1 (delta 4-3-ketosteroid-5-beta-reductase)
9.8	1.47E-13	7.720E-12	SCARB1	scavenger receptor class B, member 1
9.6	3.71E-18	7.380E-16	ABCB1LB	ATP-binding cassette, sub-family B (MDR/TAP), member 1-like B
9.5	9.13E-18	1.740E-15	RNF128	ring finger protein 128
9.2	7.41E-18	1.440E-15	SLC28A2	solute carrier family 28 (sodium-coupled nucleoside transporter), member 2
9.1	1.35E-20	7.060E-18	SH2D4A	SH2 domain containing 4°
8.7	2.97E-16	3.610E-14	SLC19A3	solute carrier family 19, member 3
8.6	1.04E-14	8.000E-13	GPR128	G protein-coupled receptor 128
8.5	7.19E-19	1.880E-16	DPP4	dipeptidyl-peptidase 4
8.3	4.78E-13	2.140E-11	ENPP3	ectonucleotide pyrophosphatase/phosphodiesterase 3
7.7	2.54E-15	2.370E-13	CYBRD1	cytochrome b reductase 1
7.5	5.59E-16	6.240E-14	HMCN1	hemicentin 1
7.5	1.29E-14	9.430E-13	ADH6	alcohol dehydrogenase 6 (class V)
7.4	7.28E-15	5.900E-13	GGT1	gamma-glutamyltransferase 1
7.3	7.67E-17	1.120E-14	CREB3L3	cAMP responsive element binding protein 3-like 3
7.3	3.11E-17	5.230E-15	SORD	sorbitol dehydrogenase
7.3	8.00E-21	4.870E-18	KLB	klotho beta
7.2	1.93E-13	9.790E-12	RBP1	retinol binding protein 1, cellular
7.2	1.07E-18	2.540E-16	HSD11B1b	hydroxysteroid (11-beta) dehydrogenase 1b
7.1	4.81E-13	2.150E-11	GRAMD3	GRAM domain containing 3
7.1	5.64E-17	8.670E-15	GPT2	glutamic pyruvate transaminase (alanine aminotransferase) 2
6.9	1.08E-14	8.180E-13	PLA2G12B	phospholipase A2, group XIIB
6.8	1.59E-20	8.090E-18	DAB1	Dab, reelin signal transducer, homolog 1 (Drosophila)
6.7	1.49E-17	2.730E-15	SOX6	SRY (sex determining region Y)-box 6
6.7	2.12E-16	2.670E-14	GDPD4	glycerophosphodiester phosphodiesterase domain containing 4
6.4	1.31E-11	3.850E-10	TMEM56	transmembrane protein 56
6.3	7.47E-16	7.990E-14	CDKL2	cyclin-dependent kinase-like 2 (CDC2-related kinase)
6.3	3.83E-16	4.470E-14	CAT	catalase
6.3	4.41E-14	2.830E-12	NMNAT3	nicotinamide nucleotide adenylyltransferase 3
6.2	1.00E-14	7.750E-13	EPHX1	epoxide hydrolase 1, microsomal (xenobiotic)
6.2	1.29E-15	1.310E-13	CES2	carboxylesterase 2 (fatty acyl-CoA hydrolase precursor, medium chain)
6.2	7.24E-17	1.070E-14	B3GNT5	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5
6.1	1.34E-16	1.800E-14	PHOSPHO1	phosphatase, orphan 1
6.0	1.78E-14	1.250E-12	TMC5	transmembrane channel-like 5
5.9	1.95E-13	9.860E-12	GLOD5	glyoxalase domain containing 5

5.8	2.28E-11	6.280E-10	MAOB	monoamine oxidase B
5.8	6.62E-12	2.120E-10	XKR9	XK, Kell blood group complex subunit-related family, member 9
5.8	1.24E-20	6.860E-18	TRIM36	tripartite motif containing 36
5.7	1.17E-13	6.370E-12	SLC16A9	solute carrier family 16, member 9
5.7	7.72E-17	1.120E-14	FMO4	flavin containing monooxygenase 4
5.7	3.33E-17	5.550E-15	ADA	adenosine deaminase
5.6	2.31E-15	2.180E-13	SEMA5B	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B
5.5	2.94E-18	6.190E-16	TBC1D24	TBC1 Domain Family Member 24: Vesicle-mediated transport and GTPase activator activity
5.3	1.70E-15	1.660E-13	CUTA	cutA divalent cation tolerance homolog (E. coli)
5.3	1.29E-15	1.310E-13	MAFB	MAF bZIP transcription factor B
5.3	1.27E-14	9.280E-13	A1CF	APOBEC1 complementation factor
5.3	2.90E-17	4.960E-15	ENPP6	ectonucleotide pyrophosphatase/phosphodiesterase 6
5.2	2.58E-13	1.260E-11	FAM83B	family with sequence similarity 83, member B
5.2	3.63E-13	1.710E-11	CL2	liver ribonuclease A
5.2	1.82E-16	2.370E-14	EPHX1L	epoxide hydrolase 1-like
5.1	4.44E-13	2.040E-11	RMDN2	regulator of microtubule dynamics 2
5.1	1.77E-12	6.630E-11	snoRNA RF00004	
5.1	1.95E-14	1.370E-12	KCNE2	potassium voltage-gated channel, Isk-related family, member 2
5.0	1.54E-12	5.850E-11	FBP1	fructose-1,6-bisphosphatase 1
5.0	3.51E-10	6.760E-09	SLC35F2	solute carrier family 35, member F2
5.0	1.81E-15	1.730E-13	AMN	amnion associated transmembrane protein
5.0	1.58E-11	4.510E-10	ANXA13	annexin A13
5.0	1.18E-13	6.410E-12	LRAT	lecithin retinol acyltransferase (phosphatidylcholine--retinol O-acyltransferase)
5.0	2.00E-08	2.300E-07	CD36	CD36 molecule (thrombospondin receptor)
4.9	4.29E-16	4.850E-14	slc27a5	Acyl-CoA synthetase involved in bile acid metabolism
4.9	7.60E-14	4.420E-12	MAN1A1	mannosidase, alpha, class 1A, member 1
4.9	1.43E-15	1.440E-13	LPGAT1	lysophosphatidylglycerol acyltransferase 1
4.8	1.12E-14	8.430E-13	IL15	interleukin 15
4.8	2.99E-17	5.060E-15	PEPD	peptidase D
4.8	8.34E-11	1.920E-09	GZMA	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)
4.7	1.95E-09	3.000E-08	FAXDC2	fatty acid hydroxylase domain containing 2
4.7	2.01E-16	2.570E-14	FER1L6	fer-1-like 6 (C. elegans)
4.7	3.64E-15	3.220E-13	GCH1	GTP cyclohydrolase 1
4.6	5.68E-16	6.300E-14	MOGAT2	2-acylglycerol O-acyltransferase 2-like
4.6	1.30E-20	7.020E-18	BAIAP2L2	BAI1-associated protein 2-like 2
4.6	1.97E-12	7.300E-11	RASGRF2	Ras protein-specific guanine nucleotide-releasing factor 2
4.6	4.32E-15	3.770E-13	FAAH	fatty acid amide hydrolase
4.6	6.61E-11	1.580E-09	SLC5A8	solute carrier family 5 (sodium/monocarboxylate cotransporter), member 8
4.5	4.32E-12	1.480E-10	PRKG2	protein kinase, cGMP-dependent, type II
4.5	2.41E-08	2.700E-07	IRG1L	immunoresponsive 1 homolog (mouse)-like
4.5	2.16E-10	4.410E-09	HSD3B7	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7

4.4	1.30E-11	3.810E-10	MLN	motilin
4.4	6.94E-17	1.030E-14	MAMDC4	MAM domain containing 4
4.4	1.03E-14	7.910E-13	LRRC58	leucine rich repeat containing 58
4.4	5.21E-11	1.280E-09	PIK3C2G	phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 gamma
4.4	8.95E-15	7.040E-13	MAP3K15	mitogen-activated protein kinase kinase kinase 15
4.4	3.84E-17	6.270E-15	ACOT2L	acyl-coenzyme A thioesterase 2, mitochondrial-like
4.3	4.84E-15	4.160E-13	DNM1	dynamin 1
4.3	5.35E-20	1.960E-17	BAAT	bile acid CoA: amino acid N-acyltransferase (glycine N-choloyltransferase)
4.3	6.15E-06	3.460E-05	DSEL	dermatan sulfate epimerase-like
4.3	1.70E-13	8.750E-12	ANPEP	aminopeptidase N
4.3	1.01E-12	4.100E-11	MLXIPL	MLX interacting protein-like
4.2	9.84E-09	1.250E-07	CES1	carboxylesterase 1 (monocyte/macrophage serine esterase 1)
4.2	3.11E-08	3.390E-07	SLC10A2	solute carrier family 10 (sodium/bile acid cotransporter), member 2
4.2	9.79E-13	3.990E-11	SLC6A4	solute carrier family 6 (neurotransmitter transporter), member 4
4.1	7.32E-12	2.310E-10	MUC2	mucin 2
4.1	6.69E-17	1.010E-14	NAPEPLD	N-acyl phosphatidylethanolamine phospholipase D
4.1	2.84E-06	1.750E-05	TMIGD1	transmembrane and immunoglobulin domain containing 1
4.1	1.79E-13	9.130E-12	FABP5	fatty acid binding protein 5 (psoriasis-associated)
4.1	6.26E-14	3.800E-12	KBTBD11	kelch repeat and BTB (POZ) domain containing 11
4.1	9.00E-17	1.280E-14	DDC	dopa decarboxylase (aromatic L-amino acid decarboxylase)
4.1	3.48E-11	9.020E-10	IAPP	islet amyloid polypeptide
4.0	6.25E-17	9.530E-15	SMPD3	sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)
3.9	7.24E-16	7.790E-14	PCSK1	proprotein convertase subtilisin/kexin type 1
3.9	1.34E-11	3.910E-10	PTPRR	protein tyrosine phosphatase, receptor type, R
3.9	1.58E-13	8.170E-12	SLC16A10	solute carrier family 16 (aromatic amino acid transporter), member 10
3.9	8.82E-10	1.500E-08	GSTT1	glutathione S-transferase theta 1
3.8	2.48E-13	1.220E-11	KYNU	kynureninase
3.8	6.21E-12	2.000E-10	SDR16C5	short chain dehydrogenase/reductase family 16C, member 5
3.8	1.13E-12	4.500E-11	ACSL5	acyl-CoA synthetase long-chain family member 5
3.8	9.40E-14	5.250E-12	SH3BP2	SH3-domain binding protein 2
3.8	2.61E-14	1.780E-12	WWP1	WW domain containing E3 ubiquitin protein ligase 1
3.7	1.23E-10	2.730E-09	NRG4	neuregulin 4
3.7	6.17E-12	1.990E-10	BST1	bone marrow stromal cell antigen 1
3.7	4.44E-10	8.250E-09	MACROD2	MACRO domain containing 2
3.7	2.01E-10	4.140E-09	TINAG	tubulointerstitial nephritis antigen
3.7	7.11E-15	5.810E-13	NR1I3	nuclear Receptor Subfamily 1 Group I Member 3
3.7	9.96E-17	1.380E-14	CHST6	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6
3.6	1.85E-11	5.200E-10	FGF19	fibroblast growth factor 19
3.6	7.09E-14	4.190E-12	GDPD1	glycerophosphodiester phosphodiesterase domain containing 1
3.6	1.41E-13	7.460E-12	PRAP1	proline-rich acidic protein 1

3.6	3.42E-09	4.950E-08	SLC51B	solute carrier family 51, beta subunit
3.6	2.83E-06	1.740E-05	RAG2	recombination activating gene 2
3.6	6.65E-18	1.310E-15	CDR2	cerebellar degeneration-related protein 2, 62kDa
3.6	6.08E-12	1.980E-10	ATP10A	ATPase, class V, type 10A
3.6	2.86E-12	1.010E-10	FOLH1	folate hydrolase (prostate-specific membrane antigen) 1
3.6	6.25E-11	1.500E-09	FLRT3	fibronectin leucine rich transmembrane protein 3
3.6	1.17E-12	4.680E-11	NPAS2	neuronal PAS domain protein 2
3.5	4.76E-11	1.180E-09	EGLN3	egl-9 family hypoxia-inducible factor 3
3.5	6.65E-14	3.980E-12	DENND5B	DENN/MADD domain containing 5B
3.5	3.95E-16	4.580E-14	TRAK1	trafficking protein, kinesin binding 1
3.5	3.97E-06	2.350E-05	GLP2R	glucagon-like peptide 2 receptor
3.5	2.83E-12	1.000E-10	CNDP2	CNDP dipeptidase 2 (metallopeptidase M20 family)
3.4	4.71E-12	1.590E-10	LAMB3	laminin, beta 3
3.4	1.13E-12	4.500E-11	ISOC1	isochorismatase domain containing 1
3.4	6.80E-08	6.720E-07	MSMO1	methylsterol monooxygenase 1
3.3	8.57E-09	1.100E-07	INSIG1	insulin induced gene 1
3.3	1.56E-12	5.920E-11	XDH	xanthine dehydrogenase
3.3	7.77E-16	8.230E-14	DMB1	MHC class II M beta chain 1
3.3	6.54E-16	7.080E-14	XCL1	lymphotactin
3.3	2.67E-16	3.310E-14	COL17A1	collagen, type XVII, alpha 1
3.3	1.00E-04	5.000E-04	LPL	lipoprotein lipase
3.3	1.55E-11	4.430E-10	GZMK	granzyme K (granzyme 3; tryptase II)
3.3	1.13E-13	6.200E-12	AUTS2	autism susceptibility candidate 2
3.3	5.97E-09	8.030E-08	LIPI	lipase, member I
3.3	5.55E-11	1.350E-09	GPR64	G protein-coupled receptor 64
3.3	1.16E-11	3.430E-10	MAP3K7CL	MAP3K7 C-terminal like
3.3	9.87E-11	2.230E-09	TMC7	transmembrane channel-like 7
3.3	1.13E-10	2.520E-09	TMEM243	transmembrane protein 243, mitochondrial
3.2	2.20E-12	8.000E-11	CRTAM	cytotoxic and regulatory T cell molecule
3.2	6.34E-13	2.730E-11	PANX1	pannexin 1
3.2	5.66E-12	1.850E-10	SELENOP	selenoprotein P, plasma, 1
3.2	2.55E-10	5.090E-09	PDZK1IP1	PDZK1 interacting protein 1
3.2	1.32E-12	5.190E-11	CROT	carnitine O-octanoyltransferase
3.2	2.56E-09	3.840E-08	DAO	D-amino acid oxidase
3.2	5.45E-15	4.600E-13	CAB39L	calcium binding protein 39-like
3.1	1.56E-14	1.130E-12	BTBD11	BTB (POZ) domain containing 11
3.1	8.99E-13	3.710E-11	SLCO4A1	solute carrier organic anion transporter family, member 4A1
3.1	2.85E-10	5.610E-09	C7H2ORF66	chromosome 7 open reading frame, human C2orf66
3.1	5.82E-08	5.830E-07	CYP2U1	cytochrome P450 Family 2 Subfamily U Member 1
3.1	2.72E-15	2.520E-13	KIAA0319L	KIAA0319-like
3.1	5.85E-13	2.550E-11	FAM8A1	family with sequence similarity 8, member A1
3.1	4.00E-12	1.370E-10	HNF4G	hepatocyte nuclear factor 4, gamma
3.1	3.16E-11	8.300E-10	FAM83F	family with sequence similarity 83, member F
3.1	1.04E-06	7.170E-06	SLC13A2	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2
3.1	1.18E-14	8.850E-13	XpNPEP2	X-prolyl aminopeptidase 2

