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GROWTH PERFORMANCE, GUT HEALTH, AND METABOLISM OF BROILERS UNDER THERMONEUTRAL AND HEAT STRESS CONDITIONS: MULTIDISCIPLINARY STUDIES ON THE EFFECTS OF NUTRITIONAL STRATEGIES AND GENOTYPE

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“In times of change, learners inherit the Earth, while the learned find themselves beautifully equipped to deal with a world that no longer exists.”

– Eric Hoffer, writer and philosopher

DEDICATION

I would like to dedicate this dissertation to God and to all the people who have gotten me to this point in my life. Professor Federico Sirri, however, deserves special mention for being an outstanding mentor and an inexhaustible source of inspiration throughout my PhD program.

TABLE OF CONTENTS

ABSTRACT

The broiler industry makes an essential contribution to food security by providing high-quality, wholesome, and inexpensive protein to an increasingly populated and affluent world. Driven by tremendous advancements at every stage, this integrated poultry supply chain has grown steadily boosting chicken meat production from less than 8 million tons in 1961 to 121.6 million tons in 2021 globally. Fast-growing, high meat-yielding broilers, the real protagonists of this success story, are the outcome of an unprecedented genetic progress in animal breeding. Although its efficiency and sustainability have improved terrifically, the broiler industry is facing difficult socioeconomic and environmental challenges that imperil its role in feeding the world. For example, raising broilers in the "post-antibiotic and global warming era" means facing an increased risk of reduced growth and feed efficiency, impaired gut function, dysbiosis and increased enteric pathogen load, heat stress onset, altered metabolism, decreased welfare, and product quality deterioration.

This PhD project was designed to fit into this context, with the aims of (i) investigating the effects of different nutritional strategies on growth performance, gut health, and metabolism of broilers fed without antibiotics under thermoneutral and heat stress conditions and (ii) exploring the impacts of heat stress on the physiological processes regulating feed intake and energy homeostasis in different chicken genotypes. The three-year project comprised four studies, each of which consisted of two experiments. Specifically, the first three studies were feeding trials set out to test a nutritional strategy (i.e., supplementation of a synbiotic, a muramidase, and arginine, respectively) to determine whether it is an effective solution to (experiment #1) enhance growth performance and gut health under optimal thermal conditions and (experiment #2) to mitigate the detrimental effects of heat stress for antibiotic-free broilers. These studies shared a multidisciplinary approach integrating poultry nutrition, physiology, and gut microbiology. Conducted during a six-month research period at Dr. Sami Dridi's lab, the fourth study was a molecular biology-based investigation of the effects of heat stress on (experiment #1) hypothalamic neuropeptides that regulate feed intake and (experiment #2) hypothalamic oxygen homeostasis and inflammatory state in three broiler lines from diverse stages of genetic selection and in the red jungle fowl, the ancestor of domestic chickens.

The present dissertation is organized into eight themed chapters. An overview of the evolution of the broiler industry and its importance for food security serves as the introductory chapter. The goal of the second chapter is to offer a literature review of the anatomy and functioning of the chicken gastrointestinal tract, the main elements that contribute to maintaining a healthy, high-performing gut, the characteristics of the microbiota, with emphasis on the environmental and dietary factors that influence it, and the adverse effects of heat stress on chickens. The third chapter is the dissertation core, in which the aims of the project are stated and its schematic structure is provided. The studies conducted as part of this project are presented in chapters four through seven. Highlights are reported at the beginning of each study so that readers can get the results at a glance. Briefly:

- **Study #1.** The synbiotic improved feed efficiency and footpad health, increased abundance of Firmicutes and reduced abundance of Bacteroidetes in the ceca of birds kept in thermoneutral conditions, while did not mitigate the impacts of heat stress on growth performance.
- **Study #2.** Under optimal thermal conditions, muramidase increased final body weight and reduced cumulative feed intake and feed conversion ratio in a dose-dependent way, with the highest dose reducing the risk of developing footpad lesions, cecal alpha diversity, the Firmicutes to Bacteroidetes ratio, and abundance of bacteria known to be butyrate producers, as well as increasing the abundance of Bacteroidaceae and Lactobacillaceae. Moreover, birds supplemented with the highest dose showed greater plasmatic levels of bioenergetic metabolites and lower levels of pro-oxidant metabolites. The same dose, however, failed to reduce the negative effects of heat stress on growth performance.
- **Study #3.** Supplementation of dietary arginine (i.e., total arginine to lysine ratio of 1.20) improved growth rate, final body weight, and feed efficiency, increased plasmatic levels of arginine, betaine, histidine, and creatine and hepatic levels of creatine and several essential amino acids, reduced alpha diversity and relative abundance of Firmicutes and Proteobacteria (especially *Escherichia coli*), and increased the abundance of Bacteroidetes and *Lactobacillus salivarius* in the ceca of thermoneutral birds. No arginine-mediated attenuation of heat stress was found, but molecular analyses revealed some physiological perturbations occurring in heat-stressed broilers, such as profound alteration in protein metabolism and accumulation of antioxidant and protective molecules in oxidative stress-

sensitive tissues. Arginine supplementation, however, may have partially counterbalanced the negative effects of heat stress on energy homeostasis.

- **Study #4.** A stable gene expression of (an)orexigenic neuropeptides was found in the four chicken populations, probably due to hypothalamic integration of circadian information, acclimation of birds to long-lasting heat stress, stable hypothalamic pathways unaffected by evolution or selection, focus on mRNA abundance, and analysis of the whole hypothalamus. On the other hand, responses to hypoxia and heat stress appeared to be related to feed intake regulation.

Lastly, the concluding chapter goes over the chief findings of the project and gives perspectives that will hopefully lay the groundwork for further investigation. It only remains for me to wish you an enjoyable and fruitful reading.

I almost forgot! The following instructions may come in handy. In addition to the typical hyperlinks in the **TABLE OF CONTENTS** or the **LIST OF FIGURES**, the document is full of bold or underlined bookmarks and cross-references to let you jump to a specific heading, figure, table, annex, or webpage; just click on them to follow the link. Don’t be fooled by the boldface though! All abbreviations/acronyms are in bold, but they don’t take you anywhere.

1 INTRODUCTION

1.1 The Broiler Industry: Nothing Short of a Success Story

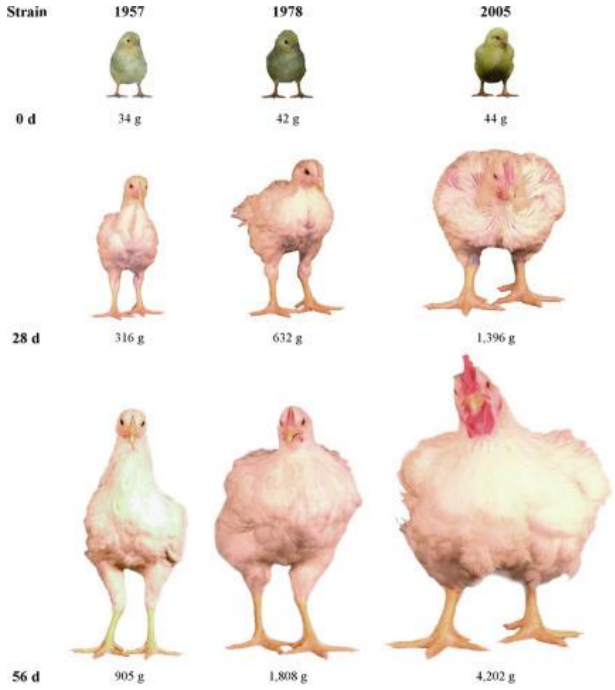
Cecile Long Steele was a forward-looking American entrepreneur from Delaware, U.S., every poultry professional and scientist should be grateful to. Her business acumen ushered in a new era for the poultry industry; a period of extraordinary development that transformed a small-scale animal farming system into a modern and prosperous agri-food sector. Indeed, until the 1920s, chicken meat production was ancillary to the activities of the egg industry and was mostly based on family farms and backyard flocks. Today, Mrs. Steele would probably be described as an astonishingly skilled woman, with problem solving and thinking outside the box driving her daring decisions. In the eyes of people living in the "Roaring 20s", however, she was *simply* able to come up with an original idea to turn a problem, namely dealing with 500 chicks mistakenly delivered to her (it was 10 times the number she had ordered), into an opportunity, that is raising those chicks specifically for their meat. Impressed by the profitable business Mrs. Steele had set up after growing that first flock of meat-type chickens, several farmers, feed mills, and egg companies realized that traditional operations, such as hatching procedures, on-farm activities, and processing, were suitable for scaling up to become part of a new, integrated and extremely efficient poultry supply chain: the broiler industry was born (Siegel, 2014; Bennett et al., 2018; Duffy, 2020; Scanes and Christensen, 2020; National Chicken Council, 2022).

Other than Mrs. Steele, numerous sharp people have contributed to building the broiler industry as we know it today and must be given credit for making a courtyard bird an excellent producer of high-quality and affordable animal proteins that are and will be essential to feeding the world. As a PhD candidate dealing with poultry science, I am fully aware that a few sentences of the present dissertation do not do justice to the efforts and achievements of all these innovators. However, as a sign of respect and gratitude, I would like to dedicate this introductory chapter to each of them; to the authors of a success story!

But let's spotlight the broiler chicken, the real protagonist of this story. Taking the cue from the superb work of Bennett et al. (2018), Van Immerseel (2022) called the broiler a "human reconfigured animal". The morphological analysis carried out by Bennett et al. (2018) substantiated, in fact, that

modern broilers (*Gallus gallus domesticus*) are radically different from their wild ancestor, the red jungle fowl (*Gallus gallus*) native to S/SE Asia, and are markedly dissimilar to their domesticated predecessors that have been reared for 8,000 years until the mid-twentieth century. Fast-growing, high meat-yielding broilers are the outcome of an unprecedented genetic progress that has taken place in the last 70 years due to human intervention (Havenstein et al., 1994, 2003a; Zuidhof et al., 2014). The pictures presented in **Figure 1** have become iconic of the striking effects of selecting broilers for rapid growth to market weight.

Figure 1 | Age-related changes in body weight and size for the 1957, 1978, and 2005 broiler line

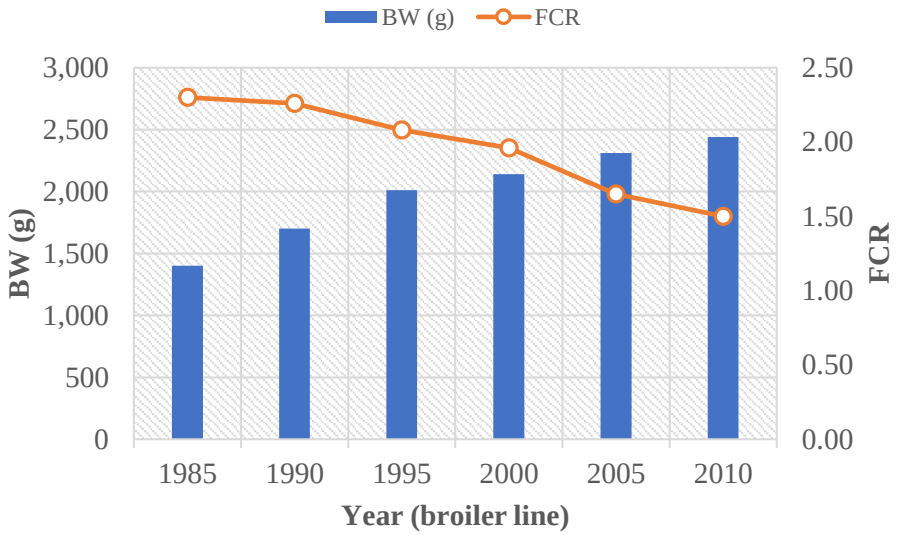


Note: For each broiler line (strain), the average mixed-sex body weight is given, while the pictures show the same representative bird at 0, 28, and 56 days of age.
Source: Zuidhof et al. (2014).

The intense genetic selection has not only caused evident changes in the phenotypic appearance of broilers (e.g., size, body mass and shape, center of gravity), but also in their feed efficiency (Siegel, 2014). Feed efficiency is a parameter of utmost importance in poultry and livestock production as it is an indicator of the ability of animals to convert feed into a certain output (i.e., human food or other goods). A performance trait widely used in animal husbandry to assess feed efficiency is feed conversion ratio (**FCR**). For meat-type poultry, FCR is the defined as the amount of feed consumed per unit of body

weight (**BW**) gain in a specific time interval (Willems et al., 2013). The modern broiler is an excellent converter of feed into BW, characterized by very high feed efficiency or, in other words, extremely low FCR (Havenstein, 2006). Although it is not updated with data from commercial lines currently used, **Figure 2** clearly shows the opposite temporal trends for BW and FCR of broilers; the former has steadily increased, while the latter has progressively dropped. As such, compared to broilers grown in 1985, those belonging to a 2010 line can reach, in 35 d, greater BW (1.40 *vs.* 2.44 kg) with lower FCR (2.30 *vs.* 1.50), namely consuming much less feed (3.22 *vs.* 3.66 kg).

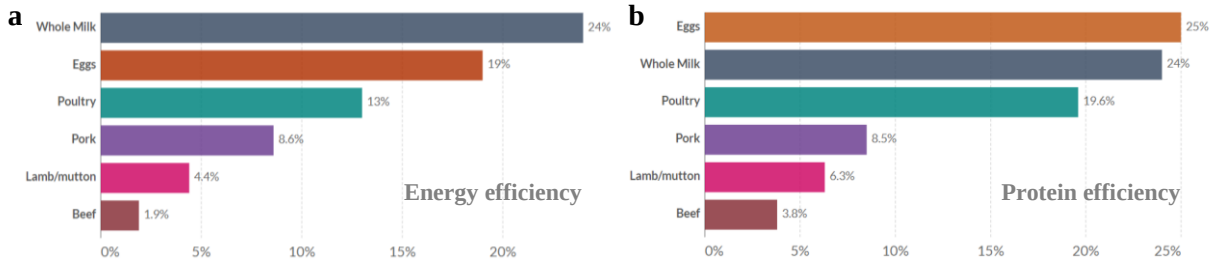
Figure 2 | Typical body weight and feed conversion ratio of different broiler lines at 35 d of age



Note: BW, body weight; FCR, feed conversion ratio.
Data source: Siegel (2014).

Already in the early 2000s, Smil (2002) pointed out that hyperselected chickens had the highest feed efficiency among meat animals. It is even more incredible, however, what emerges from the comparison between poultry meat and other meats in terms of energy and protein efficiency, which are calculated as the percentage of energy or protein input retained in the final product. According to the data reported in **Figure 3**, energy and protein efficiency of poultry meat production (13 and 19.6%, respectively) are considerably higher than those of the production of pork and ruminant meats (ranging between 1.9 and 8.6%).

Figure 3 | Energy (**a**) and protein (**b**) efficiency of livestock productions



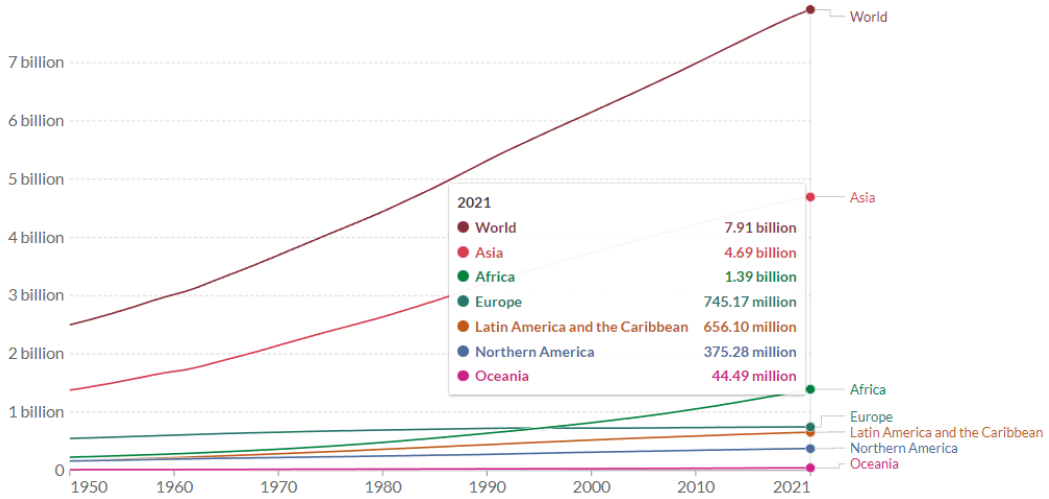
Note: Efficiency is the percentage of energy (caloric) or protein inputs as feed converted to animal product.
Source: Our World in Data based on the data of Alexander et al. (2016).

The dramatic enhancement in poultry feed efficiency over the past years has resulted in a substantial reduction in inputs required (e.g., feed, housing, utilities) and wastes produced (e.g., carbon and water footprint) per unit of poultry product (Havenstein, 2006; Willems et al., 2013). The sustainability of the poultry industry, especially the broiler one, has therefore improved terrifically. Nevertheless, this highly efficient animal-food industry is not exempt from developing more and more economically sound and eco-friendly processes to pursue a sustainable expansion in an epoch of difficult socioeconomic and environmental challenges. Keeping on improving poultry feed efficiency is therefore imperative for years to come (Zampiga et al., 2021a).

1.2 The Pivotal Role of Chicken Meat in Feeding the World

Over the last 70 years, the global population has grown steadily from 2.5 billion people in 1950 to 7.91 billion people in 2021: a threefold increase that is clearly visible in **Figure 4**. The population growth, however, is still ongoing and humans are expected to be nearly 10 billion by 2050, according to projections published by the United Nations (see **Figure 4** caption for the reference). When global data are broken down by region, the contribution of Asia is the greatest. In the past decades, Asia has witnessed the most impressive increase in population and will undoubtedly continue to be the leading continent of this "Earth demographic league" in the future.

Figure 4 | Population estimates from 1950 to 2021

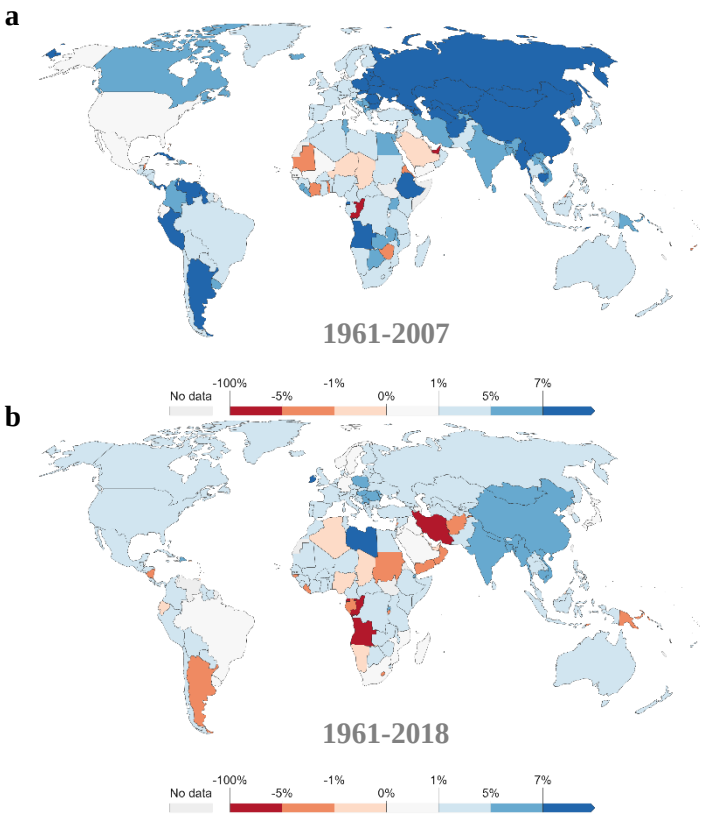


Source: Our World in Data based on The 2022 Revision of World Population Prospects released by the Population Division of the Department of Economic and Social Affairs of the United Nations.

In the same time frame, not only has humanity grown numerically, but also from an economic point of view as illustrated in **Figure 5**. Focusing on 1961-2007 and 1961-2018, two time periods that ended just before the Global Financial Crisis of 2008 and the outbreak of COVID-19 pandemic, respectively, it can be seen that GDP per capita has considerably raised, especially across developing countries.

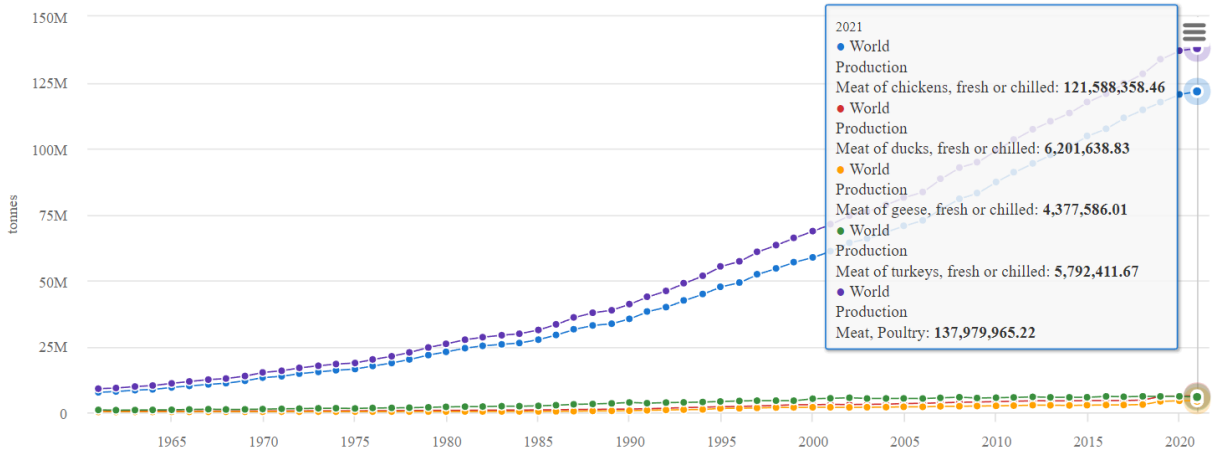
The increased wealth has reshaped eating habits and propelled the demand for animal proteins (Alexander et al., 2016; Colgrave et al., 2021), which is predicted to keep rising by almost 70% by 2050 (Searchinger et al., 2019) mostly driven by escalating consumption in low-to-middle-income countries (Boland et al., 2013). Therefore, the poultry and livestock industry has had to evolve and expand to supply enough meat, eggs, and dairy products to a progressively affluent world population that is more and more hungry for animal-based food. Driven by tremendous advancements in each of its phases, the chicken meat industry has constantly increased production as **Figure 6** indicates. Globally, chicken meat production was less than 8 million tons in 1961, 60 million tons in the early 2000s, and 121.6 million tons in 2021. The trends also reveal a large gap between chicken meat and meat from other poultry, whose production levels have remained almost unchanged and show a remarkable flatness compared to that of chicken meat.

Figure 5 | Annual percentage growth rate of GDP per capita restricted to 1961-2007 (**a**) and 1961-2018 (**b**)



Note: GDP per capita is based on constant local currency.
Source: Our World in Data based on World Bank data.

Figure 6 | Global production of poultry meat from 1961 to 2021



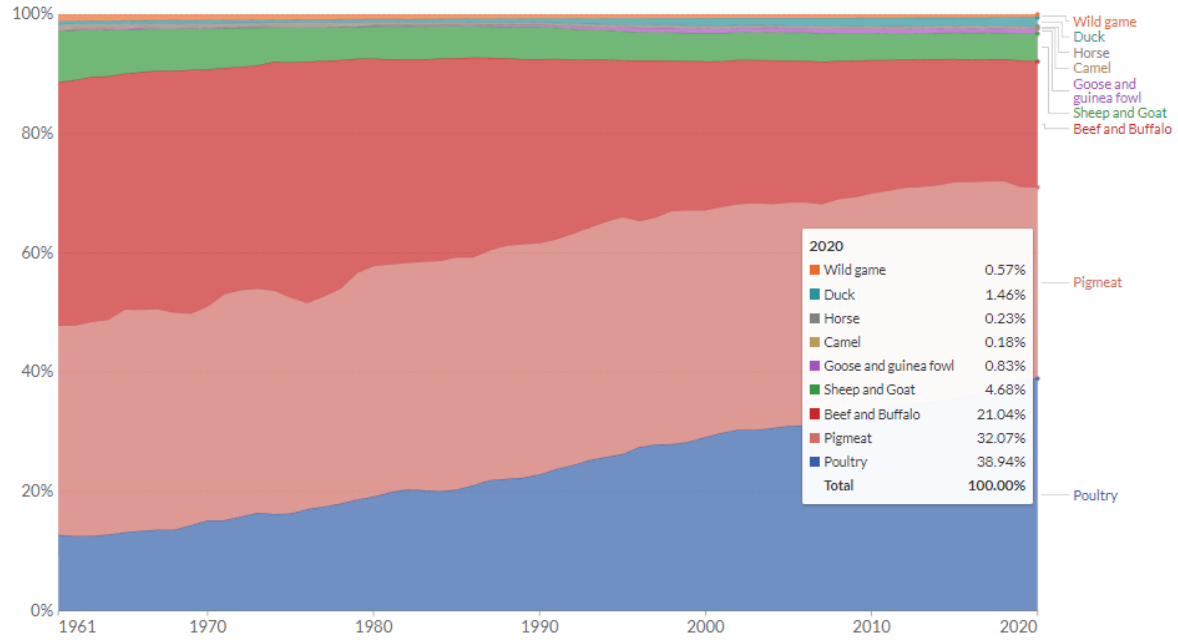
Source: FAOSTAT.

Although these figures are outstanding, the analysis of aggregate of poultry meat production data (i.e., chicken and non-chicken meat) together with data from other meat animals is even more impressive. While poultry meat accounted for just 12.7% of global meat production in 1961, it has

constantly grown to a share greater than 40% in 2020, thereby overtopping pork and beef and becoming the most produced meat in the word (**Figure 7**).

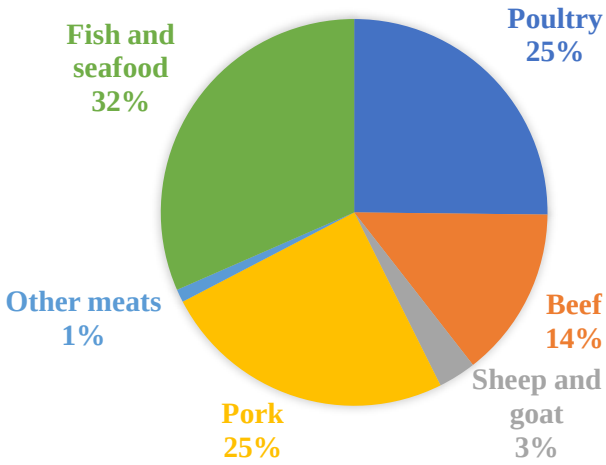
Nowadays, poultry meat represents a quarter of per capita meat consumption, as shown in **Figure 8**. When it comes to consumer preferences, the undisputed success of poultry meat has to do with its cheapness, appreciated nutritional profile and sensory properties, and simplicity and versatility of preparation. In addition, and perhaps above all else, poultry meat consumption is not restricted by religious or cultural precepts as compared with red meats (Tavárez and Solis de los Santos, 2016; Petracci et al., 2019; Baldi et al., 2020). Consequently, poultry meat makes an essential contribution to food security in the present and, looking to the future, will play a pivotal role in providing high-quality, wholesome, and inexpensive protein to an increasingly populated world (Mottet and Tempio, 2017).

Figure 7 | Global production of meat by type from 1961 to 2020



Source: Our World in Data based on FAOSTAT data.

Figure 8 | Global per capita meat consumption by type in 2019



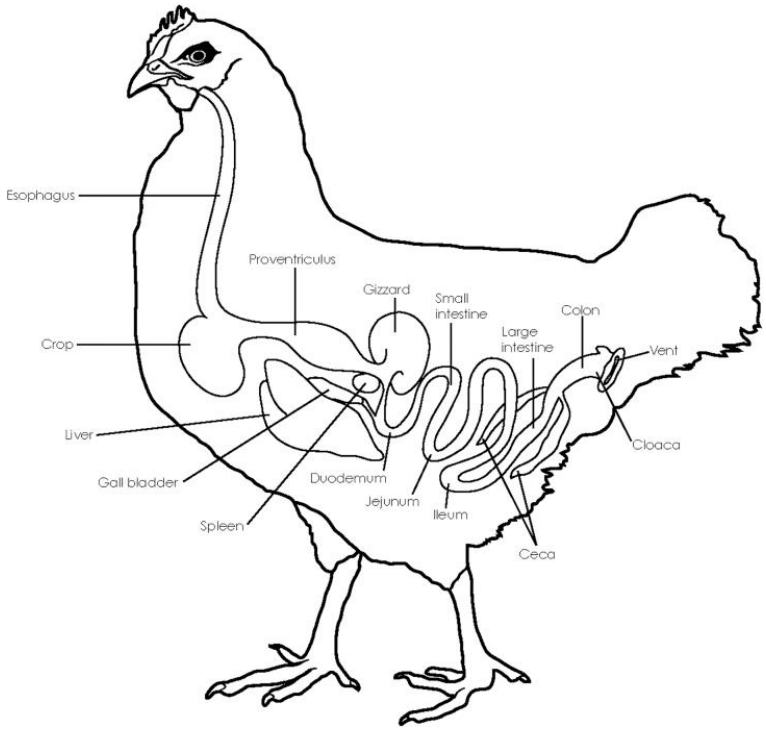
Data source: Our World in Data based on FAOSTAT data.

2 LITERATURE REVIEW

2.1 A Tour of the Chicken Gastrointestinal Tract

The modern fast-growing broiler (see section **1.1** "**The Broiler Industry: Nothing Short of a Success Story**") is an "overeater" (Siegel et al., 1984) or a "hyperphagic" (Dridi, 2017; Honda, 2021) bird whose gastrointestinal tract (**GIT**) has undergone selection-driven modifications to power extraordinary growth potential (Svihus, 2014). Nonetheless, the overall structure of the broiler GIT has basically remained equivalent to that of the red jungle fowl, the domestic chicken ancestor. With a tube configuration, the chicken GIT extends from the mouth to the anus and is divided into contiguous compartments, namely the esophagus, crop, proventriculus, gizzard, small and large intestine, ceca, and cloaca, as drawn in **Figure 9**.

Figure 9 | Sections of the chicken GIT



Source: Clavijo and Flórez (2018).

After gathering the feed with the beak, the mouth allows the bolus to move to the esophagus. In the meantime, the salivary glands release a lubricating secretion containing mucus that will aid swallowed materials in going through the first GIT sections (Scanes and Pierzchala-Koziec, 2014). The

esophagus is essentially a duct connecting the mouth, crop, and proventriculus. The crop, located just prior the thoracic cavity, is an esophagus dilation wherein ingesta is temporarily stored (Clavijo and Flórez, 2018). Here, feedstuffs undergo fermentations, carried out by commensal microorganisms as discussed below (see section **2.3** "**Exploration and Modulation of the Chicken Gastrointestinal Microbiota**"), and pre-digestion catalyzed by endogenous enzymes and those potentially given as feed additives (Olukosi et al., 2007; Scanes and Pierzchala-Koziec, 2014). It is important to note that *ad libitum*-fed broilers do not use the crop at its maximum capacity, probably because this feeding management causes ingesta to rapidly flow through or even bypass it (Svihus et al., 2010). Furthermore, broilers prefer to frequently take small amounts of feed – almost every half an hour – rather than ingesting a few, abundant meals that, oppositely, would maximize the usefulness of the crop as a storage organ (Jackson and Duke, 1995; Svihus et al., 2013; Rodrigues and Choct, 2018).

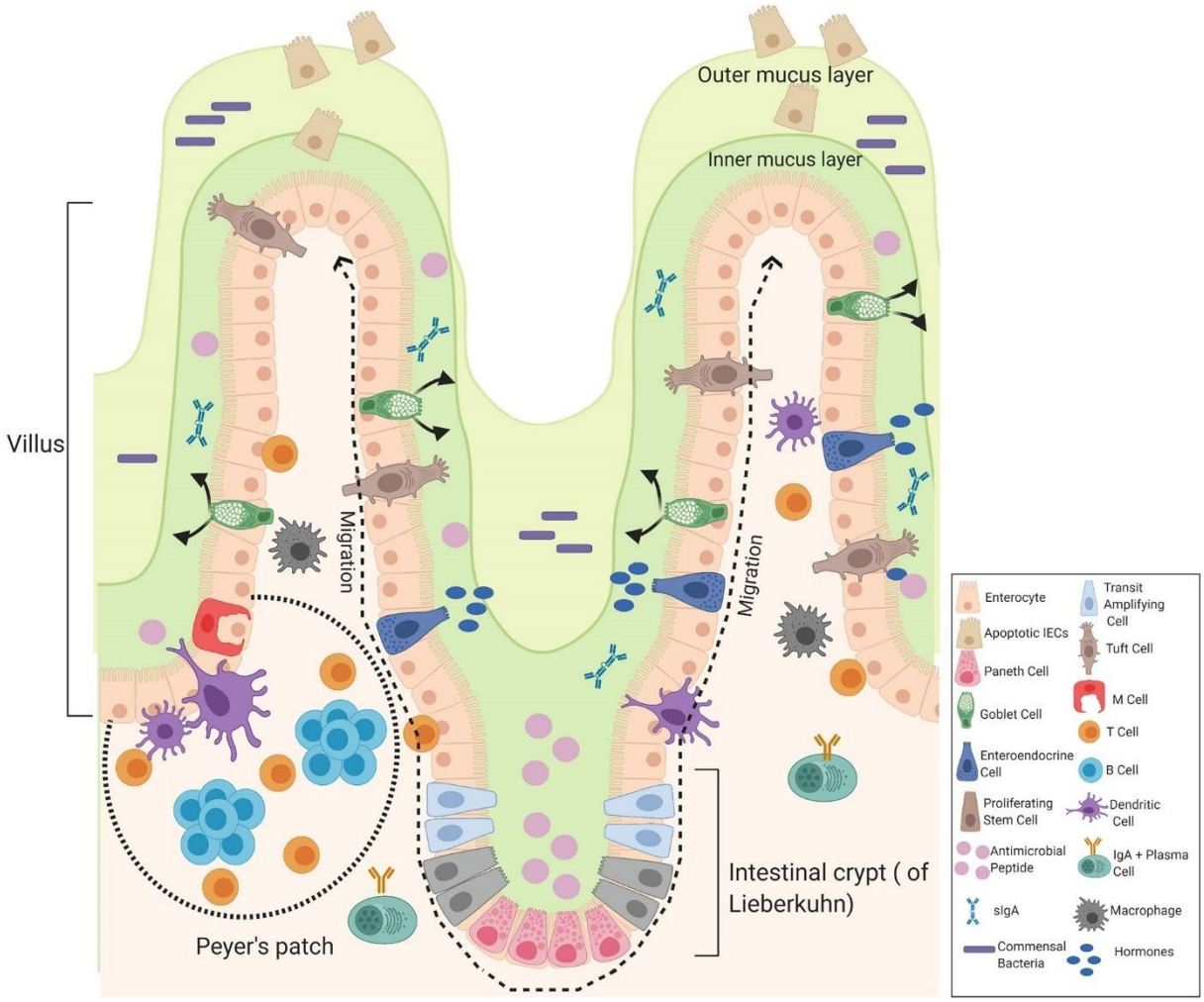
Ingesta keeps going and reaches the stomach that, in avian species, is divided into two adjacent parts: the proventriculus and the gizzard (Scanes and Pierzchala-Koziec, 2014). The function of the proventriculus, often nicknamed the "glandular stomach of birds", is the same as that of the stomach of monogastric animals, that is moistening ingesta with gastric juices secreted by oxintopeptic cells of the gastric glands. These fluids contain hydrochloric acid and pepsinogen, the latter being the precursor for pepsin that initiates protein digestion (Menzies and Fisk, 1963; Scanes and Pierzchala-Koziec, 2014). On the other hand, the gizzard is known as the "avian muscular stomach" and performs, via powerful contractions of its walls, an accurate mixing of ingesta with gastric juices as well as its mechanical breakage, which cannot be accomplished by the toothless mouth of birds. The innermost layer of the gizzard is covered with a robust and abrasive membrane, traditionally called "koilin", which bolsters the grinding activity and protects the underlying mucosa from physicochemical insults (Svihus, 2014).