3.1	1.19E-11	3.510E-10	CCK	cholecystokinin
3.1	1.02E-11	3.070E-10	TM4SF1	transmembrane 4 L six family member 1
3.1	1.51E-08	1.800E-07	ZP4	zona pellucida glycoprotein 4
3.0	6.84E-12	2.180E-10	HS3ST1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1
3.0	9.50E-13	3.890E-11	VAT1	vesicle amine transport 1
3.0	3.76E-12	1.300E-10	ZFPM1	zinc finger protein, FOG family member 1
3.0	5.76E-11	1.390E-09	SLC18B1	solute carrier family 18, subfamily B, member 1
3.0	8.97E-13	3.710E-11	ABCC10	ATP-binding cassette, sub-family C (CFTR/MRP), member 10
3.0	4.57E-11	1.140E-09	FGF9	fibroblast growth factor 9 (glia-activating factor)
3.0	7.03E-13	3.000E-11	PGAP1	post-GPI attachment to proteins 1
3.0	2.03E-13	1.020E-11	ABCD2	ATP-binding cassette, sub-family D (ALD), member 2
3.0	2.26E-13	1.120E-11	FRMD1	FERM domain containing 1
3.0	1.40E-12	5.430E-11	MAOA	monoamine oxidase A
3.0	1.09E-13	6.010E-12	OSBPL1A	oxysterol binding protein-like 1A
3.0	9.44E-10	1.590E-08	TKFC	triokinase and FMN cyclase
2.9	2.33E-13	1.150E-11	MAP3K4	mitogen-activated protein kinase kinase kinase 4
2.9	9.10E-13	3.750E-11	NEDD9	neural precursor cell expressed, developmentally down-regulated 9
2.9	8.80E-15	6.940E-13	MYRF	myelin regulatory factor
2.9	7.75E-13	3.250E-11	SCP2	sterol carrier protein 2
2.9	2.39E-11	6.510E-10	EMB	embigin
2.9	4.70E-13	2.120E-11	DAPK1	death-associated protein kinase 1 isoform 1
2.9	4.14E-11	1.040E-09	CIITA	class II, major histocompatibility complex, transactivator-like
2.9	1.53E-13	7.960E-12	STAP1	signal transducing adaptor family member 1
2.9	8.83E-10	1.500E-08	KL	klotho
2.9	1.21E-13	6.570E-12	IFFO2	intermediate filament family orphan 2
2.9	1.23E-12	4.830E-11	KY	kyphoscoliosis peptidase
2.9	1.52E-11	4.370E-10	MCF2	MCF.2 cell line derived transforming sequence
2.9	6.14E-14	3.760E-12	SLC27A4	solute carrier family 27 (fatty acid transporter), member 4
2.8	1.21E-14	8.880E-13	CD226	CD226 molecule
2.8	2.13E-13	1.070E-11	OSR2	odd-skipped related transciption factor 2
2.8	2.22E-09	3.380E-08	ATP7B	ATPase, Cu++ transporting, beta polypeptide
2.8	1.09E-08	1.360E-07	SLC6A14	solute carrier family 6 (amino acid transporter), member 14
2.8	5.14E-15	4.350E-13	GNAL	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type
2.8	1.03E-12	4.150E-11	VIPR1	vasoactive intestinal peptide receptor 1
2.8	7.83E-10	1.360E-08	EFCAB4B	EF-hand calcium binding domain 4B
2.8	2.59E-15	2.410E-13	MGAT3	mannosyl (beta-1,4-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase
2.8	2.87E-07	2.350E-06	PDK4	pyruvate dehydrogenase kinase, isozyme 4
2.8	6.70E-14	3.990E-12	RHPN1	rhophilin, Rho GTPase binding protein 1
2.8	1.44E-09	2.310E-08	ROS1	ROS proto-oncogene 1 , receptor tyrosine kinase
2.8	1.27E-13	6.820E-12	AHCYL2	adenosylhomocysteinase-like 2
2.8	1.44E-09	2.310E-08	AIFM3	apoptosis inducing factor, mitochondria associated 3
2.8	6.84E-11	1.620E-09	EPT1	ethanolaminephosphotransferase 1 (CDP-ethanolamine-specific)
2.8	2.58E-09	3.860E-08	SFXN1	sideroflexin 1

2.7	3.98E-12	1.370E-10	OSBPL6	oxysterol binding protein-like 6
2.7	5.61E-13	2.470E-11	SOAT1	sterol O-acyltransferase 1
2.7	9.80E-06	5.260E-05	TRPM6	transient receptor potential cation channel, subfamily M, member 6
2.7	4.13E-10	7.750E-09	CAPN13	calpain 13
2.7	1.84E-10	3.840E-09	CENPV	centromere protein V
2.7	1.54E-13	8.000E-12	TMPRSS7	transmembrane protease, serine 7
2.7	4.32E-10	8.050E-09	CD7	CD7 molecule
2.7	2.17E-13	1.080E-11	SGPL1	sphingosine-1-phosphate lyase 1
2.7	2.46E-10	4.930E-09	TCRD	T cell receptor delta chain
2.7	5.22E-08	5.310E-07	MAT2A	S-adenosylmethionine synthase isoform type-2-like
2.7	1.10E-11	3.270E-10	FLVCR2	feline leukemia virus subgroup C cellular receptor family, member 2
2.7	3.27E-09	4.750E-08	GK	glycerol kinase
2.7	2.47E-13	1.220E-11	LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
2.7	9.66E-10	1.630E-08	SLC23A1	Solute Carrier Family 23 Member 1
2.7	5.71E-14	3.520E-12	AGPAT3	1-acylglycerol-3-phosphate O-acyltransferase 3
2.7	3.08E-15	2.790E-13	ATRN	Attractin
2.7	5.33E-10	9.640E-09	FABP1	fatty acid binding protein 1, liver
2.7	8.51E-15	6.740E-13	ITK	IL2-inducible T-cell kinase
2.7	2.00E-09	3.080E-08	GPAM	glycerol-3-phosphate acyltransferase, mitochondrial
2.7	2.53E-12	9.030E-11	GPR126	G protein-coupled receptor 126
2.7	2.51E-10	5.000E-09	RYR3	ryanodine receptor 3
2.7	6.02E-12	1.960E-10	TDP2	tyrosyl-DNA phosphodiesterase 2
2.7	5.36E-14	3.360E-12	VIL1	villin 1
2.7	9.13E-10	1.550E-08	C5H14ORF 159	chromosome 5 open reading frame, human C14orf159
2.7	7.68E-14	4.420E-12	DOCK9	dedicator of cytokinesis 9
2.7	8.69E-10	1.490E-08	TMEM135	transmembrane protein 135
2.7	9.89E-10	1.660E-08	KIAA1211	KIAA1211
2.7	4.07E-08	4.270E-07	FALG	Fas ligand
2.7	3.53E-12	1.230E-10	PISD	phosphatidylserine decarboxylase
2.6	3.46E-13	1.630E-11	CCL1	chemokine (C-C motif) ligand 1
2.6	1.67E-09	2.620E-08	TMEM37	transmembrane protein 37
2.6	2.75E-13	1.330E-11	TJAP1	tight junction associated protein 1 (peripheral)
2.6	5.89E-15	4.950E-13	MTSS1	metastasis suppressor 1
2.6	7.85E-09	1.020E-07	PRMT8	protein arginine methyltransferase 8
2.6	2.37E-10	4.780E-09	FBXO8	F-box protein 8
2.6	1.69E-10	3.580E-09	HADH	hydroxyacyl-CoA dehydrogenase
2.6	5.17E-11	1.270E-09	CD8A	CD8a molecule
2.6	1.42E-12	5.470E-11	GNE	glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase
2.6	1.02E-11	3.070E-10	LZTFL1	leucine zipper transcription factor-like 1
2.6	2.39E-11	6.510E-10	CYP4B1L	cytochrome P450 4B1-like
2.6	2.30E-10	4.650E-09	CD200R1L	CD200 receptor 1-like
2.6	1.30E-11	3.820E-10	SLC25A16	solute carrier family 25 (mitochondrial carrier), member 16
2.6	6.79E-11	1.610E-09	AQP5	Aquaporin 5

2.6	1.96E-10	4.040E-09	IL12RB2	interleukin 12 receptor, beta 2
2.5	2.73E-11	7.290E-10	AADACL2	arylacetamide deacetylase-like 2
2.5	3.16E-10	6.160E-09	GUCY2C	guanylate cyclase 2C
2.5	1.94E-12	7.210E-11	PARP8	poly (ADP-ribose) polymerase family, member 8
2.5	6.20E-09	8.310E-08	PER2	period circadian clock 2
2.5	1.32E-12	5.190E-11	ACSL4	acyl-CoA synthetase long-chain family member 4
2.5	8.19E-12	2.540E-10	ARHGAP10	Rho GTPase activating protein 10
2.5	1.00E-12	4.070E-11	C8H1ORF168	chromosome 8 open reading frame, human C1orf168
2.5	5.67E-05	2.000E-04	FBLN5	fibulin 5
2.5	8.40E-13	3.520E-11	TOX	thymocyte selection-associated high mobility group box
2.5	5.60E-14	3.480E-12	TOM1L2	target of myb1-like 2
2.5	2.75E-09	4.060E-08	RALGPS1	Ral GEF with PH domain and SH3 binding motif 1
2.5	4.02E-11	1.020E-09	agmat	agmatinase, mitochondrial precursor
2.5	2.39E-11	6.510E-10	GPR55	G protein-coupled receptor 55
2.5	4.56E-09	6.320E-08	SAT1	spermidine/spermine N1-acetyltransferase 1
2.5	8.01E-14	4.580E-12	TESK2	testis-specific kinase 2
2.5	3.97E-09	5.620E-08	EHHADH	enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase
2.5	5.78E-14	3.550E-12	ACOT11	acyl-CoA thioesterase 11
2.5	8.42E-12	2.590E-10	ADAMTS17	ADAM metallopeptidase with thrombospondin type 1 motif, 17
2.5	7.31E-12	2.310E-10	IGSF5	immunoglobulin superfamily, member 5
2.5	1.43E-12	5.490E-11	CORO2A	coronin, actin binding protein, 2A
2.5	3.31E-07	2.650E-06	MYOM2	myomesin 2
2.5	7.87E-12	2.460E-10	PPP1R16B	protein phosphatase 1, regulatory subunit 16B
2.5	2.97E-08	3.250E-07	RASD1	RAS, dexamethasone-induced 1
2.5	1.63E-07	1.440E-06	PPARA	peroxisome proliferator-activated receptor alpha
2.5	9.15E-13	3.760E-11	PRKCH	protein kinase C eta
2.5	6.78E-13	2.910E-11	PROSER2	proline and serine rich 2
2.5	8.06E-11	1.860E-09	RUFY2	RUN and FYVE domain containing 2
2.5	4.01E-09	5.670E-08	SEC22C	SEC22 homolog C, vesicle trafficking protein
2.5	5.94E-11	1.430E-09	SLC7A6	solute carrier family 7 (amino acid transporter light chain, y+L system), member 6
2.4	4.91E-11	1.220E-09	CCSER2	coiled-coil serine-rich protein 2
2.4	2.15E-06	1.370E-05	DNAH14	dynein, axonemal, heavy chain 14
2.4	1.70E-07	1.490E-06	ATP2B2	ATPase, Ca++ transporting, plasma membrane 2
2.4	1.49E-08	1.790E-07	CIDEA	cell death-inducing DFFA-like effector a
2.4	7.37E-10	1.290E-08	F11	coagulation factor XI
2.4	2.94E-06	1.800E-05	C8ORF22	chromosome 2 open reading frame, human C8orf22
2.4	2.79E-08	3.080E-07	KCNK5	potassium channel, two pore domain subfamily K, member 5
2.4	4.47E-12	1.520E-10	NUAK2	NUAK family, SNF1-like kinase, 2
2.4	2.56E-09	3.840E-08	OIT3	oncoprotein induced transcript 3
2.4	6.76E-10	1.200E-08	SLC16A6	solute carrier family 16, member 6
2.4	6.44E-13	2.770E-11	SST	Somatostatin Somatostatin-28 Somatostatin-14
2.4	1.82E-13	9.280E-12	EXOC3L4	exocyst complex component 3-like 4
2.4	8.63E-09	1.110E-07	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
2.4	7.14E-13	3.040E-11	PNPLA2	patatin-like phospholipase domain containing 2

2.4	8.21E-10	1.420E-08	SOSTDC1	sclerostin domain containing 1
2.4	2.63E-09	3.910E-08	FOXP2	forkhead box P2
2.4	3.74E-10	7.110E-09	SLC4A7	solute carrier family 4, sodium bicarbonate cotransporter, member 7
2.4	1.64E-10	3.490E-09	GRHPR	glyoxylate reductase/hydroxypyruvate reductase
2.4	2.19E-09	3.350E-08	HERC3	hect domain and RLD 3
2.4	1.35E-13	7.210E-12	JAKMIP1	janus kinase and microtubule interacting protein 1
2.4	2.32E-11	6.380E-10	ARHGAP36	Rho GTPase activating protein 36
2.4	1.69E-08	1.990E-07	BG1	BG-like antigen 1
2.4	5.52E-12	1.820E-10	C4H4orf50	chromosome 4 open reading frame, human C4orf50
2.4	4.11E-10	7.710E-09	CCBE1	collagen and calcium binding EGF domains 1
2.4	2.02E-12	7.430E-11	RIC8B	RIC8 guanine nucleotide exchange factor B
2.4	2.30E-06	1.450E-05	SLC30A10	solute carrier family 30, member 10
2.4	2.48E-10	4.960E-09	SPG20	spastic paraplegia 20 (Troyer syndrome)
2.4	6.79E-11	1.610E-09	SYTL5	synaptotagmin-like 5
2.4	2.85E-11	7.550E-10	DHRS11	dehydrogenase/reductase (SDR family) member 11
2.4	5.45E-08	5.510E-07	F7	coagulation factor VII (serum prothrombin conversion accelerator)
2.4	3.89E-09	5.520E-08	FRMD4B	FERM domain containing 4B
2.4	5.17E-14	3.280E-12	MXI1	MAX interactor 1, dimerization protein
2.4	6.09E-13	2.640E-11	TEC	tec protein tyrosine kinase
2.3	7.86E-08	7.610E-07	ATP8B1	ATPase, class I, type 8B, member 1
2.3	8.01E-11	1.850E-09	PTBP3	polypyrimidine tract binding protein 3
2.3	3.74E-07	2.940E-06	GPR112	G protein-coupled receptor 112
2.3	1.21E-12	4.830E-11	FAM13A	family with sequence similarity 13, member A
2.3	5.47E-10	9.870E-09	ZNF502	zinc finger protein 502
2.3	5.82E-11	1.410E-09	ABHD6	abhydrolase domain containing 6
2.3	3.87E-13	1.790E-11	IL2RB	interleukin-2 receptor subunit beta-like
2.3	1.13E-12	4.500E-11	TBX3	T-box 3
2.3	8.18E-09	1.060E-07	ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2 (Junior blood group)
2.3	5.58E-12	1.840E-10	PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
2.3	3.49E-13	1.650E-11	SLC30A1	solute carrier family 30 (zinc transporter), member 1
2.3	2.12E-09	3.250E-08	DUSP16	dual specificity phosphatase 16
2.3	7.91E-10	1.380E-08	IL18	interleukin 18
2.3	5.85E-08	5.850E-07	SOT3A1L	sulfotransferase family 3A, member 1-like
2.3	9.15E-13	3.760E-11	PELI2	pellino homolog 2 (Drosophila)
2.3	2.71E-11	7.270E-10	RAB40B	RAB40B, member RAS oncogene family
2.3	6.80E-11	1.610E-09	SLC37A4	solute carrier family 37 (glucose-6-phosphate transporter), member 4
2.3	4.00E-04	1.300E-03	KIRREL3	kirre like nephrin family adhesion molecule 3
2.3	2.25E-10	4.580E-09	LCORL	ligand dependent nuclear receptor corepressor-like
2.3	1.87E-10	3.900E-09	PAFAH2	platelet activating factor acetylhydrolase 2
2.3	1.72E-09	2.710E-08	TMEM231	Transmembrane Protein 231
2.3	4.89E-08	5.020E-07	ART1	ADP-ribosyltransferase 1
2.3	5.49E-11	1.340E-09	CNDP1	carnosine dipeptidase 1 (metallopeptidase M20 family)
2.3	3.63E-06	2.170E-05	MAB21L2	mab-21-like 2 (C. elegans)
2.3	2.43E-08	2.720E-07	PHYH	phytanoyl-CoA 2-hydroxylase

2.3	7.27E-08	7.130E-07	CCDC147	coiled-coil domain containing 147
2.3	4.14E-09	5.810E-08	RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)
2.3	2.49E-07	2.070E-06	SIK1	salt-inducible kinase 1
2.2	2.30E-14	1.590E-12	INPP5B	inositol polyphosphate-5-phosphatase, 75kDa
2.2	1.98E-07	1.710E-06	TGM4	transglutaminase 4 (prostate)
2.2	3.85E-09	5.470E-08	BCHE	butyrylcholinesterase
2.2	2.74E-05	1.000E-04	CSTA	cystatin A (stefin A)
2.2	6.78E-12	2.170E-10	NRIP1	nuclear receptor interacting protein 1
2.2	4.10E-10	7.700E-09	PECR	peroxisomal trans-2-enoyl-CoA reductase
2.2	2.85E-10	5.610E-09	RIPK3	receptor-interacting serine-threonine kinase 3
2.2	2.11E-07	1.790E-06	AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9
2.2	1.10E-07	1.020E-06	ATOH7	atonal bHLH transcription factor 7
2.2	3.10E-13	1.480E-11	OTUD7A	OTU domain containing 7A
2.2	1.44E-08	1.740E-07	RALY	RALY heterogeneous nuclear ribonucleoprotein
2.2	3.94E-10	7.470E-09	SH3BP1	SH3-domain binding protein 1
2.2	6.23E-12	2.000E-10	SLC25A22	solute carrier family 25 (mitochondrial carrier: glutamate), member 22
2.2	6.19E-11	1.490E-09	TMEM181	transmembrane protein 181
2.2	2.48E-11	6.730E-10	ABHD2	abhydrolase domain containing 2
2.2	6.76E-07	4.930E-06	CH17-360D5.1	neuropeptide Y receptor Y4
2.2	7.26E-12	2.300E-10	CLCN6	chloride channel, voltage-sensitive 6
2.2	2.36E-11	6.450E-10	DISP1	dispatched homolog 1 (Drosophila)
2.2	7.55E-09	9.840E-08	GPR180	G protein-coupled receptor 180
2.2	4.94E-09	6.790E-08	NPY6R	neuropeptide Y receptor Y6
2.2	2.52E-12	9.010E-11	RNPEP	arginyl aminopeptidase (aminopeptidase B)
2.2	7.64E-13	3.220E-11	CERS1	ceramide synthase 1
2.2	5.38E-07	4.030E-06	KBP	kainate binding protein
2.2	4.32E-06	2.540E-05	LAPTM4B	lysosomal protein transmembrane 4 beta
2.2	1.12E-08	1.390E-07	WWC2	WW and C2 domain containing 2
2.2	3.56E-09	5.140E-08	CAPN8	calpain 8
2.2	2.95E-10	5.790E-09	DAW1	dynein assembly factor with WDR repeat domains 1
2.2	4.79E-10	8.800E-09	GKAP1	G kinase anchoring protein 1
2.2	5.55E-08	5.610E-07	GPCPD1	glycerophosphocholine phosphodiesterase GDE1 homolog (S. cerevisiae)
2.2	5.83E-11	1.410E-09	IKZF2	IKAROS family zinc finger 2 (Helios)
2.2	4.81E-12	1.610E-10	LGALS2	lectin, galactoside-binding, soluble, 2
2.2	1.71E-10	3.610E-09	MCU	mitochondrial calcium uniporter
2.2	9.97E-10	1.670E-08	retreg1	reticulophagy regulator 1
2.2	1.98E-10	4.090E-09	TRAT1	T cell receptor associated transmembrane adaptor 1
2.2	1.44E-10	3.130E-09	CYP2J2L3	cytochrome P450, family 2, subfamily J, polypeptide 2-like 3
2.2	3.61E-09	5.210E-08	MCUR1	mitochondrial calcium uniporter regulator 1
2.2	1.62E-07	1.440E-06	PDCD2L	programmed cell death 2-like
2.2	1.51E-12	5.770E-11	SYTL3	synaptotagmin-like 3
2.2	4.76E-12	1.600E-10	TSHZ1	teashirt zinc finger homeobox 1
2.2	7.76E-10	1.350E-08	ATL2	atlastin GTPase 2
2.2	3.81E-09	5.440E-08	BMP3	bone morphogenetic protein 3

2.2	7.30E-08	7.160E-07	F2R	coagulation factor II (thrombin) receptor
2.2	1.31E-10	2.860E-09	PXDC1	PX domain containing 1
2.1	1.59E-10	3.410E-09	DOCK5	dedicator of cytokinesis 5
2.1	3.72E-11	9.520E-10	H6PD	hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
2.1	1.20E-14	8.880E-13	SRGAP3	SLIT-ROBO Rho GTPase activating protein 3
2.1	1.38E-12	5.370E-11	ACADL	acyl-CoA dehydrogenase, long chain
2.1	2.65E-12	9.430E-11	ARHGAP18	Rho GTPase activating protein 18
2.1	1.93E-12	7.170E-11	ARHGEF18	Rho/Rac guanine nucleotide exchange factor (GEF) 18
2.1	5.42E-12	1.800E-10	ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3
2.1	4.48E-06	2.620E-05	GRID2	glutamate receptor, ionotropic, delta 2
2.1	2.61E-07	2.160E-06	P2RX1	purinergic receptor P2X, ligand-gated ion channel, 1
2.1	6.09E-07	4.500E-06	CCL20	chemokine (C-C motif) ligand 20
2.1	1.25E-10	2.760E-09	COBL	cordon-bleu WH2 repeat protein
2.1	1.35E-11	3.920E-10	FGD3	FYVE, RhoGEF and PH domain containing 3
2.1	2.53E-09	3.810E-08	FNIP1	folliculin interacting protein 1
2.1	2.66E-10	5.270E-09	IL7R	interleukin 7 receptor
2.1	5.55E-10	9.980E-09	SLC35A5	solute carrier family 35, member A5
2.1	5.47E-08	5.530E-07	FNIP2	folliculin interacting protein 2
2.1	1.32E-06	8.880E-06	HEY2	hairy/enhancer-of-split related family bHLH transcription factor with YRPW motif
2.1	3.51E-10	6.760E-09	LRRC8B	leucine rich repeat containing 8 family, member B
2.1	3.84E-12	1.330E-10	PLIN4	Perilipin 4
2.1	4.80E-10	8.810E-09	RORA	RAR-related orphan receptor A
2.1	1.15E-09	1.880E-08	SAR1B	SAR1 homolog B (S. cerevisiae)
2.1	2.00E-04	7.000E-04	snoRNA RF00431	
2.1	2.76E-09	4.080E-08	ASAP2	ArfGAP with SH3 domain, ankyrin repeat and PH domain 2
2.1	1.95E-06	1.250E-05	HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)
2.1	4.44E-12	1.520E-10	LRRC16A	leucine rich repeat containing 16A
2.1	5.77E-08	5.790E-07	ABCC6	ATP-binding cassette, sub-family C (CFTR/MRP), member 6
2.1	1.57E-10	3.380E-09	CASP3	caspase 3, apoptosis-related cysteine peptidase
2.1	5.69E-10	1.020E-08	CD3D	CD3d molecule, delta (CD3-TCR complex)
2.1	1.39E-12	5.410E-11	MYRIP	myosin VIIA and Rab interacting protein
2.1	4.42E-11	1.110E-09	PARK2	parkin RBR E3 ubiquitin protein ligase
2.1	7.83E-11	1.810E-09	TYRO3	TYRO3 protein tyrosine kinase
2.1	3.10E-03	8.500E-03	ALDH1A3	aldehyde dehydrogenase 1 family, member A3
2.1	1.11E-06	7.660E-06	CCL19	C-C Motif Chemokine Ligand 19
2.1	1.16E-11	3.440E-10	CLCN5	chloride channel, voltage-sensitive 5
2.1	4.41E-08	4.580E-07	GPR18	G protein-coupled receptor 18
2.1	8.78E-08	8.390E-07	PARP14	poly (ADP-ribose) polymerase family, member 14; poly [ADP-ribose] polymerase 14-like
2.1	3.29E-11	8.580E-10	SLC8B1	solute carrier family 8 (sodium/lithium/calcium exchanger), member B1
2.1	2.95E-08	3.240E-07	CCR9	chemokine (C-C motif) receptor 9
2.1	4.25E-08	4.440E-07	CDC14A	cell division cycle 14°
2.1	2.00E-08	2.300E-07	FASLG	Fas ligand (TNF superfamily, member 6)
2.1	1.36E-06	9.110E-06	FLVCR1	feline leukemia virus subgroup C cellular receptor 1

2.1	5.31E-14	3.340E-12	EPN2	epsin 2
2.1	1.00E-10	2.270E-09	PPP1R3B	protein phosphatase 1, regulatory subunit 3B
2.1	5.24E-09	7.150E-08	RASGEF1C	RasGEF domain family, member 1C
2.1	6.57E-15	5.390E-13	ARHGAP26	Rho GTPase activating protein 26
2.1	3.63E-08	3.860E-07	C10ORF11	chromosome 6 open reading frame, human C10orf11
2.1	3.49E-10	6.740E-09	C8H1ORF21	chromosome 8 open reading frame, human C1orf21
2.1	2.67E-11	7.180E-10	CD3E	CD3e molecule, epsilon (CD3-TCR complex)
2.1	2.53E-12	9.040E-11	CPNE2	copine II
2.1	6.90E-09	9.120E-08	DOK6	docking protein 6
2.1	2.86E-06	1.760E-05	FSHR	follicle stimulating hormone receptor
2.1	4.94E-07	3.750E-06	HEBP1	heme binding protein 1
2.1	1.12E-07	1.040E-06	TPPP	tubulin polymerization promoting protein
2.1	4.52E-13	2.070E-11	WWC1	WW and C2 domain containing 1
2.0	9.07E-08	8.620E-07	ARAP2	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2
2.0	1.54E-09	2.450E-08	IQSEC1	IQ motif and Sec7 domain 1
2.0	1.89E-08	2.200E-07	SLC22A4	solute carrier family 22 (organic cation transporter), member 4
2.0	1.13E-07	1.040E-06	TXK	TXK tyrosine kinase
2.0	9.97E-08	9.380E-07	C26H6orf222	chromosome 26 open reading frame, human C6orf222
2.0	4.66E-12	1.580E-10	GRAP2	GRB2-related adaptor protein 2
2.0	8.86E-08	8.450E-07	KLHL38	kelch-like family member 38
2.0	5.73E-10	1.030E-08	PPIP5K1	diphosphoinositol pentakisphosphate kinase 1
2.0	6.02E-13	2.620E-11	STK17B	serine/threonine kinase 17b
2.0	9.37E-10	1.590E-08	VTCN1L	V-set domain containing T cell activation inhibitor 1-like
2.0	3.15E-07	2.540E-06	XCR1	chemokine (C motif) receptor 1
2.0	5.73E-12	1.870E-10	AMDHD2	amidohydrolase domain containing 2
2.0	3.83E-08	4.050E-07	CAPN9	calpain 9
2.0	1.34E-06	8.990E-06	DHCR7	7-dehydrocholesterol reductase
2.0	1.08E-10	2.420E-09	FAM126A	family with sequence similarity 126, member A
2.0	9.49E-11	2.160E-09	PHLPP2	PH domain and leucine rich repeat protein phosphatase 2
2.0	5.20E-09	7.090E-08	SGK1	serum/glucocorticoid regulated kinase 1
2.0	3.84E-08	4.060E-07	TMEM41B	transmembrane protein 41B
2.0	3.22E-09	4.690E-08	ACSL3	acyl-CoA synthetase long-chain family member 3
2.0	5.03E-10	9.130E-09	CCL5	chemokine (C-C motif) ligand 5
2.0	4.29E-11	1.080E-09	SLC1A7	solute carrier family 1 (glutamate transporter), member 7
2.0	3.64E-07	2.870E-06	FNDC7	fibronectin type III domain containing 7
2.0	1.75E-05	8.780E-05	NELL2	NEL-like 2
2.0	5.51E-10	9.930E-09	STS	steroid sulfatase (microsomal), isozyme S
2.0	3.72E-10	7.080E-09	TMEM65	transmembrane protein 65

Supplementary Table 3. **Upregulated assigned transcripts in cecum mucosa of broiler chickens at 42 days of (n=19), compared to jejunum.**

Fold Change	P-value	FDR P-value	Gene Symbol	Description
244.4	6.170E-21	4.18E-18	CBS	cystathionine-beta-synthase
78.4	1.190E-23	7.73E-20	MAL	mal, T-cell differentiation protein
40.8	1.420E-19	4.34E-17	AQP8	aquaporin 8
24.6	6.060E-16	6.6E-14	NOXO1	NADPH oxidase organizer 1
22.5	1.760E-14	1.24E-12	CA4	carbonic anhydrase IV
18.0	3.580E-18	7.28E-16	HOXA10	homeobox A10
17.9	3.440E-20	1.4E-17	SLC38A4	solute carrier family 38, member 4 (SNAT4)
15.8	2.110E-15	2.01E-13	SLC26A4	solute carrier family 26 (anion exchanger), member 4
15.1	5.970E-22	7.8E-19	PON2	paraoxonase 2
14.2	2.930E-21	2.55E-18	SELENBP1	selenium binding protein 1; selenium-binding protein 1-A-like
14.2	4.790E-15	4.14E-13	TFCP2L1	transcription factor CP2-like 1
14.0	7.890E-15	6.28E-13	ATP6V0D2	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2
13.2	3.340E-20	1.39E-17	PADI3	peptidyl arginine deiminase, type III
12.9	5.970E-15	4.99E-13	PLET1	Placenta Expressed Transcript 1
12.9	3.030E-14	2.05E-12	GJB2	gap junction protein, beta 2, 26kDa
12.0	8.160E-18	1.57E-15	LY6E	lymphocyte antigen 6 complex, locus E-like
11.2	8.740E-17	1.25E-14	GSTA4	glutathione S-transferase alpha 4
11.0	2.260E-23	1.03E-19	B4GALNT3	beta-1,4-N-acetyl-galactosaminyltransferase 3
10.4	1.160E-15	1.19E-13	CERS4	ceramide synthase 4
10.2	8.950E-19	2.23E-16	CDHR1	cadherin-related family member 1
9.8	9.030E-19	2.23E-16	SPTSSB	serine palmitoyltransferase, small subunit B
9.4	1.370E-12	5.37E-11	CYP2W1	cytochrome P450, family 2, subfamily W, polypeptide 1
9.3	1.440E-15	1.44E-13	SCNN1A	sodium channel, non-voltage-gated 1 alpha subunit
9.2	6.050E-16	6.6E-14	ENDOD1	endonuclease domain containing 1
9.0	1.210E-18	2.83E-16	SH3BGRL2	SH3 domain binding glutamic acid-rich protein like 2
8.9	3.790E-14	2.5E-12	DSE	dermatan sulfate epimerase
8.6	1.930E-17	3.51E-15	SATB2	SATB homeobox 2
8.6	2.840E-16	3.48E-14	ATP6V0A4	ATPase, H+ transporting, lysosomal V0 subunit a4
8.5	1.520E-16	2.01E-14	INF2	inverted formin, FH2 and WH2 domain containing
8.3	8.030E-16	8.45E-14	HOXA11	Homeobox protein Hox-A11
8.2	1.430E-08	1.72E-07	SLC26A3	solute carrier family 26 (anion exchanger), member 3
8.1	2.330E-15	2.19E-13	WDR72	WD repeat domain 72
7.9	1.660E-10	3.52E-09	GPRC5A	G protein-coupled receptor, family C, group 5, member A
7.9	3.440E-15	3.09E-13	ATP6V1G3	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G3