The gastric content passes through the pylorus and enters the small intestine, which is composed of the duodenum, jejunum, and ileum and is responsible for the majority of digestive and absorptive processes (Svihus, 2014). The luminal layer of the small intestine is characterized by the presence of villi and crypts of Lieberkühn as **Figure 10** shows. Villi, finger-shaped mucosa folds pointed toward the intestinal lumen, considerably increase the nutrient absorptive surface areas (Koutsos and Arias, 2006). Additionally, microvilli that protrude from the villus apical surface forming the so called "brush

border" further extend the area committed to the uptake of nutrients (Delacour et al., 2016). On the other hand, crypts contain a pool of multipotent stem cells numerous types of intestinal cells can originate from, such as columnar epithelial cells/enterocytes, goblet cells, and enteroendocrine cells (McCracken and Lorenz, 2001). The villus height to crypt depth ratio is a morphometric parameter widely accepted in poultry science as a gold standard for assessing gut health (Ducatelle et al., 2018). In contrast to mammals, the proliferation of the chicken enterocytes has been demonstrated to occur both at the crypt level and along the crypt-villus axis (Uni et al., 1998b). During their migration to the villus tip (**Figure 10**), enterocytes gradually mature and acquire digestive and absorptive functions (Applegate and Troche, 2014). So, the enterocytes located near the villus tip are the most important for nutrient uptake (Ducatelle et al., 2018). Enterocytes have a short lifespan and undergo a special kind of apoptosis, named "anoikis", ending with their exfoliation from the intestinal epithelium (McCracken and Lorenz, 2001; Fouquet et al., 2004). Following the apoptotic process, sloughing cells (**Figure 10**) are replaced with maturing cells in a shedding process commonly described as the "intestinal turnover", lasting from less than two days to approximately six days (Imondi and Bird, 1966; Reisinger et al., 2011) depending on the villus length and the animal age (Smith et al., 2014).

In the duodenal loop, the acidic chyme coming from the gizzard is blended with pancreatic fluids and bile (Scanes and Pierzchala-Koziec, 2014). The chicken pancreas synthesizes a variety of digestive enzymes and zymogens, such as amylase, chymotrypsinogen, procarboxypeptidase, members of the cationic and anionic trypsin sub-families, and lipase (Marchaim and Kulka, 1967; Noy and Sklan, 1995; Wang et al., 1995a). On the other hand, bile acids emulsify the lipid fraction of ingesta, facilitating the action of lipases and, consequently, lipid digestion (Denbow, 2015). It is important to point out that, in chickens, nutrient uptake is largely performed by the distal jejunum, while water and mineral absorption occurs primarily in the ileum (Svihus, 2014).

Figure 10 | Overview of the small intestine epithelium



Note: IEC, intestinal epithelial cell; IgA, immunoglobulin A.
Source: Mohammad and Thiemermann (2021).

The ileo-ceco-colic junction is the caudal border of the ileum and demarcates the initial point of the large intestine (Svihus, 2014). In this crossroad, the ileum opens into the colon and ceca. Water absorption is the key role of the colon as suggested by higher water requirements of colostomized chickens (Son and Karasawa, 2001). The colonic muscular layers carry out both peristaltic contractions, pushing digesta toward the cloaca, and antiperistaltic contractions, forcing the cloacal content back to the colon and ceca (Lai and Duke, 1978). The cecal content is therefore a mixture of ileal and cloacal materials (Scanes and Pierzchala-Koziec, 2014). The chicken ceca are two blind finger-like pouches that originate from the proximal colon and can reach a length of 15-18 cm in the adult (McNab, 1973). Not only are the ceca indispensable for the hydro-electrolytic balance of chickens (Denbow, 2015), but they are also "bioreactors" harboring an extremely complex microbial population (Bäckhed et al., 2005)

whose characteristics and functions are examined below (see section **2.3** "**Exploration and Modulation of the Chicken Gastrointestinal Microbiota**").

2.2 Intestinal Epithelium, Immunity, and Microbiota: Three Pillars of Gut Health

Gut health has been defined as "the ability of the gut to perform normal physiological functions, maintain homeostasis[1], and withstand infectious and non-infectious stressors" (Kogut, 2022). This concept has taken center stage in animal science and breeding due to the increasingly evident interconnection between a healthy GIT and optimal health and performance of poultry and livestock (Kogut and Arsenault, 2016). Since numerous factors contribute to the establishment and maintenance of it, gut health should be addressed in a holistic way (Choct, 2009; Oviedo-Rondón, 2019). However, talking about gut health in poultry, Choct (2009) and Kogut et al. (2017) emphasized the fundamental role of three elements: the GI epithelium, immune system, and microbiota.

Emblematically, Klasing (1998) claimed that the gut is "the primary battleground between potential pathogens and the immune system". And, as in any battlefield, offensive and defensive maneuvers take place in the GIT. Besides fulfilling digestion and absorption of nutrients, the GI epithelium is integral to the protection of the GIT and the entire organism from detrimental physicochemical stimuli and microbiological attacks (Yegani and Korver, 2008). Firstly, it is a single-cell barrier (**Figure 10**) that prevents the passage of noxious luminal compounds and pathogens. This thin wall is strengthened by special proteins, called tight junctions (**TJ**), which seal the space between contiguous enterocytes (Farquhar and Palade, 1963). TJs have been grouped into two main categories, the transmembrane proteins (e.g., claudins and occludin) and the scaffolding/peripheral/plaque proteins (e.g., zonula occludens – **ZO**) (Turner, 2009; Ulluwishewa et al., 2011). The primary task of TJs is to control the paracellular pathway[2] and its two components known as the pore pathway and the leak pathway (Dokladny et al., 2016). Claudins are mainly involved in the pore pathway, while occludin and ZO in the leak pathway. The pore pathway restricts the passage of big, charged molecules. The leak

[1] Homeostasis is a self-regulating process by which biological systems maintain stability while adjusting to changing external conditions (Billman, 2020).
[2] Unlike the pump- and channel-dependent transports, the paracellular pathway allows a passive transepithelial diffusion of ions and molecules through the interstice between adjacent cells (Turner, 2009).

pathway, in contrast, is more "permissive" and potentially allows larger solutes, including bacterial lipopolysaccharides (**LPS**), to cross the GI epithelium (Anderson and Van Itallie, 2009; Dokladny et al., 2016; France and Turner, 2017). The consequences of an uncontrolled migration of LPS through the intestinal epithelial lining are explained below (see section **2.4** "**Raising Chickens in the "Hothouse Earth": The Effects of Heat Stress**"). Generally speaking, ZO connect the transmembrane TJs to the cytoskeleton of enterocytes with binding domains anchored to claudins and occludin on one side and to the perijunctional actomyosin ring on the other side (Ulluwishewa et al., 2011). It has been reported that the enzyme myosin light chain kinase (**MLCK**) regulates the circumferential contractions of the actomyosin ring and, consequently, affects the paracellular pathway (France and Turner, 2017). The MLCK-mediated contractions of the actomyosin ring can be induced by physiological and pathological stimuli. For example, tumor necrosis factor alpha (**TNF-α**), one of the most famous pro-inflammatory cytokines, has been shown to trigger intracellular signaling cascades resulting in contractions of the actomyosin ring and TJ internalization and disassociation (Turner et al., 2014). Moreover, González-Mariscal et al. (2011) found that oxidative stress alters the MLCK-regulated position of ZO and down-regulates their expression, weakening the paracellular pathway defense. The effects of inflammation and oxidative stress on the barrier functions of the GIT are addressed below (see section **2.4** "**Raising Chickens in the "Hothouse Earth": The Effects of Heat Stress**"). Scientists frequently use two methods to assess the stability and integrity of the epithelial paracellular pathway, that is the resistance to ion passage (better known as the transepithelial electrical resistance – **TEER**) and the passage of marker probes (e.g., fluorescein isothiocyanate-dextran – **FITC-D**) from the mucosa to the underlying serosa (Shen et al., 2011; Bischoff et al., 2014; Awad et al., 2017; Ma et al., 2018; Gilani et al., 2021). A steady paracellular pathway generally has a high TEER and resists marker penetration, and vice versa.

The GI epithelium is made up of specialized cells other than enterocytes (see section **2.1** "**A Tour of the Chicken Gastrointestinal Tract**"). Goblet cells are differentiated epithelial cells that produce mucus, a gelatinous secretion consisting of highly glycosylated glycoproteins called mucins[3] (Smith et al., 2014; Scanes and Pierzchala-Koziec, 2014). The luminal side of the GI epithelium is covered with

[3] Mucins can be divided into two categories, the neutral and acidic ones. The latter can be subdivided into sulfated and sialylated mucins according to the chemical nature of the oligosaccharide moieties (Forder et al., 2007).

a mucus bilayer having an outer, loose stratum and an inner, dense stratum that firmly adheres to the apical surface of the epithelium (**Figure 10**). The thickness of the mucus layer has been shown to change according to the GI compartment (Atuma et al., 2001) and, in chickens, Smirnov et al. (2004) measured the highest mucin production in the proventriculus and distal large intestine. Mucus is a lubricant that facilitates the flow of digesta and represents a mechanical and chemical protection for the GI epithelium: it is a physical barrier; it can retain antimicrobial and bacteriostatic substances, along with secretory immunoglobulin A (**sIgA**) produced and released by plasma cells situated in the lamina propria; its outer stratum undergoes a constant sloughing process that removes microorganisms and damaging particles, keeping them away from the GI mucosa (Applegate and Troche, 2014; Scanes and Pierzchala-Koziec, 2014). In addition, as summarized by Forder et al. (2007), mucins have been shown to compete with noxious bacteria for adhesion to the GI epithelium through their oligosaccharide chains, and to provide useful carbohydrates for the proliferation of desirable and protective microorganisms.

Paneth cells are capable of synthetizing and releasing lysozyme and antimicrobial defense peptides (**AMP**), such as β-defensins, into the lumen. Lysozyme and AMPs play a vital role in the innate immunity system exerting a broad-spectrum microbicidal activity, mainly by destabilizing or perforating the membranes of bacteria and fungi (Wellman-Labadie et al., 2007; Smith et al., 2014; Mookherjee et al., 2020). Mucus bilayer, antimicrobial proteins, and transcytosed sIgA collectively form the first defense line of the GI mucosa against pathogens (Belkaid and Hand, 2014; Broom and Kogut, 2018a). According to Broom and Kogut (2018a), if harmful microbes eluded this first barricade, they would face a multiple protecting system composed of the GI epithelium and the subjacent lamina propria housing most of the gut-associated lymphoid tissue (**GALT**).

The GALT is a component of the mucosa-associated lymphoid tissue (Smith et al., 2014) and has been described as the largest immune organ in the animal body, containing the majority of its immune cells (Jung et al., 2010; West et al., 2015). This makes the gut a core of the immune system. The chicken GALT includes lymphoid cells scattered throughout the GI epithelium and the lamina propria, as well as specialized lymphoid tissues (e.g., Peyer's patches, Meckel's diverticulum, and cecal tonsils) located in strategic sites along the GIT (Casteleyn et al., 2010; Smith et al., 2014). By means of specific signaling molecules, like cytokines and chemokines, the GI epithelial cells can interact with the GALT (Abreu,

2010) to orchestrate the enteral immune functions. This sophisticated mechanism is led by pattern recognition receptors (**PRR**) (Broom and Kogut, 2018b), among which Toll-like receptors (**TLR**) stand out. TLRs are particularly differentiated in the chicken intestine (Keestra et al., 2013) and have been shown to be crucial in maintaining gut homeostasis and evoking inflammatory responses in case of infections or other issues, such as hypoxia and tissue injury (Gribar et al., 2008). TLRs also contribute to epithelial cell proliferation, wound healing, preservation of TJs, and modulation of IgA production and AMP expression (Abreu, 2010; Iizuka and Konno, 2011). Furthermore, TLRs are rather non-responsive to the multitude of commensal microorganisms inhabiting the GIT, while they remain constantly sensitive to pathogen-associated molecular patterns (**PAMP**) and host indicators of cell damage (Harris et al., 2006; Madsen and Park, 2017; Kogut, 2017; Broom and Kogut, 2018c). The ability to distinguish between useful microbes and those that are undesirable – or can become such, like pathobionts[4] – is held to be one of the most fascinating properties of the GI immune system (Mowat, 2018).

A harmonic relationship between the GIT and its microbiota is of paramount importance for gut health (Celi et al., 2017). It is worth clarifying the apparent synonymy of two words: *microbiota* and *microbiome*. According to the definitions given by Marchesi and Ravel (2015), a microbiota is a group of microorganisms located in a certain environment, whereas the term microbiome should be used to describe the microbiota's metagenome (i.e., the collection of microbial genomes) and the environment wherein that specific microbiota resides. In practice, however, these similar-sounding words end up meaning the same thing and are frequently used interchangeably. The human GIT has been shown to harbor one of the densest microbial communities known to date (Spohn and Young, 2018; Cryan et al., 2019). This also applies to the chicken GIT, as it houses a myriad of bacteria, archaea, protozoa, fungi, and viruses (Yeoman et al., 2012). Surprisingly, the approximately 17,000 genes of chickens (Pertea and Salzberg, 2010) are significantly fewer than those of their GI microbiota (Broom and Kogut, 2018a). Therefore, almost acting as a "neglected"/"forgotten" organ (Bocci, 1992; O'Hara and Shanahan, 2006) that makes the host a "superorganism" (Lederberg, 2000), the GI microbiota extends the genome of

[4] Pathobionts are commensal GI microorganisms that, under specific conditions, can induce intestinal inflammation and cause diseases (Round and Mazmanian, 2009).

chickens and considerably influences their physiology (Koutsos and Arias, 2006) by participating in nutrient digestion and absorption, influencing the mucosal immune response, affecting energy homeostasis, and releasing a vast range of metabolites (Kogut, 2022).

Undigested carbohydrates, such as resistant starch and non-starch polysaccharides (**NSP**), serve as the primary source of carbon and energy for the microbiota of the large intestine (Cummings and Macfarlane, 1991). Bacteria hydrolyze and metabolize these substrates mainly via fermentation, releasing their catabolites in the GI lumen. Among the latter, organic acids stand out for their biological importance to the host. For example, cecal bacteria produce short-chain fatty acids (**SCFA**) that can be absorbed through the intestinal epithelium, entering several biochemical processes (Annison et al., 1968; Józefiak et al., 2004; Tellez et al., 2006). It has been reported that SCFAs provide energy to the GI mucosa (especially butyrate) and extraintestinal tissues (e.g., acetate and propionate) (Imoto and Namioka, 1978; Roediger, 1982; Panda et al., 2009). Moreover, SCFAs can act locally by: decreasing the luminal pH, thereby hindering undesirable, acid-sensitive bacteria (e.g., *Enterobacteriaceae*) and promoting the growth of desirable, acidophilic bacteria (e.g., *Bifidobacteria* and *Lactobacilli*); stimulating proliferation and development of enteric cells; modulating mucin secretion; affecting the GI immune response (Abrams et al., 1963; Van Der Wielen et al., 2000; Fukunaga et al., 2003; Broom and Kogut, 2018b). The effects of numerous microbiota-deriving molecules other than organic acids, such as vitamins and bioactive metabolites, have thoroughly been described in the excellent works of Donia and Fischbach (2015) and Turroni et al. (2018).

The microbiota has been shown to be instrumental in programming and regulating both the gastroenteric (Iyer and Blumberg, 2018; Cheng et al., 2019) and systemic (Belkaid and Hand, 2014; Zheng et al., 2020) immune system of humans and animals, including poultry (Broom and Kogut, 2018a). In this regard, results from gnotobiotic models with mice (Round and Mazmanian, 2009; Belkaid and Hand, 2014; Iyer and Blumberg, 2018) and chickens (Dibner et al., 2008) indicate that germ-free animals have severe developmental deficiencies and dysfunctions in the GI immunity. A similar phenomenon has been described in humans concerning the predisposition to Crohn's disease, a type of inflammatory bowel disease (**IBD**): according to the "hygiene hypothesis", lack of exposure to enteric pathogens during childhood makes a person more susceptible to Crohn's disease, while multiple

childhood infections and less strict hygiene protect an individual from developing the disease later in life by promoting the development of tolerance or immunity to its inciting agents (Lashner and Loftus, 2006). In addition to immunogenic and immunoregulatory activities, the microbiota considerably affects growth, morphology, and functions of the chicken intestine (Dibner et al., 2008; Pan and Yu, 2014). The commensal, beneficial microbiota also prevents pathogenic microorganisms from colonizing and proliferating in the GI ecosystem (Schneitz, 2005). This protective mechanism, conventionally termed competitive exclusion (**CE**), was observed in newly hatched chicks acquiring resistance to *Salmonella* challenges if previously treated *per os* with a suspension of crop and intestinal content collected from healthy adult chickens (Nurmi and Rantala, 1973). Although CE is essentially a blockage of GI niches carried out by beneficial bacteria to the detriment of pathogenic ones (Chichlowski et al., 2007), desirable bacteria can also compete with pathogens for nutrients as well as release microbiostatic and microbicidal substances, such as organic acids and bacteriocins (Pan and Yu, 2014; Clavijo and Flórez, 2018), as examined below (see section **2.3** "**Exploration and Modulation of the Chicken Gastrointestinal Microbiota**", subsection "**FEED ADDITIVES**").

Unfortunately, the host-microbiota relationship is not always "a bed of roses" (Stanley et al., 2014). Potential downsides, which have been symbolically described as the "microbiota cost", include: (i) competition for nutrients; (ii) alteration of bile acids and consequent reduction in fat digestibility; (iii) fermentation of dietary or endogenous proteins that results in the production of toxic compounds, such as ammonia, amines, phenols, cresol, and indoles; (iv) extra-acceleration in cellular turnover that increases energy and protein requirements of the GIT as well as the risk of accumulation of immature enterocytes unable to effectively absorb nutrients or to form a strong barrier; (v) induction of a mild, constant inflammation that impinges on nutrient intake and drains energy (Dibner and Richards, 2005).

Before proceeding to examine the chicken microbiota in detail, it is important to define two terms: *dysbiosis* and *eubiosis*. Dysbiosis, also known as dysbacteriosis, is a modification in the GI microbiota characterized by an overgrowth of harmful microorganisms or a reduction in beneficial microorganisms, which can disturb the host-microbiota balance (Walker, 2017a; Ducatelle et al., 2018). Dysbiotic states have been linked with depressed nutrient digestion, impaired intestinal barrier, and GI inflammation (Chen et al., 2015; Ducatelle et al., 2018). On the other hand, eubiosis, referred to as a stable microbial

ecosystem (Iebba et al., 2016), has been sown to be a prerequisite to produce sound, highly performant, and stress-resistant chickens (Kogut, 2019).

2.3 Exploration and Modulation of the Chicken Gastrointestinal Microbiota

Pioneering attempts to investigate the GI microbiota of chickens date back to the first half of the twentieth century (Rahner, 1901; Shapiro and Sarles, 1949). Later, in the early 1970s, Salanitro et al. (1974) were able to isolate 300 microbial strains from the ceca of broilers using culture-based techniques. However, according to Amann et al. (1995), the use of cultivation methods results in an underestimation of the microbial richness of samples. This intrinsic limitation of traditional analyses, along with unavoidable procedural obstacles microbiologists have to deal with (Stanley et al., 2014), has paved the way to culture-independent techniques. Over the last years, targeted amplicon sequencing, whole genome sequencing, and metaproteomics have become the most commonly used tools to study the chicken microbiota (Borda-Molina et al., 2018). Readers are referred to detailed publications for more information on these continuously evolving molecular techniques (Di Bella et al., 2013; Tang et al., 2014; Borda-Molina et al., 2018; Kunath et al., 2019; Brumfield et al., 2020; Bindari and Gerber, 2022). Non-cultural analyses have enabled scientists to evaluate the diversity, composition, and functions of the chicken microbiota much more accurately (Yeoman et al., 2012). For example, two labs have observed a pre-hatch vertical transmission of bacteria from the hen oviduct to the offspring (Ding et al., 2017; Lee et al., 2019), which calls to mind the microbial inoculation of human fetuses occurring *in utero* (Milani et al., 2017). The prenatal colonization of the GIT of chicken embryos predates, therefore, the penetration of bacteria through the shell pores during egg incubation (Cason et al., 1994) and debunks the theory that the GIT of newly hatched chicks is sterile (Bedford, 2000; Wielen et al., 2002). As soon as coming out from eggs, baby birds begin to peck around and ingest microorganisms from the environment (Fuller, 1989; Brisbin et al., 2008). Similarly to naturally delivered human neonates inoculated with vaginal and fecal microbes of their mothers (Mevissen-Verhage et al., 1987; Milani et al., 2017), hatchlings should first encounter GI microorganisms excreted by hens to rapidly acquire a stable microbiota typical of adult birds (Fuller, 1989). However, the poultry industry uses hatcheries to artificially incubate and hatch eggs under strictly controlled settings to produce high-

quality, healthy chicks (Scanes and Christensen, 2020). The separation of hatching eggs from parent flocks therefore interrupts the natural mother-to-chick horizontal transmission of GI microorganisms, giving to environmental microbes, both desirable and unwanted, the opportunity to find a warm shelter in the GIT of chicks (Fuller, 1989; Proszkowiec-Weglarz et al., 2022). According to Stanley et al. (2013), hatchery sanitation, including regular cleaning and disinfection (Scanes and Christensen, 2020), is the reason for the microbiota heterogeneity frequently observed between and within batches of newly hatched birds. In addition, chicks are also exposed to several hardly monitorable inoculation sources during transport to farms, such as the hatchery staff that handles them, boxes and vehicles they are transported in, and materials they are provided at placement (e.g., water, feed, and bedding material). It must be said that the first microbial settlers of the GIT markedly influence not just the postnatal period, but also later stages of the host life (Belkaid and Hand, 2014; Walker, 2017b; Milani et al., 2017). The establishment of an aberrant microbiota or early perturbations altering a desirable microbial framework can cause long-term adverse health effects, and vice versa (Iyer and Blumberg, 2018). Inspired by the concept of "metabolic imprinting[5]", Dibner et al. (2008) stressed the importance of "microbial imprinting" in the development and immune function of the poultry GIT (see section **2.2** "**Intestinal Epithelium, Immunity, and Microbiota: Three Pillars of Gut Health**").

The composition and density of the microbiota vary along the chicken GIT (Wielen et al., 2002; Yeoman et al., 2012) because each compartment has a specific combination of environmental, chemical, and physical conditions (Hillman et al., 2017). It has also been demonstrated that, even within the same GIT section, lumen and mucosa show different physicochemical properties (e.g., availability of nutrients and adhesion sites) that affect the microbial communities, leading to differentiate the luminal microbiota from the mucosal microbiota (Gong et al., 2002; Jeurissen et al., 2002; Borda-Molina et al., 2016; Proszkowiec-Weglarz et al., 2022). The GI microbiota should therefore be referred to as a metacommunity of innumerable microbial groups living in several ecological niches (Rinttilä and Apajalahti, 2013).

[5] Metabolic imprinting is the response to early nutrition that endures throughout the lifespan, even after the initiating nutritional stimulus is gone (Waterland and Garza, 1999).

Studies on the chicken microbiota have shown that the concentration of microbes increases and stabilizes as birds age, though Apajalahti et al. (2004) measured the highest microbial concentration in the ileum and ceca already on the third day post-hatch. **Table 1** shows the microbial concentrations of the luminal content according to the GIT section. The reason for the substantially different microbial density between the duodenum and both the ileum and ceca is multifactorial. In the duodenum, microbial growth is limited by the acidic and rapidly flowing chyme coming from the gizzard, the presence of a large array of digestive enzyme, and the high oxygen tension and concentrations of bile salts that have antimicrobial activities. In the ileum, however, the environment is much more favorable for bacterial growth because of lower oxygen tension and concentrations of enzymes and bile salts, the latter being partly reabsorbed and partly deconjugated by the microorganisms themselves. Lastly, a slow passage rate (i.e., a retention time of 2-20 h) of undigested, highly fermentable compounds makes the ceca an ideal habitat for microbial growth (Duke, 1986; Bedford, 2000; Gabriel et al., 2006; Gong et al., 2007; Pan and Yu, 2014; Denbow, 2015).

Table 1 | Microbial concentration of the luminal content in different sections of the chicken GIT

GIT section	Microbial concentration[†]	References
Crop	10^8-10^9	(Rehman et al., 2007)
Gizzard	10^7-10^8	(Yeoman et al., 2012; Oakley et al., 2014)
Duodenum	10^3-10^5	(Gong et al., 2007)
Ileum	10^8-10^9	(Apajalahti et al., 2004; Gong et al., 2007)
Ceca	10^{10}-10^{11}	(Gong et al., 2002; Apajalahti et al., 2004)

[†] CFU/g luminal content.

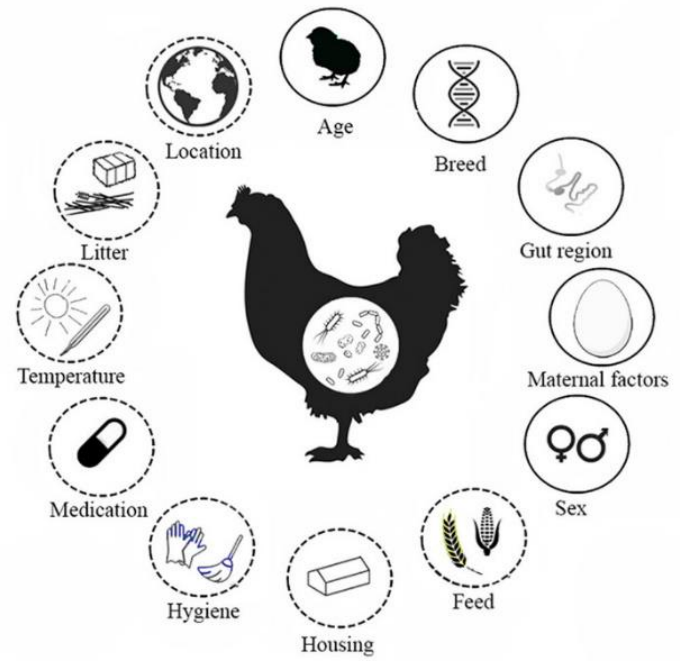
The phylogenetic structure of the chicken microbiota evolves quickly, with changes occurring over a very short time frame as in humans (Milani et al., 2017). A considerable amount of studies have investigated the compositional changes in the microbiota of chickens (Wielen et al., 2002; Pourabedin and Zhao, 2015; Oakley and Kogut, 2016; Donaldson et al., 2017; Jurburg et al., 2019) and Moore (2020) has provided an overview of these temporal changes[6]. Briefly, Enterobacteriaceae are the prevalent bacteria in the first days after hatching. A shift from Gram-negative to Gram-positive bacteria

[6] The effects of successional changes in microbiota's taxonomic composition on metabolic functioning and morphological development of the chicken GIT are not fully understood (Oakley et al., 2014).

takes place during the first week of life, with Firmicutes and Bacteroidetes outnumbering other phyla, such as Actinobacteria and Proteobacteria. In the following days, the microbiota continues to develop until an adult-like configuration is reached. According to Wei et al. (2013), the microbiota of chickens is principally composed of three bacterial phyla, namely Firmicutes, Bacteroidetes, and Proteobacteria, with *Clostridium*, *Ruminococcus*, *Lactobacillus*, and *Bacteroides* being the predominant genera. The review article authored by Bindari and Gerber (2022) lists the main bacteria usually found in the different parts of the chicken GIT.

A plethora of host- and environment-related variables affects the chicken microbiota other than the maternal factors, section of the GIT, and animal age, as indicated in **Figure 11**. The following subsections narrow in on some environmental factors that have been shown to influence the microbiota in chickens.

Figure 11 | Set of factors affecting the chicken GI microbiota



Note: Solid lines indicate host-related factors, while dashed lines indicate environmental factors. Maternal factors comprise horizontal transmission, vertical transmission, and maternal antibodies.
Source: Kers et al. (2018).

LOCATION AND ENVIRONMENTAL TEMPERATURE

Climatic conditions, generally referred to as the macroclimate, have been shown to influence the microclimate inside the poultry houses, with considerable effects on the GI microbiota and growth

performance of chickens. However, except for rare cases as the work of Xu et al. (2016), the geographical position of commercial or experimental barns is almost always undeclared in published studies, thereby complicating the evaluation of the real contribution of location to differences in the microbiota (Videnska et al., 2014; Zhou et al., 2016; Kers et al., 2018). Temperature is one of the most important environmental variables affecting poultry production (Yahav et al., 2004). For instance, high environmental temperatures have been shown to significantly affect the chicken microbiota, as examined in detail below (see section **2.4** "**Raising Chickens in the "Hothouse Earth": The Effects of Heat Stress**", subsection "**GASTROINTESTINAL MICROBIOTA**").

HOUSING

It has been demonstrated that the rearing system is an important factor affecting the chicken GI microbiota. Substantial differences have been detected between broilers reared under standard commercial conditions and those kept at low stoking density in sheds with both indoor and outdoor zones (Bjerrum et al., 2004; Gabriel et al., 2006). High stoking densities have been associated to reduction in performance and profound modifications in the microbiota of broilers. In addition, overcrowding has been found to favor the growth of harmful bacteria in the chicken GIT (Suzuki et al., 1989; Guardia et al., 2011), whereas access to an outdoor area has been linked to increased abundance of beneficial *Bifidobacteria* in the ileum and ceca (Gong et al., 2008).

The consequences of rigorous hygiene on the establishment and development of the chicken microbiota have already been discussed in this section with respect to hatcheries. Oddly enough, paying meticulous attention to hygiene and biosecurity measures at the farm can be counterproductive too, as demonstrated in an experiment comparing broilers conventionally reared with those kept in a low-bacterial load environment (Forder et al., 2007). In the latter study, birds raised in the intentionally extra-clean environment showed altered mucin profile and mucosal morphology, such as shorter villi and lower crypt depth, whose importance to protection and functions of the chicken GIT has been discussed earlier (see section **2.2** "**Intestinal Epithelium, Immunity, and Microbiota: Three Pillars of Gut Health**").

One of the most important sources of microbial inoculation chickens are exposed to throughout their life is the litter (Oakley et al., 2014). During a growth cycle, litter becomes a complex ecosystem harboring countless microbial communities, mainly of GI origin, which can shape the chicken microbiota (Pan and Yu, 2014). It has been shown that samples of litter, excreta, and carcasses share many bacterial genera (Oakley et al., 2013) and that the microbiota composition of litter samples clustered with that of cecal samples (Mancabelli et al., 2016). In recent years, there has been a growing interest in the effects of litter management on the chicken microbiota. The widespread practice of recycling litter to reduce the costs of purchasing fresh bedding material and disposing of used litter (Coufal et al., 2006) increases the carryover of environmental microorganisms from one flock to the next. This is a double-edged sword because young birds can take advantage from beneficial microbes excreted by their healthy predecessors, but can also be exposed to undesirable microorganisms and pathogens (e.g., coliforms and coccidia) as soon as they are placed in poultry barns with recycled litter from unhealthy flocks (Stanley et al., 2004; Cressman et al., 2010; Wang et al., 2016).

DIET

Diet has been known as a major driver of the chicken GI microbiota for a long time (Klasing, 1998; Bedford, 2000). In his enlightening review, Bedford (2000) argued that diet is as much a source of nutrients for the microbiota as it is for the animal to which it is offered. Several dietary factors can influence the microbiota of chickens, such as feed form and processing, particle size, quality and digestibility of dietary components, digesta viscosity and passage rate, and source and level of dietary protein and fat. For example, considerable differences in GI bacterial populations arose from the comparison between a pelleted, wheat-based feed and the same diet fed as mash; broilers receiving pellets had increased ileal concentration of coliforms and *Enterococci*, a lower number of *Lactobacilli* in the ileum, ceca, and rectum, and reduced concentration of *Clostridium perfringens* at the cecal and rectal level (Engberg et al., 2002). Interestingly, in another broiler trial performed by the same lab a few years later, the negative association between pelleted diets and *Lactobacilli* count was partially confirmed (Engberg et al., 2004). Extensive research has also shown that diets rich in water-soluble NSP (mainly from wheat, barley, oats, and rye) are poorly digestible, increase viscosity and retention time of

digesta, and promote *C. perfringens* propagation, ultimately predisposing chickens to necrotic enteritis[7] (**NE**) (Klasing, 1998; Timbermont et al., 2011; Shojadoost et al., 2012). The results obtained by Tellez et al. (2014) confirmed that broilers fed on a rye-based diet show increased intestinal viscosity compared to their corn-fed counterparts. In addition, these researchers observed that rye consumption altered the mucosal barrier and microbiota composition (e.g., a greater number of coliforms and lactic acid bacteria – **LAB**). Moving to the effects of dietary protein, the study of Drew et al. (2004) revealed that the inclusion of fishmeal as a replacement for soy protein concentrate stimulated, in a dose-dependent manner, the growth of *C. perfringens* in the broiler gut. Similarly, comparing a diet containing a blend of lard and tallow to a diet with soy oil, Knarreborg et al. (2002) showed that the abundance of *C. perfringens* was higher in the ileum of broilers fed the diet formulated with animal fat. Other studies reporting comparable findings have been discussed in the reviews by Shojadoost et al. (2012) and Bindari and Gerber (2022).

Recently, scientists have shown increased interest in the presence of biological and chemical contaminants in feed, such as pathogens, mycotoxins, heavy metals, pesticides, and herbicides, to name but a few. According to Stanley and Bajagai (2022), harmful microorganisms and noxious compounds carried by feed can adversely affect the GI microbiota, especially in chicks during the critical stages of microbiota establishment and maturation.

However, it is not only a matter of physicochemical properties and safety of diet, but also of access to feed (Kers et al., 2018). The investigation conducted by Shapiro and Sarles (1949) brought to light a fast increase in the number of bacteria in the GIT of newly hatched chickens after the first ingestion of feed. Five decades later, Uni et al. (1998a) found that the first feed provided to chicks has a pivotal role in the development of their GIT, while a delayed feed access results in a significant slowing down of the morpho-functional growth of the intestine. The negative consequences of delaying access to feed post-hatch also affect the microbiota. Proszkowiec-Weglarz et al. (2022) observed increased

[7] *Clostridium perfringens* is a commensal bacterium of the chicken GIT, but it can become an opportunistic pathogen under certain conditions that promote its excessive growth and production of toxins (Collier et al., 2003). Despite occurring most commonly in broiler flocks, *C. perfringens*-caused necrotic enteritis has also been reported in pullets and layers (Collier et al., 2003) and represents one of the greatest health problems for the poultry industry: it considerably reduces the productive performance and causes tremendous economic losses amounting to 2 billion US dollars yearly (Van der Sluis, 2000, cited in Van Immerseel et al., 2009).

relative abundance of Clostridiaceae (e.g., *C. perfringens*) and reduced level of Enterobacteriaceae in the gut of chicks without access to feed for the first 48 h after hatch compared with their broodmates immediately fed. Although these findings were obtained under experimental conditions, it is more than likely that the abovementioned, or similar, undesirable effects take place in commercial operations where hatchlings are deprived of feed and water for up to 72 h after emerging from eggs due to the hatching window, hatchery procedures, and transport to farms. The effects of post-hatch deprivation of food and water on chickens have been analyzed meticulously by de Jong et al. (2017). There is a growing body of literature that recognizes early nutrition as an effective strategy to alleviate these problems and optimize gut health, well-being, and performance of chickens right from the beginning of the growth cycle (Uni and Ferket, 2004; Willemsen et al., 2010; Noy and Uni, 2010; Jha et al., 2019).