7.6	1.020E-16	1.39E-14	STMN2	stathmin-like 2
7.4	3.550E-15	3.17E-13	TLL2	tolloid like 2
7.3	8.690E-14	4.88E-12	SCNN1B	sodium channel, non voltage gated 1 beta subunit
7.2	7.340E-14	4.3E-12	NOV	nephroblastoma overexpressed
6.8	4.820E-17	7.54E-15	SFXN5	sideroflexin 5
6.7	1.670E-13	8.62E-12	SMOC1	SPARC related modular calcium binding 1
6.6	2.580E-09	3.86E-08	SPIC	Spi-C transcription factor (Spi-1/PU.1 related)
6.5	1.390E-17	2.57E-15	FAM101B	family with sequence similarity 101, member B
6.4	5.760E-16	6.36E-14	SLC16A14	solute carrier family 16, member 14
6.3	1.860E-16	2.4E-14	ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
6.2	1.010E-11	3.07E-10	TSPAN7	tetraspanin 7
6.2	2.830E-20	1.23E-17	HIGD1A	HIG1 domain family member 1A, mitochondrial
6.1	1.060E-09	1.75E-08	FNDC1	fibronectin type III domain containing 1
6.1	4.620E-17	7.3E-15	PAPSS2	3-phosphoadenosine 5-phosphosulfate synthase 2
5.8	1.890E-20	8.64E-18	HOXD8	Homeobox protein Hox-D8
5.7	3.830E-13	1.77E-11	BEST4	bestrophin 4
5.7	4.320E-14	2.78E-12	VILL	villin-like
5.6	1.650E-10	3.51E-09	LUM	lumican
5.5	6.580E-20	2.19E-17	HOXD3	homeobox D3
5.4	8.650E-13	3.61E-11	KCNJ15	potassium inwardly-rectifying channel, subfamily J, member 15
5.4	4.700E-13	2.12E-11	EMP1	epithelial membrane protein 1
5.4	5.460E-20	1.96E-17	LRRC42	leucine rich repeat containing 42
5.3	2.870E-15	2.64E-13	CHRDL1	chordin-like 1
5.3	3.620E-16	4.31E-14	GLIPR2	GLI pathogenesis-related 2
5.3	1.140E-09	1.87E-08	OGN	osteoglycin
5.2	3.450E-14	2.31E-12	HOXA10-AS	HOXA10 antisense RNA
5.1	3.650E-13	1.71E-11	SCNN1G	sodium channel, non voltage gated 1 gamma subunit
5.1	7.630E-13	3.22E-11	SCD	stearoyl-CoA desaturase (delta-9-desaturase)
5.0	3.730E-16	4.37E-14	LDHB	lactate dehydrogenase B
5.0	4.410E-17	7.09E-15	HOXD9	homeobox D9; homeobox protein Hox-D10
4.9	9.120E-17	1.28E-14	STEAP3	STEAP family member 3, metalloreductase
4.9	2.260E-18	5.04E-16	SEMA3G	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G
4.9	3.520E-17	5.81E-15	FRAS1	Fraser extracellular matrix complex subunit 1
4.8	1.070E-14	8.17E-13	EPHB3	EPH receptor B3
4.8	3.260E-13	1.55E-11	GUCY1A2	guanylate cyclase 1, soluble, alpha 2
4.7	2.150E-15	2.04E-13	FRY	furry homolog (Drosophila)
4.7	1.020E-16	1.39E-14	LIPG	lipase, endothelial
4.7	3.140E-16	3.75E-14	HOXD4	homeobox D4
4.7	2.750E-17	4.76E-15	APITD1	apoptosis-inducing, TAF9-like domain 1

4.7	2.720E-08	3.02E-07	ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide
4.7	4.750E-15	4.12E-13	MPST	mercaptopyruvate sulfurtransferase
4.6	3.980E-15	3.51E-13	LRP8	low density lipoprotein receptor-related protein 8
4.6	8.370E-12	2.58E-10	ITGB6	integrin, beta 6
4.6	3.220E-10	6.25E-09	ACTA2	actin, alpha 2, smooth muscle, aorta
4.5	4.920E-15	4.21E-13	HK1	hexokinase 1
4.5	5.020E-09	6.87E-08	ABI3BP	ABI family, member 3 (NESH) binding protein
4.5	1.410E-12	5.46E-11	VNN1	vanin 1
4.5	1.650E-14	1.18E-12	HK2	hexokinase-2
4.4	2.690E-18	5.8E-16	HSPB8	heat shock 22kDa protein 8
4.3	1.110E-10	2.47E-09	FAM26E	family with sequence similarity 26, member E
4.3	1.340E-14	9.71E-13	POSTN	periostin, osteoblast specific factor
4.3	8.680E-06	4.72E-05	LYGL	lysozyme g-like
4.3	8.640E-14	4.87E-12	DIO2	deiodinase, iodothyronine, type II
4.2	1.270E-10	2.79E-09	NRG1	neuregulin 1
4.2	5.020E-14	3.19E-12	HOXA9	homeobox A9; homeobox protein Hox-A9-like
4.2	1.500E-10	3.24E-09	KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)
4.2	2.620E-18	5.7E-16	PPARD	peroxisome proliferator-activated receptor delta
4.1	1.480E-12	5.69E-11	WLS	wntless Wnt ligand secretion mediator
4.1	1.010E-09	1.69E-08	S100B	S100 calcium binding protein B
4.1	1.600E-14	1.15E-12	DDAH1	dimethylarginine dimethylaminohydrolase 1
4.1	1.710E-13	8.76E-12	PRDX6	peroxiredoxin 6
4.0	1.390E-13	7.36E-12	CDCA7L	cell division cycle associated 7-like
4.0	3.760E-13	1.75E-11	SALL1	sal-like 1 (Drosophila)
4.0	1.600E-15	1.59E-13	ATP6V1C2	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C2
4.0	2.080E-12	7.58E-11	CMBL	carboxymethylenebutenolidase homolog (Pseudomonas)
4.0	2.320E-16	2.89E-14	BAG3	BCL2-associated athanogene 3
4.0	2.200E-17	3.87E-15	TXN	thioredoxin
4.0	5.620E-13	2.47E-11	BMP7	bone morphogenetic protein 7
4.0	2.320E-11	6.38E-10	GLDC	glycine dehydrogenase (decarboxylating)
3.9	2.970E-15	2.71E-13	TFRC	transferrin receptor (p90, CD71)
3.9	8.590E-12	2.63E-10	MYB	v-myb avian myeloblastosis viral oncogene homolog
3.9	8.950E-13	3.71E-11	FAM83D	family with sequence similarity 83, member D
3.9	5.300E-11	1.3E-09	KCNG3	potassium channel, voltage gated modifier subfamily G, member 3
3.9	1.930E-13	9.79E-12	TRPA1	transient receptor potential cation channel, subfamily A, member 1
3.8	1.420E-08	1.72E-07	AQP1	ACQUAPORIN 1
3.8	5.120E-07	3.87E-06	C4BPA	complement component 4 binding protein, alpha
3.8	4.930E-08	5.05E-07	PLK1	polo-like kinase 1

3.8	7.380E-08	7.21E-07	MKI67	marker of proliferation Ki-67
3.8	9.830E-15	7.69E-13	SESTD1	SEC14 and spectrin domains 1
3.8	1.950E-12	7.24E-11	PPARG	peroxisome proliferator-activated receptor gamma
3.8	6.460E-14	3.88E-12	HOXA6	homeobox A6; homeobox protein Hox-A6-like
3.8	5.800E-12	1.89E-10	MOXD1	monooxygenase, DBH
3.8	9.440E-14	5.26E-12	NDE1	nudE neurodevelopment protein 1
3.7	2.520E-11	6.82E-10	GEM	GTP binding protein overexpressed in skeletal muscle
3.7	2.970E-13	1.43E-11	PAQR5	progestin and adipoQ receptor family member V
3.7	8.520E-13	3.56E-11	LINGO1	leucine rich repeat and Ig domain containing 1
3.6	2.220E-12	8.07E-11	EPHB2	EPH receptor B2
3.6	1.280E-17	2.41E-15	KCNQ1	potassium channel, voltage gated KQT-like subfamily Q, member 1
3.6	4.370E-17	7.08E-15	FAM3D	family with sequence similarity 3, member D
3.6	1.020E-14	7.85E-13	GPR85	G protein-coupled receptor 85
3.6	9.100E-12	2.78E-10	HS3ST3B1L	heparan sulfate glucosamine 3-O-sulfotransferase 3B1-like
3.6	6.020E-09	8.09E-08	CCNB2	cyclin B2
3.6	3.050E-16	3.67E-14	FABP3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
3.6	4.700E-13	2.12E-11	LYPD6B	LY6/PLAUR domain containing 6B
3.6	1.750E-09	2.74E-08	CA7	carbonic anhydrase VII
3.6	1.830E-11	5.15E-10	SVEP1	sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1
3.6	5.100E-11	1.26E-09	GNG4	guanine nucleotide binding protein (G protein), gamma 4
3.6	6.290E-14	3.8E-12	NAALADL2	N-acetylated alpha-linked acidic dipeptidase-like 2
3.5	1.980E-12	7.31E-11	FOXI1	forkhead box I1
3.5	7.870E-09	1.02E-07	KIF4A	kinesin family member 4A
3.5	5.630E-12	1.85E-10	IGFBP5	Insulin Like Growth Factor Binding Protein 5
3.5	3.000E-10	5.86E-09	CYGB	cytoglobin
3.5	2.800E-10	5.52E-09	hoxa13	homeobox A13
3.5	5.680E-14	3.51E-12	ZBTB10	zinc finger and BTB domain containing 10
3.5	1.000E-04	0.0005	GAL3ST2	galactose-3-O-sulfotransferase 2
3.5	4.530E-13	2.07E-11	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1
3.5	1.260E-10	2.78E-09	AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)
3.5	8.680E-07	6.13E-06	GSTT1L	glutathione S-transferase theta 1-like
3.5	2.640E-13	1.29E-11	SASH1	SAM and SH3 domain containing 1
3.4	4.930E-12	1.65E-10	SLITRK2	SLIT and NTRK-like family, member 2
3.4	2.820E-13	1.36E-11	ATP8A2	ATPase, aminophospholipid transporter, class I, type 8A, member 2
3.4	1.340E-11	3.91E-10	AURKA	aurora kinase A
3.4	1.100E-11	3.28E-10	FRZB	frizzled-related protein
3.4	4.720E-11	1.18E-09	DUSP14	dual specificity phosphatase 14
3.4	5.280E-09	7.18E-08	THY1	Thy-1 cell surface antigen

3.4	3.180E-09	4.64E-08	LAMB1	laminin, beta 1
3.4	4.570E-12	1.55E-10	RXFP1	relaxin/insulin-like family peptide receptor 1
3.4	6.980E-11	1.65E-09	BMP4	bone morphogenetic protein 4
3.4	1.220E-09	1.99E-08	ROBO1	roundabout, axon guidance receptor, homolog 1 (Drosophila)
3.4	3.350E-11	8.71E-10	CSRP1	cysteine and glycine-rich protein 1
3.4	9.530E-13	3.89E-11	SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1
3.4	4.720E-13	2.12E-11	ETV4	ETS translocation variant 4-like; ets variant 4
3.4	1.880E-11	5.27E-10	NCAM1	neural cell adhesion molecule 1
3.3	2.910E-12	1.03E-10	GPRC6A	G protein-coupled receptor, class C, group 6, member A
3.3	2.660E-13	1.29E-11	PGD	phosphogluconate dehydrogenase
3.3	3.680E-08	3.9E-07	BUB1	BUB1 mitotic checkpoint serine/threonine kinase
3.3	2.900E-08	3.18E-07	TAGLN	Transgelin
3.3	7.250E-14	4.27E-12	SLC8A1	solute carrier family 8 (sodium/calcium exchanger), member 1
3.3	4.960E-15	4.23E-13	HDAC1	histone deacetylase 1
3.3	2.170E-13	1.08E-11	RNF223	ring finger protein 223
3.3	3.430E-10	6.64E-09	TTC38	tetratricopeptide repeat domain 38
3.3	8.100E-14	4.62E-12	ESYT3	extended synaptotagmin-like protein 3
3.3	6.800E-09	9.01E-08	ifitm1	interferon-induced transmembrane protein 3-like
3.3	4.610E-13	2.1E-11	WIF1	WNT inhibitory factor 1
3.2	1.240E-09	2.01E-08	VIPR2	vasoactive intestinal peptide receptor 2
3.2	5.230E-11	1.28E-09	CTGF	connective tissue growth factor
3.2	2.050E-16	2.61E-14	ARHGEF10L	Rho guanine nucleotide exchange factor (GEF) 10-like
3.2	3.870E-14	2.54E-12	ATP2A3	ATPase, Ca++ transporting, ubiquitous
3.2	2.100E-17	3.73E-15	CA9	carbonic anhydrase IX
3.2	4.050E-10	7.64E-09	PHLDA2	pleckstrin homology-like domain, family A, member 2
3.2	1.030E-08	1.29E-07	TOP2A	topoisomerase (DNA) II alpha 170kDa
3.2	3.390E-07	2.7E-06	USP18	ubiquitin specific peptidase 18
3.2	1.110E-15	1.15E-13	SMS	spermine synthase
3.2	3.800E-11	9.69E-10	ANXA1	annexin A1
3.2	6.210E-12	2E-10	ASL2	argininosuccinate lyase
3.2	2.590E-09	3.87E-08	CYR61	cysteine-rich, angiogenic inducer, 61
3.2	7.090E-10	1.25E-08	WNT5A	wingless-type MMTV integration site family, member 5A
3.2	4.120E-07	3.2E-06	CRISPLD2	cysteine-rich secretory protein LCCL domain containing 2
3.2	6.050E-15	5.03E-13	HSPB11	heat shock protein family B (small), member 11
3.2	4.910E-10	8.98E-09	LGR5	leucine-rich repeat containing G protein-coupled receptor 5
3.2	6.220E-13	2.69E-11	UGDH	UDP-glucose 6-dehydrogenase
3.1	6.130E-09	8.22E-08	RRM2	ribonucleotide reductase M2
3.1	1.340E-10	2.93E-09	ECT2	epithelial cell transforming 2
3.1	5.520E-10	9.94E-09	ITGA8	integrin, alpha 8