Besides the timing of the first exposure to feed, feed withdrawal later in life has been shown to influence the microbiota. Thompson et al. (2008) observed a decrease in microbial diversity as the duration of feed withdrawal increased, while Burkholder et al. (2008) detected a greater intestinal colonization of *Salmonella* due to fasting. Once again, it is possible that these variations can occur in commercial practice, especially where restrictive feeding programs[8] rather than *ad libitum* regimens are adopted. In their review article covering the effects of different feeding practices on the chicken GIT, Rodrigues and Choct (2018) remind us that chickens fed intermittently undergo modifications in feeding behavior, location of feed storage along the GIT, and retention time. Consequently, microbial populations experience, specially under time-restricted feeding, considerable changes in the ecosystem they live in, such as fluctuations in physicochemical parameters and nutrient availability; a phenomenon that Kers et al. (2018) explained in light of the "circadian lock" notion that Johnson et al. (2017) had previously extended to GI microbes.

[8] Restrictive feeding programs, also known as intermittent feeding regimes, are intended to manipulate feed intake and can be applied by a physical removal of feed or the use of intermittent lighting, the latter being easier to manage (Rodrigues and Choct, 2018).

ANTIBIOTICS

In the second half of the 1940s, Moore et al. (1946) published a paper turning out to be a game changer in the history of animal husbandry. Observing that the inclusion of antibiotics in the diet resulted in growth stimulation of chickens, these scientists unintentionally paved the way for the use of in-feed antibiotics (**IFA**) as growth promoters in poultry and livestock nutrition. Also known as "antibiotic growth promoters" (**AGP**) to effectively convey the main goal they began to be employed for, IFAs have been an essential factor in the intensification and success of animal farming by significantly improving animals' performance (e.g., increased growth/yield and enhanced feed efficiency), health (e.g., prevention of GI disorders and reduced risk of enteric infection outbreaks[9]), and uniformity at market age (Thomke and Elwinger, 1998a; Bedford, 2000; Gadde et al., 2017). Historically, AGPs have been incorporated in meat-type poultry diets at sub-therapeutic doses – typically 5-50 g/ton feed – with a periodic rotation of different preparations to prevent the microbiota from adapting to a specific molecule (Thomke and Elwinger, 1998a; Scanes and Christensen, 2020). Indeed, the growth-promoting effects of AGPs have largely been ascribed to a modulation of the microbiota (Thomke and Elwinger, 1998b; Dibner and Richards, 2005; Gadde et al., 2017) as their presumed mechanisms of action suggest: (i) inhibition of sub-clinical infections; (ii) reduced formation of performance-depressing microbial metabolites; (iii) decreased microbial competition for nutrients; (iv) thinning of the intestinal wall that allows for increased absorption of nutrients (Gaskins et al., 2002). Several antimicrobials used as AGPs, such as avilamycin, bacitracin, enramycin, salinomycin, tylosin, and virginiamycin, have been shown to affect the chicken microbiota (Engberg et al., 2000; Knarreborg et al., 2002; Collier et al., 2003; Dumonceaux et al., 2006; Pedroso et al., 2006; Gong et al., 2008; Danzeisen et al., 2011). In contrast, no performance improvement has been observed in germ-free chickens treated with these molecules, further corroborating the assumption that the microbiota is the principal mediator for the performance-promoting effect of AGPs rather than direct changes in animal physiology (Bedford, 2000).

[9] Enteric diseases have always been an important concern to the poultry industry because of production losses, increased mortality, reduced health, and increased risk of contamination of poultry products intended for human consumption (Timbermont et al., 2011).

In addition to their use as AGPs, antibiotics have been and are administered to poultry as therapeutic means to treat bacterial infections (Scanes and Christensen, 2020), such as NE caused by *C. perfringens*. However, sub-therapeutic levels of AGPs have particularly been suitable for keeping under control the sub-clinical forms of NE (Bedford, 2000; Collier et al., 2003), which are difficult to detect and may persist in the flock without any clinical sign or increase in mortality, frequently causing greater economic losses than the clinical forms (Choct, 2009; Timbermont et al., 2011; M'Sadeq et al., 2015). Ironically, the scientific community and professionals working in the poultry industry realized that antibiotics had been a silver bullet to control *C. perfringens* immediately after the first adoptions of bans on the use of AGPs (Bedford, 2000; Collier et al., 2003; Van Immerseel et al., 2009).

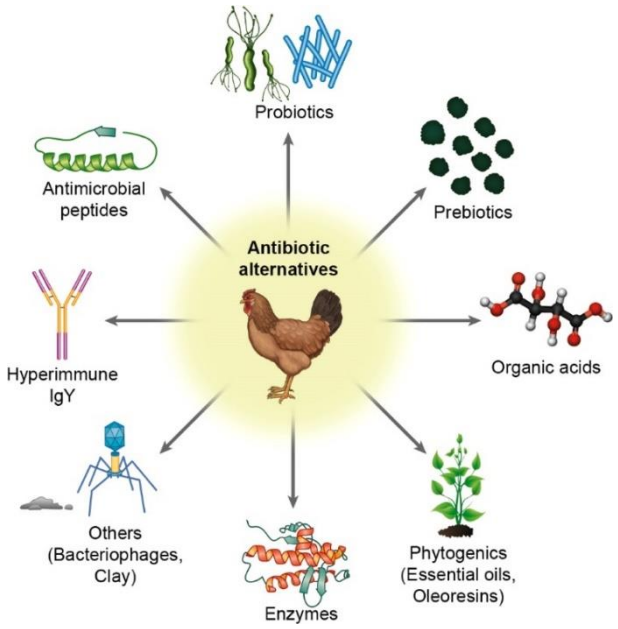
The overuse of AGPs – certainly classifiable as a misuse of antibiotics – in poultry and livestock production has contributed to the spread of antibiotic-resistant bacteria in the environment and food supply chains, posing a serious threat to human health (Ritchie, 2017; Rahman et al., 2022; Stanley et al., 2022). Although the onset of antimicrobial resistance in GI bacteria of food-producing animals had long been known (Dibner and Richards, 2005), the phase-out of AGPs has only recently been implemented, especially in some countries and not globally anyway (Castanon, 2007; Rahman et al., 2022). Consumers, retailers, and restaurant and fast food chains have been putting pressure on the poultry and livestock industry to produce food using antibiotic-free (**ABF**) systems (Scanes and Christensen, 2020). This has been challenging the animal-food industry from a sustainability perspective (e.g., reduced feed efficiencies imply a larger carbon footprint and a greater environmental impact) and farmers from a management (e.g., increased importance of biosecurity measures) and nutritional (e.g., urgent need for affordable and reliable alternatives to AGPs) standpoint (Gadde et al., 2017; Tabler et al., 2020b).

FEED ADDITIVES

Numerous feed additives have been shown to shape the GI microbiota in chickens. This particularly applies to those products that have been conceived to deal with the previously mentioned withdrawal of AGPs from many markets. Ideally, a great non-pharmaceutical alternative to AGPs should somehow mimic the beneficial effects antibiotics have on the GIT, ultimately translating into a tangible

improvement in growth performance (Huyghebaert et al., 2011). Over the last years, academia and industry have worked tirelessly to develop preparations that can successfully replace AGPs. Some of them are listed in **Figure 12**, though it should be pointed out that it is not uncommon to chance on inconsistent results concerning their efficacy while examining the literature (Gadde et al., 2017). The present subsection is by no means exhaustive as only the classes of feed additives relevant to this dissertation have briefly been presented below. Readers are therefore referred to the comprehensive reviews by Thomke and Elwinger (1998c), Yang et al. (2009), Huyghebaert et al. (2011), Gadde et al. (2017a), and Rahman et al. (2022) to find out more about these and other promising products.

Figure 12 | Potential antibiotic alternatives showing modulatory effects on the GI microbiota of chickens



Note: IgY, immunoglobulin Y.
Source: Gadde et al. (2017a).

Probiotics

More than a hundred years ago, Metchnikoff (1907) – an eminent scientist awarded the Nobel Prize in Physiology or Medicine – studying the eating habits of long-lived Bulgarians suggested that consumption of specific bacteria promotes human health. He wrote: "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes". Since then, research on these beneficial bacteria, known today as probiotics, has increased dramatically, and has led to real breakthroughs in many fields,

including animal nutrition. Although the term *probiotics* was used for the first time by Lilly and Stillwell (1965) to describe the stimulatory effects that some substances produced by microbes can have on other microorganisms, its meaning has been revised several times as knowledge about them progressed. Guarner and Schaafsma (1998) reported a definition that was subsequently rephrased in a Joint FAO/WHO Expert Consultation (2001), ending up being widely used by the scientific community: "Live microorganisms which when consumed in adequate amounts confer a health effect on the host". Speaking of their features, Fuller (1989) argued that probiotics should be non-pathogenic and non-toxic, give benefits to the host (e.g., performance improvement and increased resistance to disease), survive the transit through the GIT (e.g., tolerance to very low pH and bile salts), and withstand long storage and a variety of conditions (e.g., feed processing). Complying with these guidelines, Klose (2005) proposed an assay for probiotics intended to be used as feed additives in chicken nutrition. This evaluation is based on three classes of requirements the product in question should meet, as shown in **Table 2**.

Table 2 | Requirements a probiotic preparation should ideally meet to be used in chicken nutrition

Class	Requirements
Safety	• Chicken origin (i.e., isolated from the chicken GIT) for excellent host adaptability • Precise strain identification • Documented safe use • Tested for antibiotic resistance
Technology	• Suitable for industrial production (e.g., by fermentation) • Long shelf-life
Function	• Survival along the GIT (e.g., resistance to extreme acidity and bile salt attacks) • Ability to adhere to the mucosa and/or remain in the GIT

Source: Klose (2005).

Probiotics influence the GI microbiota and can (re-)establish eubiosis through a complex mode of action involving: (i) competition with pathogenic bacteria for space, adhesion sites, and nutrients; (ii) strengthening of the epithelial barrier integrity; (iii) secretion of antimicrobial and bacteriostatic compounds; (iv) release of organic acids that affect the GI ecosystem; (v) support to the GI immune function (Mohnl, 2014). The central importance of these aspects has been discussed in detail before (see section **2.2** "**Intestinal Epithelium, Immunity, and Microbiota: Three Pillars of Gut Health**").

Among them, however, CE (in a broad sense) is worth focusing on. Probiotics produce several substances that hinder undesirable bacteria. For instance, the undissociated form of organic acids (e.g., lactate and SCFAs) penetrate the bacterial membrane and dissociate into protons and anions in the cytoplasm, acidifying it. Bacteria try to keep the cytoplasmic pH roughly neutral to prevent conformational and functional changes in vital macromolecules, such as enzymes and nucleic acids. Excess protons must then be removed through active transport that consumes ATP. Depletion of cellular energy, coupled with an alteration in membrane structure and cellular function, results in a bacteriostatic or even bactericidal effect on sensitive bacteria (Ricke, 2003; Marteau et al., 2012; Gomez-Osorio et al., 2021). Hydrogen peroxide produced by some probiotics is another powerful agent that counteracts undesirable bacteria (Vesterlund, 2008). Lastly, an appreciated ability probiotics are often screened for is the production of bacteriocins, peptides that inhibit or kill other microorganisms (Dobson et al., 2012). For example, enterocin, a broad-spectrum bacteriocin, has been shown to inhibit several Gram-positive pathogenic bacteria. Enterocin is synthesized by and can be isolated from *Enterococcus faecium*, a LAB widely used as a probiotic supplement in livestock and poultry nutrition (Line et al., 2008; Mohnl, 2014).

Prebiotics

According to Bindels et al. (2015), a *prebiotic* is "a non-digestible compound that, through its metabolization by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host". Although this definition is very clear, it does not stress a critical point that, in contrast, was emphasized by Gibson and Roberfroid (1995) when they introduced the concept of feeding prebiotics: the chief goal of this "microbiota-targeted nutritional strategy" is to selectively give health-promoting bacteria (e.g., *Bifidobacteria* and LAB) a fitness advantage over unwanted or pathogenic microorganisms.

Similarly to AGPs, the modulatory effect on the microbiota seems therefore to be the principal mechanism by which prebiotics provide benefits to the host (Pineiro et al., 2008). Nevertheless, as Bindels et al. (2015) pointed out, the key difference is that prebiotics are essentially substrates for GI microorganisms and must be metabolized by them to exert their positive effects, whereas the mode of action of antibiotics does not depend on metabolization by microorganisms. The same authors, however,

added that prebiotics can also act without being metabolized, such as by hindering the adhesion of pathogens to host cells and by performing an immunomodulatory activity.

The list of prebiotics – with documented, or presumed and thereby under assessment, beneficial properties for the microbiota and gut health – is long and continuously updated because of the growing interest in them (Bindels et al., 2015; Hutkins et al., 2016; Singh et al., 2017). Fructo-oligosaccharides (**FOS**) are, for example, a class of prebiotics a considerable literature has grown up around. They belong to the category of inulin-type fructans, are primarily composed of fructose monomers, and can be obtained via many techniques (Roberfroid et al., 2010; Bali et al., 2015; Singh et al., 2017). Like other prebiotics, FOS cannot be hydrolyzed by digestive enzymes of birds or mammals, thereby reaching virtually intact the large intestine where they can become nourishment for the microbiota. Bacteria able to utilize FOS and other prebiotics as substrates can produce a wide range of catabolites the host may take advantage of. Besides SCFAs, whose beneficial functions have been explained earlier (see section **2.2** "**Intestinal Epithelium, Immunity, and Microbiota: Three Pillars of Gut Health**"), other "scrap" from bacterial metabolism is supposed to have positive effects, some of which are still unknown (Roberfroid, 1998; Pourabedin and Zhao, 2015).

Synbiotics

Probiotics and prebiotics can be blended to make a product that Gibson and Roberfroid (1995) named *synbiotic*. This combined preparation benefits from the synergy between its components, amplifying the positive effects probiotics and prebiotics have on the host when administered alone. Synbiotics seed the GIT with probiotic bacteria and selectively stimulate the growth and/or metabolism of beneficial bacteria, both the exogenous (i.e., the probiotics carried by the synbiotic itself) and endogenous (i.e., already present in the GIT) ones (Roberfroid, 1998).

Enzymes

Feed digestibility has always been a primary concern for poultry nutritionists. In addition to formulating diets with high-quality ingredients (e.g., low quantity of antinutritional factors) and applying feed processing (e.g., pelleting), the supplementation of enzymes has been instrumental in

boosting digestibility of chicken diets. In his informative review, Bedford (2000) described a double-phase activity for dietary enzymes, which influences not only nutrient digestibility, but also the GI microbiota. The "ileal phase" can be interpreted considering (i) an increased ileal digestibility – in other words, birds extract more nutrients from the diet and, consequently, can grow more efficiently – and (ii) a reduced availability of nutrients for the ileal microbiota, bearing in mind the abovementioned host-microbiota competition for nourishing substances (see section **2.2** "**Intestinal Epithelium, Immunity, and Microbiota: Three Pillars of Gut Health**"). On the other hand, the "cecal phase" should be understood considering the response of cecal bacteria to the end products of dietary enzyme activity, especially small oligomers and free sugars from plant cell wall. The latter are generally poorly absorbed by chickens and end up being a valuable fermentation source for some cecal bacteria (e.g., *Bifidobacteria*), acting just like the previously described prebiotics. SCFAs produced by these bacteria can be useful in controlling pathogens and providing the host with an important source of energy, as discussed before (see section **2.2** "**Intestinal Epithelium, Immunity, and Microbiota: Three Pillars of Gut Health**").

So, although they have primarily been used to improve feed digestibility, dietary enzymes have also been shown to indirectly control the proliferation of undesirable bacteria, reduce irritation of the GI mucosa that would lead to inflammation, and favor the formation of immunomodulators and gut health-promoting compounds (Bedford and Cowieson, 2012; Kiarie et al., 2013; Gadde et al., 2017; Oviedo-Rondón, 2019). Some enzymes, however, have purposely been fed to farm animals for their indirect or even "digestion-unrelated" effects, as is the case of lysozymes. Lysozymes are naturally produced by prokaryotes and eukaryotes (Wellman-Labadie et al., 2007). In the animal kingdom, they are secreted with tears, saliva, airway fluid, and breast milk, among others (Callewaert and Michiels, 2010). The chicken lysozyme, isolated for the first time by Sir Alexander Fleming, is considered the quintessential lysozyme (Wellman-Labadie et al., 2007). Avian lysozymes are mainly found in the egg albumen (Wellman-Labadie et al., 2007; Callewaert and Michiels, 2010), but chicken small intestine enterocytes are also capable of synthesizing them (Nile et al., 2004). Indeed, as stated above (see section **2.2** "**Intestinal Epithelium, Immunity, and Microbiota: Three Pillars of Gut Health**"), lysozymes are crucial effectors for the innate immunity because they act as bacteriolytic, broad-spectrum antimicrobial

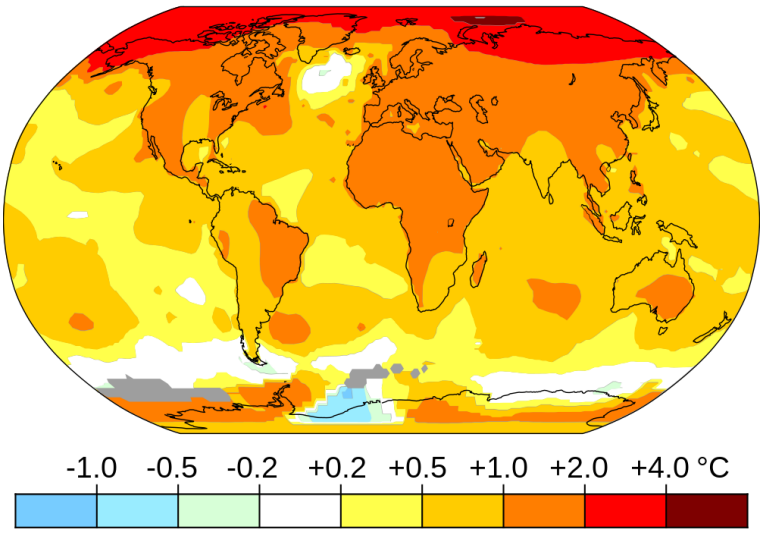
agents by hydrolyzing the β-(1,4)-glycosidic bond between *N*-acetylmuramic acid (**NAM**) and *N*-acetylglucosamine (**NAG**) of the bacterial peptidoglycan (**PGN**). Being PGN *N*-acetylmuramoylhydrolase, lysozymes are also known as *muramidases* (Masschalck and Michiels, 2003; Wellman-Labadie et al., 2007; Callewaert and Michiels, 2010). Feed additives containing muramidases have been shown to exert benefits on the health and performance of pigs (May et al., 2012; Ma et al., 2017; Xu et al., 2018c), rabbits (EL-Deep et al., 2020), and chickens (Liu et al., 2010; Gong et al., 2017), which have largely been ascribed to their modulatory effect on the microbiota (Liu et al., 2010; Gong et al., 2017; Xia et al., 2019; EL-Deep et al., 2020).

2.4 Raising Chickens in the "Hothouse Earth": The Effects of Heat Stress

Human-induced climate change, especially global warming[10], is one of the hardest challenges the poultry and livestock industry have been facing (Nardone et al., 2010; Bezner Kerr et al., 2022). As shown in **Figure 13**, our planet is getting warmer and warmer due to anthropogenic greenhouse gas emissions. Experts claim that the likelihood of the annual average global temperature temporarily reaching 1.5 °C above the pre-industrial level as well as the occurrence of devastating, extreme heat waves has steadily increased over the last years (CarbonBrief, 2022; WMO, 2022). Alarmingly, the analysis carried out by Steffen et al. (2018) suggests that the possibility that a cascade of feedbacks pushing the Earth system irreversibly toward a "Hothouse Earth pathway" cannot be ruled out. Regardless of the industrialization level of the agricultural system, poultry and livestock farming is expected to become an increasingly difficult activity that will necessitate multidisciplinary solutions to endure such a worrying climate scenario (Nardone et al., 2010).

[10] *Climate change* and *global warming* are often used interchangeably but, scientifically, they have different meanings. According to NASA (2018), climate change refers to a broad range of global phenomena created predominantly by burning fossil fuels, which add heat-trapping gases to Earth's atmosphere. These phenomena include the increased temperature trends described as global warming, but also encompass changes such as sea level rise, ice mass loss, and extreme weather events. Global warming – which use has spread after NASA climate scientist James Hansen used it in his testimony before the U.S. Senate in 1988 – describes the upward temperature trend across the Earth since the early 20th century due to the increase in fossil fuel emissions.

Figure 13 | Average Earth surface temperature from 2011 to 2021 compared to 1956-1976 baseline



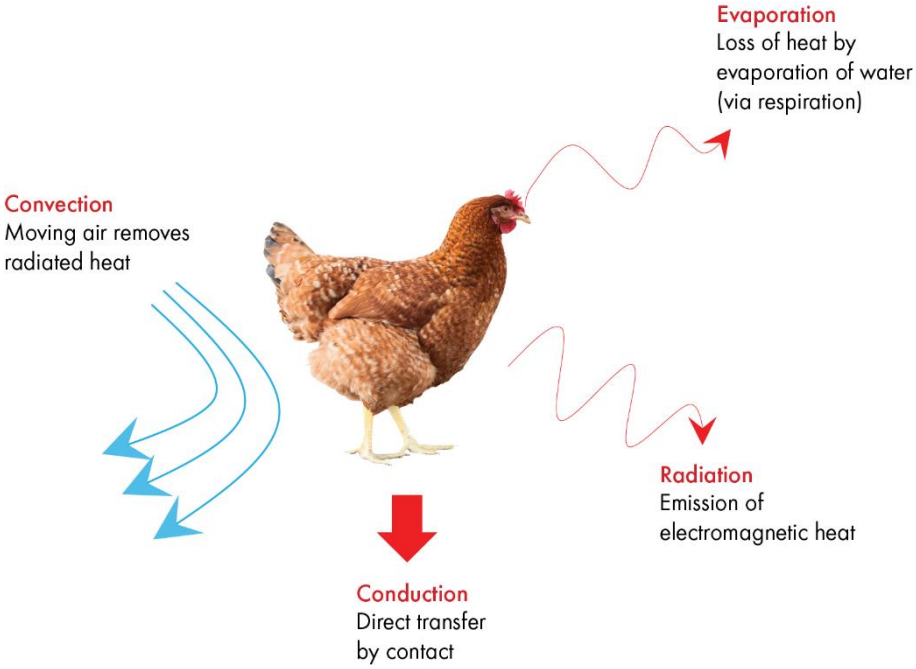
Source (adapted): Wikipedia based on NASA data.

Being homeothermic, chickens can regulate their body temperature within a narrow range irrespective of the ambient temperature. To keep a normal body temperature between 40 and 42 °C, chickens adopt autonomic and behavioral thermoregulatory processes. The autonomic processes are involuntary responses to heat and cold that modify the rates of heat production (thermogenesis) and heat loss, while the behavioral processes are any coordinated movement of an organism in search of a condition to optimize heat exchange with the environment (IUPS Thermal Commission, 1987). Chickens dissipate heat through conduction, convection, radiation, and evaporation, as illustrated in **Figure 14**.

It is worth noting that the ability of chickens to lose heat is rather limited due to the plumage, which insulates the body and restricts sensible heat loss[11], and the phylogenetic absence of sweat glands, which makes chickens incapable of perspiring and limits evaporative heat loss[12] to heat dissipated by exhalation of air saturated with water vapor (Nichelmann et al., 1986; Yunis and Cahaner, 1999; Renaudeau et al., 2012; Rostagno, 2020).

[11] Sensible heat loss is the sum of heat flows or fluxes by radiation, convection, and conduction from a body to the environment (IUPS Thermal Commission, 1987).
[12] Evaporative heat loss is the evaporative heat transfer from the body to the ambient by evaporation of water from the skin and the surfaces of the respiratory tract (IUPS Thermal Commission, 1987).

Figure 14 | Heat loss mechanisms of the chicken body



Source: Munters.

Farm animals can achieve maximal performance as long as they are kept in a thermoneutral zone (**TNZ**[13]; Kadzere et al., 2002), namely within a range of ambient temperature at which the regulation of body temperature is accomplished by control of sensible heat loss without changes in metabolic heat production or evaporative heat loss (IUPS Thermal Commission, 1987). The TNZ varies depending on the animal age, species/breed, tissue insulation (e.g., fat, skin), external insulation (e.g., coat, feathering), behavior, productive/physiological stage, feed intake (**FI**), diet composition, previous acclimation, and housing conditions (Kadzere et al., 2002). With respect to poultry, young birds tolerate low temperatures less than adults because of their unfavorable body surface to volume ratio and incomplete feathering, whereas tolerance to high temperatures decreases as birds age (Tzschentke et al., 1996). Breeding companies therefore recommend adjusting the environmental temperature of poultry houses at animal level according to the rearing cycle phase, as exemplified in **Table 3**. The breeding company in question provided target environmental temperatures not only as a function of the flock age, but also depending on relative humidity (**RH**). Indeed, along with temperature, RH and air velocity are the main

[13] The TNZ ranges from the lower critical temperature to the upper critical temperature. The first is the ambient temperature below which thermogenesis must be increased, while the second is the ambient temperature above which the rate of evaporative heat loss must be increased (e.g., by thermal tachypnea or thermal sweating) to maintain thermal balance (IUPS Thermal Commission, 1987).

environmental factors affecting poultry performance, especially for broilers (Yahav et al., 2004). "Effective temperature", an arbitrary index that combines in a single value the effects of temperature, RH, and air velocity on the sensation of warmth or cold felt by animals (Bligh and Johnson, 1973), should therefore be considered in poultry husbandry rather than the ambient temperature alone. For instance, sensible heat loss is primarily driven by the ambient temperature and depends upon the thermal gradient between the animal body and the environment, while RH affects water evaporation and, consequently, influences evaporative heat loss (Renaudeau et al., 2012). So, the simultaneous occurrence of high environmental temperatures and RH constrains heat dissipation and considerably increases the risk of manifesting hyperthermia[14] and heat stress (**HS**).

Table 3 | Target environmental temperatures for a commercial broiler line according to flock age

Age (Days)	Dry Bulb Temperature °C (°F)			
	40 RH%	50 RH%	60 RH%	70 RH%
Day-old	36.0 (96.8)	33.2 (91.8)	30.8 (87.4)	29.2 (84.6)
3	33.7 (92.7)	31.2 (88.2)	28.9 (84.0)	27.3 (81.1)
6	32.5 (90.5)	29.9 (85.8)	27.7 (81.9)	26.0 (78.8)
9	31.3 (88.3)	28.6 (83.5)	26.7 (80.1)	25.0 (77.0)
12	30.2 (86.4)	27.8 (82.0)	25.7 (78.3)	24.0 (75.2)
15	29.0 (84.2)	26.8 (80.2)	24.8 (76.6)	23.0 (73.4)
18	27.7 (81.9)	25.5 (77.9)	23.6 (74.5)	21.9 (71.4)
21	26.9 (80.4)	24.7 (76.5)	22.7 (72.9)	21.3 (70.3)
24	25.7 (78.3)	23.5 (74.3)	21.7 (71.1)	20.2 (68.4)
27	24.8 (76.6)	22.7 (72.9)	20.7 (69.3)	19.3 (66.7)

Note: Within a specific age, optimal dry bulb temperatures[15] at the ideal relative humidity (RH)[16] are colored in red.
Source: Ross® Broiler Management Handbook 2018.

[14] Hyperthermia is the condition of a temperature regulator (i.e., an organism that regulates its body temperature to some extent by autonomic and/or behavioral processes) when the core temperature (i.e., ideally, the mean temperature of the thermal core) is above the range specified for the normal active state of the species. Hyperthermia is the consequence of the temporary or permanent imbalance between heat load and the capability to dissipate heat (IUPS Thermal Commission, 1987).
[15] Dry bulb temperature is the temperature of a gas or mixture of gases indicated by a thermometer shielded from radiation (IUPS Thermal Commission, 1987).
[16] Relative humidity is the ratio of the mol fraction of water vapor present in a volume of air to the tool fraction present in saturated air, both at the same temperature and pressure (IUPS Thermal Commission, 1987).

It has been said earlier that poultry become increasingly susceptible to high environmental temperatures during their lifespan. When it comes to fast-growing broilers (see section **1.1** "**The Broiler Industry: Nothing Short of a Success Story**"), besides the *natural* obstacles to losing heat listed before, the *artificial selection*-caused increase in metabolic rates and inadequate development of the cardiovascular and respiratory systems have exacerbated their inability to withstand hot conditions, making them poorly thermotolerant and extremely vulnerable to HS (Cahaner and Leenstra, 1992; Yunis and Cahaner, 1999; Havenstein et al., 2003b; Gous and Morris, 2005; Yahav et al., 2005; Lu et al., 2007; Tickle et al., 2014; Xu et al., 2018b). This is evident not only when comparing fast-growing to slow-growing broilers, as reported by some of the studies just cited, but also within the same line when comparisons between heavy birds and their lighter counterparts equal in age are made (Gogoi et al., 2021).

Despite the marked sensitivity to high temperatures, fast-growing broilers share several evolutionary conserved responses to HS with other animals, including humans (Lambert et al., 2002; Pearce et al., 2012; Snipe et al., 2018; Koch et al., 2019; Kaufman et al., 2021). These reactions have negative implications for behavior, metabolism, homeostasis, and the GIT, as summarized in **Figure 15** and examined in the following subsections.

BEHAVIOR

When exposed to environmental temperatures above the upper limit of the TNZ (see **Footnote 13** for more information), chickens typically show a set of "cooling behaviors" (Wang et al., 2018b) to avoid overheating and preserve or promptly restore normothermia. Respiratory evaporative heat loss becomes the predominant way to dissipate heat, with thermal tachypnea[17] characterizing the early stages of the heat load reaction (Jukes, 1971; Teeter et al., 1985). If hot conditions persist, thermal tachypnea turns into a slower and deeper panting called thermal hyperpnea[18] (Hales, 1973; Renaudeau et al., 2012).

[17] Thermal tachypnea/polypnea/panting is a rapid respiratory frequency accompanied by an increase in respiratory minute volume and, commonly, a decrease in tidal volume, in response to a thermoregulatory need to dissipate heat (IUPS Thermal Commission, 1987).

[18] Thermal hyperpnea is an increase in tidal volume associated with an increase in alveolar ventilation occurring during severe heat stress which has caused a large rise in core temperature. It is also named second panting phase

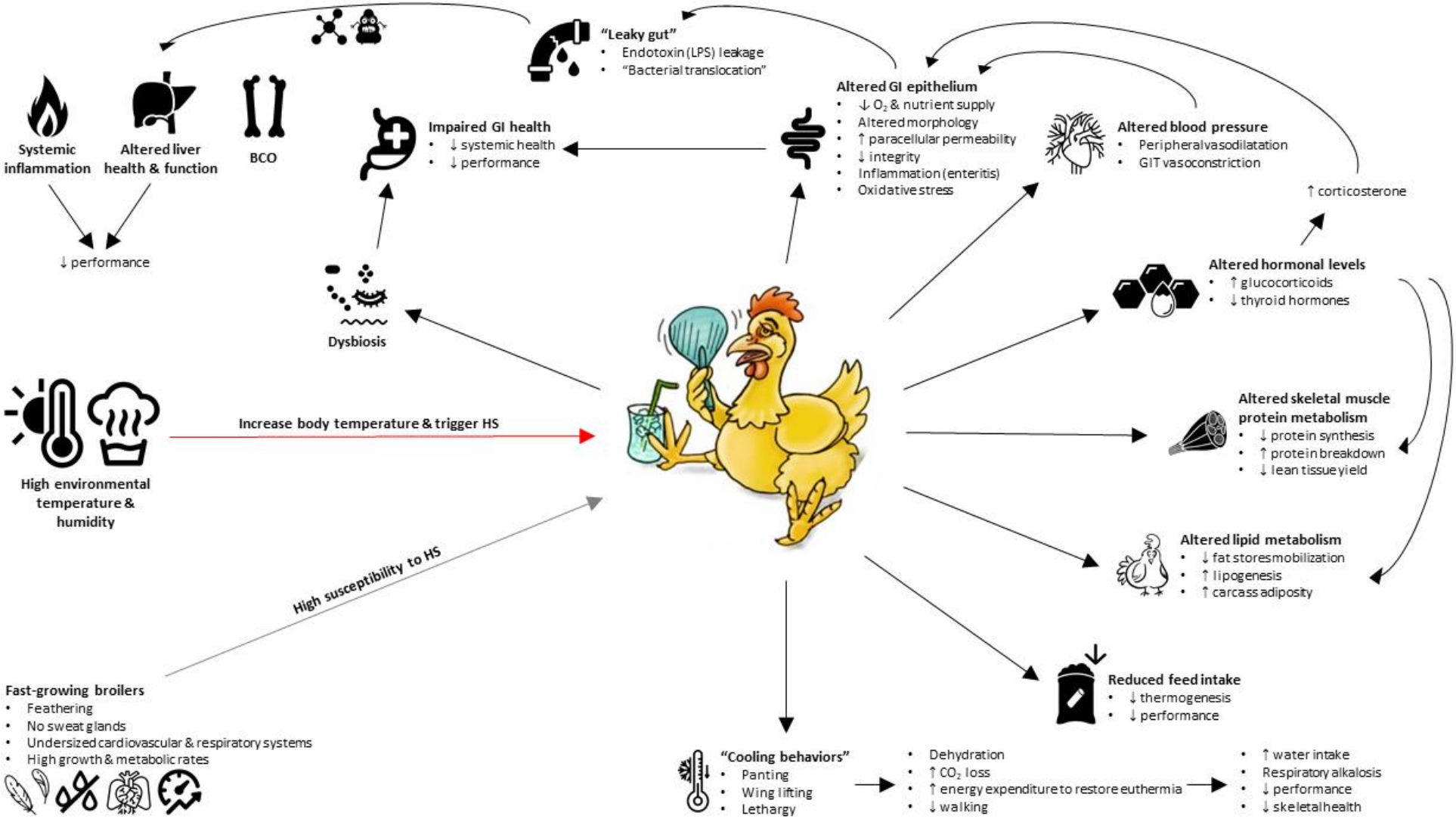
In addition, chickens start lifting their wings and keeping them spread away from the body to expose unfeathered areas to the surrounding air in an attempt to increase sensible heat loss (Borges et al., 2007). Despite being vital behavioral adaptations to HS, panting and frequently raising wings have important drawbacks for commercially-reared chickens. Panting can cause dehydration, which usually results in increased requirement and consumption of water (Wang et al., 2018b), and excessive exhalation of CO_2, which leads to hypocapnia and, potentially, respiratory alkalosis (Richards, 1970; Marder and Arad, 1989; Renaudeau et al., 2012; Wasti et al., 2020; Beckford et al., 2020). Alkalosis, a disturbance in acid-base balance, poses a real threat to the egg industry as it reduces blood ionized calcium levels, thereby impairing eggshell mineralization (Odom et al., 1986). However, heat-stressed broilers are also affected by the negative effects of respiratory alkalosis, such as lowered FI that compromises their growth (Teeter et al., 1985; Borges et al., 2007). Furthermore, both panting and wing lifting are energy-intensive activities (Brackenbury and Avery, 1980; Dale and Fuller, 1980) that deplete energy that can be allocated to production purposes (Yahav et al., 2004; Baumgard and Rhoads, 2013).