3.1	1.740E-11	4.95E-10	LGALS1	lectin, galactoside-binding, soluble, 1
3.1	1.030E-13	5.7E-12	DIAPH3	diaphanous-related formin 3
3.1	4.010E-07	3.12E-06	TNFAIP2	tumor necrosis factor, alpha-induced protein 2
3.1	1.360E-11	3.96E-10	MLLT3	myeloid/lymphoid or mixed-lineage leukemia translocated to chromosome 3
3.1	5.300E-09	7.2E-08	TUBA3E	tubulin, alpha 3e; anche introne di csrp1 (Cysteine and glycine-rich protein 1)
3.1	3.190E-11	8.34E-10	MET	met proto-oncogene; hepatocyte growth factor receptor-like
3.1	5.870E-08	5.87E-07	GTSE1	G2 and S phase-expressed protein 1
3.1	3.740E-12	1.3E-10	CA2	carbonic anhydrase II
3.1	6.690E-12	2.14E-10	NUF2	NUF2, NDC80 kinetochore complex component
3.1	1.500E-11	4.31E-10	OSR1	odd-skipped related transciption factor 1
3.1	2.570E-11	6.96E-10	PARVB	parvin, beta
3.0	3.250E-13	1.55E-11	B3GAT2	beta-1,3-glucuronyltransferase 2
3.0	4.600E-14	2.94E-12	SP8	Sp8 transcription factor
3.0	4.710E-12	1.59E-10	CCDC80	coiled-coil domain containing 80
3.0	2.330E-05	0.0001	CXCR5	chemokine (C-X-C motif) receptor 5
3.0	2.710E-14	1.85E-12	FANCA	Fanconi anemia, complementation group A
3.0	2.040E-12	7.48E-11	GLIPR1L	GLI pathogenesis-related 1-like
3.0	3.510E-07	2.79E-06	IL8L1	interleukin 8-like 1
3.0	1.050E-09	1.74E-08	CAPN5	calpain 5
3.0	3.550E-08	3.79E-07	HOXA3	homeobox A3
3.0	1.540E-11	4.43E-10	FGF7	fibroblast growth factor 7
3.0	6.640E-10	1.18E-08	RRM1	ribonucleotide reductase M1
3.0	2.400E-10	4.83E-09	RBPMS2	RNA binding protein with multiple splicing 2
3.0	3.290E-05	0.0002	RSAD2	radical S-adenosyl methionine domain containing 2
3.0	6.350E-15	5.23E-13	TXNRD3	thioredoxin reductase 3
3.0	3.640E-15	3.22E-13	ANKRD22	ankyrin repeat domain 22
3.0	2.200E-11	6.07E-10	TTK	TTK protein kinase
3.0	1.960E-14	1.37E-12	UNC119	unc-119 homolog (C. elegans)
3.0	5.610E-17	8.67E-15	PLA2G4F	phospholipase A2, group IVF; vacuolar protein sorting 39 homolog (S. cerevisiae)
3.0	4.020E-10	7.57E-09	SMC2	structural maintenance of chromosomes 2
3.0	2.400E-06	1.51E-05	AVD	Avidin
3.0	6.840E-08	6.76E-07	CASC5	cancer susceptibility candidate 5
3.0	2.090E-08	2.4E-07	TPX2	TPX2, microtubule-associated
2.9	2.770E-11	7.38E-10	RXFP2	relaxin/insulin-like family peptide receptor 2,)
2.9	2.660E-13	1.29E-11	SULT1E1	sulfotransferase family 1E, estrogen-preferring, member 1
2.9	3.670E-09	5.27E-08	VWF	von Willebrand factor
2.9	4.090E-09	5.76E-08	ADAMTS15	ADAM metallopeptidase with thrombospondin type 1 motif, 15
2.9	1.690E-07	1.49E-06	B3GALT5	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5
2.9	1.010E-06	7.01E-06	PDGFD	platelet derived growth factor D

2.9	1.780E-11	5.04E-10	PSD3	pleckstrin and Sec7 domain containing 3
2.9	3.190E-12	1.12E-10	ETV1	ets variant 1
2.9	4.890E-13	2.17E-11	MID1	midline 1 (Opitz/BBB syndrome)
2.9	2.870E-07	2.35E-06	NUSAP1	nucleolar and spindle associated protein 1
2.9	6.820E-17	1.02E-14	AOX1	aldehyde oxidase 1
2.9	5.130E-10	9.31E-09	ARL14	ADP-ribosylation factor-like protein 14
2.9	6.850E-14	4.07E-12	FAM155B	family with sequence similarity 155, member B
2.9	1.450E-13	7.63E-12	FAR1	fatty acyl CoA reductase 1
2.9	1.800E-11	5.1E-10	ADAMTSL2	ADAMTS-like 2
2.9	3.820E-11	9.7E-10	APOH	apolipoprotein H (beta-2-glycoprotein I)
2.9	5.150E-13	2.27E-11	EPB41L2	erythrocyte membrane protein band 4.1-like 2
2.9	6.320E-09	8.42E-08	HMMR	hyaluronan-mediated motility receptor (RHAMM)
2.9	3.320E-14	2.23E-12	HOMER1	homer scaffolding protein 1
2.9	2.780E-18	5.92E-16	HOXB9	homeobox B9
2.9	1.510E-16	2.01E-14	ERO1L	ERO1-like (S. cerevisiae)
2.9	3.670E-08	3.9E-07	AIFM2	apoptosis-inducing factor, mitochondrion-associated, 2
2.9	6.090E-13	2.64E-11	ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1
2.9	6.440E-06	3.61E-05	CYYR1	cysteine/tyrosine-rich 1
2.9	1.520E-12	5.8E-11	KHDRBS3	KH domain containing, RNA binding, signal transduction associated 3
2.9	3.700E-14	2.45E-12	PLEKHA6	pleckstrin homology domain containing, family A member 6
2.8	3.770E-13	1.75E-11	FAM20A	family with sequence similarity 20, member A
2.8	3.640E-14	2.42E-12	SLC16A3	solute carrier family 16 (monocarboxylate transporter), member 3
2.8	2.930E-09	4.3E-08	CKAP2	cytoskeleton associated protein 2
2.8	5.130E-12	1.71E-10	CTNND2	catenin (cadherin-associated protein), delta 2
2.8	1.350E-13	7.19E-12	EML1	echinoderm microtubule associated protein like 1
2.8	1.140E-15	1.18E-13	ICA1	islet cell autoantigen 1, 69kDa
2.8	2.500E-12	8.96E-11	KIAA1210	KIAA1210
2.8	1.080E-13	5.93E-12	BEND6	BEN domain containing 6
2.8	1.560E-09	2.48E-08	CTH	cystathionine gamma-lyase
2.8	2.800E-08	3.09E-07	SYNPO2	synaptopodin 2
2.8	7.520E-15	6.06E-13	SLC16A1	solute carrier family 16 (monocarboxylate transporter), member 1
2.8	4.290E-09	5.98E-08	LMNB2	lamin B2
2.8	1.050E-08	1.31E-07	ATP10B	ATPase, class V, type 10B
2.8	2.450E-12	8.81E-11	CCDC60	coiled-coil domain containing 60
2.8	7.390E-11	1.73E-09	CDK1	cyclin-dependent kinase 1
2.8	3.260E-13	1.55E-11	EMILIN2	elastin microfibril interfacer 2
2.8	7.570E-12	2.38E-10	FSTL4	follistatin-like 4
2.8	8.320E-11	1.92E-09	MPP6	membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)
2.8	3.820E-06	2.27E-05	MXRA5	matrix-remodelling associated 5

2.8	1.620E-10	3.46E-09	ANLN	anillin actin binding protein
2.8	2.070E-08	2.37E-07	ASS1	argininosuccinate synthase
2.8	8.030E-17	1.16E-14	MYO10L	myosin-X-like
2.7	4.610E-08	4.77E-07	CENPF	centromere protein F, 350/400kDa
2.7	2.080E-07	1.77E-06	FGFBP1	fibroblast growth factor binding protein 1
2.7	1.150E-08	1.42E-07	CDH13	cadherin 13
2.7	5.460E-12	1.81E-10	LPCAT2	lysophosphatidylcholine acyltransferase 2
2.7	5.940E-09	8.01E-08	FHL2	four and a half LIM domains 2
2.7	1.510E-10	3.26E-09	SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A
2.7	1.390E-06	9.3E-06	CXCL13L2	chemokine
2.7	1.630E-15	1.61E-13	GMDS	GDP-mannose 4,6-dehydratase
2.7	2.510E-08	2.8E-07	KIF20A	kinesin family member 20A
2.7	9.360E-09	1.19E-07	NDC80	NDC80 kinetochore complex component
2.7	6.510E-11	1.56E-09	PBK	PDZ binding kinase
2.7	4.410E-09	6.12E-08	TPBG	trophoblast glycoprotein
2.7	1.670E-09	2.63E-08	CDC20	cell division cycle 20
2.7	8.360E-12	2.58E-10	HSBP1L1	heat shock factor binding protein 1-like 1
2.7	2.270E-10	4.61E-09	TMEM213	transmembrane protein 213
2.7	2.720E-11	7.28E-10	CHPT1	choline phosphotransferase 1
2.7	1.190E-11	3.51E-10	PLAC9	placenta-specific 9
2.7	2.220E-11	6.13E-10	PYCR1	pyrroline-5-carboxylate reductase 2
2.7	4.980E-08	5.1E-07	ARHGEF39	Rho guanine nucleotide exchange factor (GEF) 39
2.7	1.890E-09	2.92E-08	BUB1B	BUB1 mitotic checkpoint serine/threonine kinase B
2.7	2.900E-13	1.4E-11	METRNL	meteorin, glial cell differentiation regulator-like
2.7	8.220E-09	1.06E-07	PLK4	polo-like kinase 4
2.7	3.160E-12	1.11E-10	ST5	suppression of tumorigenicity 5
2.7	3.280E-08	3.54E-07	MYBL1	v-myb avian myeloblastosis viral oncogene homolog-like 1
2.7	4.680E-09	6.47E-08	RACGAP1	Rac GTPase activating protein 1
2.7	4.680E-10	8.62E-09	TPH1	tryptophan hydroxylase 1
2.6	4.700E-13	2.12E-11	ATOH8	protein atonal homolog 8
2.6	1.400E-12	5.43E-11	TPST1	tyrosylprotein sulfotransferase 1
2.6	3.520E-10	6.76E-09	arsh	arylsulfatase family, member H
2.6	4.770E-13	2.14E-11	MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)
2.6	5.110E-08	5.2E-07	GCNT4	glucosaminyl (N-acetyl) transferase 4, core 2
2.6	5.410E-11	1.32E-09	TSPO	translocator protein (18kDa)
2.6	1.680E-10	3.55E-09	BORA	bora, aurora kinase A activator
2.6	1.060E-11	3.19E-10	CLCNKB	chloride channel, voltage-sensitive Kb
2.6	1.240E-11	3.67E-10	INTU	inturned planar cell polarity protein
2.6	2.160E-12	7.89E-11	PPP1R9A	protein phosphatase 1, regulatory subunit 9A
2.6	1.270E-13	6.82E-12	AMD1	adenosylmethionine decarboxylase 1
2.6	2.210E-09	3.37E-08	SLC16A5	solute carrier family 16 (monocarboxylate transporter), member 5

2.6	1.620E-09	2.56E-08	KIF2C	kinesin-like protein KIF2C-like; kinesin family member 2C
2.6	1.630E-06	1.07E-05	ME1	malic enzyme 1, NADP(+)-dependent, cytosolic
2.6	1.360E-10	2.96E-09	SERTAD4	SERTA domain containing 4
2.6	7.070E-06	3.93E-05	COL6A2	collagen, type VI, alpha 2
2.6	1.820E-10	3.8E-09	KLF5	Kruppel-like factor 5 (intestinal)
2.6	3.100E-07	2.5E-06	NEK2	NIMA-related kinase 2
2.5	1.310E-12	5.16E-11	AGPAT5	1-acylglycerol-3-phosphate O-acyltransferase 5
2.5	3.510E-08	3.76E-07	KIF15	kinesin family member 15
2.5	1.820E-10	3.8E-09	LPHN2	latrophilin 2
2.5	1.660E-11	4.72E-10	CDC7	cell division cycle 7
2.5	1.440E-09	2.31E-08	NEXN	nexilin (F actin binding protein)
2.5	1.300E-10	2.85E-09	SMOC2	SPARC related modular calcium binding 2
2.5	1.050E-09	1.74E-08	AHNAK2	protein AHNAK2-like
2.5	5.930E-06	3.35E-05	MGP	matrix Gla protein
2.5	4.290E-14	2.78E-12	PTDSS2	phosphatidylserine synthase 2
2.5	8.470E-11	1.95E-09	CEP55	centrosomal protein 55kDa
2.5	1.160E-07	1.07E-06	ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)
2.5	5.660E-11	1.37E-09	GREM2	gremlin 2, DAN family BMP antagonist
2.5	2.000E-12	7.35E-11	HOXA4	homeobox A4; homeobox protein Hox-A4-like
2.5	1.270E-10	2.79E-09	MLF1	myeloid leukemia factor 1
2.5	2.710E-06	1.68E-05	ACTG2	Actin, gamma-enteric smooth muscle
2.5	1.280E-05	0.000066	AKAP12	A kinase (PRKA) anchor protein 12
2.5	4.220E-09	5.89E-08	CBLN1	cerebellin 1 precursor
2.5	2.420E-10	4.87E-09	ELOVL6	ELOVL fatty acid elongase 6
2.5	2.050E-07	1.76E-06	MCM5	minichromosome maintenance complex component 5
2.5	1.830E-05	9.12E-05	PAX5	paired box 5
2.5	5.800E-13	2.54E-11	RAPGEF5	Rap guanine nucleotide exchange factor (GEF) 5
2.5	2.460E-12	8.84E-11	CLSTN1	calsyntenin 1
2.5	4.930E-08	5.05E-07	DLGAP5	discs, large (Drosophila) homolog-associated protein 5
2.5	1.880E-10	3.91E-09	ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5
2.5	3.060E-10	5.98E-09	CTNS	cystinosin, lysosomal cystine transporter
2.5	3.020E-11	7.97E-10	SPC25	SPC25, NDC80 kinetochore complex component
2.5	3.330E-09	4.83E-08	EAF2	ELL associated factor 2
2.5	1.060E-13	5.84E-12	GOLM1	golgi membrane protein 1
2.5	7.650E-08	7.44E-07	MCM3	minichromosome maintenance complex component 3
2.5	7.310E-13	3.09E-11	C16ORF45	chromosome 14 open reading frame, human C16orf45
2.5	9.080E-09	1.16E-07	CENPK	centromere protein K
2.5	8.390E-07	5.95E-06	CXCL12	chemokine (C-X-C motif) ligand 12
2.5	4.160E-09	5.83E-08	DACT2	dishevelled-binding antagonist of beta-catenin 2

2.5	1.250E-08	1.54E-07	FAM3B	family with sequence similarity 3, member B
2.5	3.740E-09	5.35E-08	LAMA5	laminin, alpha 5
2.5	4.930E-09	6.78E-08	MELK	maternal embryonic leucine zipper kinase; maternal embryonic leucine zipper kinase-like
2.5	6.740E-06	3.76E-05	MYH11	Myosin-11
2.5	2.650E-11	7.13E-10	NMRAL1	NmrA-like family domain containing 1
2.5	8.490E-09	1.09E-07	TMEM248	transmembrane protein 248
2.5	4.900E-07	3.72E-06	VCAN	versican core protein precursor
2.4	7.220E-15	5.87E-13	ENAH	enabled homolog (Drosophila)
2.4	2.710E-06	1.67E-05	HVCN1	hydrogen voltage gated channel 1
2.4	1.790E-09	2.79E-08	PRC1	protein regulator of cytokinesis 1
2.4	1.330E-11	3.88E-10	TACC3	transforming, acidic coiled-coil containing protein 3
2.4	9.170E-11	2.09E-09	CENPL	centromere protein L
2.4	1.370E-08	1.66E-07	S100A11	S100 calcium binding protein A11
2.4	8.420E-10	1.45E-08	CLMN	calmin (calponin-like, transmembrane)
2.4	2.770E-14	1.88E-12	PARM1	prostate androgen-regulated mucin-like protein 1
2.4	7.670E-14	4.42E-12	WISP1	WNT1 inducible signaling pathway protein 1
2.4	1.540E-13	7.99E-12	ENY2	enhancer of yellow 2 homolog (Drosophila)
2.4	3.520E-12	1.23E-10	FASN	fatty acid synthase; serine/arginine repetitive matrix protein 2-like
2.4	3.780E-11	9.66E-10	PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase
2.4	1.960E-10	4.04E-09	PLAC8L1	Placenta-specific 8-like 1
2.4	1.100E-09	1.81E-08	TRPC6	transient receptor potential cation channel, subfamily C, member 6
2.4	7.130E-12	2.26E-10	VLDLR	very low density lipoprotein receptor
2.4	1.370E-08	1.67E-07	ACPP	acid phosphatase, prostate
2.4	2.630E-11	7.08E-10	CHST10	carbohydrate sulfotransferase 10
2.4	1.500E-13	7.84E-12	DEPTOR	DEP domain containing MTOR-interacting protein
2.4	1.760E-10	3.7E-09	IGF2	insulin-like growth factor 2
2.4	2.840E-11	7.54E-10	MYO1C	myosin IC
2.4	6.910E-13	2.95E-11	WHSC1	Wolf-Hirschhorn syndrome candidate 1
2.4	1.510E-10	3.26E-09	PCDH18	protocadherin 18
2.4	1.400E-09	2.25E-08	PDLIM3	PDZ and LIM domain 3
2.4	4.680E-06	2.73E-05	EBF1	early B-cell factor 1
2.4	1.300E-06	8.77E-06	NID1	nidogen 1
2.4	1.230E-07	1.13E-06	NOX1	NADPH oxidase 1
2.4	2.170E-08	2.47E-07	ANXA6	annexin A6
2.4	7.560E-11	1.76E-09	AXIN2	axin 2
2.4	1.080E-05	5.71E-05	COL6A1	collagen, type VI, alpha 1
2.4	4.360E-08	4.54E-07	FST	follistatin
2.4	3.160E-08	3.43E-07	INCENP	inner centromere protein antigens 135/155kDa