Lethargy is another behavior commonly observed in chickens experiencing HS, with listless birds squatting on the ground or litter to maximize sensible heat loss via conduction. However, apathy is negatively related to walking and the desire to move in search of feed or toward the feeders. The undesirable consequences of lethargy on skeletal health (Hester et al., 2013) and FI (Wang et al., 2018b) are therefore obvious. Anyway, it should be noted that reducing feed consumption is a conserved thermoregulation strategy to decrease postprandial heat production[19] due to nutrient digestion, absorption, and utilization (Baumgard and Rhoads, 2013). In the past, there was a consensus among poultry scientists and professionals that the considerable reduction in performance caused by the exposure to HS was mostly, or even solely, ascribable to reduced FI (Dale and Fuller, 1980; Teeter et al., 1985).

because it is usually preceded by a phase of tachypnea (rapid, shallow breathing) (IUPS Thermal Commission, 1987).

[19] Postprandial heat production is the increase in metabolic heat production, relative to its post-absorptive resting level, in the hours following food intake (IUPS Thermal Commission, 1987).

Figure 15 | Overview of heat stress effects on chickens



Note: HS, heat stress; LPS, lipopolysaccharide; GI, gastrointestinal; BCO, bacterial chondronecrosis with osteomyelitis.
Source: Brugaletta et al. (2022).

Pair-feeding[20] models applied in HS trials with broilers, however, have brought to light that 60 to 99% of body weight gain (**BWG**) loss can be attributed to drops in FI, while the remaining part has to do with impaired digestibility and/or physiological and metabolic changes caused by HS (Dale and Fuller, 1980; Geraert et al., 1996a; Ain Baziz et al., 1996; Lu et al., 2007, 2018; Zuo et al., 2015; Teyssier et al., 2022b; a). Interestingly, BWG loss broilers show under HS is often greater than expected due to reduced FI alone (Renaudeau et al., 2012). This has also been reported for milk production loss of heat-stressed dairy cows (Baumgard and Rhoads, 2013) suggesting, once again, that many shared mechanisms steer the response of homeothermic animals to high environmental temperatures and HS.

LIPID AND PROTEIN METABOLISM

Animals under HS have shown to have limited fat mobilization despite a negative energy balance and catabolic state (Baumgard and Rhoads, 2013). A gradual reduction in circulating non-esterified fatty acids (**NEFA**, also known as free fatty acids), a reliable biomarker of lipid metabolism, indicates a restricted use of fat for energy in chickens (Bobek et al., 1997; Lu et al., 2018), pigs (Pearce et al., 2013a; Victoria Sanz Fernandez et al., 2015), and dairy cattle (Rhoads et al., 2009) experiencing a thermal stress. In addition, many papers have documented increased accumulation of visceral/abdominal, subcutaneous, and intramuscular fat in heat-stressed chickens (Kleiber and Dougherty, 1934; Kubena et al., 1972; Ain Baziz et al., 1996; Yunianto et al., 1997; He et al., 2015; Lu et al., 2018, 2019). It is worth noting that peripheral retention of lipids can obstruct the dissipation of sensible heat (Renaudeau et al., 2012), increasing the risk of hyperthermia. Focusing on pigs and cattle, Baumgard and Rhoads (2013) hypothesized that the scarce fat mobilization is a metabolic response due to hyperinsulinemia induced by HS. Unlike mammals, however, chickens have shown no rise in blood insulin when exposed to HS (Geraert et al., 1996b; Tang et al., 2013; Belhadj Slimen et al., 2016), although Lu et al. (2019) have reported an increase and a decrease in circulating insulin after 7 and 14 d of HS, respectively. Avian insulin has also been shown not to have a potent antilipolytic activity and, besides, the relevance of

[20] Pair-feeding is used to minimize the confounding effect due to different feed intake of thermoneutral and heat-stressed animals. In practice, pair-fed animals are given daily the amount of feed consumed the day before by their thermoneutral, *ad libitum*-fed counterparts (Dale and Fuller, 1980).

insulin signaling cascades in chicken adipose tissue has been the subject of intense debate in the scientific community (Dupont et al., 2012, 2015). The role of insulin in fat metabolism of heat-stressed chickens remains therefore unclear. Anyway, the altered lipid metabolism in chickens subjected to HS has also been ascribed to increased expression of proteins involved in hepatic lipogenesis, resulting in fat accumulation in the liver (Flees et al., 2017; Lu et al., 2019).

Not only an increase in fat, but also a reduction in lean tissue yield has frequently been found in carcasses of heat-stressed broilers (Howlider and Rose, 1989; Geraert et al., 1996a; Ain Baziz et al., 1996; Temim et al., 2000; Zuo et al., 2015; Lu et al., 2018; Qaid and Al-Garadi, 2021; Zampiga et al., 2021b). Temim et al. (2000) first hypothesized that the decrease in protein deposition under HS conditions is related to reduced protein synthesis rather than intensified protein degradation. However, according to Zuo et al. (2015), the reasons for the reduction in protein accretion can be muscle-specific, with the breast mainly showing reduced protein synthesis, while the thigh increased protein degradation[21]. According to the latter researchers, the decrease in protein synthesis is caused by reduced mRNA expression of insulin-like growth factor 1 (**IGF-1**), phosphatidylinositol 3-kinase (**PI3K**), and ribosomal protein S6 kinase beta-1 (**S6K**), while the intensification of protein degradation is a result of increased expression of muscle atrophy F-box (**MAFbx**, also called atrogin-1). Ma et al. (2021) confirmed that HS alters gene expression of S6K and MAFbx in broilers. S6K plays a critical role in the control of protein synthesis and muscle development in chickens (Bigot et al., 2003; Duchêne et al., 2008; Everaert et al., 2010) and its signaling pathway has been shown to respond only partially to anabolic stimuli during HS (Boussaid-Om Ezzine et al., 2010). On the other hand, the expression of MAFbx, an ubiquitin ligase that participates in muscle protein breakdown, was found to be up-regulated during muscle atrophy (Zuo et al., 2015). Curiously, analyzing the blood of heat-stressed broilers, Lu et al. (2018) revealed a reduction in glucose and NEFA as well as an increase in uric acid, urea, and proteinogenic amino acids (**AA**), although those birds had also showed a substantial decrease in FI and breast muscle yield compared to the thermoneutral (**TN**) control. They then assumed that chickens undergoing HS obtain energy from the degradation of skeletal muscle protein, mainly from pectoral

[21] The intensity of protein degradation in skeletal muscle can be assessed by measuring several biomarkers, such as blood levels of creatine, 3-methylhistidine, and urea (Rhoads et al., 2013).

muscle reserves, to compensate for the limited ability to get energy from fat. Heat-stressed animals have been shown to become highly dependent on glucose for their energy needs (Baumgard and Rhoads, 2013). Interestingly, Ma et al. (2021) reported that, in broilers, HS decreased plasmatic level of glucogenic AAs, increased liver uptake of AAs and its glucogenic potential, and improved the activity of hepatic transaminases that deaminize AAs to transform them into precursors for gluconeogenesis. Zampiga et al. (2021b) also found reduced levels of glucogenic precursors in the blood and free AAs in the pectoral muscle of heat-stressed broilers, probably associated with a prioritization of hepatic gluconeogenesis at the expense of muscle-derived glucogenic AAs.

HORMONES

The exposure to HS stimulates the hypothalamic-pituitary-adrenal axis, resulting in a significant increase in circulating glucocorticoids, especially corticosterone (Geraert et al., 1996b; Yunianto et al., 1997; Quinteiro-Filho et al., 2010, 2012; Rajaei-Sharifabadi et al., 2017; Lu et al., 2019; Beckford et al., 2020; Ma et al., 2021). It has been reported that high corticosterone levels reduce growth, trigger proteolysis and lower protein synthesis in skeletal muscle, and promote fat deposition in chickens (Decuypere and Buyse, 1988; Dong et al., 2007; Yuan et al., 2008). These are all common effects of HS as said before (Rhoads et al., 2013). Ma et al. (2021) suggested that corticosterone alters protein metabolism in skeletal muscle by inducing the previously described changes in gene expression of S6K and MAFbx. However, Furukawa et al. (2021) demonstrated that treating chicken myotubes subjected to HS with corticosterone does not increase the expression of MAFbx or makes proteolysis worse in comparison to HS exposure alone. As these results have been obtained *in vitro*, further studies should be undertaken to clarify the role of corticosterone in altered protein metabolism of heat-stressed chickens. In addition, corticosterone has been shown to act as a lipogenic hormone that up-regulates the expression of fatty acid synthase (**FAS**) in hepatocytes and adipocytes (Gonzalez-Rivas et al., 2020).

According to Quinteiro-Filho et al. (2010), hypercorticosteronemia is immunosuppressive. Indeed, HS has been shown to compromise the immunocompetence in chickens, making them more susceptible to infectious diseases (Renaudeau et al., 2012; Farag and Alagawany, 2018; Chauhan et al., 2021). Severe immunological disorders have been observed in heat-stressed broilers, such as decreased

immunoglobulin production against a prototype antigen and atrophy and dysfunction of lymphoid tissues accompanied by depression of lymphocyte levels (Hirakawa et al., 2020). In the latter paper, hypercorticosteronemia was recognized as a likely cause of these immune system abnormalities.

Reduced hematic levels of triiodothyronine ($\mathbf{T_3}$) and thyroxine ($\mathbf{T_4}$) have also been reported in heat-stressed broilers (Geraert et al., 1996b; Yunianto et al., 1997; Sohail et al., 2010; Rajaei-Sharifabadi et al., 2017; Beckford et al., 2020) and laying hens (de Andrade et al., 1977; Bobek et al., 1997). Surprisingly, these modifications, potentially due to reduced thyroid size and decreased thyroid activity (Huston and Carmon, 1962; Dale and Fuller, 1980; Yunianto et al., 1997), were found in heat-stressed dairy cows too (Chen et al., 2018). It is a widely held view that the thyroid responds in this way to high environmental temperatures to enable homeothermic animals to reduce basal metabolism and thermogenesis to avert hyperthermia (Renaudeau et al., 2012; Chen et al., 2018; Gonzalez-Rivas et al., 2020). When it comes to chicken performance, the hypothyroid-like state caused by HS can justify part of BWG loss (McNabb and Darras, 2015), increased adiposity (Decuypere and Buyse, 1988; Geraert et al., 1996b), and reduced egg production and shell quality (de Andrade et al., 1977).

GASTROINTESTINAL TRACT

The complex compensatory response to HS also involves the cardiovascular system with as immediate as rather unforeseen repercussions on the GIT. Peripheral vasodilation, intended to increase circulatory convection[22] and sensible heat dissipation, takes place at the expense of visceral circulation that must be reduced to balance blood pressure (Hales, 1973; Borges et al., 2007; Lambert, 2009). By reducing the supply of nutrients and O_2 to splanchnic tissues, visceral hypoperfusion compromises the health and regular functions of the GIT (Lambert, 2009; Baumgard and Rhoads, 2013; Rostagno, 2020). The sub-optimal trophism challenges the metabolically demanding GI epithelium altering its cellular turnover and preventing it from keeping a robust mucosal barrier (Koutsos and Arias, 2006), while

[22] Circulatory convection is the convective transfer of heat with the blood stream from the thermal core to heat dissipating surfaces of the body, namely the skin and respiratory mucosal surfaces (IUPS Thermal Commission, 1987).

hypoxia[23] is a direct effect of inadequate tissue oxygenation. Hypoxic states impinge on cellular bioenergetic pathways and boost processes that generate reactive oxygen and nitrogen species (**ROS** and **RNS**, respectively) (Hall et al., 1999). Hyperthermia has also been shown to increase production of ROS and RNS *per se* (Hall et al., 2001) and to damage the enzymatic antioxidant system (Farag and Alagawany, 2018). The progress of pro-oxidative conditions seems therefore to be almost inevitable under HS. In chickens, it has been demonstrated that the exposure to high environmental temperatures leads to oxidative stress and that HS can impair the mitochondrial respiratory chain activity, with consequent overproduction and accumulation of ROS (Lin et al., 2006; Tan et al., 2010; Akbarian et al., 2016). Oxidative damage affects several tissues, such as skeletal muscle and the GIT. Analyses of *Pectoralis major* of broilers have detected an increase in mitochondrial membrane potential, production of mitochondrial superoxide and ROS, and malondialdehyde level (used as a biomarker for lipid peroxidation) due to HS (Mujahid et al., 2006; Wang et al., 2009; Azad et al., 2010; Kikusato and Toyomizu, 2013). In the GIT, however, oxidative stress has been shown to destabilize the TJ-regulated paracellular barrier and increase intestinal permeability by triggering circumferential contractions of the actomyosin ring of enterocytes and by reducing TJ expression (Rao, 2008; Bischoff et al., 2014; Liu et al., 2022), as examined in detail above (see section **2.2** "**Intestinal Epithelium, Immunity, and Microbiota: Three Pillars of Gut Health**").

In addition to oxidative stress, increased corticosterone levels, already discussed in this section, have been shown to weaken the GI barrier in broilers experiencing HS (Quinteiro-Filho et al., 2012). Exposure of lab animals (Dokladny et al., 2016), pigs (Pearce et al., 2013b), and broilers (Song et al., 2014; Tabler et al., 2020a) to high environmental temperatures has been found to cause a significant reduction in TEER and a substantial increase in permeation of markers through the GI epithelium, which are two gold standards for assessing gut health, as said before (see section **2.2** "**Intestinal Epithelium, Immunity, and Microbiota: Three Pillars of Gut Health**"). These are typical features of a "leaky gut" that is insufficiently hermetic to avoid infiltration of harmful luminal compounds to the underlying

[23] Hypoxia is a condition in which oxygen availability of a tissue is not enough to maintain homeostasis. It can be caused by insufficient oxygen supply to tissues due to low blood supply or low blood oxygen content (hypoxemia) (Bhutta et al., 2022).

lamina propria (Shen et al., 2011; Awad et al., 2017; Ma et al., 2018; Ruff et al., 2020). The intestinal content abounds in PAMPs, specially LPSs detaching from the wall of Gram-negative bacteria (Wassenaar and Zimmermann, 2018), which can cross a leaky barrier and bind to the TLR4–MD-2 receptor complex at the basolateral membrane of the GI epithelium (Shimazu et al., 1999; Abreu, 2010; Keestra et al., 2013). As previously mentioned (see section **2.2** "**Intestinal Epithelium, Immunity, and Microbiota: Three Pillars of Gut Health**"), TLR activation results in intracellular signaling cascades that induce the expression of pro-inflammatory cytokines (Vaure and Liu, 2014), initiating an inflammatory response in the GIT. These cytokines are also released by LPS-stimulated innate immune cells and have been reported to fuel a vicious cycle leading to GI barrier degradation (Lambert, 2009). TNF-α, interleukin 1 beta (**IL-1β**), and interferon gamma (**IFN-γ**) have been shown to damage the paracellular barrier and increase LPS leakage from the GIT (Turner et al., 2014; Dokladny et al., 2016; Awad et al., 2017; Ma et al., 2018). A common, easily recognizable inflammatory state of the GIT in heat-stressed broilers is multifocal enteritis (Quinteiro-Filho et al., 2010, 2012). Irrespective of the form, however, enteric inflammation has been shown to reduce enterocyte lifespan and cause villus atrophy and crypt hyperplasia in chickens (Smith et al., 2014). These modifications in the GI epithelium morphology, along with increased cell apoptosis and reduced cell proliferation, have been found in broilers undergoing HS (He et al., 2018a, 2018b; Liu et al., 2020, 2022; Nanto-Hara et al., 2020). Adopting a pair-feeding model along the lines of those already mentioned in this section, Nanto-Hara et al. (2020) found that the morphological alterations and increased permeability affecting the GI epithelium of heat-stressed broilers are direct effects of HS instead of the reduction in FI caused by HS itself. In their review article on the roles and impacts of inflammation in farm animals, Broom and Kogut (2018c) pointed out that GI inflammation induces nutrient malabsorption and a rearrangement of energy partition to prioritize the immune response in the GIT; this undesirably affects performance and may prelude more serious health problems.

In addition to posing a local threat to the GIT, enteral LPSs can reach the liver via the portal circulation (Wang et al., 2015) and, after exceeding the hepatic detoxification, can diffuse systemically with the bloodstream and cause endotoxemia (Baumgard and Rhoads, 2013; Alhenaky et al., 2017; Epstein and Yanovich, 2019; Nanto-Hara et al., 2020). Once again, but this time amplified,

inflammatory reactions induced by these noxious bacterial products are energy-consuming and depress performance (Broom and Kogut, 2018c; Ruff et al., 2020). If worst comes to worst, endotoxemia can cause multi-organ failures and lethal septic shocks (Wassenaar and Zimmermann, 2018).

Moreover, it is important to note that cell injury and failure of TJ-independent barriers can worsen permeability problems in the GI epithelium (France and Turner, 2017). Enteric bacteria can leak from the GIT and, eventually, get to the liver or even spread to other extraintestinal tissues and organs, as demonstrated in heat-stressed broilers showing serious hepatic invasions of *Salmonella* (Alhenaky et al., 2017). "Bacterial translocations" can result in impaired health and function of the liver (Ilan, 2012; Ducatelle et al., 2018) and bacterial chondronecrosis with osteomyelitis (**BCO**; Wideman, 2016).

GASTROINTESTINAL MICROBIOTA

Speaking of GI bacteria, HS has been shown to perturb the microbiota in rats (Suzuki et al., 1983, 1989), pigs (Le Sciellour et al., 2019; He et al., 2019a; Xiong et al., 2020), dairy cattle (Chen et al., 2018), and poultry (Suzuki et al., 1989, 1983; Lan et al., 2004; Burkholder et al., 2008; Song et al., 2014; Wang et al., 2018a, 2020c; Zhu et al., 2019; He et al., 2019b, 2021; Shi et al., 2019; Xing et al., 2019; Liu et al., 2020, 2022; Goel et al., 2022), pushing it to dysbiosis. Although sophisticated techniques to study the microbiota are available (Borda-Molina et al., 2018), its alterations remain to be elucidated in heat-stressed chickens (He et al., 2021; Liu et al., 2022). It can be assumed that changes in morphology of the GI epithelium, quantity and composition of mucus, and attachment sites, along with accumulation of poorly digested or even undigested dietary components, are all likely reasons for dysbiosis during HS. Increased susceptibility to intestinal colonization by *Salmonella* Enteritidis has been observed in heat-stressed broilers (Burkholder et al., 2008; Soliman et al., 2009). Moreover, it has been demonstrated that *C. perfringens* can flourish in the GIT of broilers under HS (Tsiouris et al., 2018), significantly increasing the risk of NE outbreaks. Enterotoxins released by *C. perfringens* or other bacteria can also damage TJs, weakening the GI barrier (Awad et al., 2017). Dysbiosis, barrier disorders, and inflammation are interrelated, fuel each other (Ducatelle et al., 2018), and contribute to the worsening of the injurious effects of HS on gut health and performance of chickens.

3 PHD PROJECT GOALS AND STRUCTURE

Previous chapters have emphasized the negative aspects of the AGP withdrawal and the consequences of HS for broiler production. Raising broilers in the "post-antibiotic and global warming era" means facing an increased risk of reduced growth and feed efficiency, impaired gut function, dysbiosis and increased enteric pathogen load, HS onset, altered metabolism, and decreased welfare. The resulting profit losses and product quality deterioration jeopardize the sustainability of a food industry called upon to play a central role in feeding our increasingly populous planet. Interdisciplinary research is therefore needed to help broiler integrators tackle these many-sided problems. This PhD project was designed to fit into this context, with the aims of (i) investigating the effects of different nutritional strategies on growth performance, gut health, and metabolism of ABF broilers reared under TN and HS conditions and (ii) exploring the impacts of HS on the physiological processes regulating FI and energy homeostasis in different chicken genotypes. The three-year project comprised four studies (**S**), each of which consisted of two experiments (**E**), hereafter referred to as **S*n*E1** and **S*n*E2** according to the study number. The project structure is schematized in **Figure 16**.

Specifically, S1, S2, and S3 were feeding trials set out to test a nutritional strategy (i.e., dietary supplementation of a synbiotic, a muramidase, and arginine, respectively) to determine whether it is an effective solution to (S*n*E1) enhance growth performance and gut health and to (S*n*E2) mitigate the detrimental effects of HS for ABF broilers. These studies shared a multistep and multidisciplinary approach integrating poultry nutrition, physiology, and gut microbiology. The *modus operandi* for S1, S2, and S3 was as follows:

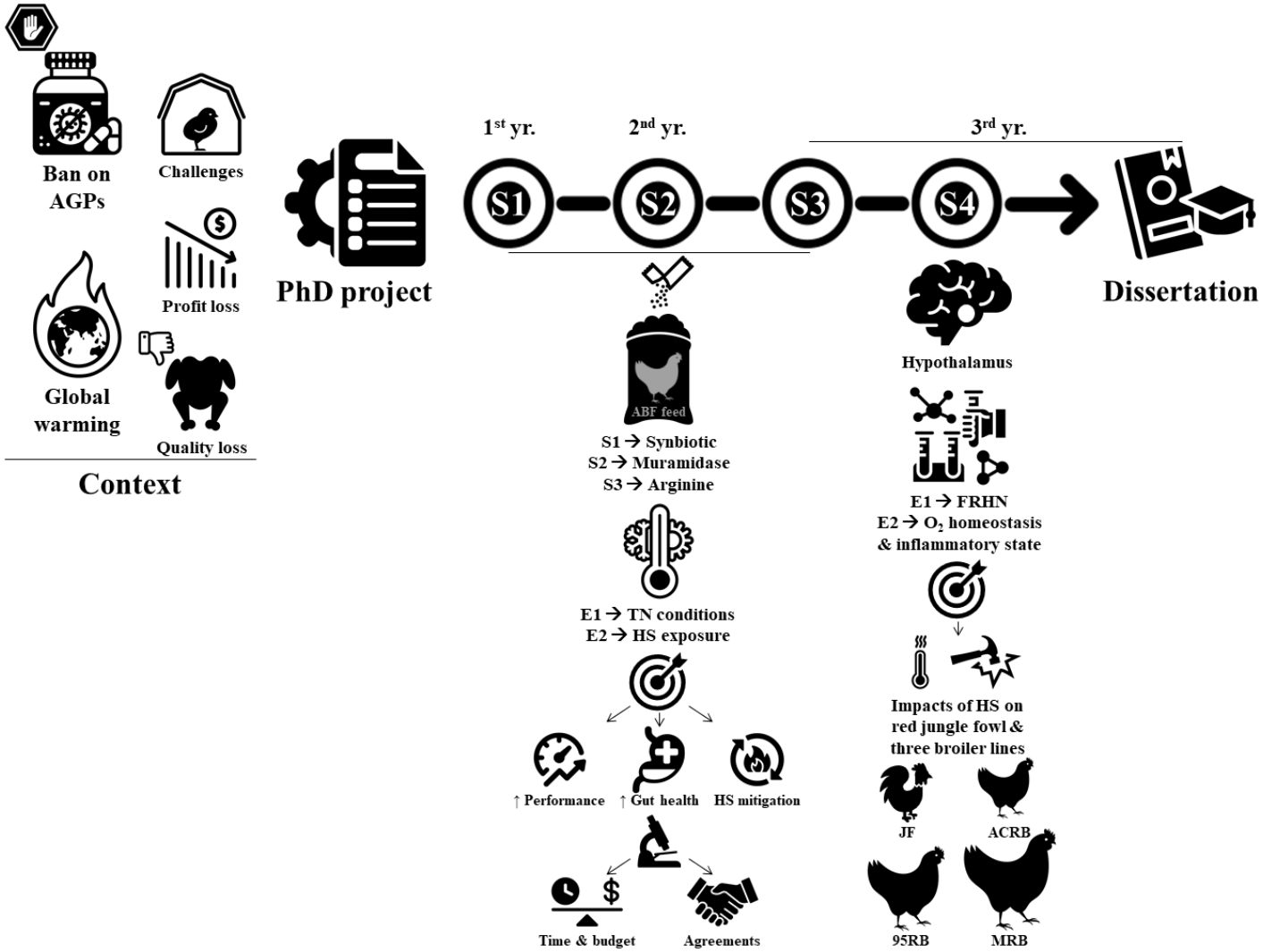
i. Running a feeding trial with ABF broilers under experimental settings (TN conditions throughout S*n*E1, whereas HS exposure during a limited period of S*n*E2);
ii. Collection of growth performance data (e.g., FI, BW, and FCR);
iii. Collection and storage of biological samples (e.g., blood, intestinal content, liver, and pectoral muscle);
iv. Analysis of growth performance data;

v. Allocation of budget and time for lab analyses of stored biological samples depending on the relevance of growth performance results and the terms of the agreement with the study sponsor, if any.

On the other hand, S4 was not part of the abovementioned series of feeding trials; it was a "freestanding" study instead. Conducted during a six-month research period the PhD candidate spent at Dr. Sami Dridi's lab, S4 was a molecular biology-based investigation of the effects of HS on (S4E1) hypothalamic neuropeptides that regulate FI and (S4E2) hypothalamic oxygen homeostasis and inflammatory state in three broiler lines from diverse stages of genetic selection and in the red jungle fowl, the ancestor of domestic chickens. Carrying out S4 allowed the PhD candidate to expand his knowledge of chicken physiology and learn cutting-edge molecular techniques to study the response of chickens to HS. Furthermore, performing tasks outside the purposes of S4, such as running a trial to assess a tannin-containing feed additive as a dietary treatment to help broilers counteract chronic cyclic HS, enabled the PhD candidate to gain expertise to improve the quality of his research and make it more sophisticated.

Dissemination of the results obtained throughout this PhD project was attained by means of research articles published in peer-reviewed journals and presentations at scientific conferences (see **Annex 1** and **Annex 2**).

Figure 16 | Schematic representation of the structure of the three-year PhD project



Note: AGP, antibiotic growth promoter; S1/2/3/4, study #1/2/3/4; ABF, antibiotic-free; E1/2, experiment #1/2 of study #*n*; TN, thermoneutral; HS, heat stress; FRHN, feeding-related hypothalamic neuropeptides; JF, red jungle fowl; ACRB, Athens Canadian Random Bred; 95RB, 1995 random bred; MRB, modern random bred.
Icon source: Noun Project.

4 STUDY #1: EFFECTS OF A SYNBIOTIC ON BROILERS EXPOSED TO THERMONEUTRAL AND HEAT STRESS CONDITIONS

Note: The results of experiment S1E1 have been published. For more information, please refer to **Annex 1**.

4.1 Highlights

- A nutritional strategy combining post-hatch and in-feed supplementations of a synbiotic preparation was tested.
- Under thermoneutral conditions, the synbiotic improved feed efficiency and footpad health and modified the cecal microbiota composition.
- Supplemented birds showed increased abundance of Firmicutes (e.g., *Bifidobacterium longum*, *Lactobacillus panis*, *Lactobacillus reuteri*, and Clostridia) and reduced abundance of Bacteroidetes.
- More information about the effects of the synbiotic on the gastrointestinal microbiota is needed to establish whether the differences observed were the causes or consequences of improved feed efficiency.
- In a small-scale experiment, the synbiotic did not mitigate the impacts of heat stress on body temperature or growth performance.

4.2 Background and Aims

As discussed in detail before (see section **2.3** "**Exploration and Modulation of the Chicken Gastrointestinal Microbiota**", subsection "**FEED ADDITIVES**"), probiotics, prebiotics and synbiotics, hereafter referred to as "biotics", have been shown to positively affect the GI ecosystem and favor eubiosis, benefiting the host they are administered to. These products have been included in poultry diets to compensate for the drawbacks of phasing AGPs out, such as increased occurrence of enteric infections and losses of growth performance (Patterson and Burkholder, 2003; Yang et al., 2009; Gaggìa et al., 2010; Gadde et al., 2017; Tayeri et al., 2018). Over the last years, biotics have also drawn the attention of poultry nutritionists looking for solutions to alleviate the negative effects of HS on birds, showing promising results (Sugiharto et al., 2017; Ringseis and Eder, 2022).

Additives containing probiotic bacteria have usually been administered through feed or drinking water during the growth cycle of poultry (Jha et al., 2020; Abd El-Hack et al., 2020); that is why they have traditionally been called "direct-fed microbials" (Miles and Bootwalla, 1991). However, growing awareness of the long-term impacts of stressors to which young birds may be exposed during the early stages of life, imprint of the microbiota on the development of a healthy, well-functioning GIT, and advantages of early nutrition for gut health and growth performance of poultry (see section **2.3** "**Exploration and Modulation of the Chicken Gastrointestinal Microbiota**") has led scientists to study other delivery methods for these products, which can be chick- or embryo-targeted. The pioneers in this field were Goren et al. (1984), who proposed spraying a probiotic preparation on the feathering of newly hatched chicks to protect them against *Salmonella* infection, and Cox et al. (1992), who injected probiotics *in ovo* to make chicks, also in this case, more resistant to *Salmonella*.

Postnatal treatments with "biotics" have been shown to be particularly effective as the immature microbiota of baby chicks is malleable and, consequently, very susceptible to modulating interventions (Baldwin et al., 2018; Jurburg et al., 2019; Proszkowiec-Weglarz et al., 2022; Kayal et al., 2022). Nevertheless, rather than replacing the traditional dietary administrations, perinatal applications of "biotics" may be part of multi-step treatment plans to permanently shape the microbiota and constantly support the growth of healthier, more efficient, and stress-resistant chickens. For example, Blankenship et al. (1993) sprayed a CE preparation at the hatchery and administered the same product via drinking water at the farm, showing that this two-step treatment was effective in controlling *Salmonella* infection. Anyway, these authors did not establish whether the double intervention was essential to fully exploiting the CE effect of the probiotic they were testing. A few years later, however, Chen et al. (1998) were able to demonstrate that combining a spray application of a CE preparation onto broiler hatchlings with a subsequent treatment by oral gavage was more effective in reducing intestinal colonization by *Salmonella* than using the delivery methods one at a time, suggesting that the double administration produced a synergistic effect.

Besides pursuing a protection against pathogens, however, early applications of these microbiota modulators have been shown to enhance gut health, well-being, and growth performance of chickens (Rubio, 2019). Therefore, S1E1 was conceived to evaluate the effects of a treatment program combining

post-hatch and in-feed supplementations of a synbiotic preparation on growth performance, pododermatitis (used as a welfare indicator), and cecal microbiota of broilers. In addition, the ability of this nutritional strategy to mitigate the effects of HS on broiler performance was tested in S1E2. It should be noted, however, that the PhD candidate conducted S1E2 primarily to familiarize himself with the management of experimental facilities to be used in later phases of his project to run trials with broilers under HS conditions. Therefore, despite being obtained with scientific rigor, the results of S1E2 should be interpreted with caution, especially due to the limited number of replicates used.

4.3 Materials and Methods

EXPERIMENTAL DESIGN, HOUSING, AND HUSBANDRY CONDITIONS

In S1, approved by the Ethical Committee of the University of Bologna (ID: 1049), birds were reared, monitored, and slaughtered in compliance with EU legislation (i.e., Dir. 2007/43/EC, Reg. 2009/1099/EC, and Dir. 2010/63/EU). Feed and water were provided *ad libitum* throughout the trials of this study.

For S1E1, 500 one-day-old male Ross 308 broilers, obtained from the same breeder flock and hatching batch, were supplied by a commercial hatchery, and vaccinated against infectious bronchitis, Marek's, Newcastle and Gumboro diseases, and coccidiosis. At the hatchery, half of the chicks (i.e., 250) were also treated with a synbiotic (PoultryStar® Hatchery, Biomin Holding GmbH, Getzersdorf, Austria) containing *Bifidobacterium animalis* ssp. *animalis*, *Lactobacillus salivarius* ssp. s*alivarius*, *Enterococcus faecium*, and FOS. Following the manufacturer's instructions, the synbiotic preparation was dissolved in water (100 g preparation/10,000 chicks) and sprayed on the feathering of chicks. Treated and untreated chicks were transported in distinct boxes to an experimental poultry barn. At placement, chicks were assigned to 2 experimental groups (10 replicate pens/group) according to the post-hatch treatment with the synbiotic: those untreated were fed a commercial ABF basal diet in mash form (**CON1**), while those previously treated were fed the same basal diet supplemented with the microencapsulated form of the synbiotic for feed application (**SYN1**; PoultryStar® me, Biomin). The three-phase feeding trial lasted 42 d (i.e., starter, 0-14 d; grower, 15-28 d; finisher, 29-42 d). For each

feeding phase, the basal diet (**Annex 3**) consisted of a single batch of coarsely ground mash feed[24], and the sub-batches intended for SYN1 replicates were supplemented on top with the microencapsulated synbiotic at 1,000, 500, and 250 g/ton feed during the starter, grower, and finisher phase, respectively. The floor pens were arranged in a block design and equipped with wood shavings as bedding material, a bell feeder, and nipple drinkers. The environmental temperature was modified according to the flock age and the breeding company's instructions (Aviagen, 2018). The artificial photoperiod was 23L:1D during the first 7 and last 3 d, while 18L:6D for the remainder days following EU legislation (i.e., Dir. 2007/43/EC) and the breeding company's guidelines for lightning and pre-processing management (Aviagen, 2018).

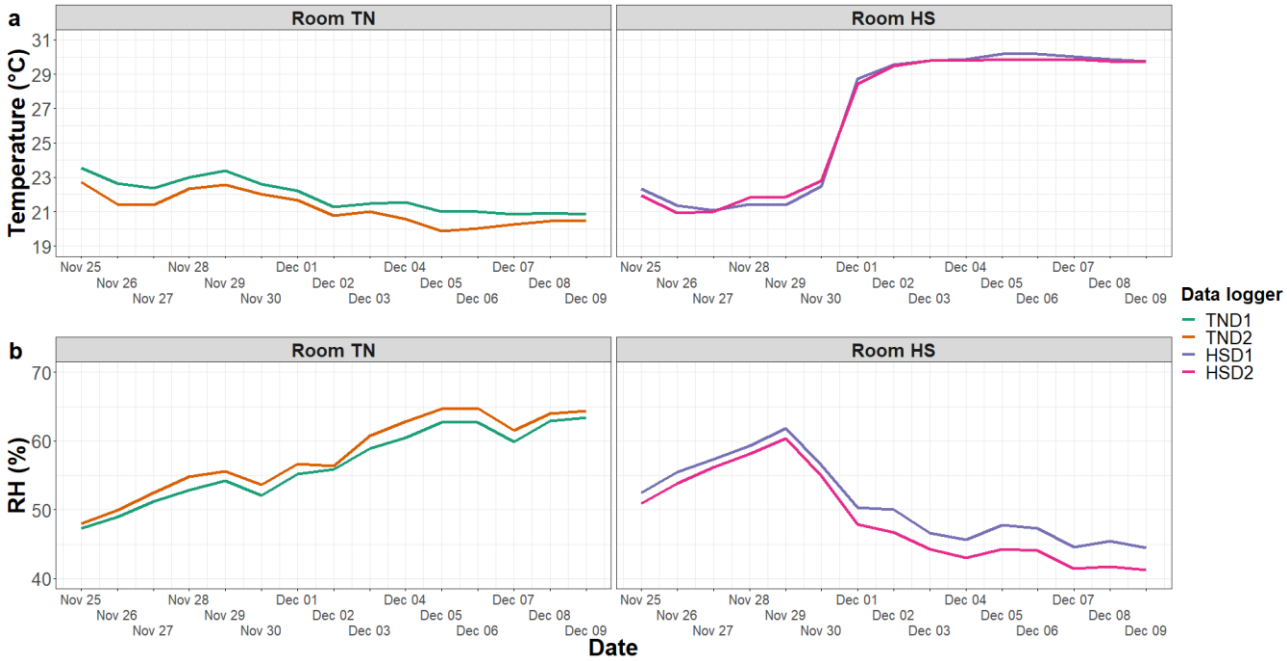
For S1E2, 300 one-day-old male Ross 308 broilers, procured and vaccinated as detailed above, were used. At the hatchery, half of the chicks (i.e., 150) were also treated with the same synbiotic tested in S1E1. Treated and untreated chicks were transported in distinct boxes to an experimental facility consisting of 2 identical environmental chambers, hereafter referred to as *rooms*. Each room was divided into 6 equally sized floor pens equipped as previously described. Chicks were assigned to one of two experimental groups (i.e., 3 replicate pens/group/room arranged in a block design) according to the post-hatch treatment with the synbiotic: those untreated were fed a commercial ABF basal diet in mash form (**CON2**), while those previously treated were fed the same basal diet supplemented on top with the microencapsulated form of the synbiotic (**SYN2**) as in S1E1. In this case, however, the inclusion levels were 1,000 g/ton feed during the starter phase (0-15 d) and 500 g/ton feed during both the grower (16-29 d) and finisher phase (30-41 d). **Annex 3** provides the formula and composition of the basal diet, which, as before, was a coarsely ground mash feed (for information on particle size, see **Footnote 24**). The artificial photoperiod was adjusted as described earlier. Environmental temperature and RH were recorded with climate data loggers (Trotec GmbH, Heinsberg, Germany) located at animal level (2 data

[24] The particle size of the mash feed was as follows (in %).