2.4	4.080E-09	5.75E-08	KIF14	kinesin family member 14
2.4	5.330E-10	9.64E-09	MEIS1	lINGrna
2.4	6.930E-07	5.04E-06	PDGFRB	platelet-derived growth factor receptor, beta polypeptide
2.4	5.330E-08	5.41E-07	DGUOK	Deoxyadenosine kinase
2.4	9.640E-09	1.22E-07	MBOAT2	membrane bound O-acyltransferase domain containing 2
2.4	1.520E-08	1.81E-07	SLC12A2	solute carrier family 12 (sodium/potassium/chloride transporter), member 2
2.3	2.210E-09	3.37E-08	PRRT1B	Proline Rich Transmembrane Protein 1B
2.3	4.420E-13	2.04E-11	TST	thiosulfate sulfurtransferase (rhodanese)
2.3	8.820E-10	1.5E-08	ACRC	acidic repeat containing
2.3	4.780E-07	3.65E-06	DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides
2.3	1.100E-08	1.38E-07	P2RY14	purinergic receptor P2Y, G-protein coupled, 14
2.3	4.970E-09	6.82E-08	PIK3C2B	phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 beta
2.3	3.080E-05	0.0001	SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)
2.3	1.000E-04	0.0004	snoRNA RF00138	
2.3	8.750E-09	1.12E-07	BIRC5	baculoviral IAP repeat containing 5
2.3	2.090E-10	4.29E-09	CD200L	CD200 molecule-like
2.3	2.250E-10	4.58E-09	CENPI	centromere protein I
2.3	2.880E-06	1.77E-05	GPC1	glypican 1
2.3	1.970E-08	2.27E-07	HGF	hepatocyte growth factor (hepapoietin A; scatter factor)
2.3	2.100E-08	2.4E-07	PPDPF	pancreatic progenitor cell differentiation and proliferation factor
2.3	1.740E-08	2.04E-07	PRELP	proline/arginine-rich end leucine-rich repeat protein
2.3	5.550E-14	3.46E-12	SLC26A5	solute carrier family 26 (anion exchanger), member 5
2.3	2.090E-10	4.29E-09	STON2	stonin 2
2.3	7.060E-10	1.25E-08	CIT	citron rho-interacting serine/threonine kinase
2.3	4.240E-07	3.27E-06	GRTP1	growth hormone regulated TBC protein 1
2.3	2.250E-07	1.89E-06	MBOAT1	membrane bound O-acyltransferase domain containing 1
2.3	8.690E-11	1.99E-09	RHOC	ras homolog family member C
2.3	9.700E-10	1.63E-08	TMTC2	transmembrane and tetratricopeptide repeat containing 2
2.3	3.270E-09	4.74E-08	DMD	dystrophin
2.3	4.820E-09	6.64E-08	DUT	deoxyuridine triphosphatase
2.3	1.740E-12	6.54E-11	MFGE8	milk fat globule-EGF factor 8 protein
2.3	8.420E-07	5.97E-06	SLBP	stem-loop binding protein
2.3	7.290E-10	1.28E-08	GAD1	glutamate decarboxylase 1 (brain, 67kDa)
2.3	3.810E-11	9.69E-10	ATRNL1	attractin-like 1
2.3	7.050E-07	5.11E-06	CCNA2	cyclin A2
2.3	8.880E-13	3.69E-11	DAGLA	diacylglycerol lipase, alpha
2.3	4.020E-05	0.0002	GJA1	gap junction protein, alpha 1, 43kDa

2.3	8.700E-11	1.99E-09	ITGAV	integrin, alpha V
2.3	1.590E-08	1.89E-07	LAMP5	lysosomal-associated membrane protein family, member 5
2.3	1.940E-06	1.25E-05	CAP2	CAP, adenylate cyclase-associated protein, 2 (yeast)
2.3	3.250E-13	1.55E-11	CASP8	caspase 8, apoptosis-related cysteine peptidase
2.3	3.590E-11	9.27E-10	FOXP4	Forkhead Box P4
2.3	4.150E-07	3.22E-06	GNG12	guanine nucleotide binding protein (G protein), gamma 12
2.3	5.740E-08	5.77E-07	PNAT3	Arylamine N-acetyltransferase, pineal gland isozyme NAT-3
2.3	2.430E-09	3.66E-08	SHCBP1	SHC SH2-domain binding protein 1
2.3	3.830E-07	0.000003	CDCA7	cell division cycle associated 7
2.3	3.760E-06	2.24E-05	FSTL1	follistatin-like 1
2.3	2.420E-08	2.71E-07	NCOA7	nuclear receptor coactivator 7
2.3	3.580E-08	3.82E-07	STEAP2	STEAP family member 2, metalloreductase
2.3	3.560E-05	0.0002	EDNRB	endothelin receptor type B
2.3	1.020E-08	1.28E-07	GAS2L3	growth arrest-specific 2 like 3
2.3	1.190E-07	1.09E-06	GPR20	G protein-coupled receptor 20
2.3	1.100E-05	5.83E-05	HIST1H2B7	Histone H2B 1/2/3/4/6
2.3	6.770E-07	4.93E-06	JAM2	junctional adhesion molecule 2
2.2	1.230E-08	1.51E-07	FAM20C	family with sequence similarity 20, member C
2.2	6.390E-09	8.51E-08	KNSTRN	kinetochore-localized astrin/SPAG5 binding protein
2.2	7.600E-11	1.77E-09	PAQR8	progestin and adipoQ receptor family member VIII
2.2	1.080E-09	1.78E-08	PDE1A	phosphodiesterase 1A, calmodulin-dependent
2.2	5.490E-07	4.11E-06	TUBB2A	tubulin, beta 2A class IIa
2.2	1.870E-11	5.25E-10	BARD1	BRCA1 associated RING domain 1
2.2	6.760E-11	1.61E-09	EPB41L1	erythrocyte membrane protein band 4.1-like 1
2.2	3.950E-08	4.16E-07	KIF11	kinesin family member 11
2.2	9.930E-10	1.66E-08	RHOB	ras homolog family member B
2.2	1.090E-05	5.78E-05	TAAR1	trace amine associated receptor 1
2.2	8.270E-10	1.43E-08	KIAA1524	KIAA1524
2.2	4.830E-08	4.98E-07	NT5DC2	5-nucleotidase domain containing 2
2.2	5.730E-10	1.03E-08	PTTG1	pituitary tumor-transforming 1
2.2	3.360E-08	3.61E-07	RARRES1	retinoic acid receptor responder (tazarotene induced) 1
2.2	9.590E-06	5.16E-05	SLC7A5	solute carrier family 7 (amino acid transporter light chain, L system), member 5
2.2	4.930E-10	9E-09	AASS	aminoadipate-semialdehyde synthase
2.2	1.570E-09	2.5E-08	CCDC13	coiled-coil domain containing 13
2.2	5.070E-09	6.93E-08	CDX2	caudal type homeobox 2
2.2	1.180E-05	6.18E-05	E2F7	E2F transcription factor 7
2.2	4.190E-10	7.84E-09	HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B member 1

2.2	2.160E-07	1.83E-06	MFSD10	major facilitator superfamily domain containing 10
2.2	1.810E-12	6.75E-11	SLC17A9	solute carrier family 17 (vesicular nucleotide transporter), member 9
2.2	3.630E-09	5.23E-08	ST6GAL2	ST6 beta-galactosamide alpha-2,6-sialyltranferase 2
2.2	1.460E-13	7.69E-12	ATP2B4	ATPase, Ca++ transporting, plasma membrane 4
2.2	1.340E-06	9.02E-06	MYL9	myosin, light chain 9, regulatory
2.2	7.290E-14	4.28E-12	PLEKHB2	pleckstrin homology domain containing, family B (evectins) member 2
2.2	6.510E-07	4.77E-06	SDPR	serum deprivation response
2.2	9.480E-12	2.89E-10	TERF1	telomeric repeat binding factor (NIMA-interacting) 1
2.2	3.590E-10	6.88E-09	TIMP4	TIMP metallopeptidase inhibitor 4
2.2	2.800E-11	7.43E-10	PRRX2	paired related homeobox 2
2.2	2.650E-11	7.13E-10	RNF144B	ring finger protein 144B
2.2	7.910E-08	7.65E-07	ROPN1L	rhophilin associated tail protein 1-like
2.2	3.840E-09	5.47E-08	SLC9A3R2	solute carrier family 9, subfamily A (NHE3, cation proton antiporter 3), member 3 regulator 2
2.2	1.330E-07	1.21E-06	AFF3	AF4/FMR2 family, member 3
2.2	7.750E-08	7.53E-07	ARVCF	armadillo repeat gene deleted in velocardiofacial syndrome
2.2	1.210E-08	1.5E-07	LHFP	lipoma HMGIC fusion partner
2.2	2.300E-08	2.6E-07	NPM3	nucleophosmin/nucleoplasmin 3
2.2	3.510E-05	0.0002	PDGFRA	platelet derived growth factor receptor alpha
2.2	3.640E-11	9.34E-10	TTC26	tetratricopeptide repeat domain 26
2.2	1.460E-08	1.75E-07	VIM	vimentin
2.2	2.200E-10	4.47E-09	BRCA1	breast cancer 1, early onset
2.2	3.040E-08	3.32E-07	IL22RA2	interleukin 22 receptor, alpha 2
2.2	9.670E-08	9.14E-07	LRRC26	leucine rich repeat containing 26
2.2	3.710E-07	2.92E-06	NOS2	nitric oxide synthase 2, inducible
2.2	2.110E-06	1.34E-05	PTCH2	patched 2
2.2	6.740E-06	3.76E-05	CYBB	cytochrome b-245, beta polypeptide
2.2	3.280E-10	6.36E-09	GRIP1	glutamate receptor interacting protein 1
2.2	1.380E-09	2.22E-08	MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)
2.2	6.560E-07	4.81E-06	NXN	nucleoredoxin
2.2	8.690E-12	2.66E-10	UNC79	unc-79 homolog (C. elegans)
2.2	6.410E-14	3.86E-12	CAMTA1	calmodulin binding transcription activator 1
2.2	4.770E-11	1.18E-09	CDC45	cell division cycle 45
2.2	1.260E-09	2.05E-08	ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2
2.2	1.100E-07	1.03E-06	FOXM1	forkhead box protein M1
2.2	4.480E-10	8.31E-09	GTF2H4	general transcription factor IIH, polypeptide 4, 52kDa
2.2	7.860E-07	5.62E-06	MYBL2	v-myb avian myeloblastosis viral oncogene homolog-like 2
2.2	4.090E-08	4.28E-07	TWSG1	twisted gastrulation BMP signaling modulator 1

2.1	2.240E-08	2.54E-07	AK4	adenylate kinase 4
2.1	2.090E-07	1.78E-06	ANGPT1	angiopoietin 1
2.1	2.620E-09	3.9E-08	CENPP	centromere protein P
2.1	5.480E-11	1.34E-09	DCK	deoxycytidine kinase
2.1	1.590E-09	2.52E-08	GALNT15	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 15
2.1	3.590E-12	1.25E-10	HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
2.1	5.020E-08	5.13E-07	KCNJ8	potassium inwardly-rectifying channel, subfamily J, member 8
2.1	3.460E-11	8.96E-10	LGALS3	lectin, galactoside-binding, soluble, 3
2.1	7.460E-10	1.31E-08	NCAPD3	non-SMC condensin II complex, subunit D3
2.1	4.930E-12	1.65E-10	PHEX	phosphate regulating endopeptidase homolog, X-linked
2.1	5.690E-07	4.24E-06	PPP1R17	protein phosphatase 1, regulatory subunit 17
2.1	7.240E-10	1.27E-08	PTTG1IP	PTTG1 interacting protein
2.1	9.220E-06	4.98E-05	RGS5	regulator of G-protein signaling 5
2.1	1.890E-09	2.92E-08	SLC35B2	solute carrier family 35 (adenosine 3-phospho 5-phosphosulfate transporter), member B2
2.1	5.500E-14	3.43E-12	SLC35C1	solute carrier family 35 (GDP-fucose transporter), member C1
2.1	1.520E-08	1.81E-07	SULT1C3	sulfotransferase family, cytosolic, 1C, member 3
2.1	1.850E-09	2.87E-08	ZDHHC2	zinc finger, DHHC-type containing 2
2.1	1.080E-09	1.78E-08	CAPN6	calpain 6
2.1	7.390E-07	5.32E-06	CKAP4	cytoskeleton-associated protein 4
2.1	4.050E-08	4.25E-07	ERBB4	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4
2.1	5.590E-07	4.17E-06	MYLK	myosin light chain kinase
2.1	2.290E-06	1.45E-05	PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta
2.1	1.640E-10	3.49E-09	PYGO1	pygopus family PHD finger 1
2.1	1.030E-08	1.29E-07	RAD51	RAD51 recombinase
2.1	2.710E-07	2.23E-06	RERG	RAS-like, estrogen-regulated, growth inhibitor
2.1	9.200E-06	4.97E-05	CALD1	caldesmon 1
2.1	5.000E-04	0.0018	FIGF	c-fos induced growth factor (vascular endothelial growth factor D)
2.1	1.840E-09	2.86E-08	KNTC1	kinetochore associated 1
2.1	2.000E-04	0.0006	MRC2	mannose receptor, C type 2
2.1	7.330E-11	1.72E-09	PPAT	phosphoribosyl pyrophosphate amidotransferase
2.1	4.890E-13	2.17E-11	TBC1D16	TBC1 domain family, member 16
2.1	5.270E-09	7.17E-08	YBX3	Y-box binding protein 3
2.1	2.600E-11	7.02E-10	BCL2L10	Anti-apoptotic protein NR13
2.1	1.550E-07	1.38E-06	CENPN	centromere protein N
2.1	8.590E-06	4.68E-05	FAM72A	family with sequence similarity 72, member A
2.1	1.850E-09	2.87E-08	NKD1	naked cuticle homolog 1 (Drosophila)