Feeding phase	Particle size (mm)						
	2.80	**2.40**	**1.70**	**1.20**	**0.85**	**0.60**	**0.25**
Starter	2.8	13.8	18.9	17.2	13.1	9.5	24.6
Grower	3.4	15.9	20.0	16.0	12.1	8.9	23.7
Finisher	4.6	18.3	21.4	15.7	11.0	7.9	21.1

loggers/room having a recording time of 900 s). As for the temperature program, the rooms were kept at TN conditions until D32 and at constant HS from D33 onwards (i.e., temperature was raised to and maintained at ~30 °C all day long from D33 to D41). During the HS period, RH was adjusted with humidifiers (Trotec GmbH) and ranged between 55 and 65% in "room TN" and between 55 and 40% in "room HS" (**Figure 17**).

Figure 17 – S1E2 | Average daily temperature (**a**) and RH (**b**) of the two rooms



Note: December 01, constant HS period start (D33); December 09, constant HS period and trial end (D41).

DATA AND SAMPLE COLLECTION

For S1E1, on a replicate basis, the number and BW of birds were recorded at placement (D0), at every feeding phase switch (D15/30), and at slaughter (D42), while FI was measured for each feeding phase. Daily weight gain (**DWG**), daily feed intake (**DFI**), and FCR were calculated for the feeding phases separately and cumulative performance traits were calculated for the entire rearing period (0-42 d). The number and BW of dead or culled birds were recorded daily to compute the mortality rate and correct the performance data for mortality. At slaughter (D42) in a commercial abattoir, groups were processed individually. Footpad dermatitis (**FPD**) were measured by specially trained personnel on one foot per processed bird (i.e., more than 200 observations/group) using a 3-point scale: score 0, no lesions;

score 1, mild lesions (≤ 0.8 cm); score 2, severe lesions (> 0.8 cm) (Ekstrand et al., 1998). This scoring was performed in a blinded fashion. In addition, one bird per replicate (i.e., 10 birds/group) with a BW close to the flock average was selected for collection of cecal content samples, which were frozen in LN_2 and stored at -80 °C until DNA extraction for 16S rRNA amplicon sequencing.

For S1E2, four representative birds per pen (i.e., 12 birds/group/room) were randomly chosen and labeled to measure the rectal temperature with a veterinary thermometer (Scala Electronic GmbH, Stahnsdorf, Germany). The rectal temperature was taken at three time points: one day before the beginning of the HS period, on the third day of the HS period, and at the end of the trial at 9:00 AM. Growth performance data were collected as described before, but in this case cumulative FI and cumulative FCR were computed for the period consisting of a feeding phase and its previous one/ones (i.e., starter + grower; starter + grower + finisher).

LAB ANALYSIS

For S1E, DNA was extracted from cecal content samples using a bead-beating procedure, as illustrated in the work by De Cesare et al. (2017). Libraries were prepared following the 16S Metagenomic Sequencing Library Preparation protocol released by Illumina (San Diego, CA, U.S.), amplifying the V3 and V4 hypervariable regions of 16S rRNA gene to obtain a single amplicon of approximately 460 bp. Sequencing was performed in paired-end employing the MiSeq System with the MiSeq Reagent kit v2 500 cycles (Illumina).

DATA ANALYSIS

For S1E1, a one-way ANOVA with blocks was used to evaluate the effect of diet (i.e., the group factor) on growth performance, using the replicate as the experimental unit. One replicate of group CON1 was excluded from statistical analysis due to a failure of the drinking system. Mortality rate data were transformed using the arcsine transformation before being analyzed with inferential statistics. Count data of FPD were analyzed with Pearson's chi-squared test using the sampled animal as the experimental unit. Count data were also arranged in 2 by 2 contingency tables aligning the levels of the group factor (i.e., CON1 and SYN1) and having binarily aggregated scores of FPD in columns (i.e.,

"presence" as a sum of counts of score 1 and score 2, while "absence" as score 0 counts). The incidence risk ratio was computed on these tables with *epiR* package (Stevenson et al., 2021) of R (R Core Team, 2020). The risk of developing FPD was calculated as incidence risk ratio minus 1 and expressed in percentage. A confidence interval of 95% and Pearson's chi-squared test were used to test the incidence risk ratio significance. These analyses were carried out with R (R Core Team, 2020). With respect to the analysis of cecal microbiota data, the MG-RAST analysis server (Meyer et al., 2008) was utilized for taxonomic identification by mapping the sequencing reads against the SILVA database (Pruesse et al., 2007). The bacterial abundance matrix down to the species level was downloaded from MG-RAST for statistical analysis with STAMP v2.1.3 (Parks et al., 2014) using Welch's *t*-test with group as the experimental factor and the sampled animal as the experimental unit. *P*-values less than 0.05 were considered significant, while those ranging between 0.05 and 0.1 were considered tendencies.

For S1E2, the effect of diet (i.e., the group factor) on growth performance of the starter and grower phases was assessed with a two-way blocked ANOVA without interaction, considering the room as a fixed factor and using the replicate as the experimental unit. The finisher phase data, however, were analyzed as a factorial arrangement of group and room using a two-way blocked ANOVA with interaction. Tukey's HSD post-hoc test was used if needed. Mortality rate data were handled as before. Rectal temperature data were analyzed through a three-way mixed ANOVA, a type of repeated-measures ANOVA that includes between-subject factors (i.e., group and room) and within-subject factors (i.e., time point). After verifying that there was no statistically significant three-way interaction, rectal temperature data were grouped by the factors group and room to run paired *t*-tests with Bonferroni adjustment between time points. These analyses were carried out using R (R Core Team, 2020) and considering 0.05 as the significance threshold. *P*-values ranging between 0.05 and 0.1 were considered tendencies.

4.4 Results

Table 4 provides the growth performance of group CON1 and group SYN1 in the three-phase feeding trial of S1E1. At placement, chicks showed an average weight of approximately 49 g with no significant differences attributable to the post-hatch treatment with the synbiotic. A mortality of 2.33%

was recorded for group CON1, while no birds of group SYN1 died from D0 to D14; mortality therefore differed ($P < 0.05$) between groups during the starter phase. In the later phases, FCR was the only performance trait influenced by the group factor, with SYN1 birds showing lower values than CON1 birds at the end of the grower (1.570 *vs.* 1.509; $P < 0.05$) and finisher phase (1.809 *vs.* 1.753; $P = 0.01$). As also shown in **Figure 18**, FCR was the only performance parameter that significantly differed between groups in the entire trial too (1.643 *vs.* 1.596 for CON1 and SYN1, respectively; $P < 0.01$).

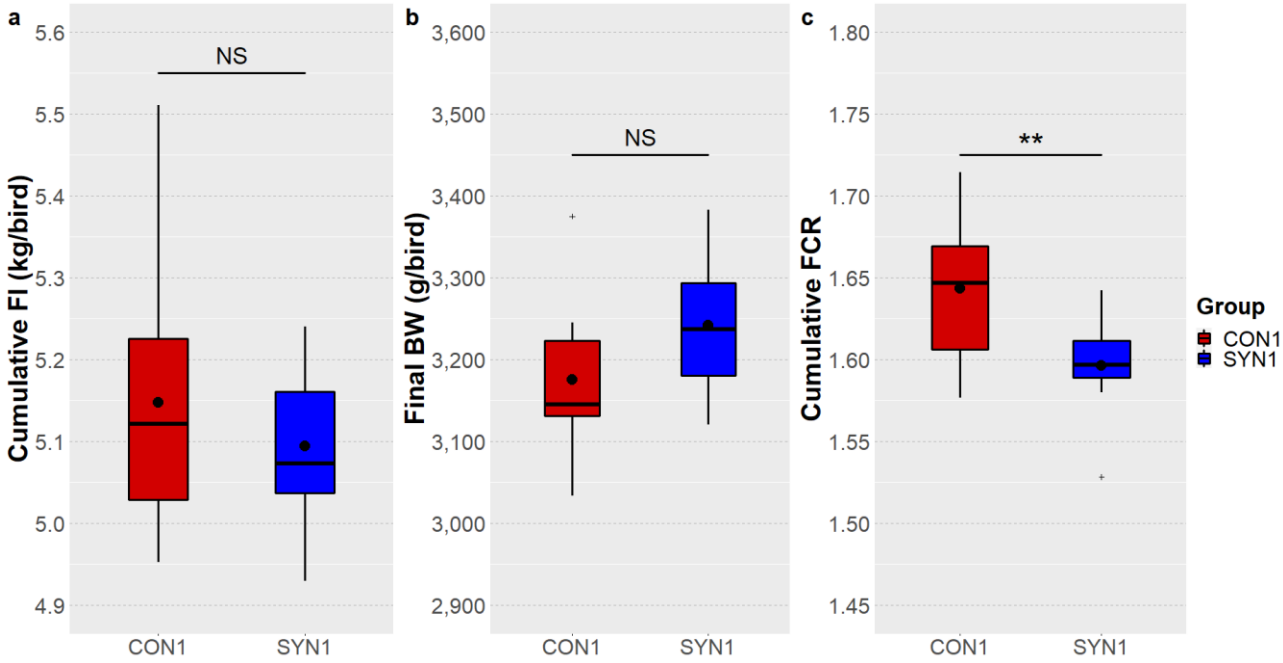
Table 4 – S1E1 | Growth performance of groups CON1 and SYN1 at the end of each feeding phase and in the entire trial (0-42 d)

Dependent variable	Group[†]		SE	*P*-value
	CON1	SYN1		
Chick weight (g/bird)	49.60	49.23	0.80	0.339
Starter (0-14 d)				
BW (g/bird)	470.0	462.1	23.34	0.482
DWG[§] (g/bird/d)	29.81	29.49	1.69	0.690
DFI[§] (g/bird/d)	39.49	39.50	3.04	0.998
FI[§] (kg/bird)	0.553	0.553	0.04	0.998
FCR[§]	1.325	1.340	0.04	0.453
Mortality %	2.33	0.00	0.08	**0.012**
Grower (15-29 d)				
BW (g/bird)	1,731	1,755	55.33	0.355
DWG[§] (g/bird/d)	83.91	85.81	2.94	0.198
DFI[§] (g/bird/d)	131.7	129.5	5.48	0.407
FI[§] (kg/bird)	1.975	1.942	0.08	0.407
FCR[§]	1.570	1.509	0.06	**0.047**
Mortality (%)	0.00	0.83	0.06	0.200
Finisher (30-42 d)				
BW (g/bird)	3,175	3,242	85.59	0.128
DWG[§] (g/bird/d)	111.5	114.2	5.00	0.268
DFI[§] (g/bird/d)	201.5	200.0	6.80	0.633
FI[§] (kg/bird)	2.620	2.599	0.09	0.633
FCR[§]	1.809	1.753	0.04	**0.010**
Mortality (%)	0.00	0.87	0.07	0.200
Entire trial (0-42 d)				
BW (g/bird)	3,175	3,242	85.59	0.128
DWG[§] (g/bird/d)	74.27	75.88	2.01	0.120
DFI[§] (g/bird/d)	120.7	119.4	3.95	0.514
FI[§] (kg/bird)	5.147	5.094	0.17	0.512
FCR[§]	1.643	1.596	0.03	**0.004**
Mortality (%)	2.32	1.67	0.14	0.615

[†] Means of 10 replicate pens/group (1 replicate pen of group CON1 was excluded due to a drinking system failure).
[§] Corrected for mortality.
Note: P-values less than 0.05 are in bold.

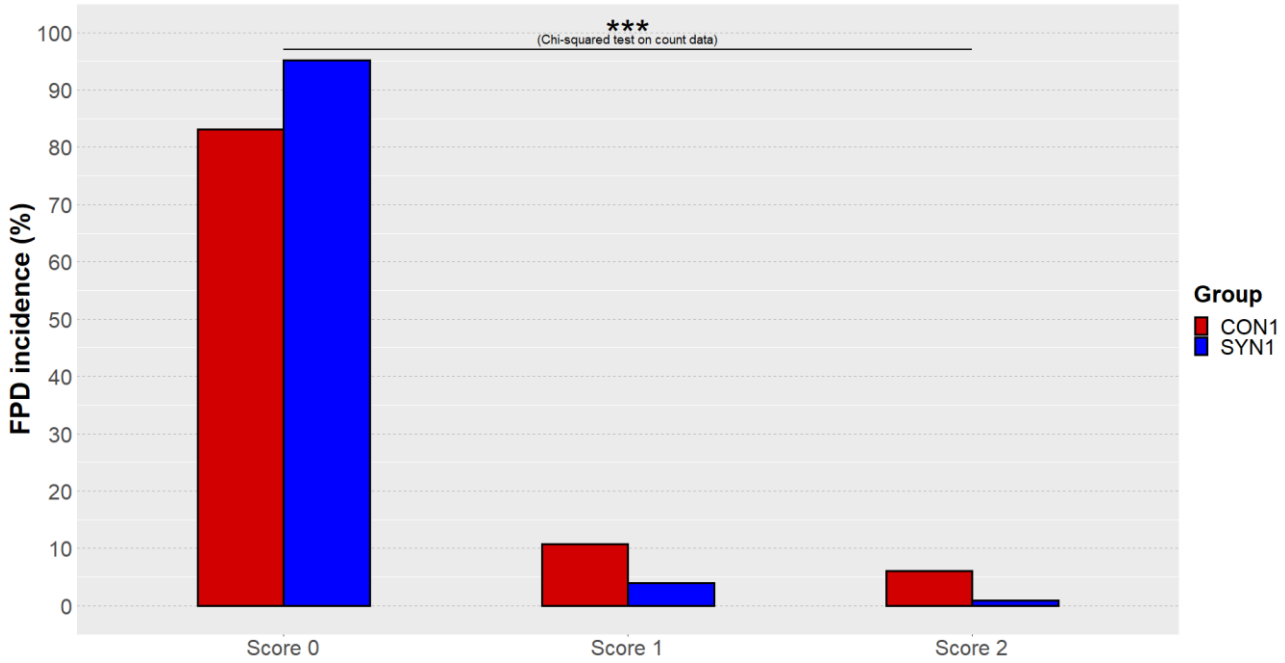
In S1E1, the occurrence of FPD was found to be related to the group factor, with group SYN1 showing a greater percentage of healthy footpad, namely a higher incidence of FPD with score 0 than group CON1 ($P < 0.001$; **Figure 19**). In addition, the incidence risk ratio analysis showed that SYN1 birds were 0.29 (95% confidence interval of 0.15 to 0.56) times less likely to develop FPD than CON1 birds; that is, treating birds with the synbiotic reduced by 71% the relative risk of developing FPD ($P < 0.001$).

Figure 18 – S1E1 | Cumulative FI (**a**), final BW (**b**), and cumulative FCR (**c**) of groups CON1 and SYN1 in the entire trial (0-42 d)



Note: Means of 10 replicate pens/group are the black dots inside the boxes (1 replicate pen of group CON1 was excluded due to a failure of the drinking system). NS, not significant. **, $P < 0.01$.

Figure 19 – S1E1 | Incidence and severity of FPD of CON1 and SYN1 birds at D42

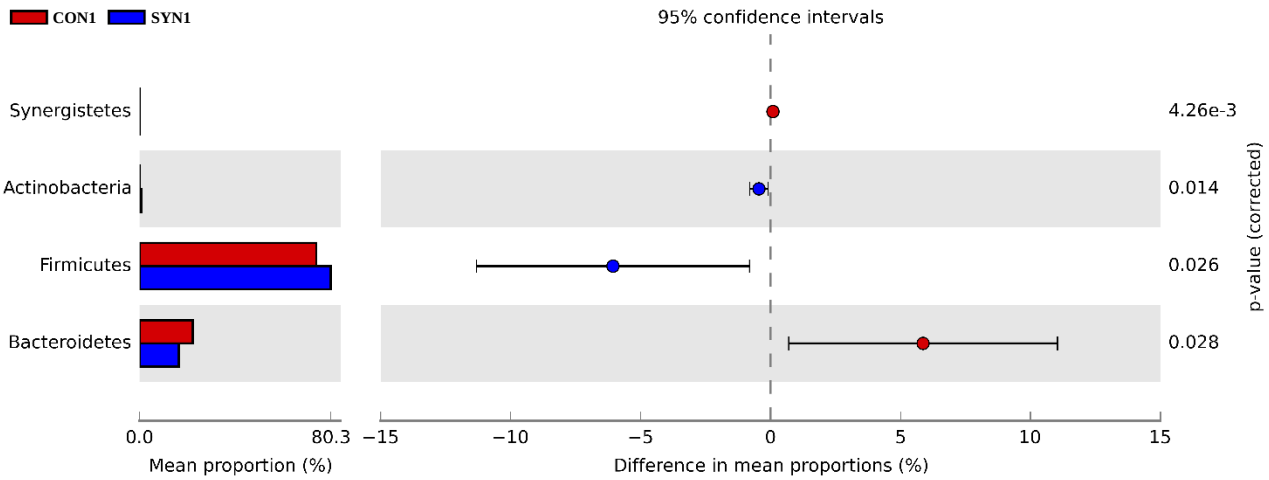


Note: Score 0, no lesions; score 1, mild lesions (≤ 0.8 cm); score 2, severe lesions (> 0.8 cm). ***, $P < 0.001$.

Analysis of taxonomical data of cecal samples collected in S1E1[25] showed that the phyla (**Figure 20**) Actinobacteria and Firmicutes were more abundant in group SYN1 ($P < 0.05$), whereas Synergistetes and Bacteroidetes had a higher abundance in group CON1 ($P < 0.01$ and $P < 0.05$, respectively). Similarly, at the class level (**Figure 21**), the abundances of Actinobacteria and Clostridia were garter in SYN1 birds, while Bacteroidia were more abundant in CON1 birds ($P < 0.05$). In the order Bacteroidales, the genera *Bacteroides*, *Parabacteroides*, and *Prevotella* were significantly less abundant in group SYN1 than group CON1, with the abundance of the genus *Collinsella* varying oppositely. Focusing on bacterial species, the abundances of *Bifidobacterium longum*, *Collinsella intestinalis*, *Lactobacillus panis*, *Lactobacillus reuteri*, uncultured *Streptococcus* sp., *Clostridium cocleatum*, *Clostridium difficile*, *Clostridium innocuum*, *Eubacterium ramulus*, *Peptoniphilus asaccharolyticus*, *Ruminococcus obeum*, *Blautia producta*, *Blautia* sp. Ser5, and *Eggerthella lenta* were significantly greater in group SYN1, whereas *Eubacterium rectale*, *Bacteroides fragilis*, *Bacteroides* sp. 1AL, *Finegoldia magna*, *Prevotella pallens*, and *Synergistetes bacterium* SGP1 showed higher abundances in group CON1.

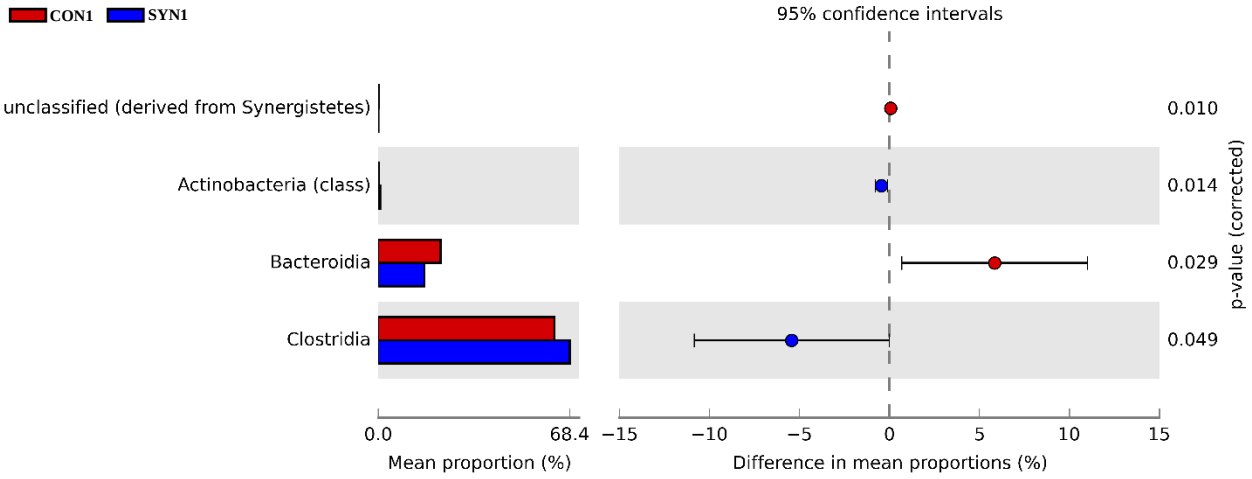
[25] Sequencing data are available upon request.

Figure 20 – S1E1 | Mean relative abundance of biologically relevant bacterial phyla in the cecal content of CON1 and SYN1 birds at D42



Note: The colored bars indicate the mean relative abundance of bacterial phyla (n = 10 cecal samples/group). The colored dots inside the 95% confidence interval mark the differences in mean relative abundance between groups. *P*-values of Welch's *t*-test are reported on the right side of the figure.

Figure 21 – S1E1 | Mean relative abundance of biologically relevant bacterial classes in the cecal content of CON1 and SYN1 birds at D42



Note: The colored bars indicate the mean relative abundance of bacterial classes (n = 10 cecal samples/group). The colored dots inside the 95% confidence interval mark the differences in mean relative abundance between groups. *P*-values of Welch's *t*-test are reported on the right side of the figure.

Turning now to the results of S1E2, **Table 5** presents the growth performance of groups CON2 and SYN2 in the three-phase feeding trial. Group SYN2 showed higher FCR ($P < 0.05$) than group CON2 in the starter (+2.9%) and grower (+5.6%) phase, as well as in the period that combines these two feeding phases (+4.6%). Furthermore, BW and DWG of SYN2 birds were lower than those of CON2 birds in the grower phase, with *P*-values approaching or reaching statistical significance (1,539

and 73.07 g *vs.* 1,477 and 69.22 g, for CON2 and SYN2, respectively). The factorial arrangement of the data revealed no significant interaction between group and room on growth performance of the finisher phase, though DWG and FCR showed a weak interactive effect heading toward significance ($P = 0.08$). The main effect of room, however, was consistently significant on all performance traits, except for mortality. Indeed, birds kept in "room HS" showed reduced BW, DWG, and (D)FI, while increased FCR compared to their counterparts reared in "room TN" (*P*-values less than 0.01 for most of the dependent variables). Cumulative FI and cumulative FCR mirrored the just mentioned differences in FI and FCR between rooms. On the other hand, the group factor did not influence the performance parameters of the finisher phase in a significant manner.

The rectal temperature of CON2 and SYN2 birds are reported in **Figure 22**. The raw data ranged between 40.4 and 43.0 °C. The three-way mixed ANOVA used to analyze these data did not show a significant interaction between group, room, and time point ($P > 0.05$). The effect of group was also not significant ($P > 0.05$), but those of room, time point, and their interaction were considerable ($P < 0.001$). Only in "room HS" the pairwise comparisons indicated a significant increase in rectal temperature between one time point and the next for either group (*P*-values even less than 0.0001), except for the T2-T3 contrast for group SYN2 ($P > 0.05$). In "room TN", however, no change in rectal temperature between time points was found ($P > 0.05$).

4.5 Discussion

Feeding chickens microbial supplements immediately after hatch has been shown to promote early colonization of their GIT by desirable bacteria, with positive effects on health and productivity (Ritzi et al., 2016; Baldwin et al., 2018). Therefore, S1E1 aimed at evaluating how a supplementation program of a synbiotic combining a post-hatch treatment (i.e., preparation sprayed onto newly hatched chicks) and dietary administrations during the growth cycle (i.e., microencapsulated form of the preparation added to the feed) affects growth performance, footpad health, and cecal microbiota of broilers. The treated group, SYN1, showed significant improvements in mortality in the starter phase, FCR in the grower and finisher phases as well as in the entire trial (-2.9%), and FPD at processing (D42). Despite being of interest for the industry, the reduction in mortality potentially ascribable to the synbiotic

supplementation should be interpreted with caution due to the quite modest number of birds per pen (i.e., 25), which may have made mortality rate a parameter very susceptible to change. On the other hand, the enhancement in FCR and FPD can be considered a sound result because there were many replicates per experimental group (i.e., 10), as well as footpad observations at processing (i.e., more than 200/group). According to Shepherd and Fairchild (2010), FPD is a multifactorial problem in poultry, but litter quality and litter management play key roles in its etiology. It can be hypothesized that the reduction in pododermatitis incidence for SYN1 birds was an indirect consequence of their better FCR potentially leading to a mitigation in FPD predisposing factors associated to litter, such as high moisture levels as Sirri et al. (2011) suggested. Further studies on this synbiotic with a greater focus on litter quality are therefore recommended.

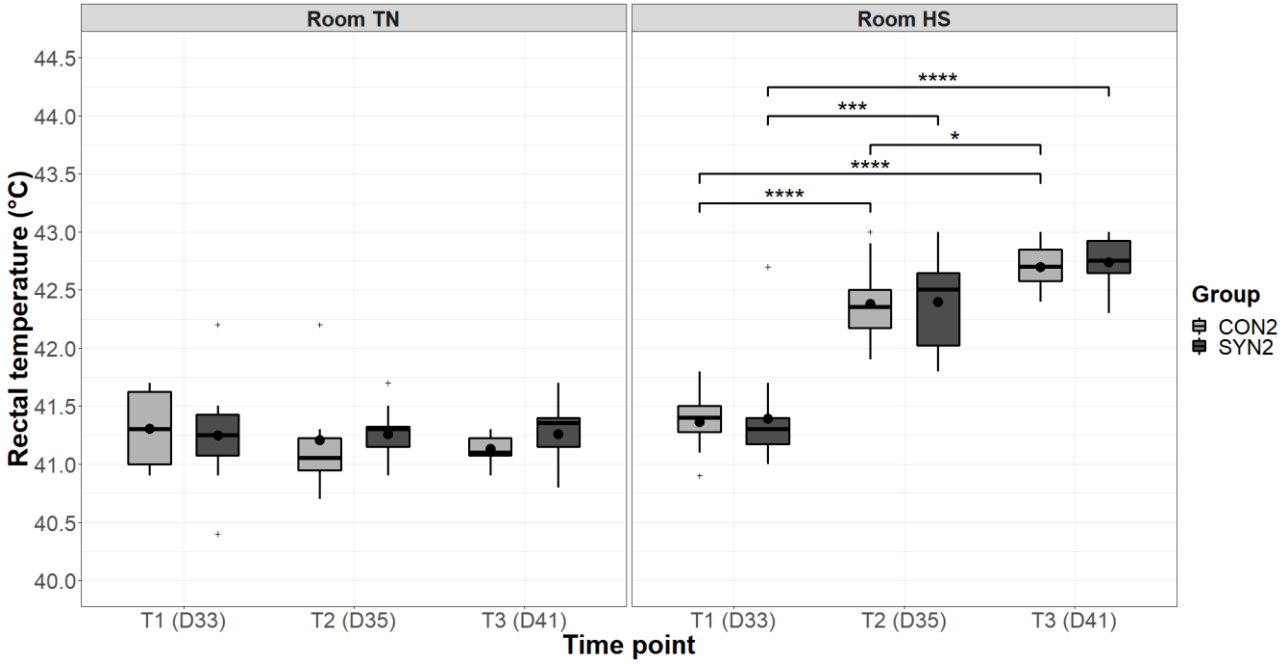
It is intriguing to examine the improvement in FCR in light of the modulatory activity synbiotics have been shown to have on the microbiota (see section **2.3** "**Exploration and Modulation of the Chicken Gastrointestinal Microbiota**", subsection "**FEED ADDITIVES**"). It has been demonstrated that broiler flocks with different feed efficiencies have distinct intestinal bacterial compositions (Torok et al., 2011; Stanley et al., 2012). Stanley et al. (2014) have also claimed that the microbiota influences the productivity of chickens and factors that shape the microbiota may, in turn, affect bird performance. It has been reported that the microbiota plays a pivotal role in energy harvesting processes from the diet, thereby affecting the energy balance of the host (Bäckhed et al., 2005). In S1E1, significant changes in the microbiota composition between control and supplemented birds were observed. The relative abundance of Actinobacteria and Firmicutes increased, while that of Bacteroidetes decreased in response to the synbiotic administration. The Firmicutes to Bacteroidetes ratio was therefore higher in group SYN1 than in group CON1. This parameter has become a hot topic for scientists who study the relationship between the microbiota and human health. Ley et al. (2006) found a shift toward Firmicutes in obese individuals, whereas other researchers suggested that this ratio has no association with overweight or obesity (Duncan et al., 2008; Schwiertz et al., 2010; Delzenne and Cani, 2011).

Table 5 – S1E2 | Growth performance of groups CON2 and SYN2 in the three-phase feeding trial

Feeding phase	Factor	Dependent variable								
		Chick weight (g/bird)	**BW (g/bird)**	**DWG[§] (g/bird/d)**	**DFI[§] (g/bird/d)**	**FI[§] (kg/bird)**	**Cum. FI[§, ¶] (kg/bird)**	**FCR[§]**	**Cum. FCR[§, ¶]**	**Mortality (%)**
Starter[†] (0-15 d)	**Group**									
	CON2	42.41	514.1	31.39	44.85	0.673	/	1.429	/	2.00
	SYN2	41.93	508.1	31.02	45.58	0.684	/	1.470	/	2.00
	SE	0.69	20.93	1.35	1.89	0.03	/	0.00	/	0.09
	***P*-value**	0.274	0.641	0.659	0.534	0.534	/	**0.015**	/	1.000
Grower[†] (16-29 d)	**Group**									
	CON2	/	1,539	73.07	120.5	1.687	2.360	1.650	1.580	0.69
	SYN2	/	1,477	69.22	120.2	1.682	2.366	1.742	1.652	0.67
	SE	/	46.29	2.46	3.99	0.06	0.07	0.06	0.04	0.09
	***P*-value**	/	0.068	**0.042**	0.894	0.894	0.875	**0.042**	**0.026**	0.990
Finisher[‡] (30-41 d; constant HS from D33 onwards)	**Group**									
	CON2	/	2,626	90.58	181.7	2.180	4.540	2.035	1.763	0.00
	SYN2	/	2,590	92.45	175.4	2.104	4.470	1.962	1.765	0.69
	Room									
	TN	/	2,818	109.1	194.2	2.330	4.684	1.784	1.689	0.00
	HS	/	2,399	73.93	162.8	1.954	4.326	2.214	1.840	0.69
	Group × Room									
	CON2-TN	/	2,821	105.2	196.4	2.357	4.738	1.867	1.708	0.00
	SYN2-TN	/	2,814	113.0	192.0	2.304	4.629	1.700	1.671	0.00
	CON2-HS	/	2,431	75.94	166.9	2.003	4.341	2.203	1.819	0.00
	SYN2-HS	/	2,366	71.92	158.7	1.905	4.311	2.225	1.860	1.39
	SE	/	32.95	4.47	11.36	0.14	0.16	0.07	0.05	0.06
	***P*-value**									
	Group	/	0.132	0.509	0.390	0.390	0.495	0.156	0.944	0.374
	Room	/	**< 0.001**	**< 0.001**	**0.009**	**0.009**	**0.018**	**< 0.001**	**0.007**	0.374
	Group × Room	/	0.204	0.084	0.793	0.793	0.690	0.085	0.244	0.374

[†] Means of 6 replicate pens/group.
[‡] *n* = 3 replicate pens/group/room. In "room HS", temperature was raised to and maintained at ~30 °C all day long from D33 to D41.
[¶] Computed for the period consisting of a feeding phase and its previous one/ones. Cum., cumulative.
Note: P-values less than 0.05 are in bold.

Figure 22 – S1E2 | Rectal temperature of CON2 and SYN2 birds in the two rooms at three time points



Note: The rectal temperature of 12 labeled birds/group/room was measured at three time points at 9:00 AM: T1, one day before the beginning of the HS period (D33); T2, the third day of the HS period (D35); T3, the trial end (D41). Group means are the black dots inside the boxes. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

In this heated scientific debate, the results of S1E1 seem to support the hypothesis that high Firmicutes to Bacteroidetes ratios are positively related to the efficiency with which the microbiota collects energy from the diet and makes it available to the host. Also in this case, however, there is no lack of opposite results, such as those of Han et al. (2016) who found no relationship between the Firmicutes to Bacteroidetes ratio and BW in broilers. More thorough investigations are therefore needed to clarify whether and how this parameter, and the possible effects of synbiotics on it, affects energy balance, BW, and FCR in chickens.