2.1	2.230E-12	8.07E-11	OAZ2	ornithine decarboxylase antizyme 2
2.1	8.480E-07	6.01E-06	STK17A	serine/threonine kinase 17a
2.1	2.260E-14	1.57E-12	TINAGL2	tubulointerstitial nephritis antigen like 1
2.1	2.730E-07	2.24E-06	BEST1	bestrophin 1
2.1	2.350E-10	4.74E-09	CENPE	centromere protein E, 312kDa
2.1	4.530E-08	4.69E-07	GAR1	GAR1 homolog, ribonucleoprotein
2.1	1.900E-05	9.42E-05	NRP2	neuropilin 2
2.1	3.300E-09	4.79E-08	UNC13C	unc-13 homolog C (C. elegans)
2.1	2.620E-07	2.16E-06	NT5C2	5-nucleotidase, cytosolic II
2.1	2.440E-09	3.67E-08	ACER1	alkaline ceramidase 1
2.1	2.080E-06	1.33E-05	CHTF8	chromosome transmission fidelity factor 8
2.1	4.570E-07	3.5E-06	NCAPH	condensin complex subunit 2
2.1	3.080E-11	8.1E-10	PQLC1	PQ loop repeat containing 1
2.1	1.520E-12	5.8E-11	SLC51A	solute carrier family 51, alpha subunit
2.1	1.880E-05	9.31E-05	SPON1	spondin 1, extracellular matrix protein
2.1	3.840E-10	7.29E-09	TBC1D4	TBC1 domain family, member 4
2.1	2.000E-04	0.0006	TNC	tenascin C
2.1	2.420E-11	6.58E-10	C7ORF50	chromosome 14 open reading frame, human C7orf50
2.1	1.390E-08	1.69E-07	CENPJ	centromere protein J
2.1	7.610E-06	0.000042	FOXF1	forkhead box F1
2.1	3.260E-08	3.52E-07	LMNB1	lamin B1
2.1	4.280E-06	2.51E-05	LRRC32	leucine rich repeat containing 32
2.1	6.660E-11	1.59E-09	BPNT1	3(2), 5-bisphosphate nucleotidase 1
2.1	3.340E-12	1.17E-10	CCT8	chaperonin containing TCP1, subunit 8 (theta)
2.1	2.620E-09	3.9E-08	DCTD	dCMP deaminase
2.1	7.730E-09	1.01E-07	MYO5C	myosin VC
2.1	2.990E-09	4.39E-08	PPA1	pyrophosphatase (inorganic) 1
2.1	1.030E-11	3.09E-10	PTPRK	protein tyrosine phosphatase, receptor type, K
2.1	3.910E-08	4.13E-07	S100A6	Protein S100-A6
2.1	1.690E-07	1.49E-06	TMEM164	transmembrane protein 164
2.1	1.260E-06	8.54E-06	UNC5C	unc-5 homolog C (C. elegans)
2.1	1.710E-10	3.61E-09	DEPDC1	DEP domain containing 1
2.1	7.280E-10	1.28E-08	FKBP9	FK506 binding protein 9, 63 kDa
2.1	2.070E-07	1.76E-06	HSPB1	heat shock 27kDa protein 1
2.1	2.000E-07	1.72E-06	MCM6	minichromosome maintenance complex component 6
2.1	8.860E-11	2.02E-09	PKN3	protein kinase N3
2.1	1.230E-06	8.39E-06	PTRF	polymerase I and transcript release factor
2.0	1.340E-08	1.64E-07	ADCY9	adenylate cyclase 9
2.0	1.960E-05	9.69E-05	ARHGAP19	Rho GTPase activating protein 19
2.0	1.220E-08	1.5E-07	C4ORF19	chromosome 4 open reading frame, human C4orf19
2.0	4.250E-10	7.94E-09	CAV1	caveolin-1
2.0	1.180E-07	1.09E-06	CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3

2.0	5.080E-09	6.94E-08	FAXC	failed axon connections homolog (Drosophila)
2.0	6.860E-08	6.77E-07	GPC2	glypican 2
2.0	6.370E-10	1.14E-08	HELLS	helicase, lymphoid-specific
2.0	9.830E-10	1.65E-08	IL31RA	interleukin 31 receptor A
2.0	6.130E-06	3.45E-05	NT5E	5-nucleotidase, ecto (CD73)
2.0	3.990E-07	3.11E-06	SLC29A1	solute carrier family 29 (equilibrative nucleoside transporter), member 1
2.0	8.990E-07	6.32E-06	SLC37A2	solute carrier family 37 (glucose-6-phosphate transporter), member 2
2.0	2.080E-11	5.78E-10	ZNF516	zinc finger protein 516
2.0	5.000E-08	5.11E-07	CACNB2	calcium channel, voltage-dependent, beta 2 subunit
2.0	4.550E-08	4.71E-07	ENC1	ectodermal-neural cortex 1 (with BTB domain)
2.0	2.340E-12	8.44E-11	MGST3	microsomal glutathione S-transferase 3
2.0	3.490E-11	9.02E-10	MYO3AL	myosin IIIA-like
2.0	1.920E-06	1.24E-05	SOX9	SRY (sex determining region Y)-box 9
2.0	1.330E-05	6.84E-05	THBS1	thrombospondin 1
2.0	2.300E-12	8.31E-11	TKT	transketolase
2.0	5.920E-08	5.91E-07	TRIM71	tripartite motif containing 71, E3 ubiquitin protein ligase
2.0	1.670E-14	1.18E-12	DUS1L	dihydrouridine synthase 1-like (S. cerevisiae)
2.0	4.640E-11	1.16E-09	GOT2	glutamic-oxaloacetic transaminase 2, mitochondrial
2.0	4.670E-10	8.61E-09	HAUS1	HAUS augmin-like complex, subunit 1
2.0	4.320E-08	4.5E-07	HIST1H111R	histone cluster 1, H1.11R (similar to human histone cluster 1, class H1 genes)
2.0	4.600E-05	0.0002	HISTH1	histone H1
2.0	2.660E-10	5.27E-09	JAZF1	JAZF zinc finger 1
2.0	1.820E-08	2.13E-07	RHPN2	rhophilin, Rho GTPase binding protein 2
2.0	1.880E-08	2.19E-07	ARNT2	aryl-hydrocarbon receptor nuclear translocator 2
2.0	5.550E-05	0.0002	COL12A1	collagen, type XII, alpha 1
2.0	1.820E-05	9.06E-05	DES	DESMIN
2.0	7.440E-11	1.74E-09	HHATL	hedgehog acyltransferase-like
2.0	3.580E-13	1.69E-11	ITPR3	inositol 1,4,5-trisphosphate receptor, type 3
2.0	1.080E-07	1.01E-06	MAT1A	methionine adenosyltransferase I, alpha
2.0	8.880E-11	2.03E-09	GCNT1	glucosaminyl (N-acetyl) transferase 1, core 2
2.0	2.040E-07	1.74E-06	GOLT1B	golgi transport 1B
2.0	1.240E-09	2.01E-08	HMGA2	high mobility group AT-hook 2
2.0	5.380E-08	5.45E-07	IDUA	iduronidase, alpha-L-
2.0	6.440E-07	4.73E-06	KDELC2	KDEL (Lys-Asp-Glu-Leu) containing 2
2.0	1.850E-06	0.000012	KIF18B	kinesin family member 18B
2.0	1.230E-07	1.13E-06	MFAP5	microfibrillar associated protein 5
2.0	2.690E-08	2.98E-07	MTFR2	mitochondrial fission regulator 2
2.0	3.130E-09	4.59E-08	PTGFR	prostaglandin F receptor (FP)
2.0	7.410E-11	1.73E-09	SLC4A9	solute carrier family 4, sodium bicarbonate cotransporter, member 9

2.0	1.120E-08	1.39E-07	TOR4A	torsin family 4, member A

Supplementary Table 4. **Gene sets of KEGG-derived list upregulated in jejunum mucosa of broiler chickens at 42 days of (n=19), compared to cecum, with g FDR q value ≤ 0.05**

GENE SET - JEJUNUM	FDR q value
PRIMARY_IMMUNODEFICIENCY	0.000
PEROXISOME	0.000
T_CELL_RECEPTOR_SIGNALING_PATHWAY	0.001
NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY	0.001
PPAR_SIGNALING_PATHWAY	0.006
HEMATOPOIETIC_CELL_LINEAGE	0.009
RENIN_ANGIOTENSIN_SYSTEM	0.010
FATTY_ACID_METABOLISM	0.010
MATURITY_ONSET_DIABETES_OF_THE_YOUNG	0.011
HISTIDINE_METABOLISM	0.020
INOSITOL_PHOSPHATE_METABOLISM	0.022
PRIMARY_BILE_ACID_BIOSYNTHESIS	0.042
TRYPTOPHAN_METABOLISM	0.042
STEROID_BIOSYNTHESIS	0.044

Supplementary Table 5. **Gene sets of KEGG-derived list upregulated in cecum mucosa of broiler chickens at 42 days of (n=19), compared to jejunum, with g FDR q value ≤ 0.05**

GENE SET - CECUM	FDR q value
CELL_CYCLE	0.000
RIBOSOME	0.000
DNA_REPLICATION	0.000
PYRIMIDINE_METABOLISM	0.000
BASAL_CELL_CARCINOMA	0.001
OXIDATIVE_PHOSPHORYLATION	0.002
ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC	0.002
HEDGEHOG_SIGNALING_PATHWAY	0.004
ECM_RECEPTOR_INTERACTION	0.004
HOMOLOGOUS_RECOMBINATION	0.006
PROTEASOME	0.008
VIBRIO_CHOLERAE_INFECTION	0.008
MISMATCH_REPAIR	0.008
SPLICEOSOME	0.009
RNA_POLYMERASE	0.012
FOCAL_ADHESION	0.014
PATHOGENIC_ESCHERICHIA_COLI_INFECTION	0.018
PURINE_METABOLISM	0.021
CYSTEINE_AND_METHIONINE_METABOLISM	0.022
TASTE_TRANSDUCTION	0.022
OOCYTE_MEIOSIS	0.028
BASE_EXCISION_REPAIR	0.040
CARDIAC_MUSCLE_CONTRACTION	0.041
SYSTEMIC_LUPUS_ERYTHEMATOSUS	0.048

Supplementary. Table 6. **The first 20 gene sets of Gene Ontology - Biological Processes -derived list upregulated in cecum mucosa of broiler chickens at 42 days of (n=19), compared to jejunum, with g FDR q value ≤ 0.05.**

GENE SET - CECUM	FDR q value
EXTRACELLULAR_MATRIX_STRUCTURAL_CONSTITUENT	0.001
NUCLEOSOME_BINDING	0.013
SNORNA_BINDING	0.017
NUCLEOSOMAL_DNA_BINDING	0.014
OXIDOREDUCTASE_ACTIVITY_ACTING_ON_A_SULFUR_GROUP_OF_DONORS	0.012
RRNA_BINDING	0.013
DISULFIDE_OXIDOREDUCTASE_ACTIVITY	0.011
INSULIN_LIKE_GROWTH_FACTOR_BINDING	0.012
LAMININ_BINDING	0.011
STRUCTURE_SPECIFIC_DNA_BINDING	0.013
UNFOLDED_PROTEIN_BINDING	0.016
GROWTH_FACTOR_BINDING	0.017
PROTEIN_DISULFIDE_OXIDOREDUCTASE_ACTIVITY	0.020
CHROMATIN_DNA_BINDING	0.024
TRANSLATION_INITIATION_FACTOR_ACTIVITY	0.028
FRIZZLED_BINDING	0.027
STRUCTURAL_MOLECULE_ACTIVITY	0.026
GLYCOSAMINOGLYCAN_BINDING	0.026
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	0.027
DNA_DEPENDENT_ATPASE_ACTIVITY	0.033

Supplementary Table 7. **Non-coding genes of upregulated in jejunum or cecum mucosa of broiler chickens at 42 days of (n=19 per intestinal tissue), with a log2-fold change ratio ≥2**

Cecum Avg (log2)	Jejunum Avg (log2)	Fold Change	Name
Jejunum			
4.56	*6.91*	*-5.1*	snoRNA RF00004
3.9	5.21	-2.48	gga-mir-1783
2.53	3.82	-2.45	gga-mir-1654-1
5.88	6.96	-2.11	snoRNA RF00431
3.58	4.64	-2.08	gga-mir-1416
2.15	3.16	-2.02	gga-mir-215
Cecum			
7.71	4.16	11.7	gga-mir-196-4
4.09	2.14	3.87	gga-mir-196-1
6.88	5.49	2.61	gga-mir-1732-1
7.8	6.58	2.33	snoRNA RF00138
5.05	4.02	2.05	gga-mir-1800

Study 2 – Supplementary Tables and Figures

Supplementary Table 1. **Composition of the commercial diets.**

Item	STARTER (0-10 d)	GROWER (11-25 d)	FINISHER (26-42 d)
Corn	42.17	34.96	12.73
White Corn	0.00	0.00	15.00
Wheat	10.00	20.00	25.01
Sorghum	0.00	0.00	5.00
Soybean Meal	23.11	20.63	17.60
Expanded Soybean	10.00	10.00	13.00
Sunflower	3.00	3.00	3.00
Corn Gluten	4.00	3.00	0.00
Soybean Oil	3.08	4.43	5.48
Dicalcium phosphate	1.52	1.20	0.57
Calcium carbonate	0.91	0.65	0.52
Sodium bicarbonate	0.15	0.10	0.15
Salt	0.27	0.27	0.25
Choline chloride	0.10	0.10	0.10
Lysine sulphate	0.59	0.55	0.46
Dl-Methionine	0.27	0.29	0.30
Threonine	0.15	0.14	0.14
Enzyme - Roxazyme G2G	0.08	0.08	0.08
Phytase 0.1%	0.10	0.10	0.10
Vitamin - Mineral Premix [1]	0.50	0.50	0.50
Calculated chemical composition:			
Dry Matter,%	88.57	88.65	88.64
Crude Protein,%	22.70	21.49	19.74
Lipid,%	7.06	8.24	9.74
Crude Fibre,%	3.08	3.04	3.07
Ash,%	5.85	5.17	4.49
Lysine,%	1.38	1.29	1.21
Methionine,%	0.67	0.62	0.59
Methionine + Cysteine,%	1.03	0.97	0.91
Calcium,%	0.91	0.80	0.59
Phosphate,%	0.63	0.57	0.46
Metabolizable Energy (Kcal/Kg)	3.076	3.168	3.264

[1] Provided the following per kg of diet: vitamin A (retinyl acetate), 13,000 IU; vitamin D3 (cholecalciferol), 4,000 IU; vitamin E (DL-α_tocopheryl acetate), 80 IU; vitamin K (menadione sodium bisulfite), 3 mg; riboflavin, 6.0 mg; pantothenic acid, 6.0 mg; niacin, 20 mg; pyridoxine, 2 mg; folic acid, 0.5 mg; biotin, 0.10 mg; thiamine, 2.5 mg; vitamin B12 20 μg; Mn, 100 mg; Zn, 85 mg; Fe, 30 mg; Cu, 10 mg; I, 1.5 mg; Se, 0.2 mg; ethoxyquin, 100 mg.

Supplementary Table 2. ***P*** **values for abundances of phyla in broiler chickens *in ovo* injected with a single dose (0.2 ml) of physiological saline (0.9% NaCl), control (CON) versus 0.9 % physiological saline+3.5 mg galacto-oligosaccharide prebiotic/egg, prebiotic (GOS) at 32 days of age and CON group vs GOS group at 42 days of age under thermoneutral condition (TN, 25°C) and heat stress (HS, 30°C constantly) groups (n=24 per *in ovo* treatment)**

	CON vs GOS		CON TN vs GOS TN		CON HS vs GOS HS	
Phylum	32 days		42 days		42 days	
	p-value	FDR	*p*-value	FDR	*p*-value	FDR
Firmicutes	0.7092	0.7092	0.6596	0.839	0.2966	0.395
Actinobacteria	0.4044	0.7092	0.4467	0.839	0.2816	0.395
Proteobacteria	0.6298	0.7092	0.8185	0.839	0.538	0.538
Tenericutes	0.1276	0.5104	0.8389	0.839	0.2047	0.395

Significant differences with *p*-value and adjusted *p*-value (FDR) < 0.05

Adjusted *p*-value by FDR (false discovery rate) correction

Supplementary Table 3. ***P*** **values for abundances of classes in broiler chickens *in ovo* injected with a single dose (0.2 ml) of physiological saline (0.9% NaCl), control (CON) versus 0.9 % physiological saline+3.5 mg galacto-oligosaccharide prebiotic/egg, prebiotic (GOS) at 32 days of age and CON group vs GOS group at 42 days of age under thermoneutral condition (TN, 25°C) and heat stress (HS, 30°C constantly) groups (n=24 per *in ovo* treatment).**

Class	CON vs GOS 32 days		CON vs GOS TN 42 days		CON HS vs GOS HS 42 days	
	p-value	FDR	*p*-value	FDR	*p*-value	FDR
Clostridia	0.5242	0.73388	0.8462	0.9047	0.6795	0.8483
Bacilli	0.6364	0.7425	0.9047	0.9047	0.8483	0.8483
Actinobacteria	0.4044	0.7077	0.4467	0.9047	0.2816	0.6571
Erysipelotrichia	0.0763	0.4466	0.3134	0.9047	0.7619	0.8483
Gammaproteobacteria	0.9641	0.9641	0.8866	0.9047	0.6207	0.8483
Mollicutes	0.1276	0.4466	0.8389	0.9047	0.2047	0.6571
Deltaproteobacteria	0.2327	0.5430	0.214	0.9047	0.1257	0.6571

Significant differences with *p*-value and adjusted *p*-value (FDR) < 0.05

Adjusted *p*-value by FDR (false discovery rate) correction

Supplementary Table 4. ***P*** **values for abundances of orders in broiler chickens *in ovo* injected with a single dose (0.2 ml) of physiological saline (0.9% NaCl), control (CON) versus 0.9 % physiological saline+3.5 mg galacto-oligosaccharide prebiotic/egg, prebiotic (GOS) at 32 days of age and CON group vs GOS group at 42 days of age under thermoneutral condition (TN, 25°C) and heat stress (HS, 30°C constantly) groups (n=24 per *in ovo* treatment).**

Order	CON vs GOS 32 days		CON TN vs GOS TN 42 days		CON HS vs GOS HS 42 days	
	p-value	FDR	*p*-value	FDR	*p*-value	FDR
Clostridiales	0.52	0.7083	0.8574	0.8866	0.6951	0.8707
Lactobacillales	0.5312	0.7083	0.7825	0.8866	0.8949	0.8949
Erysipelotrichales	0.0763	0.436	0.3134	0.8357	0.7619	0.8707
Bacillales	0.1635	0.436	0.0811	0.6488	**0.0009**	0.0072
Coriobacteriales	0.7303	0.8346	0.3045	0.8357	0.5919	0.8707
Enterobacteriales	0.9641	0.9641	0.8866	0.8866	0.2047	0.5459
Anaeroplasmatales	0.1276	0.436	0.8399	0.8866	0.2047	0.5459
Bifidobacteriales	0.473	0.7083	0.7466	0.8866	0.2786	0.5572

*Values in bold refer to significative *p* values

Significant differences with *p*-value and adjusted *p*-value (FDR) < 0.05

Adjusted *p*-value by FDR (false discovery rate) correction

Supplementary Table 5. ***P*** **values for abundances of families in broiler chickens *in ovo* injected with a single dose (0.2 ml) of physiological saline (0.9% NaCl), control (CON) versus 0.9 % physiological saline+3.5 mg galacto-oligosaccharide prebiotic/egg, prebiotic (GOS) at 32 days of age and CON group vs GOS group at 42 days of age under thermoneutral condition (TN, 25°C) and heat stress (HS, 30°C constantly) groups (n=24 per *in ovo* treatment).**

Family	CON vs GOS 32 days		CON TN vs GOS TN 42 days		CON HS vs GOS HS 42 days	
	p-value	FDR	*p*-value	FDR	*p*-value	FDR
Ruminococcaceae	0.4458	0.7652	0.5137	0.8967	0.5534	0.7937
Lachnospiraceae	0.2739	0.7652	0.6154	0.8967	0.7937	0.7937
Erysipelotrichaceae	0.0763	0.7652	0.3134	0.8619	0.7619	0.7937
Lactobacillaceae	0.6021	0.7652	0.6286	0.8967	0.7644	0.7937
Coriobacteriaceae	0.7303	0.8033	0.2897	0.8619	0.6671	0.7937
Eubacteriaceae	0.2161	0.7652	0.7521	0.8967	0.7264	0.7937
Peptostreptococcaceae	0.5394	0.7652	0.8967	0.8967	0.4499	0.7937
Enterococcaceae	0.6261	0.7652	**0.0457**	0.5027	0.3907	0.7937
Enterobacteriaceae	0.9641	0.9641	0.8866	0.8967	0.6207	0.7937
Streptococcaceae	0.4762	0.7652	0.1523	0.8377	0.3794	0.7937
Bifidobacteriaceae	0.473	0.7652	0.7466	0.8967	0.2786	0.7937

*Values in bold refer to significative *p* values

Significant differences with *p*-value and adjusted *p*-value (FDR) < 0.05

Adjusted *p*-value by FDR (false discovery rate) correction

Supplementary Table 6. ***P* values for abundances of genera in broiler chickens *in ovo* injected with a single dose (0.2 ml) of physiological saline (0.9% NaCl), control (CON) versus 0.9 % physiological saline+3.5 mg galacto-oligosaccharide prebiotic/egg, prebiotic (GOS) at 32 days of age and CON group vs GOS group at 42 days of age under thermoneutral condition (TN, 25°C) and heat stress (HS, 30°C constantly) groups (n=24 per *in ovo* treatment).**

Genus	CON vs GOS 32 days		CON TN vs GOS TN 42 days		CON HS vs GOS HS 42 days	
	p-value	FDR	*p*-value	FDR	*p*-value	FDR
Clostridium_IV	0.5065	0.8373	0.1893	0.8831	0.3718	0.9138
Clostridium_XlVb	0.8277	0.9508	0.8715	0.8831	0.4300	0.9138
Lactobacillus	0.6021	0.8373	0.6286	0.8831	0.7644	0.9789
Blautia	0.2849	0.8373	0.8831	0.8831	0.2604	0.9138
Faecalibacterium	0.6056	0.8373	0.2957	0.8831	0.7033	0.9789
Ruminococcus	0.1689	0.8373	0.7486	0.8831	0.1178	0.9138
Butyricicoccus	0.3265	0.8373	0.7734	0.8831	0.5139	0.9707
Oscillibacter	0.4784	0.8373	0.4687	0.8831	0.2248	0.9138
Clostridium_XVIII	0.2334	0.8373	0.6053	0.8831	0.9789	0.9789
Clostridium_XlVa	0.911	0.9508	0.3569	0.8831	0.8402	0.9789
Lachnospiracea_incertae_sedis	0.9508	0.9508	0.6433	0.8831	0.9057	0.9789
Anaerostipes	0.9273	0.9508	0.7896	0.8831	0.8193	0.9789
Eubacterium	0.2136	0.8373	0.7632	0.8831	0.7245	0.9789
Enterococcus	0.6261	0.8373	**0.0457**	0.7769	0.3907	0.9138
Streptococcus	0.4762	0.8373	0.1523	0.8831	0.3794	0.9138
Pseudoflavonifractor	0.6403	0.8373	0.6273	0.8831	0.9713	0.9789
Bifidobacterium	0.473	0.8373	0.7466	0.8831	0.2786	0.9138

*Values in bold refer to significative *p* values

Significant differences with *p*-value and adjusted *p*-value (FDR) < 0.05

Adjusted *p*-value by FDR (false discovery rate) correction

Supplementary Table 7. ***P* values for abundances of species in broiler chickens *in ovo* injected with a single dose (0.2 ml) of physiological saline (0.9% NaCl), control (CON) versus 0.9 % physiological saline+3.5 mg galacto-oligosaccharide prebiotic/egg, prebiotic (GOS) at 32 days of age and CON group vs GOS group at 42 days of age under thermoneutral condition (TN, 25°C) and heat stress (HS, 30°C constantly) groups (n=24 per *in ovo* treatment).**

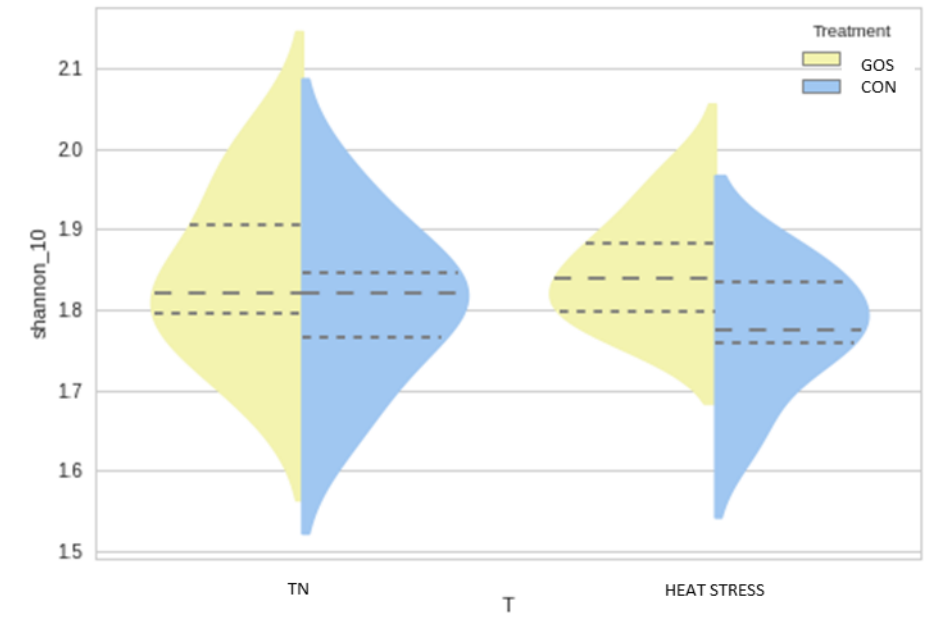
Species	CON vs GOS 32 days		CON TN vs GOS TN 42 days		CON HS vs GOS HS 42 days	
	p-value	FDR	*p*-value	FDR	*p*-value	FDR
Clostridium_lactatifermentans	0.7721	0.8824	0.8834	0.8834	0.0795	0.3180
Faecalibacterium_prausnitzii	0.6056	0.8537	0.2957	0.8669	0.7033	0.9713
Butyricicoccus_pullicaecorum	0.3265	0.6530	0.7734	0.8834	0.5139	0.9713
Eubacterium_coprostanoligenes	0.0848	0.6530	0.5131	0.8669	0.7264	0.9713
Eubacterium_hallii	0.9553	0.9553	0.6502	0.8669	0.8841	0.9713
Blautia_glucerasea	0.2355	0.6530	0.4963	0.8669	**0.0334**	0.2672
Pseudoflavonifractor_capillosus	0.6403	0.8537	0.6273	0.8669	0.9713	0.9713
Eubacterium_desmolans	0.3261	0.6530	0.4422	0.8669	0.8520	0.9713

*Values in bold refer to significative *p* values

Significant differences with *p*-value and adjusted *p*-value (FDR) < 0.05

Adjusted *p*-value by FDR (false discovery rate) correction

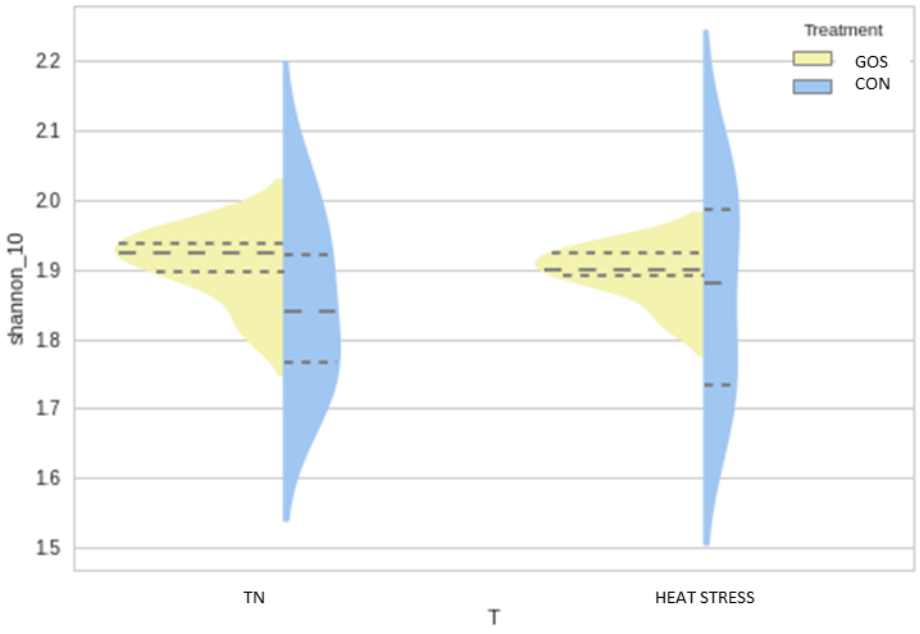
Supplementary Fig. 1. **Alpha diversity calculated for the tested groups at 32 (a) and 42 (b) days by Shannon index**.



a

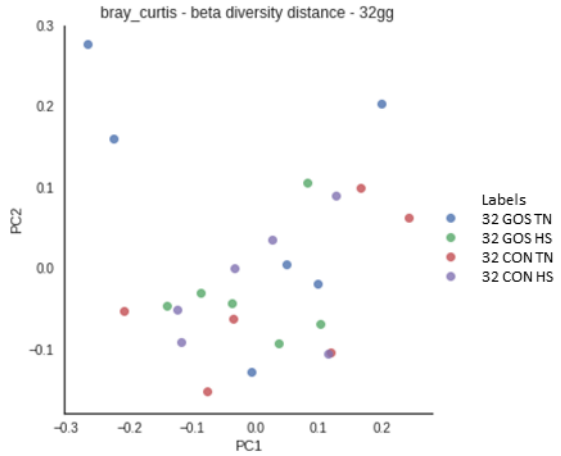
a

b

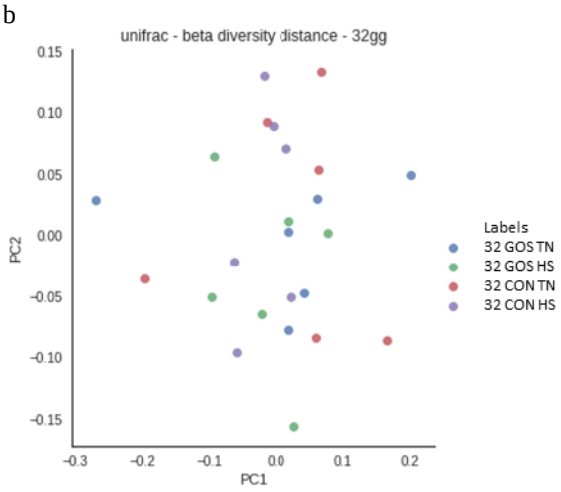


Supplementary Fig. 2. **Beta diversity calculated for the tested groups at 32days using the Bray Curtis (a) and Unifrac (b) indexes and at 42 (b) days using the Bray Curtis (c) and Unifrac (c) indexes**

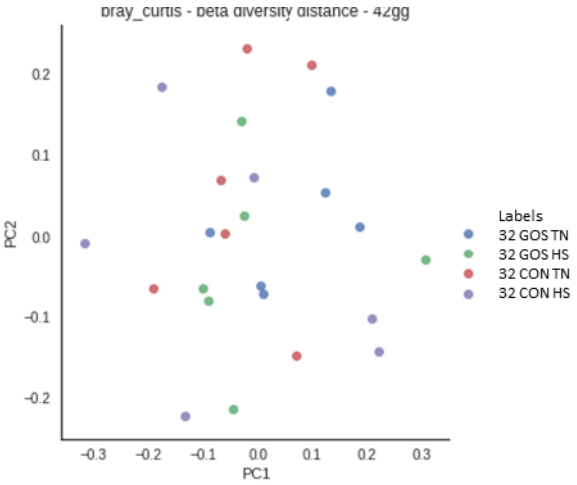
a



b



c



d