The establishment and development of an advantageous bacterial consortium in the ceca of synbiotic-supplemented birds may have promoted their feed efficiency. The abundances of *Bifidobacterium longum* and *Collinsella intestinalis* were higher in SYN1 birds. The beneficial effects of the genus *Bifidobacterium* for poultry and livestock has extensively been documented (FAO, 2016) and *Collinsella intestinalis* has been shown to produce formate and lactate via fermentation (Kageyama and Benno, 2000). Moreover, SYN1 birds showed a greater abundance of *Lactobacillus panis*, *Lactobacillus reuteri*, and uncultured *Streptococcus* sp, all of which are LAB. A huge number of studies have assessed members of the genus *Lactobacillus* as probiotic candidates for animal nutrition,

frequently obtaining positive results in relation to health and performance promotion (FAO, 2016). The extraordinary importance of organic acids of microbial origin and the crucial role of *Bifidobacteria* and LAB for the GI ecosystem, gut health, and host physiology have been explained in detail before (see sections **2.2** "**Intestinal Epithelium, Immunity, and Microbiota: Three Pillars of Gut Health**" and **2.3** "**Exploration and Modulation of the Chicken Gastrointestinal Microbiota**", subsection "**FEED ADDITIVES**"). The abundance of several Clostridia significantly increased due to the synbiotic treatment, such as *Clostridium difficile*, *Eubacterium ramulus*, *Peptoniphilus asaccharolyticus*, *Ruminococcus obeum*, *Blautia producta*, *Blautia* sp. Ser5. Additionally, *Clostridium cocleatum* and *Clostridium innocuum* (belonging to the class Erysipelotrichi) were also more abundant in the synbiotic-fed group. Although no confirmation of potential benefits from these bacteria have been found in the literature, especially regarding *Clostridium difficile* (Czepiel et al., 2019), most of them have been shown to produce useful SCFAs (Moore et al., 1976; Ezaki et al., 1994, 2001; Liu et al., 2008a; Lawson and Finegold, 2015). So, their increased abundance might have been beneficial for the health and performance of SYN1 birds. Within the class Clostridia, the decreased abundance of *Eubacterium rectale* in the ceca of group SYN1 was also observed. In humans, this bacterial species, along with the renowned *Faecalibacterium prausnitzii*, has been shown to produce large quantities of butyrate (Rivière et al., 2016). Its reduction may therefore be considered an unfavorable effect of the synbiotic treatment because butyrate has been shown to be essential for GI homeostasis (Liu et al., 2018). Nevertheless, the significant increase in the abundance of *Eubacterium ramulus*, another butyrate producer (Moore et al., 1976), may have compensated for the reduction in *Eubacterium rectale* in group SYN1. Cecal bacteria able to produce butyrate have been associated with high performance in broilers (Torok et al., 2011; Stanley et al., 2012). Interestingly, it has been suggested that the ability of cecal butyrate producers to promote broiler growth can be linked to cross-feeding mechanisms involving them and probiotic bacteria supplemented to birds (Huyghebaert et al., 2011; De Cesare et al., 2017). Therefore, it is possible that the probiotics ingested with the supplement tested here might have indirectly supported the growth of SYN1 birds through mediation of butyrate-producing bacteria stimulated by themselves. Anyway, the possibly higher concentration of useful organic acids in the ceca of birds treated with the

synbiotic is to be confirmed in future studies by means of specific analyses of the chemical composition of the cecal digesta.

Supplemented birds of group SYN1 were also characterized by significantly lower abundances of *Bacteroides fragilis* and *Bacteroides* sp. 1AL than their control counterparts. It has been reported that propionate produced by *Bacteroides* positively affect gut health in humans (Rios-Covian et al., 2017; Jacobson et al., 2018) and some *Bacteroides* species, like *B. thetaiotaomicron* and *B.* vulgatus, have been tested *in vitro* as components of a probiotic preparation intended to re-establish gut eubiosis after antibiotic treatments (El Hage et al., 2019). It had therefore been hypothesized that an increase in *Bacteroides* abundance would have been desirable for synbiotic-supplemented birds. The results, however, appear to contradict this hypothesis as birds with greater performance, and thus likely in a better health state, showed a decreased abundance of *Bacteroides*. Besides *Bacteroidetes*, the synbiotic treatment might have reduced pathogen load in the ceca. The abundances of *Finegoldia magna*, *Prevotella pallens*, and *Synergistetes bacterium* SGP1 were found to be lower in the supplemented group. These bacteria, or at least the genus they belong to, have been associated to infections and severe diseases (Vartoukian et al., 2007; Debrah and Feingold, 2012; Rosenthal et al., 2012; Uzal et al., 2015; Ley, 2016; Brook, 2017). SYN1 birds may therefore have benefited from their decreased abundance. On the other hand, the increased abundance of *Eggerthella lenta* (formerly known as *Eubacterium lentum*) is controversial and difficult to interpret because this bacterium has been shown to be involved in numerous illnesses (Gardiner et al., 2015).

Taken together, the results of S1E1 confirm that early applications of "biotics" combined with their dietary administration during the growth cycle is beneficial to the health and performance of broilers. The two-step synbiotic treatment tested here improved feed efficiency and, likely because of this, reduced pododermatitis. It can be supposed that the differences between the cecal microbiota of control and supplemented birds were related to these positive results. Nonetheless, Pan and Yu (2014) have urged the importance of conducting more and more in-depth investigations to determine if modifications in the microbiota are the causes or consequences of changes in feed efficiency of poultry. More information about the effects of the synbiotic studied on the microbiota of broilers is therefore needed.

Although the primary aim of S1E2 was to gain expertise in managing experimental facilities to run HS trials in, it is still worth briefly commenting on its results. Unexpectedly, before HS was applied, synbiotic-supplemented birds of group SYN2 showed worse growth rate and feed efficiency than control birds of group CON2, thereby partially disagreeing with the improvement in FCR observed in S1E1. The literature, however, offers inconsistent findings about the performance response of broilers to "biotics" supplementation, which may contradict the efficacy of this product category (Blajman et al., 2014; Ducatelle et al., 2015). The variability in the outcomes depends on several factors that are believed to considerably affect the activity of "biotics" once fed to birds, such as storage conditions, administration level, frequency of application, diet, farm hygiene, animal age, health and physiological state, conditions of the GI microbiota, and persistency of the additive components in the gut (Yang et al., 2009; Huyghebaert et al., 2011; Blajman et al., 2014; Gadde et al., 2017). Additionally, it has been reported that feed additives intended to modulate the composition of GI microbial communities, like the synbiotic used here as the microbiota analysis of S1E1 suggests, may produce inconsistent performance responses if they fail to influence the abundance of specific bacterial taxa under different trial conditions (Walsh et al., 2021). Further studies on this synbiotic with broilers kept in TN environments can therefore be necessary to validate the positive performance results obtained in S1E1 but contradicted by data from the first two feeding phases of S1E2.

As expected, the application of HS during the finisher phase of S1E2 caused substantial deterioration in growth performance, confirming what has previously been discussed (see section **2.4** "**Raising Chickens in the "Hothouse Earth": The Effects of Heat Stress**"). However, synbiotic supplementation did not improve performance or mitigate the negative effects of HS on them or rectal temperature. Although a multitude of studies have seen that "biotics" can help broilers withstand HS (Sugiharto et al., 2017; Ringseis and Eder, 2022), the synbiotic in question was first tested under HS conditions in the present study. Facility limitations led to design S1E2 as an experiment that, upon the beginning of HS, would have caused a considerable reduction in the number of replicates. It was known that this statistical constraint would have undoubtedly impinged on the reliability of the performance data collected during the HS period, but, as stated earlier, demonstrating the efficacy of the synbiotic against HS was not the chief goal for S1E2. Care must therefore be taken when interpreting the HS-

related results of S1E2 or when drawing the conclusion that this synbiotic does not definitely work to counteract HS in broilers. For this reason, S1E2 would be worth repeating with more replicates to get sounder performance data and, possibly, with the analysis of the GI microbiota to make useful comparisons with other studies.

5 STUDY #2: EFFECTS OF A MURAMIDASE ON BROILERS EXPOSED TO THERMONEUTRAL AND HEAT STRESS CONDITIONS

Note: The results of experiment S2E1 have been published. For more information, please refer to **Annex 1**.

5.1 Highlights

- Dietary supplementation of a novel feed-grade muramidase was investigated.
- The muramidase increased final body weight and reduced cumulative feed intake and feed conversion ratio in a dose-dependent way (i.e., 450 > 250 > 0 g/ton feed) and the highest dose reduced the risk of developing footpad lesions in broilers reared in a thermoneutral environment.
- The highest dose decreased cecal alpha diversity, the Firmicutes to Bacteroidetes ratio, and abundance of bacteria known to be butyrate producers (e.g., Clostridia, *Roseburia intestinalis*, *Ruminococcus albus*, and *Eubacterium rectale*), as well as increased the abundance of Bacteroidaceae and Lactobacillaceae.
- The enrichment in bioenergetic metabolites and reduction in pro-oxidant ones in plasma indicate that a more balanced energy metabolism may have favored the growth of birds supplemented at the highest dose.
- A muramidase-based feed additive was first tested in a pilot study with the aim of alleviating the effects of heat stress on broilers, but it failed to meet expectations.

5.2 Background and Aims

As mentioned earlier (see section **2.3** "**Exploration and Modulation of the Chicken Gastrointestinal Microbiota**", subsection "**FEED ADDITIVES**"), supplementing dietary muramidases has been shown to be beneficial for monogastric food animals, such as pigs (May et al., 2012; Ma et al., 2017; Xu et al., 2018c), rabbits (EL-Deep et al., 2020), and chickens (Liu et al., 2010; Gong et al., 2017). The positive effects of muramidases have predominantly been attributed to a modulation of the microbiota of treated animals (Liu et al., 2010; Gong et al., 2017; Xia et al., 2019; EL-Deep et al., 2020). These enzymes, however, have also been shown to have anti-inflammatory and

immunomodulatory properties (Lee et al., 2009; Ragland and Criss, 2017), as confirmed in studies on broilers (Abdel-Latif et al., 2017; Wang et al., 2020a).

A novel feed-grade muramidase, obtained through a biotech process (Lichtenberg et al., 2017), has been found to degrade bacterial cell debris containing PGN. It is thought that the removal of luminal PGN results in optimization of nutrient digestion and absorption and in favorable regulation of the intestinal inflammatory response, with consequent improvements in gut health and performance of broilers (Goodarzi Boroojeni et al., 2019; Sais et al., 2020; Wang et al., 2020b; Pirgozliev et al., 2021). Nevertheless, the mechanisms of action of this muramidase are yet to be fully understood and, besides, no studies have tested its supplementation, or the use of other muramidase-based feed additives, as a potential nutritional solution to help broilers combat HS[26]. The aims of S2 were therefore: (S2E1) to investigate how ABF broilers respond to different inclusion levels of this muramidase in terms of growth performance, welfare indicators (i.e., FPD), breast muscle myopathies, cecal microbiota, and cecal and plasmatic metabolomes to extended our knowledge of the mechanisms by which this dietary enzyme operates; (S2E2) to conduct a pilot study to see if this muramidase is worth administering to broilers facing HS conditions.

5.3 Materials and Methods

EXPERIMENTAL DESIGN, HOUSING, AND HUSBANDRY CONDITIONS

In S2, approved by the Ethical Committee of the University of Bologna (ID: 1277), birds were reared, monitored, and slaughtered in compliance with EU legislation (i.e., Dir. 2007/43/EC, Reg. 2009/1099/EC, and Dir. 2010/63/EU). Feed and water were provided *ad libitum* throughout the trials of this study.

For S2E1, 2,340 one-day-old male Ross 308 broilers, obtained from the same breeder flock and hatching batch, were supplied by a commercial hatchery, and vaccinated against infectious bronchitis,

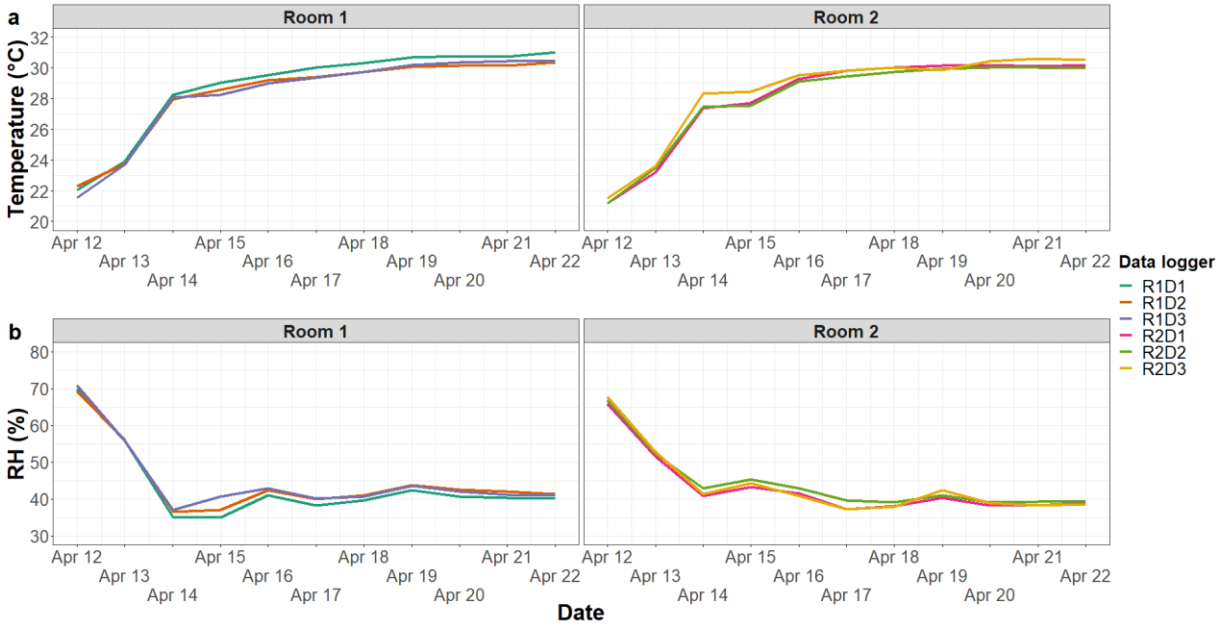
[26] The literature search was carried out by searching the Scopus database on March 01, 2021, entering the following query string: (TITLE ((chicken OR broiler) AND (muramidase OR lysozyme) AND supplementation AND heat AND stress AND NOT serum) OR ABS ((chicken OR broiler) AND (muramidase OR lysozyme) AND supplementation AND heat AND stress AND NOT serum)). No restriction on the date and type of publication was set.

Marek's, Newcastle and Gumboro diseases, and coccidiosis. At placement in an experimental poultry barn, birds were randomly assigned to 3 experimental groups (12 replicate pens/group) that were fed a commercial ABF basal diet in mash form (**CON1**), or the same basal diet supplemented with a feed-grade muramidase (Balancius®, DSM Nutritional Products) at 250 (low-dose group – **MUL1**) or 450 g/ton feed (high-dose group – **MUH1**) for the entire trial (0-42 d). **Annex 3** provides the formula and composition of the basal diet according to the four-phase feeding program used (i.e., starter, 0-9 d; grower I, 10-21 d; grower II, 22-28 d; finisher, 29-42 d). For each feeding phase, the basal diet (**Annex 3**) consisted of a single batch of coarsely ground mash feed (for information on particle size, see **Footnote 24**), and the muramidase was added on top to the sub-batches intended for MUL1 and MUH1 replicates. The analytical inclusion levels of muramidase in the feed met the abovementioned targets. The floor pens were arranged in a randomized complete block design and equipped with chopped straw as bedding material, two bell feeders, and nipple drinkers. The environmental temperature was modified according to the flock age and the breeding company's instructions (Aviagen, 2018). The artificial photoperiod was 23L:1D during the first 7 and last 3 d, while 18L:6D for the remainder days following EU legislation (i.e., Dir. 2007/43/EC) and the breeding company's guidelines for lightning and pre-processing management (Aviagen, 2018).

For S2E2, 300 one-day-old male Ross 308 broilers, procured and vaccinated as detailed above, were randomly placed in 2 identical environmental chambers, hereafter referred to as *rooms*. The rooms were divided into 10 equally sized floor pens equipped as previously described. The pens were assigned to one of two experimental groups (i.e., 5 replicate pens/group) and were arranged in a block design. The control group (**CON2**) was given a commercial ABF basal diet in mash form, while the treated group was fed the same basal diet supplemented on top with muramidase at 450 g/ton feed (**MUH2**) throughout the trial (0-42 d). **Annex 3** provides the formula and composition of the basal diet according to the three-phase feeding program used (i.e., starter, 0-14 d; grower, 15-27 d; finisher, 28-42 d). As before, the basal diet was a coarsely ground mash feed (for information on particle size, see **Footnote 24**). The artificial photoperiod was adjusted as described above. Environmental temperature and RH were recorded with climate data loggers (Trotec GmbH, Heinsberg, Germany) located at animal level (3 data loggers/room having a recording time of 900 s). As for the temperature program, the rooms were

kept at TN conditions until D33 and at constant HS from D34 onwards (i.e., temperature was raised to and maintained at ~31 °C all day long from D34 to D42). During the HS period, RH was kept at ~40% by means of humidifiers (Trotec GmbH) (**Figure 23**).

Figure 23 – S2E2 | Average daily temperature (**a**) and RH (**b**) of the two rooms



Note: April 14, constant HS period start (D34); April 22, constant HS period and trial end (D42).

DATA AND SAMPLE COLLECTION

For S2E1, on a replicate basis, the number and BW of birds were recorded at placement (D0), at every feeding phase switch (D10/22/29), and at slaughter (D42), while FI was measured for each feeding phase. DWG, DFI, and FCR were calculated for the feeding phases separately and cumulative performance traits were calculated for the entire rearing period (0-42 d). The number and BW of dead or culled birds were recorded daily to compute the mortality rate and correct the performance data for mortality. At the end of each feeding phase, fresh excreta samples were collected from every pen (i.e., 12 samples/group for a total of 36 samples/feeding phase) to evaluate the hydrolysis of bacterial PGN. At slaughter (D42) in a commercial abattoir, carcass and cut-up yields were measured on a group basis according to standard commercial procedures. Breast muscle myopathies, namely white striping (**WS**), woody breast (**WB**), and spaghetti meat (**SM**), were assessed blindly by specially trained staff 24 h post-mortem – after chilling, deboning, and skin removal – on a randomly selected sample of approximately

150 breast fillets per group with a 3-point-scale: score 0, normal; score 1, mild myopathy; score 2, severe myopathy (Sirri et al., 2016). FPD were measured by specially trained personnel on one foot per processed bird (i.e., more than 670 observations/group) using a 3-point scale: score 0, no lesions; score 1, mild lesions (≤ 0.8 cm); score 2, severe lesions (> 0.8 cm) (Ekstrand et al., 1998). As with the grading of myopathies, this evaluation was performed blind. One bird per replicate (i.e., 12 birds/group) with a BW close to the flock average was selected at the slaughterhouse (D42) for collection of blood and cecal content samples. Blood was kept at RT before being centrifuged to get plasma that was subsequently stored at -80 °C until metabolomics analysis through proton nuclear magnetic resonance (**^{1}H-NMR**). The content of both ceca was collected in duplicate, frozen in LN_2, and stored at -80 °C until ^{1}H-NMR analysis and DNA extraction for shotgun metagenomic sequencing.

For S2E2, four representative birds per pen (i.e., 20 birds/group) were randomly chosen and labeled to measure the rectal temperature with a veterinary thermometer (Scala Electronic GmbH, Stahnsdorf, Germany). The rectal temperature was taken at three time points: one day before the beginning of the HS period and on the third and the sixth day of the HS period at 9:00 AM. Growth performance data were collected as described before.

LAB ANALYSIS

For S2E1, the freeze-dried excreta samples were resuspended and centrifuged. While the supernatant contained soluble PGN, the precipitate was high in insoluble PGN. These samples were subjected to acid hydrolysis to measure total and soluble PGN through liquid chromatography-mass spectrometric quantification of muramic acid, the latter being used as a marker to estimate the amount of hydrolyzed PGN (Novozymes A/S Biologiens, Lyngby, Denmark). The insoluble fraction of PGN was calculated as the difference between the total and the soluble PGN expressed as muramic acid. This muramidase has been shown to hydrolyze the PGN of bacterial debris both *in vitro* and in *ex vivo* digesta samples of broilers (Frederiksen et al., 2021). Therefore, the previously described assay was carried out to test, via a non-invasive method, the hypothesis of a larger proportion of hydrolyzed PGN in excreta from muramidase-treated birds. Turning now to shotgun metagenomic sequencing, DNA extraction from cecal content samples was performed adopting a bead-beating procedure and using the QIAmp®

DNA Stool Mini Kit (Qiagen, Milan, Italy), as detailed in the work by De Cesare et al. (2017). Total DNA was fragmented and tagged with sequencing indexes and adapters using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, U.S.). Shotgun metagenomic sequencing was performed with NextSeq500 (Illumina) 2×149 bp in paired-end mode generating, on average, 6 Gbp per sample. Two out of the 36 cecal content samples were excluded from subsequent bioinformatics analysis due to low sequencing yield. Moving to metabolomics analysis, an ^{1}H-NMR solution with D_2O, containing TSP 10 mmol/L and NaN_3 2 mmol/L, was created. Phosphate buffer 1 M was used to achieve a pH of 7.00 ± 0.02, while TSP was used as a reference for NMR chemical-shift and NaN_3 avoided bacterial proliferation. Cecal content samples were prepared by vortex mixing approximately 80 mg of each sample with 1 mL of bi-distilled water. Plasma and cecal content samples were centrifuged (18,630 g; 900 s; 4 °C) and 0.7 mL of supernatant were mixed with 0.1 mL of the ^{1}H-NMR solution. Samples were centrifuged as before. The ^{1}H-NMR spectra were registered (600.13 MHz; 298 K) with an AVANCE™ III spectrometer (Bruker, Milan, Italy) equipped with TopSpin software v3.5 (Bruker). The signals from broad resonances due to large molecules were suppressed with CPMG-filter (400 echoes with a τ of 400 μs and a 180° pulse of 24 μs, for a total filter of 330 ms), while the residual signal of water was suppressed by means of presaturation. This was done employing the *cpmgpr1d* sequence, part of the standard pulse sequence library. Each spectrum was acquired summing up 256 transients constituted by 32,000 data points encompassing a window of 7,184 Hz, separated by a relaxation delay of 5 s. The ^{1}H-NMR spectra were phase-adjusted in TopSpin v3.5 (Bruker) and then exported to ASCII format by means of the built-in script *convbin2asc*. Spectra were processed with R (R Core Team, 2020) through home-made scripts. Signal assignment was performed comparing their chemical shift and multiplicity with the Human Metabolome Database (Wishart et al., 2007) and Chenomx software library (Chenomx Inc., Edmonton, Canada, v10), by means of Chenomx software routines. Concentrations of plasmatic molecules were assessed by quantifying the molecules of the first sample analyzed by means of an external standard. Differences in water content between samples were then taken into consideration by probabilistic quotient normalization (Dieterle et al., 2006). Molecule concentrations in cecal samples were assessed as described for plasma by considering as reference the sample with the median water

dilution assessed via probabilistic quotient normalization. The quantification of each molecule was performed through rectangular integration, considering one of its signals free from interferences.

DATA ANALYSIS

For S2E1, a one-way ANOVA with blocks was used to evaluate the effect of diet (i.e., the group factor) on growth performance, using the replicate as the experimental unit. Tukey's HSD post-hoc test was used to separate the means. Furthermore, polynomial contrasts were carried out to look for linear and quadratic trends in growth performance data from the entire trial. Mortality rate data were transformed using the arcsine transformation before being analyzed with inferential statistics. For each feeding phase, differences in soluble muramic acid, total muramic acid, and their ratio were analyzed through a one-way ANOVA and Tukey's HSD post-hoc test, as described above. Pearson's correlation coefficient between soluble to total muramic acid ratio data and FCR data from each feeding phase was computed and tested for significance. Carcass and cut-up yields data were not statistically analyzed because they were recorded on a group basis without considering the replicates at the slaughterhouse. Count data of WS, WB, SM, and FPD were analyzed with Pearson's chi-squared test using the sampled animal as the experimental unit. Count data were also arranged in 2 by 2 contingency tables aligning a combination of levels of the group factor (i.e., CON1 and MUL1; CON1 and MUH1; MUL1 and MUH1) and having binarily aggregated scores of a myopathy or FPD in columns (i.e., "presence" as a sum of counts of score 1 and score 2, while "absence" as score 0 counts). The incidence risk ratio was computed on these tables with *epiR* package (Stevenson et al., 2021) of R (R Core Team, 2020). The risk of developing a myopathy or FPD was calculated as incidence risk ratio minus 1 and expressed in percentage. A confidence interval of 95% and Pearson's chi-squared test were used to test the incidence risk ratio significance. With respect to the analysis of cecal microbiota data, the MG-RAST analysis server (Meyer et al., 2008) was utilized for taxonomic identification by mapping the sequencing reads against the RefSeq database (O'Leary et al., 2016). Moreover, the Kyoto Encyclopedia of Genes and Genome (**KEGG**) database (Kanehisa and Goto, 2000) was used for hierarchical analysis of sequencing reads. The bacterial abundance matrix and KEGG matrix (down to the species level and the third KEGG level, respectively) were downloaded from MG-RAST for statistical analysis. Ecological diversity

indices were analyzed with *vegan* package (Oksanen et al., 2020) of R (R Core Team, 2020): Shannon, Simpson, and Inverse Simpson indices were used for alpha diversity, while Bray-Curtis distance matrix was used for beta diversity analysis at the genus level. Alpha indices were subjected to a one-way ANOVA and Tukey's HSD post-hoc test by considering the group as the experimental factor and the sampled animal as the experimental unit. Beta diversity was graphically explored through a PCoA and analyzed with a PERMANOVA – *adonis2* function with 10,000 permutations – followed by a pairwise permutation MANOVA with *RVAideMemoire* package (Hervé, 2021) of R (R Core Team, 2020). The matrix of abundances of cecal bacteria was normalized for the total read number in each sample and analyzed with STAMP v2.1.3 (Parks et al., 2014) using Kruskal-Wallis H-test and Games-Howell post-hoc test with group as the experimental factor and the sampled animal as the experimental unit. The Firmicutes to Bacteroidetes ratio was calculated for every group and analyzed with a one-way ANOVA and orthogonal contrasts (i.e., CON1 *vs.* MUL1 + MUH1; MUL1 *vs.* MUH1). KEGG level 3 matrix was filtered for "KEGG level 2": this subset was then normalized and analyzed as described above. Regarding the analysis of metabolomics data, a one-way ANOVA and Tukey's HSD post-hoc test were carried out by considering the group as the experimental factor and the sampled animal as the experimental unit. Metabolomics data deviating from normality in Shapiro-Wilk test were transformed with Box-Cox transformation (Box and Cox, 1964). In addition, an rPCA (Hubert et al., 2005) was performed on the molecules showing significantly different concentrations between groups in the aforementioned univariate analysis. Initially, the PcaHubert algorithm detected outlying samples according to their distance from others along and orthogonally to the PCA plane. Later, the optimal number of PCs was determined. A score plot and a correlation plot summarized the main features of the rPCA models. The former represented the samples in the PC space, thus evidencing the overall structure of the data. The latter reported Pearson's correlations between the concentration of each molecule and the model components, thereby showing which molecule mostly affected the data structure. These analyses were carried out using R (R Core Team, 2020). *P*-values less than 0.05 were considered significant, while those ranging between 0.05 and 0.1 were considered tendencies.

For S2E2, a one-way ANOVA was used to evaluate the effect of diet (i.e., the group factor) on growth performance, accounting for the effects of block and room and considering the replicate as the

experimental unit. Mortality rate data were handled as before. Rectal temperature data were analyzed through a three-way mixed ANOVA, a type of repeated-measures ANOVA that includes between-subject factors (i.e., group and room) and within-subject factors (i.e., time point). After verifying that there was no statistically significant three-way interaction, rectal temperature data were grouped by the factors group and room to run paired *t*-tests with Bonferroni adjustment between time points. These analyses were carried out using R (R Core Team, 2020) and considering 0.05 as the significance threshold. *P*-values ranging between 0.05 and 0.1 were considered tendencies.

5.4 Results

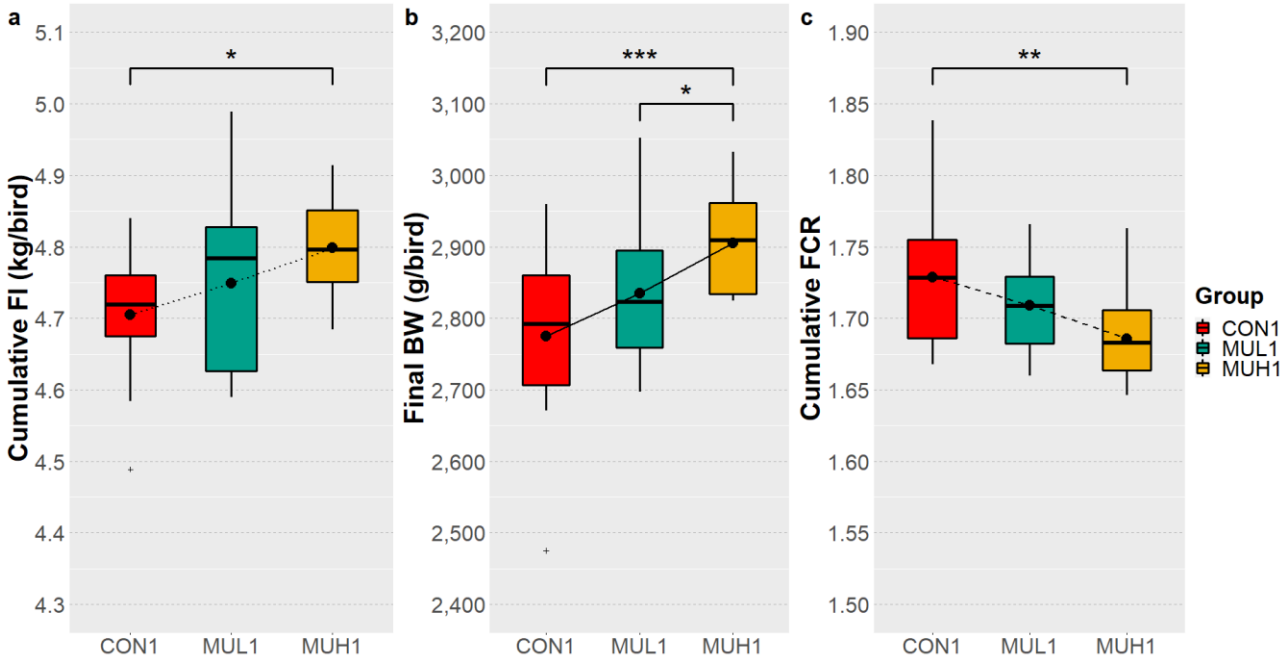
For S2E1, **Table 6** compares the growth performance of CON1, MUL1, and MUH1 birds in the four feeding phases and in the whole trial. At placement, chicks weighed approximately 42 g with no significant differences between groups. At the end of the starter phase, muramidase-supplemented birds exhibited higher BW than control birds (199.5, 204.8, and 205.8 g for CON1, MUL1, and MUH1, respectively; $P < 0.01$), while only MUH1 birds showed lower FCR than control birds (1.267 *vs.* 1.240 for CON1 and MUH1, respectively; $P < 0.05$). Birds of MUH1 group reached the greatest BW at the end of the first grower phase (765.3, 785.1, and 818.8 g for CON1, MUL1, and MUH1, respectively; $P < 0.001$), as well as higher FI than control birds (0.866 and 0.908 kg for CON1 and MUH1, respectively; $P < 0.05$). At the end of the second grower phase, MUH1 birds showed the highest BW (1,344, 1,375, and 1,443 g for CON1, MUL1, and MUH1, respectively; $P < 0.001$) and the lowest FCR (1.673, 1.651, and 1.590 for CON1, MUL1, and MUH1, respectively; $P = 0.001$). At the end of the finisher phase, MUH1 birds exhibited greater BW than control birds (2,775 and 2,906 g for CON1 and MUH1, respectively; $P < 0.05$) and MUL1 birds (2,835 and 2,906 g for MUL1 and MUH1, respectively; $P = 0.05$), while the other performance traits were unaffected. Results of the entire trial indicated that, in addition to final BW, MUH1 birds outperformed control birds in terms of cumulative FI and cumulative FCR (4.705 g and 1.729, and 4.798 kg and 1.686 for CON1 and MUH1, respectively; $P < 0.05$). Polynomial contrasts also revealed that cumulative FI and final BW significantly increased but cumulative FCR significantly decreased across groups in a linear fashion (**Figure 24**).

Table 6 – S2E1 | Growth performance of groups CON1, MUL1, and MUH1 at the end of each feeding phase and in the entire trial (0-42 d)

Dependent variable	Group[†]			SE	*P*-value
	CON1	MUL1	MUH1		
Chick weight (g)	42.18	42.11	42.20	0.28	0.685
Starter (0-9 d)					
BW (g/bird)	199.5^{b}	204.8^{a}	205.8^{a}	4.48	**0.005**
DWG[§] (g/bird/d)	17.48^{b}	18.08^{a}	18.18^{a}	0.51	**0.005**
DFI[§] (g/bird/d)	22.15	22.61	22.53	0.47	0.053
FI[§] (kg/bird)	0.199	0.204	0.203	0.00	0.053
FCR[§]	1.267^{a}	1.251^{ab}	1.240^{b}	0.02	**0.016**
Mortality (%)	0.00	0.00	0.00	/	/
Grower I (10-21 d)					
BW (g/bird)	765.3^{b}	785.1^{b}	818.8^{a}	22.20	**< 0.001**
DWG[§] (g/bird/d)	47.15^{b}	48.36^{b}	51.10^{a}	1.66	**< 0.001**
DFI[§] (g/bird/d)	72.17^{b}	73.22^{ab}	75.65^{a}	3.26	**0.044**
FI[§] (kg/bird)	0.866^{b}	0.879^{ab}	0.908^{a}	0.04	**0.044**
FCR[§]	1.532	1.515	1.482	0.06	0.108
Mortality (%)	0.00	0.00	0.13	0.02	0.384
Grower II (22-28 d)					
BW (g/bird)	1,344^{b}	1,375^{b}	1,443^{a}	39.85	**< 0.001**
DWG[§] (g/bird/d)	82.69^{b}	83.88^{b}	89.13^{a}	3.70	**0.001**
DFI[§] (g/bird/d)	138.0	138.3	141.6	3.94	0.062
FI[§] (kg/bird)	0.966	0.968	0.991	0.03	0.062
FCR[§]	1.673^{a}	1.651^{a}	1.590^{b}	0.05	**0.001**
Mortality (%)	0.26	0.39	0.26	0.06	0.877
Finisher (29-42 d)					
BW (g/bird)	2,775^{b}	2,835^{b}	2,906^{a}	69.52	**0.001**
DWG[§] (g/bird/d)	101.6	103.5	103.2	2.93	0.235
DFI[§] (g/bird/d)	191.0	192.8	192.6	3.29	0.349
FI[§] (kg/bird)	2.674	2.699	2.696	0.05	0.349
FCR[§]	1.883	1.864	1.866	0.05	0.547
Mortality (%)	1.41	1.41	1.80	0.08	0.948
Entire trial (0-42 d)					
BW (g/bird)	2,775^{b}	2,835^{b}	2,906^{a}	69.52	**0.001**
DWG[§] (g/bird/d)	65.03^{b}	66.46^{ab}	68.13^{a}	1.65	**0.001**
DFI[§] (g/bird/d)	111.8^{b}	112.8^{ab}	114.0^{a}	2.01	**0.043**
FI[§] (kg/bird)	4.705^{b}	4.749^{ab}	4.798^{a}	0.08	**0.034**
FCR[§]	1.729^{a}	1.709^{ab}	1.686^{b}	0.03	**0.012**
Mortality (%)	1.68	1.80	2.18	0.07	0.746

[†] Means of 12 replicate pens/group.
[§] Corrected for mortality.
Note: P-values less than 0.05 are in bold. Within a row, means with distinct superscripts are significantly different ($P < 0.05$).

Figure 24 – S2E1 | Cumulative FI (**a**), final BW (**b**), and cumulative FCR (**c**) of groups CON1, MUL1, and MUH1 in the entire trial (0-42 d)



Note: Means of 12 replicate pens/group are the black dots inside the boxes. *, $P \leq 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Linear trends are the black lines connecting group means: dotted line, $P < 0.05$; dashed line, $P < 0.01$; solid line, $P < 0.001$.

Concentrations of muramic acid in excreta samples at the end of each feeding phase are reported in **Table 7**. In every feeding phase, muramidase-supplemented birds exhibited a higher soluble fraction of muramic acid and a greater soluble to total muramic acid ratio than control birds ($P < 0.05$). Moreover, it was found a weak, negative correlation between the soluble to total muramic acid ratio and FCR (r = -0.30; $P < 0.001$).

Although they were not statistically analyzable as mentioned before, processing data of S2E1 showed that muramidase-supplemented birds had a greater numerical eviscerated carcass yield (70.1, 70.4, and 70.8% for CON1, MUL1, and MUH1, respectively) and breast yield calculated as percentage of the eviscerated carcass weight (30.6, 30.9, and 31.3% for CON1, MUL1, and MUH1, respectively).

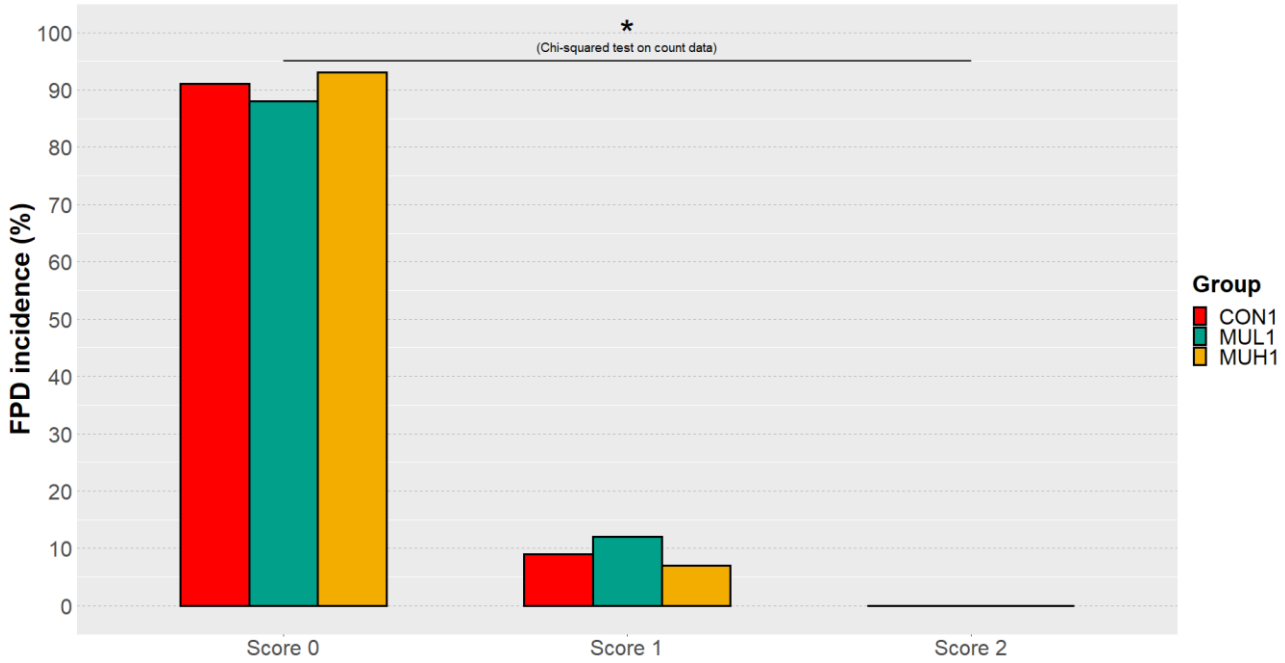
Table 7 – S2E1 | Total muramic acid, soluble muramic acid, and their ratio in excreta samples of CON1, MUL1, and MUH1 birds at the end of each feeding phase

Variable	Group[†]			SE	*P*-value
	CON1	MUL1	MUH1		
Starter (0-9 d)					
Total muramic acid (mg/kg)	497.8	398.8	444.0	54.7	0.453
Soluble muramic acid (mg/kg)	121.7^{b}	214.6^{a}	256.6^{a}	19.0	< **0.001**
Soluble/total muramic acid (%)	25.3^{b}	55.6^{a}	62.2^{a}	3.2	< **0.001**
Grower I (10-21 d)					
Total muramic acid (mg/kg)	932.2^{a}	747.0^{b}	897.6^{a}	37.4	**0.005**
Soluble muramic acid (mg/kg)	284.6^{b}	575.7^{a}	539.8^{a}	31.3	< **0.001**
Soluble/total muramic acid (%)	30.8^{c}	78.5^{a}	62.8^{b}	3.6	< **0.001**
Grower II (22-28 d)					
Total muramic acid (mg/kg)	660.4	666.1	648.7	38.1	0.947
Soluble muramic acid (mg/kg)	148.2^{b}	325.3^{a}	335.6^{a}	27.6	< **0.001**
Soluble/total muramic acid (%)	23.5^{b}	49.6^{a}	51.2^{a}	2.7	< **0.001**
Finisher (29-42 d)					
Total muramic acid (mg/kg)	830.6^{a}	606.3^{b}	740.4^{ab}	58.6	**0.041**
Soluble muramic acid (mg/kg)	127.6^{b}	272.7^{a}	284.4^{a}	20.2	< **0.001**
Soluble/total muramic acid (%)	17.1^{b}	47.3^{a}	40.9^{a}	4.1	< **0.001**

[†] Means of 12 replicate pens/group.
Note: P-values less than 0.05 are in bold. Within a row, means with distinct superscripts are significantly different ($P < 0.05$).

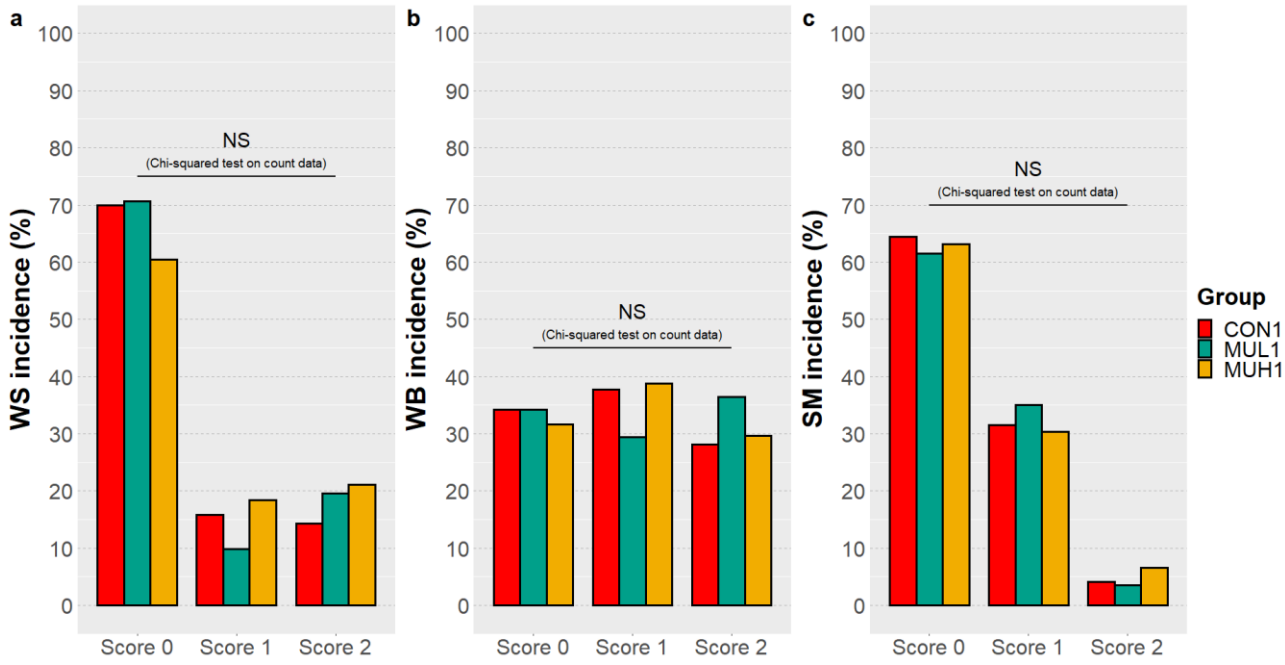
In S2E1, the occurrence of FPD was found to be related to the group factor, with group MUH1 showing the highest proportion of birds without footpad lesions, namely a greater incidence of FPD with score 0 than the other groups ($P < 0.05$; **Figure 25**). Besides, the incidence risk ratio analysis showed that MUH1 birds were 0.58 (95% confidence interval of 0.41 to 0.82) times less likely to develop FPD than MUL1 birds; that is, supplementing birds with the highest dose of muramidase decreased by 42% the relative risk of developing FPD compared to their counterparts supplemented at the lowest dose ($P < 0.001$). On the other hand, MUL1 diet tended to increase by 33% the relative risk of FPD development compared to CON1 diet ($P = 0.07$). Breast muscle myopathies, however, did not show a significant relationship with the group factor (**Figure 26**).

Figure 25 – S2E1 | Incidence and severity of FPD of CON1, MUL1, and MUH1 birds at D42



Note: Score 0, no lesions; score 1, mild lesions (≤ 0.8 cm); score 2, severe lesions (> 0.8 cm). *, $P < 0.05$.

Figure 26 – S2E1 | Incidence and severity of WS (**a**), WB (**b**), and SM (**c**) of CON1, MUL1, and MUH1 birds at D42



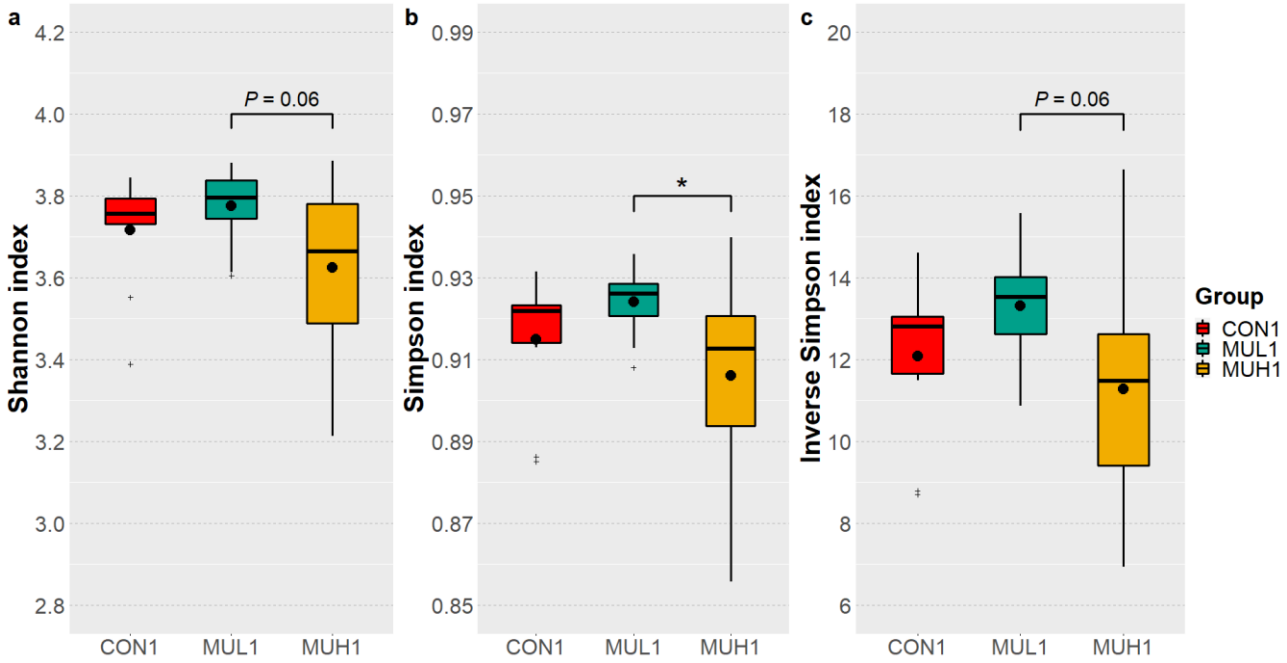
Note: Score 0, normal; score 1, mild myopathy; score 2, severe myopathy. NS, not significant.

As for the cecal microbiota of S2E1[27], MUH1 birds showed lower alpha diversity than MUL1 birds ($P < 0.05$ for Simpson index and $P = 0.06$ for Shannon and Inverse Simpson indices), while CON1 birds did not differ from muramidase-supplemented groups (**Figure 27**). Regarding beta diversity, the PCoA revealed an evident segregation of MUH1 samples (**Figure 28**). Besides this graphic distinction, the PERMANOVA confirmed the effect of group on beta diversity ($P = 0.004$; $R^2 = 0.22$), while the pairwise permutation MANOVA revealed a differentiation of MUH1 samples from those of the other groups ($P < 0.05$). At the phylum level (**Figure 29**), MUH1 birds showed a lower relative abundance of Firmicutes and a greater relative abundance of Bacteroidetes than MUL1 birds (69.9% and 17.9%, and 59.2% and 28.4% for MUL1 and MUH1, respectively; $P < 0.05$). Similarly, the Firmicutes to Bacteroidetes ratio differed between MUH1 and MUL1 samples (2.7 and 4.5, respectively; $P = 0.05$). At the family level, Clostridiaceae were underrepresented in group MUH1 compared to the other groups (21.1, 21.3, and 17.4% for CON1, MUL1, and MUH1, respectively; $P < 0.05$). Lachnospiraceae were less abundant in MUH1 than MUL1 birds (5.7% and 4.7% for MUL1 and MUH1, respectively; $P < 0.05$). Cecal content samples of MUH1 group exhibited a higher relative abundance of Bacteroidaceae than those of MUL1 group (13.2% and 21.5% for MUL1 and MUH1, respectively; $P < 0.05$). The relative abundance of Lactobacillaceae was greater in MUH1 than CON1 birds (4.4% and 1.8%, respectively; $P < 0.05$). At the species level, the relative abundances of *C. phytofermentans*, *C. saccharolyticum*, *C. cellulolyticum*, *C. butyricum*, *C. perfringens*, *C. botulinum*, *Eubacterium rectale*, *Roseburia intestinalis*, *Ruminococcus albus*, and *Listeria monocytogenes* were lower in MUH1 group than the other groups ($P < 0.1$ or $P < 0.05$). The relative abundance of *Bacteroides thetaiotaomicron*, however, was higher in MUH1 than MUL1 birds ($P < 0.05$). At the glycan biosynthesis and metabolism KEGG level, MUH1 samples showed a greater relative abundance of genes associated to glycosaminoglycan degradation pathway than MUL1 samples ($P < 0.05$), while the relative abundance of genes involved in peptidoglycan biosynthesis pathway was affected in the opposite way ($P < 0.05$). Genes involved in starch and sucrose metabolism and amino sugar and nucleotide metabolism were

[27] The metagenomes have been uploaded to the MG-RAST repository (project entitled "Muramidase_UniBO_project_2020_34WGS" available at https://www.mg-rast.org/linkin.cgi?project=mgp98274).

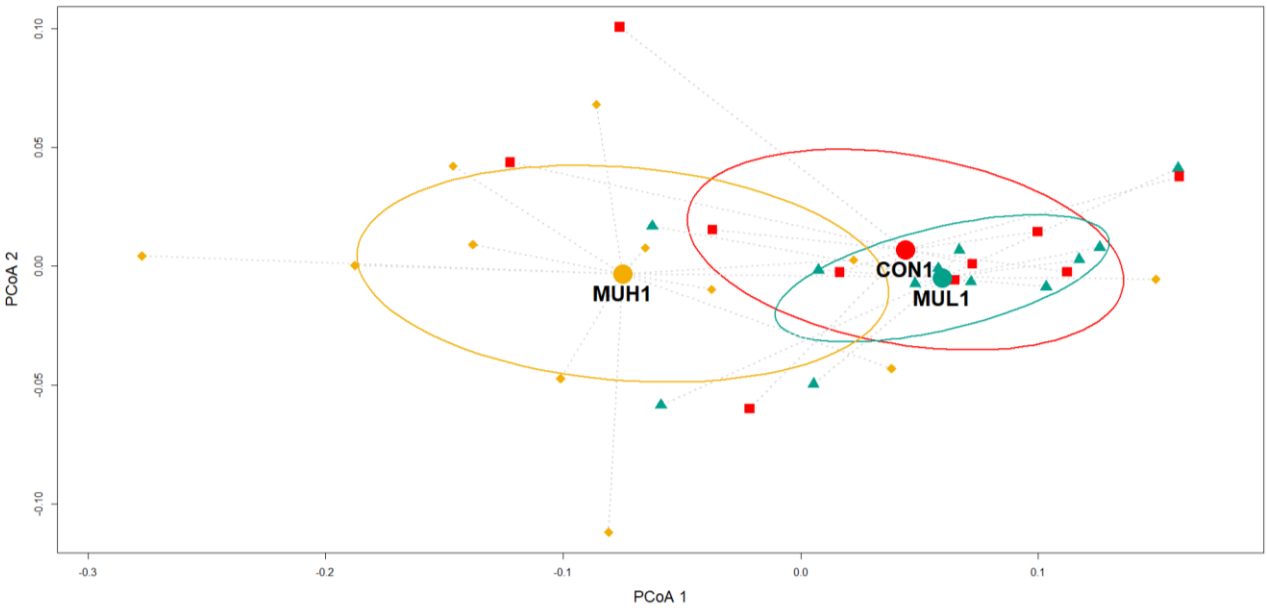
affected by group: the former had a higher relative abundance in CON1 and MUL1 than MUH1 samples ($P < 0.01$), whereas the latter showed an increasing trend in MUL1 compared to MUH1 samples ($P < 0.1$). Genes involved in seleno-compound metabolism pathway showed an increase in MUL1 compared to MUH1 cecal content samples ($P < 0.05$). However, MUH1 samples exhibited a higher relative abundance of genes involved in glutathione metabolism pathway than CON1 and MUL1 samples ($P < 0.05$). Two transport and catabolism pathways revealed a difference between groups: genes involved in lysosome path had a greater relative abundance in MUH1 than MUL1 samples ($P < 0.05$), while the abundance of genes linked to peroxisome path changed the other way around ($P < 0.05$).

Figure 27 – S2E1 | Shannon index (**a**), Simpson index (**b**), and Inverse Simpson index (**c**) of the cecal content of CON1, MUL1, and MUH1 birds at D42



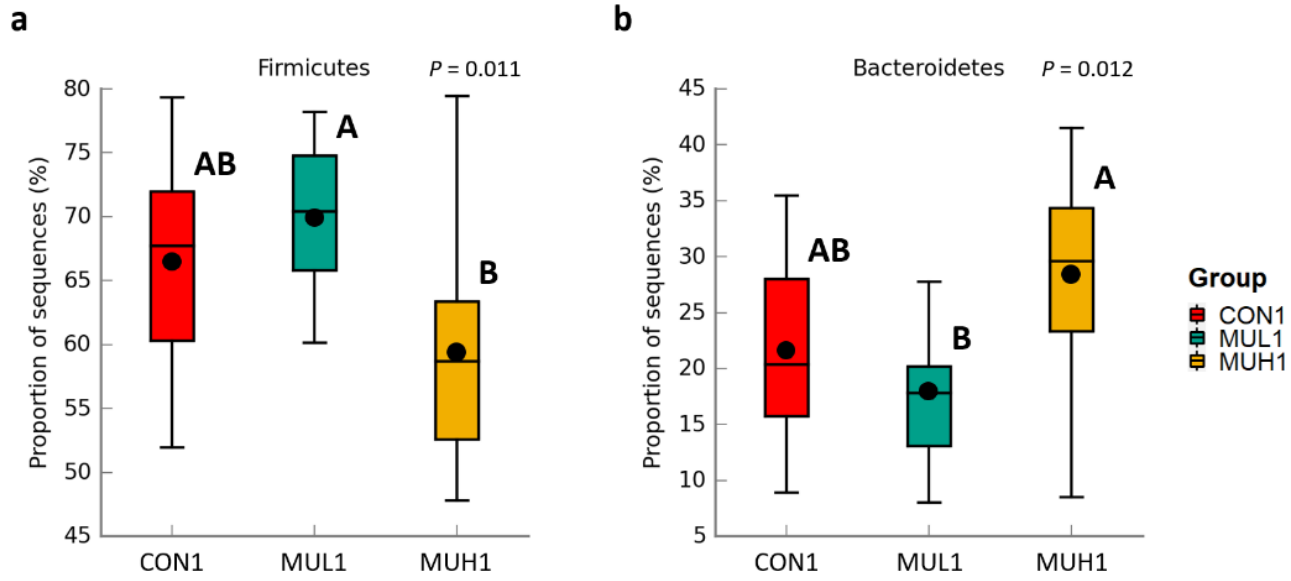
Note: Means of 12 birds/group are the black dots inside the boxes. *, $P < 0.05$.

Figure 28 – S2E1 | PCoA plot based on Bray-Curtis distance matrix used to compute beta diversity of the cecal content of CON1, MUL1, and MUH1 birds at D42



Note: CON1 samples are the red squares, MUL1 samples are the green triangles, and MUH1 samples are the yellow diamonds. The large, colored dots are group centroids, while the colored plane curves are standard deviational ellipses.

Figure 29 – S2E1 | Relative abundance of Firmicutes (**a**) and Bacteroidetes (**b**) in the cecal content of CON1, MUL1, and MUH1 birds at D42



Note: Means of 12 birds/group are the black dots inside the boxes. *P*-values of Kruskal-Wallis H-test are reported in the top-right corner of each boxplot. Different capital letters indicate a $P < 0.01$.

After the registration of ^{1}H-NMR spectra for plasma and cecal content samples of S2E1, 54 and 78 molecules were assigned and quantified, respectively. In the cecal content, the concentration of 4 metabolites showed a significant difference between groups (**Table 8**): while the levels of acetate,

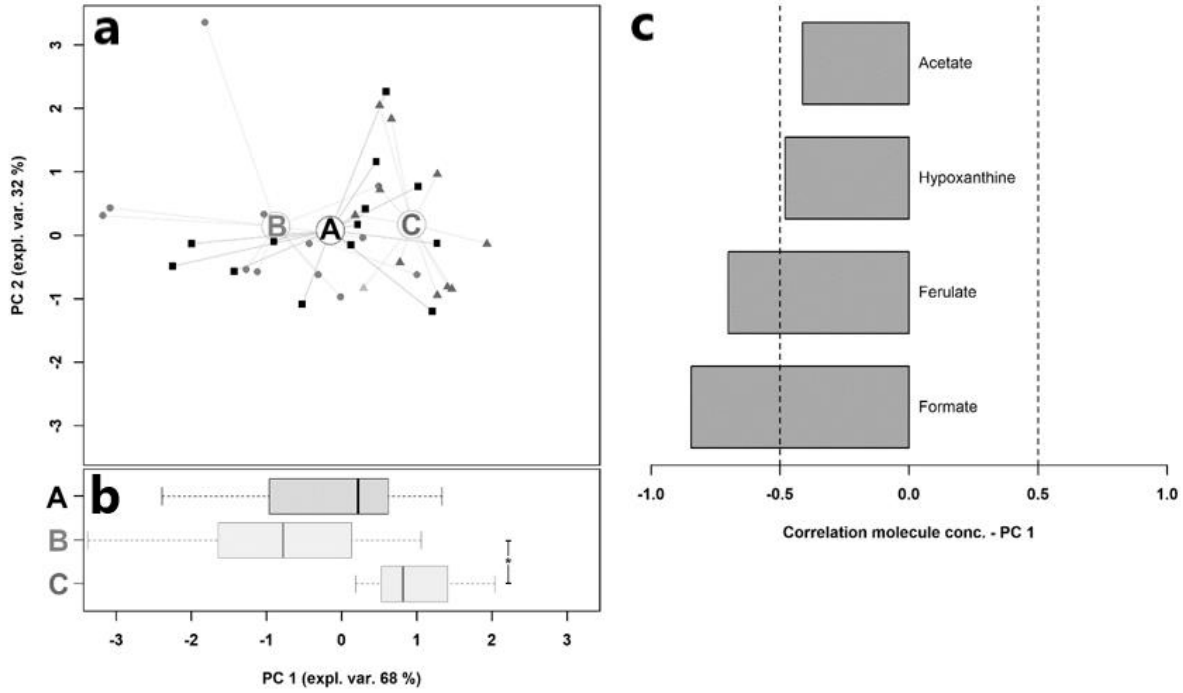
ferulate, and formate were greater in MUL1 than MUH1 birds ($P < 0.05$), that of hypoxanthine was higher in CON1 than MUH1 birds ($P < 0.05$). The rPCA model shown in **Figure 30** was built on these molecules. The PC1 scores of MUH1 samples are substantially or marginally higher than those of the other groups, resulting in a group-based clustering of samples mainly led by ferulate and formate ($r < -0.5$). In plasma, the concentration of 9 metabolites showed a significant variation between groups (**Table 9**). Specifically, a higher concentration of pyruvate was observed in MUH1 than CON1 and MUL1 birds ($P < 0.05$), while those of 2-oxoglutarate, glucose, and uridine were greater in MUH1 than MUL1 birds ($P < 0.05$). Contrarily, MUH1 birds showed a higher concentration of myo-inositol than CON1 birds ($P < 0.05$), whereas CON1 birds exhibited a greater concentration of histidine than MUH1 birds ($P < 0.05$). CON1 and MUL1 birds had a higher level of hypoxanthine than MUH1 birds ($P < 0.05$), while MUL1 birds showed a greater concentration of uracil than MUH1 birds ($P < 0.05$). **Figure 31** illustrates an rPCA model produced as described above. Samples of MUH1 group are characterized by higher PC1 scores and segregate ($P < 0.05$): this separation is predominantly driven by pyruvate, 2-oxoglutarate, glucose, uracil, and hypoxanthine ($r > 0.5$ or < -0.5).

Table 8 – S2E1 | Metabolites showing different concentrations (mmol/L) in the cecal content of CON1, MUL1, and MUH1 birds at D42

Metabolite	Group[†]		
	CON1	**MUL1**	**MUH1**
Acetate	4.70E-02^{ab} (2.79E-02)	6.38E-02^{a} (1.65E-02)	4.82E-02^{b} (1.81E-02)
Hypoxanthine	6.67E-05^{a} (3.71E-05)	5.46E-05^{ab} (3.91E-05)	3.22E-05^{b} (2.05E-05)
Ferulate	6.54E-05^{ab} (1.64E-05)	8.04E-05^{a} (2.99E-05)	5.16E-05^{b} (2.75E-05)
Formate	7.17E-05^{ab} (1.52E-05)	7.86E-05^{a} (2.65E-05)	6.08E-05^{b} (1.38E-05)

[†] Means of 12 birds/group. Standard deviation is given in brackets.
Note: Within a row, means with distinct superscripts are significantly different ($P < 0.05$).

Figure 30 – S2E1 | rPCA model based on cecal content metabolites of **Table 8**



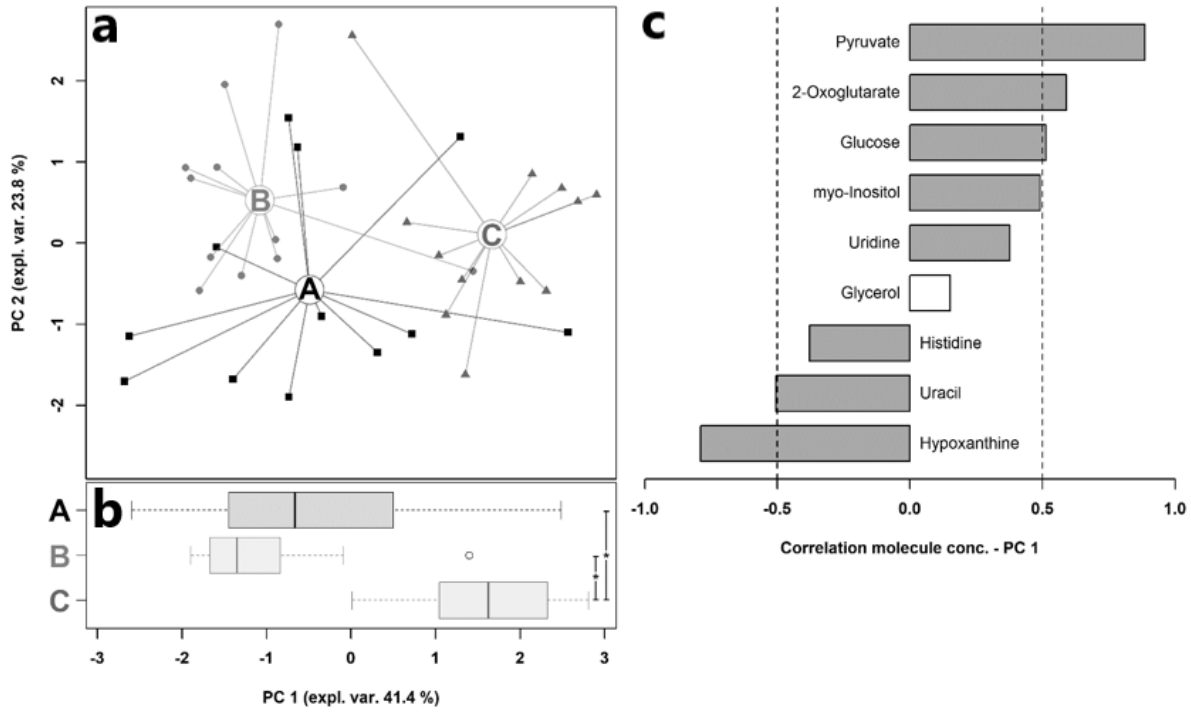
Note: In the score plot (**a**), cecal content samples of groups CON1 ("A"), MUL1 ("B"), and MUH1 ("C") are squares, dots, and triangles, respectively. Wide circles are group medians. The boxplot (**b**) summarizes the sample position along the PC1. The loading plot (**c**) reports the correlations between the concentration of each metabolite and its importance over the PC1. Grey bars indicate significant correlations ($P < 0.05$).

Table 9 – S2E1 | Metabolites showing different concentrations (mmol/L) in plasma of CON1, MUL1, and MUH1 birds at D42

Metabolite	Group†		
	CON1	**MUL1**	**MUH1**
Pyruvate	4.97E-02^{b} (1.92E-02)	4.19E-02^{b} (8.92E-03)	6.45E-02^{a} (2.50E-02)
2-Oxoglutarate	7.31E-03^{ab} (2.17E-03)	6.07E-03^{b} (2.60E-03)	8.97E-03^{a} (2.61E-03)
Glucose	4.73E+00^{ab} (3.20E-01)	4.76E+00^{b} (4.69E-01)	5.12E+00^{a} (3.75E-01)
myo-Inositol	1.13E-01^{b} (2.27E-02)	1.10E-01^{ab} (1.66E-02)	1.29E-01^{a} (2.42E-02)
Uridine	4.36E-03^{ab} (1.90E-03)	3.26E-03^{b} (1.13E-03)	5.40E-03^{a} (1.38E-03)
Glycerol	2.11E-02^{b} (4.29E-03)	2.63E-02^{a} (4.01E-03)	2.33E-02^{ab} (6.32E-03)
Histidine	2.17E-02^{a} (6.97E-03)	2.09E-02^{ab} (5.59E-03)	1.97E-02^{b} (4.91E-03)
Uracil	3.37E-03^{ab} (9.47E-04)	4.27E-03^{a} (8.40E-04)	3.33E-03^{b} (4.25E-04)
Hypoxanthine	4.49E-03^{a} (3.35E-03)	5.47E-03^{a} (2.02E-03)	2.83E-03^{b} (1.70E-03)

† Means of 12 birds/group. Standard deviation is given in brackets.
Note: Within a row, means with distinct superscripts are significantly different ($P < 0.05$).

Figure 31 – S2E1 | rPCA model based on plasma metabolites of **Table 9**



Note: In the score plot (**a**), plasma samples of groups CON1 ("A"), MUL1 ("B"), and MUH1 ("C") are squares, dots, and triangles, respectively. Wide circles are group medians. The boxplot (**b**) summarizes the sample position along the PC1. The loading plot (**c**) reports the correlations between the concentration of each metabolite and its importance over the PC1. Grey bars indicate significant correlations ($P < 0.05$).

Moving to the results of S2E2, **Table 10** provides the growth performance of CON2 and MUH2 birds in the three-phase feeding trial. At placement, chicks weighed approximately 47 g with no significant differences between groups. Growth performance traits were not significantly affected by the group factor neither at the end of each feeding phase nor in the entire trial ($P > 0.05$). Even the application of constant HS for part of the finisher phase did not make the gap between the groups significant ($P > 0.05$).

Figure 32 compares the rectal temperature of representative birds of groups CON2 and MUH2 one day before the beginning of the HS period and on the second and the sixth day of the HS period of S2E2. Rectal temperature data ranged between 40.8 and 44.0 °C. The mixed ANOVA applied to these data revealed that the interaction between the three factors (i.e., group, room, and time point) and the main effects of group and room were not significant ($P > 0.05$), while the effect of time point was highly significant ($P < 0.001$). In addition, the pairwise comparisons of time points made it possible to find a

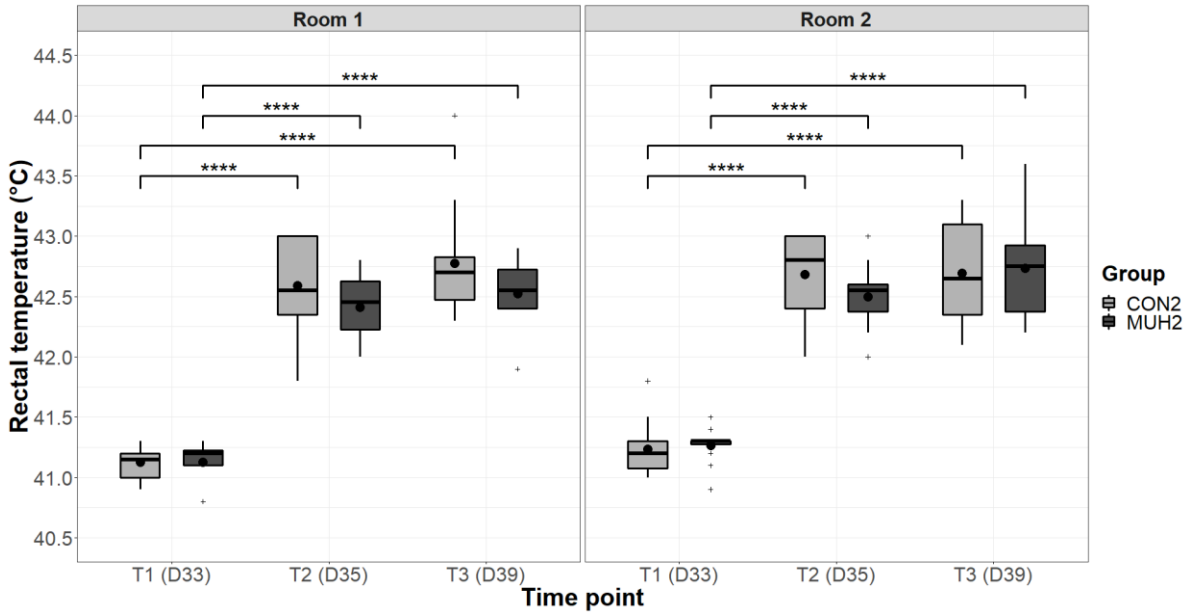
considerable increase in rectal temperature from T1 to later time points in either room ($P < 0.001$). The differences between T2 and T3, however, were not significant ($P > 0.05$).

Table 10 – S2E2 | Growth performance of groups CON2 and MUH2 at the end of each feeding phase and in the entire trial (0-42 d)

Dependent variable	Group[†]		SE	*P*-value
	CON2	MUH2		
Chick weight (g)	47.05	46.52	0.78	0.361
Starter (0-14 d)				
BW (g/bird)	455.5	460.1	27.34	0.807
DWG[§] (g/bird/d)	28.91	29.40	2.17	0.746
DFI[§] (g/bird/d)	42.14	41.90	1.89	0.852
FI[§] (kg/bird)	0.590	0.587	0.03	0.852
FCR[§]	1.460	1.427	0.09	0.595
Mortality (%)	1.60	2.40	0.03	0.126
Grower (15-27 d)				
BW (g/bird)	1,463	1,487	67.43	0.618
DWG[§] (g/bird/d)	77.13	78.70	3.84	0.565
DFI[§] (g/bird/d)	119.6	121.0	1.28	0.185
FI[§] (kg/bird)	1.555	1.573	0.02	0.185
FCR[§]	1.551	1.538	0.06	0.765
Mortality (%)	2.47	1.63	0.11	0.603
Finisher[‡] (28-42 d; constant HS from D34 onwards)				
BW (g/bird)	2,579	2,611	81.70	0.574
DWG[§] (g/bird/d)	76.07	75.94	0.22	0.388
DFI[§] (g/bird/d)	146.8	144.3	4.02	0.401
FI[§] (kg/bird)	2.201	2.164	0.06	0.401
FCR[§]	1.932	1.900	0.06	0.464
Mortality (%)	0.00	2.54	0.09	0.185
Entire trial (0-42 d)				
BW (g/bird)	2,579	2,611	81.70	0.574
DWG[§] (g/bird/d)	60.06	60.80	1.95	0.588
DFI[§] (g/bird/d)	102.7	102.1	1.76	0.649
FI[§] (kg/bird)	4.346	4.324	0.06	0.593
FCR[§]	1.704	1.678	0.04	0.401
Mortality (%)	4.00	6.40	0.15	0.545

[†] Means of 5 replicate pens/group.
[§] Corrected for mortality.
[‡] Temperature was raised to and maintained at ~31 °C all day long from D34 to D42.

Figure 32 – S2E2 | Rectal temperature of CON2 and MUH2 birds in the two rooms at three time points



Note: The rectal temperature of 20 labeled birds/group was measured at three time points at 9:00 AM: T1, one day before the beginning of the HS period (D33); T2, the third day of the HS period (D35); T3, the sixth day of the HS period (D39). Group means are the black dots inside the boxes. ****, $P < 0.0001$.

5.5 Discussion

The objective of S2E1 was to integrate performance and multi-omics data to shed light on the mechanisms of action of a novel feed-grade muramidase fed to broilers. The group receiving muramidase at the highest dose (i.e., 450 g/ton feed) significantly outperformed the control group in terms of cumulative FI (+2.0%), final BW (+4.7%), and cumulative FCR (-2.5%). The low-dose group (i.e., 250 g/ton feed), however, exhibited intermediate performance and did not differ from the control in a significant manner. The trend analysis also showed that the abovementioned performance traits improved proportionately with muramidase dose. These results broadly support those from previous studies assessing the administration of the same muramidase, wherein broilers fed diets supplemented at high inclusion levels (i.e., 350-450 g/ton feed) performed better than their control and low-dose counterparts (Lichtenberg et al., 2017; Goodarzi Boroojeni et al., 2019; Sais et al., 2020; Pirgozliev et al., 2021).

Taken together, these findings suggest that the beneficial effects of this muramidase on broiler performance intensify as a function of the dose administered. For this reason, the highest inclusion level used in S2E1 (i.e., 450 g/ton feed) was tested in S2E2 under HS, a condition that considerably challenges the health and growth performance of broilers, as discussed in detail earlier (see section **2.4** "**Raising**

Chickens in the "Hothouse Earth": The Effects of Heat Stress"). Although the differences between group CON2 and group MUH2 were appreciable, they did not reach the significance threshold neither during the TN feeding phases nor in the entire trial that included the HS period applied in the finisher phase (e.g., +1.2% final BW and -1.5% cumulative FCR for group MUH2). Thus, in general, performance responses to muramidase were not consistent with those observed in S2E1 or reported in the literature. Furthermore, the muramidase appeared to be ineffective for broilers undergoing HS, both in terms of preventing an increase in body temperature – an outcome rather unsurprising – or limiting performance losses. A note of caution, however, is due here since the number of replicates used in S2E2 was quite small because of inevitably facility constraints. Increasing the replicate number may therefore be a viable idea to repeat S2E2 with a greater statistical power. Another possible reason for the limited effect of muramidase on cumulative performance of group MUH2 is the substantial reduction in FI due to the exposure to HS: in the finisher phase of S2E2, birds daily consumed about 25% less feed than those of S2E1. Increasing the inclusion level of this muramidase to make up for such a large reduction in FI may be another aspect to consider when designing new HS studies on this dietary enzyme.

Returning to the results of S2E1, the non-invasive technique used to measure the PGN hydrolysis this muramidase potentially carries out in excreta samples seems to be a reliable alternative to the *ex vivo* analysis illustrated in the work of Frederiksen et al. (2021). The method proposed here confirmed that this muramidase effectively hydrolyzes bacterial PGN causing the release of fragments that, according to recent reports, favor intestinal health and increase growth performance of broilers (Goodarzi Boroojeni et al., 2019; Sais et al., 2020; Wang et al., 2020b; Pirgozliev et al., 2021). The reduction in FPD occurrence associated with muramidase supplementation, especially at the highest dose, is consistent with the results obtained by Pirgozliev et al. (2021). The welfare of poultry's feet depends largely on the quality of the litter (e.g., moisture and ammonia levels) and its management (Shepherd and Fairchild, 2010). Although the measurement of litter parameters was beyond the scope of S2E1, it is possible to hypothesize that the abovementioned improvement in FCR resulted in better nutrient utilization and less watery excreta, thereby playing an indirect role in the attenuation of FPD risk (Sirri et al., 2011). Analysis of breast fillets revealed that muramidase did not affect WS, WB, or SM. Therefore, under the experimental settings of S2E1, muramidase was able to enhance broiler

performance without exerting negative effects on the occurrence of breast muscle myopathies, with positive consequences for the sustainability of chicken meat production.

Studies on pigs (Maga et al., 2012; Wells et al., 2015; Ma et al., 2017; Xiong et al., 2019; Xu et al., 2020), rabbits (EL-Deep et al., 2020), and broilers (Liu et al., 2010; Gong et al., 2017; Xia et al., 2019) have suggested that the beneficial effects of dietary muramidases can be ascribed to their modulatory effect on the microbiota. In S2E1, muramidase supplemented at the highest dose produced a drop in cecal alpha diversity, a result that is in agreement with those from studies with pigs (Xu et al., 2020) and, above all, with broilers fed the same dietary enzyme (Wang et al., 2020a; Sais et al., 2020). Moreover, MUH1 birds had a different bacterial community structure in the cecal content, especially compared to MUL1 birds. This is in accord with research on piglets and lactating sows (Xiong et al., 2019; Xu et al., 2020) and supports findings obtained in broiler trials testing the same muramidase (Wang et al., 2020b). Not only was changed the overall bacterial community structure, but also its taxonomic composition. The underrepresentation of Firmicutes and outgrowth of Bacteroidetes, particularly evident when comparing groups MUH1 and MUL1, seem to be consistent with the results of Maga et al. (2012). These researchers fed pigs with milk produced by transgenic goats expressing the gene encoding for human muramidase and found that the abundance of fecal Firmicutes fell whereas that of Bacteroidetes raised over time. In the current study, MUH1 birds also showed a significant decrease in the Firmicutes to Bacteroidetes ratio compared with MUL1 birds. A considerable amount of papers on the potential association of the Firmicutes to Bacteroidetes ratio with the microbiota-to-host energy supply and development of obesity have been published. Since the results available in the literature are rather contradictory, Magne et al. (2020) have advocated that a direct effect of this ratio on the health state of the host is hard to be attested. A possible explanation for the differences in the abundance of Firmicutes and Bacteroidetes is that bacteria positive for Gram stain, such as Firmicutes, are generally more vulnerable to the hydrolytic action of muramidases on PGN: unlike Bacteroidetes, Firmicutes possess an undefended, thicker cell wall lacking an outer protecting lipid membrane and offering up to 40 PGN layers as a substrate to muramidases (Masschalck and Michiels, 2003). The lower abundance of Firmicutes showed by group MUH1 may explain the decreased abundance of genes related to the peptidoglycan biosynthesis pathway. The reduction in the abundance of genes involved in amino

sugar and nucleotide metabolism pathway, in which NAG is directly involved (KEGG, 2022), can be taken as another indicator for the inhibition of bacteria with a high ability to synthesize PGN, particularly Firmicutes.

Group MUH1 also showed a considerable drop in Clostridiaceae. Interestingly, studies on pigs (Maga et al., 2012) and rabbits (EL-Deep et al., 2020) have established that dietary muramidase supplementation causes a depression in GI Clostridia. Moreover, Sais et al. (2020) found a decreasing trend in *Clostridium* count in the ileum of broilers fed with the same muramidase tested here, but at 350 g/ton feed. It may be argued that the reduction in several Clostridia, Lachnospiraceae (i.e., *Roseburia intestinalis* and *Ruminococcus albus*), and *Eubacterium rectale*, which are all able to produce butyrate (Rivière et al., 2016), is contrary to expectations because butyrogenic bacteria have traditionally been linked to gut health (Koh et al., 2016; Vital et al., 2017; Milani et al., 2017) and chickens have been shown to benefit from SCFAs, especially butyrate, released by GI bacteria (Torok et al., 2011; Stanley et al., 2012, 2016; De Maesschalck et al., 2015). Further research on this topic is therefore recommended. As discussed in greater detail before (see section **2.3** "**Exploration and Modulation of the Chicken Gastrointestinal Microbiota**", subsections "**DIET**" and "**ANTIBIOTICS**"), control of *C. perfringens* – the causative agent of NE – is of utmost importance in poultry production, particularly for ABF rearing systems. Even though its relative abundances were low, a reduction in *C. perfringens* was found in MUH1 birds. This is consistent with Liu et al. (2010) who demonstrated that treating *C. perfringens*-challenged broilers with a dietary muramidase hindered the intestinal colonization by this pathogen. Poultry are also susceptible to *C. botulinum* neurotoxins and sporadically manifest avian botulism, a flaccid paralytic disease (Souillard et al., 2014). Although its abundances in S2E1 were low, the reduction in *C. botulinum* potentially caused by the highest muramidase dose is an issue that would merit further investigations. *Listeria monocytogenes*, an extremely important human pathogen as pointed out in an EFSA/ECDC joint report on zoonoses (2021), showed a lower abundance in cecal samples of group MUH1, though the measured values were rather low. According to Rothrock et al. (2017), poultry can be a reservoir of *L. monocytogenes*, thereby contributing to contamination of processing facilities. It is worth noting that the inhibition of *C. botulinum* and *L. monocytogenes* supports

earlier studies indicating that muramidases are effective solutions against these food-borne pathogenic bacteria (Hughey and Johnson, 1987; Hughey et al., 1989).

The increased abundance of Bacteroidaceae found in cecal samples of MUH1 birds agrees with studies on pigs (Maga et al., 2012; Xiong et al., 2019), although opposite findings have also been reported (Xu et al., 2020). The genus *Bacteroides* has been associated to improved gut health due to its ability to produce propionate (Rios-Covian et al., 2017; Jacobson et al., 2018). The species *B. thetaiotaomicron* has even been included in a probiotic preparation intended to restore intestinal eubiosis after antibiotic therapies in humans (El Hage et al., 2019). Hence, it may be hypothesized that cecal Bacteroidaceae promotes intestinal health in broilers as well. The increase in Lactobacillaceae is also comparable to previous studies on pigs (Maga et al., 2012; Xiong et al., 2019; Xu et al., 2020), rabbits (EL-Deep et al., 2020), and broilers (Lichtenberg et al., 2017; Sais et al., 2020), the latter supplemented with the same muramidase used here. This result, however, differs from those from other investigations (Ma et al., 2017; Wang et al., 2020a) and can be contradictory due to the aforementioned proneness of Gram-positive bacteria to the hydrolytic action of muramidases on PGN. Nonetheless, some LAB employed in the dairy industry have been shown to be or gradually become resistant to muramidases (Bester and Lombard, 1990; Neviani et al., 1996). Several members of Lactobacillaceae have also probiotic properties (FAO, 2016) that may have supported the growth performance of birds supplemented with muramidase, particularly those at the highest dose. Further work, however, needs to be done to establish whether this feed-grade muramidase has stimulating or, at least, non-inhibiting effects on Lactobacillaceae in the chicken intestine.

Analysis of KEGG data helped to interpret the changes occurred in the cecal metabolome. In group MUH1, the decreased abundance of genes of starch and sucrose metabolism path may have been related to the reduction in fermentation-deriving organic acids, such as acetate, ferulate, and formate measured in the cecal content. Furthermore, the enrichment in genes linked to glutathione metabolism path – an important antioxidant tool for bacteria (Masip et al., 2006) – was potentially associated with the reduction in hypoxanthine level in the ceca. Hypoxanthine is a noxious end product of purine catabolism and is used as a biomarker for oxidative stress (Beauclercq et al., 2016; Abasht et al., 2016; Soglia et al., 2019). So, the lower concentration of hypoxanthine in the ceca may have positively

influenced the GI ecosystem in MUH1 birds. It is also intriguing to associate the reduced hypoxanthine level in the ceca to the lower concentration of this metabolite in plasma. Lower levels of circulating hypoxanthine, histidine, and uracil – the latter two being shown to be end products of protein and nucleotide catabolism, respectively (Abasht et al., 2016; Soglia et al., 2019) – suggest that the degradation of proteins and nucleotides to generate energy may have occurred to a lesser extent in the body of MUH1 birds. Additionally, due to their considerable production of propionate, the abovementioned increased abundance of Bacteroidaceae in the ceca may have caused a greater supply of propionate for hepatic gluconeogenesis (Schwiertz et al., 2010), thereby potentially causing the increase in plasmatic levels of energetic compounds (e.g., pyruvate, 2-oxoglutarate, and glucose) observed in MUH1 birds. The enrichment in bioenergetic compounds and reduction in pro-oxidant catabolites indicate that a more balanced energy metabolism may have stimulated the growth performance of high-dose supplemented broilers.

Overall, the results of S2E1 show that muramidase affected cumulative FI, final BW, and cumulative FCR of broilers in a dose-dependent way (i.e., MUH1 > MUL1 > CON1), but the most marked differences in the cecal microbiota and cecal and plasmatic metabolomes were those between the two muramidase-supplemented groups (i.e., MUL1 and MUH1). A possible explanation for this apparently weird outcome might be that cumulative growth performance benefited from the positive effects muramidase exerted in each feeding phase (resembling an additive effect), whereas the results of molecular analyses at slaughter cannot fully justify the complex GI and metabolic dynamics potentially affected by muramidase throughout a 42-day grow cycle. S2E1, however, contributes to our understanding of the mechanism of action of this dietary muramidase and lays the groundwork for further investigations on its effects on the GIT and metabolism of broilers. In contrast to the thoroughness of S2E1, S2E2 was only the first basic attempt to assess the administration of muramidase-based feed additives as a potential nutritional solution to help heat-stressed broilers. Recognizing and fixing the limitations reported at the beginning of the present discussion is a crucial precondition for re-testing this promising muramidase in broilers under HS conditions.

6 STUDY #3: EFFECTS OF ARGININE SUPPLEMENTATION ON BROILERS EXPOSED TO THERMONEUTRAL AND HEAT STRESS CONDITIONS

Note: The results of experiments S3E1 and S3E2 have been published. For more information, please refer to **Annex 1**.

6.1 Highlights

- This study set out to assess the effects of feeding broilers diets supplemented with arginine (i.e., total arginine to lysine ratio of 1.20).
- Under optimal thermal conditions (experiment #1), arginine supplementation improved growth rate, final body weight, and feed efficiency.
- In a cyclic heat stress model (experiment #2), before the application of the thermal stressor, supplemented birds performed better in terms of body weight (gain) and feed efficiency. Arginine supplementation, however, did not mitigate the negative effects of heat stress on growth performance.
- In experiment #1, supplemented birds showed increased plasmatic levels of arginine, betaine, histidine, and creatine and hepatic levels of creatine and several essential amino acids, reduced levels of glutamine in plasma and glutathione in liver, as well as reduced alpha diversity and relative abundance of Firmicutes and Proteobacteria (especially *Escherichia coli*) and increased abundance of Bacteroidetes and *Lactobacillus salivarius* in the ceca.
- In experiment #2, arginine supplementation increased the levels of arginine and creatine in plasma, liver, and pectoral muscle and methionine in liver, while reduced those of glutamine in plasma, liver, and pectoral muscle and glutathione in liver and pectoral muscle. Arginine supplementation may have partially counterbalanced the negative effects of heat stress on energy homeostasis, as well as influenced the cecal microbiota in a similar way to experiment #1. Data indicate that heat stress altered protein metabolism, promoted the accumulation of antioxidant and protective molecules (e.g., histidine-containing dipeptides, beta-alanine, and choline) in specific tissues, and affected the cecal microbiota.

6.2 Background and Aims

Arginine is a multivalent AA that plays proteinogenic, trophic, and functional roles in the animal body and affects metabolism, growth, immunity, and health in several ways (Wu and Morris, 1998; Wu, 2010; Khajali and Wideman, 2010; Bortoluzzi et al., 2018; Zampiga, 2019; Castro and Kim, 2020; Hassan et al., 2021; Lee et al., 2022). For instance, it is the substrate for the biosynthesis of nitric oxide, polyamines, proline, and glutamate (Wu and Morris, 1998). Nitric oxide is involved in many physiological processes, such as the regulation of the cardiovascular and renal functions, inhibition of tumor growth, and modulation of the immune response (Wu et al., 2000, 2021). Polyamines (i.e., putrescine, spermine, and spermidine) have been shown to regulate gene expression and protein synthesis, as well as cellular proliferation, differentiation, and apoptosis (Pegg, 1986; Lenis et al., 2017). Proline is a key regulator of cellular metabolism and physiology (Wu et al., 2011), while glutamate is an essential component of glutathione, the potent antioxidant tripeptide (Wu et al., 2004). Arginine has also been demonstrated to induce the expression and secretion of anabolic hormones, such as insulin, growth hormone (**GH**), and IGF-1 (Barbul, 1986; Collier et al., 2005; Zajac et al., 2010; Oh et al., 2017). Moreover, arginine affects skeletal muscle development through the mechanistic target of rapamycin (**mTOR**) pathway (Wang et al., 2022) and is used to generate creatine, an AA derivative that is important for function and energy homeostasis of muscles (Oviedo-Rondón and Córdova-Noboa, 2020). Over the last two decades, there has also been an increasing interest in the effects of arginine on the GIT. Arginine and its derivatives have been shown to possess gut health promoting and re-establishing properties, such as acceleration of mucosal regeneration and recovery from gastroenteric disorders, improvement of epithelial integrity and barrier function, immunomodulation, anti-inflammatory activity, inhibition of enteric pathogens, and restoration of a desirable microbiota (Wu et al., 2000; Rhoads et al., 2004; Liu et al., 2008b; Rhoads and Wu, 2009; Coburn et al., 2012; Fritz, 2013; Xia et al., 2016; Zhang et al., 2018, 2017; Singh et al., 2019; Baier et al., 2020).

Arginine is commonly considered a semi-essential or conditionally essential AA for adult mammals (Rose, 1937; Barbul, 1986; Wu and Morris, 1998), while chickens depend exclusively on dietary arginine to meet their needs for this AA (Khajali and Wideman, 2010; Castro and Kim, 2020). Indeed, mammals are ureotelic (i.e., urea-excreting) animals that can endogenously produce arginine *de*

novo with urea cycle enzymes, whereas avian species are uricotelic (i.e., uric acid-excreting) organisms unable to complete the urea cycle (Khajali and Wideman, 2010; Castro and Kim, 2020). In the early 1960s, Tamir and Ratner (1963) found that chickens lack carbamoyl phosphate synthase I, which would catalyze ammonia fixation, and have a scarcely active ornithine transcarbamylase that transfers the fixed nitrogen to ornithine to generate citrulline, an indispensable intermediate in the urea cycle. However, pioneering studies conducted almost 30 years before had already suggested that arginine is an essential nutrient for chickens (Arnold et al., 1936; Klose et al., 1938). According to the well-known Nutrient Requirements of Poultry published by the National Research Council (**NRC**, 1994), broilers should be given diets containing 1.25, 1.10, and 1.00% of arginine up to the 3rd, from the 3rd to 6th, and from the 6th to 8th week of age, respectively, with a constant arginine to lysine ratio of 1.04. Although these guidelines have been adequate for a long time, extensive research has demonstrated that arginine requirements of modern broilers are substantially influenced by diet composition and environmental conditions (Khajali and Wideman, 2010; Hassan et al., 2021). For example, consistent requirements for broilers facing HS cannot be easily found in the literature (Teyssier et al., 2022a). The reason for this might be that the previously examined modifications in FI, physiology, metabolism, and gut health and function induced by HS (see section **2.4** "**Raising Chickens in the "Hothouse Earth": The Effects of Heat Stress**") complicate the computation of arginine or other nutrient needs for heat-stressed broilers. In this regard, considering the "ideal protein" concept widely adopted in poultry nutrition (Wiseman e Garnsworthy, 1999), an increase in the arginine to lysine ratio for broilers undergoing HS has been attributed to a likely reduction in intestinal absorption of arginine (Balnave and Brake, 2002).

Interestingly, it has been shown that feeding broilers and other poultry arginine above the recommended levels, such as those released by the NRC (1994) or the breeding companies, is beneficial for their health, growth performance, and slaughtering yields, both under TN and HS conditions (Hassan et al., 2021; Teyssier et al., 2022a). Given the properties of arginine discussed earlier, high dietary arginine levels could reasonably produce an alleviation of the negative effects of HS, such as immunodeficiency, inflammation and oxidative stress, protein turnover alteration indicating a catabolic state, gut health degradation, and perturbation in the microbiota (see section **2.4** "**Raising Chickens in the "Hothouse Earth": The Effects of Heat Stress**"). Nevertheless, very little attention has been paid

to the biological roles of arginine and its dietary supplementation in heat-stressed broilers[28], suggesting that there is abundant room for further progress in these research areas. An essential step to move forward is to fully comprehend the involvement of arginine in metabolism and intestinal health of broilers. According to Morris (2007), "omics" technologies can help advance our knowledge of how arginine affects and modulates animal metabolism. Therefore, the aims of S3 were to evaluate the effects of arginine supplementation on growth performance of broilers exposed to TN and HS conditions and to explore the impacts of this nutritional strategy on their hepatic, muscular, and blood metabolic profiles, as well as on their intestinal microbiota. Specifically, arginine supplementation was tested in a TN environment in S3E1, while in S3E2 birds were exposed to a cyclic HS period to simulate field conditions typical of the summer in temperate areas of the world, namely a succession of diurnal hot temperatures and cooler nights.

6.3 Materials and Methods

EXPERIMENTAL DESIGN, HOUSING, AND HUSBANDRY CONDITIONS

In S3, approved by the Ethical Committee of the University of Bologna (ID: 4387), birds were reared, monitored, and slaughtered in compliance with EU legislation (i.e., Dir. 2007/43/EC, Reg. 2009/1099/EC, and Dir. 2010/63/EU). Feed and water were provided *ad libitum* throughout the trials of this study.

For S3E1, 630 one-day-old male Ross 308 broilers, obtained from the same breeder flock and hatching batch, were supplied by a commercial hatchery, and vaccinated against infectious bronchitis, Marek's, Newcastle and Gumboro diseases, and coccidiosis. At placement in an experimental poultry barn, birds were randomly assigned to 2 experimental groups (7 replicate pens/group) that were fed a commercial ABF basal diet in mash form (**CON1**) or the same basal diet supplemented with crystalline *L*-arginine (**ARG1**) for the entire trial (0-49 d). The basal diet was formulated to meet the nutrition

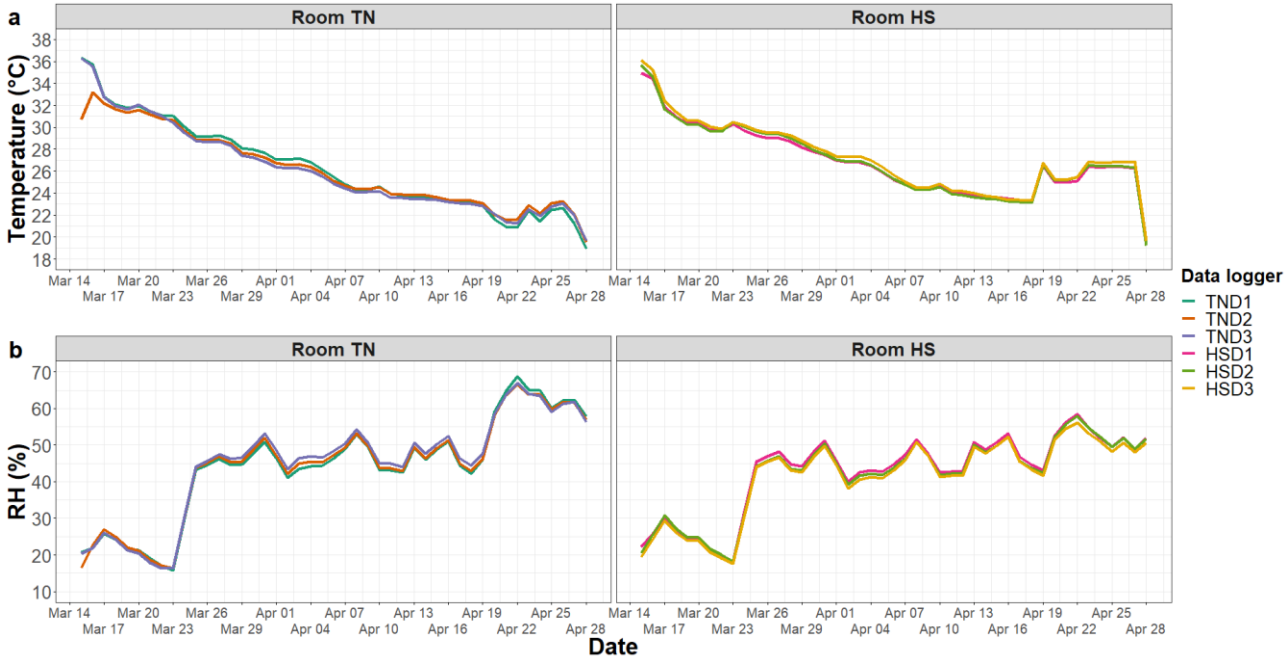
[28] The literature search was carried out by searching the Scopus database on September 12, 2022, entering the following query string: (TITLE ((chicken OR broiler) AND (arginine OR arg) AND heat AND stress AND NOT (arginine AND vasopressin) AND NOT plant AND NOT polymorphism) OR ABS ((chicken OR broiler) AND (arginine OR arg) AND heat AND stress AND NOT (arginine AND vasopressin) AND NOT plant AND NOT polymorphism)). No restriction on the date and type of publication was set.

specifications released by the breeding company (Aviagen, 2022a). Analysis of AA concentration of the experimental diets was outsourced to Evonik Industries AG (Hanau, Germany). **Annex 3** provides the formula and composition of the basal diet according to the four-phase feeding program used (i.e., starter, 0-9 d; grower I, 10-21 d; grower II, 22-35 d; finisher, 36-49 d). For each feeding phase, the basal diet (**Annex 3**) consisted of a single batch of coarsely ground mash feed (for information on particle size, see **Footnote 24**), and the sub-batches intended for ARG1 replicates were supplemented on top with crystalline *L*-arginine (about 1.5 g/kg feed; purity of 98%; BESTAMINO^{TM}, CJ BIO, Seoul, Korea). Consequently, the total arginine level of the basal diet fed to group CON1 was 1.59, 1.42, 1.32, and 1.25%, while that of the supplemented diet fed to group ARG1 was 1.75, 1.57, 1.47, and 1.39% in the starter, grower I, grower II, and finisher phase, respectively. The total arginine to lysine ratio of the former diet ranged between 1.07 and 1.08 and was consistent with the breeding company's guidelines (Aviagen, 2022a), whereas that of the latter diet was 1.20 throughout the trial. The floor pens were arranged in a block design and equipped with chopped straw as bedding material, two bell feeders, and nipple drinkers. The environmental temperature was modified according to the flock age and the breeding company's instructions (Aviagen, 2018). The artificial photoperiod was 23L:1D during the first 7 and last 3 d, while 18L:6D for the remainder days following EU legislation (i.e., Dir. 2007/43/EC) and the breeding company's guidelines for lightning and pre-processing management (Aviagen, 2018).

For S3E2, 240 one-day-old male Ross 308 broilers, procured and vaccinated as detailed above, were randomly placed in 2 identical environmental chambers, hereafter referred to as *rooms*. Each room was divided into 12 equally sized floor pens equipped as previously described. The pens were assigned to one of two experimental groups (i.e., 6 replicate pens/group/room) and were arranged in a block design. The control group (**CON2**) was given a commercial ABF basal diet in mash form, while the treated group was fed the same basal diet supplemented on top with crystalline *L*-arginine (**ARG2**) throughout the trial (0-44 d), as detailed above. **Annex 3** provides the formula and composition of the basal diet according to the three-phase feeding program used (i.e., starter, 0-14 d; grower, 15-27 d; finisher, 28-44 d). As before, the basal diet was a coarsely ground mash feed (for information on particle size, see **Footnote 24**). In this case, however, the total arginine level of the basal diet fed to group CON2 was 1.50, 1.38, and 1.23%, while that of the supplemented diet fed to group ARG2 was 1.74, 1.56, and

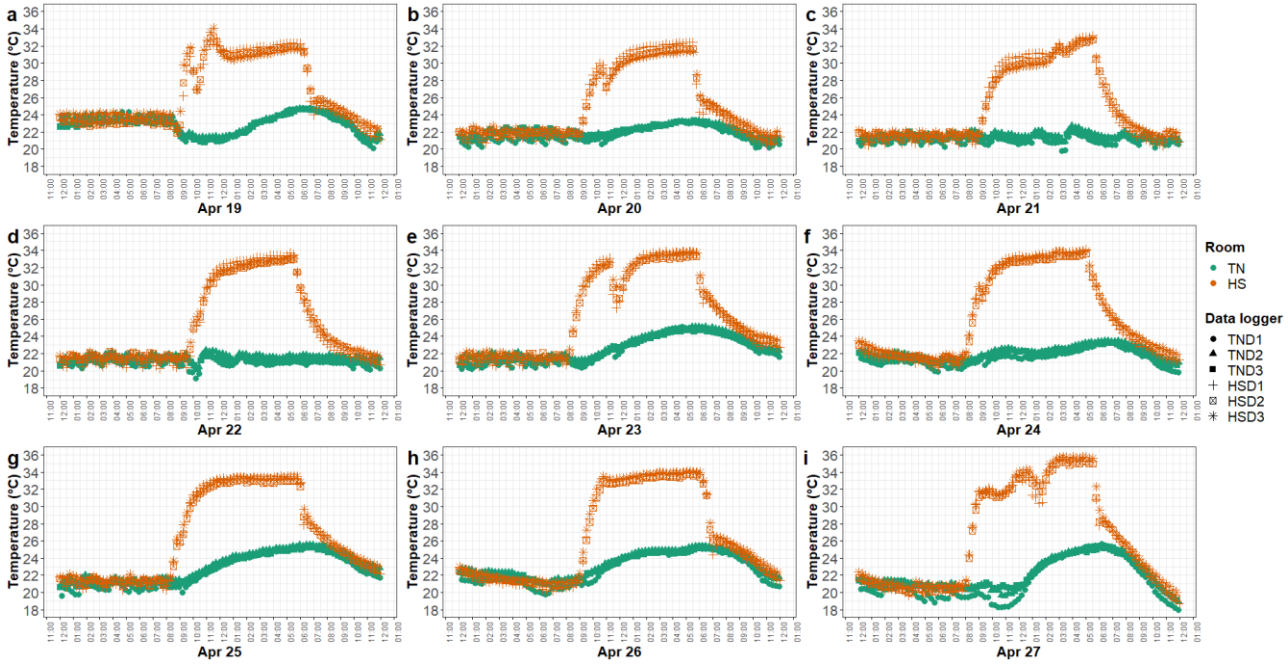
1.37% in the starter, grower, and finisher phase, respectively. The total arginine to lysine ratio of the former diet ranged between 1.02 and 1.07 and was consistent with the breeding company's guidelines (Aviagen, 2022a), whereas that of the latter diet was 1.20 throughout the trial. The artificial photoperiod was adjusted as previously described. Environmental temperature and RH were recorded with climate data loggers (Trotec GmbH, Heinsberg, Germany) located at animal level (3 data loggers/room having a recording time of 900 s). As for the temperature program, one room was constantly kept at TN conditions, while the other room was kept at TN conditions until D34 and subjected to cyclic HS from D35 onwards (i.e., temperature was raised daily to ~34 °C from 9:00 AM to 6:00 PM from D35 to D43). During the HS period, RH ranged between 40 and 70% in both rooms. These RH values were maintained by means of humidifiers (Trotec GmbH) installed on the ninth day of the trial (**Figure 33** and **Figure 34**).

Figure 33 – S3E2 | Average daily temperature (**a**) and RH (**b**) of the two rooms



Note: March 15, trial start (D0); April 19-27, cyclic HS period (D35-D43); April 28, trial end (D44).

Figure 34 – S3E2 | Hourly temperature (**a**-**i**) of the two rooms during the cyclic HS period



Note: April 19-27, cyclic HS period (D35-D43).

DATA AND SAMPLE COLLECTION

For S3E1, on a replicate basis, the number and BW of birds were recorded at placement (D0), at every feeding phase switch (D10/22/36), and at slaughter (D49), while FI was measured for each feeding phase. DWG, DFI, and FCR were calculated for the feeding phases separately and cumulative performance traits were calculated for the entire rearing period (0-49 d). The number and BW of dead or culled birds were recorded daily to compute the mortality rate and correct the performance data for mortality. At slaughter (D49) in a commercial abattoir, carcass and cut-up yields were measured on a group basis according to standard commercial procedures. Breast muscle myopathies, namely WS, WB, and SM, were assessed blindly by specially trained staff 24 h post-mortem – after chilling, deboning, and skin removal – on a randomly selected sample of approximately 290 breast fillets per group with a 3-point-scale: score 0, normal; score 1, mild myopathy; score 2, severe myopathy (Sirri et al., 2016). Two birds per replicate (i.e., 14 birds/group) with a BW close to the flock average were selected at the slaughterhouse (D49) for collection of blood, liver, and cecal content samples. Blood was kept at RT before being centrifuged to get plasma that was subsequently stored at -80 °C until metabolomics analysis through ^{1}H-NMR. Hepatic tissue (~1 cm^{3}) was dissected from the right caudal lobe of the liver,

frozen in LN_2, and stored at -80 °C until ^{1}H-NMR analysis. Similarly, the content of both ceca was collected in duplicate, frozen in LN_2, and stored at -80 °C until ^{1}H-NMR analysis and DNA extraction for shotgun metagenomic sequencing.

For S3E2, three representative birds per pen (i.e., 18 birds/group/room) were randomly chosen and labeled to measure the rectal temperature with a veterinary thermometer (Scala Electronic GmbH, Stahnsdorf, Germany). The rectal temperature was taken on the first and eighth day of the HS period (i.e., D35 and D42, respectively) at two time points, that is 9:00 AM and 6:00 PM. Growth performance data were collected as described before, but in this case cumulative FI and cumulative FCR were computed for the period consisting of a feeding phase and its previous one/ones (i.e., starter + grower; starter + grower + finisher). At the slaughterhouse (D44), blood, liver, and cecal content were collected from two of the three birds previously labeled in each pen (i.e., 12 birds sampled/group/room), as explained above. Besides, breast muscle (~1 cm^3) was collected from the left cranial portion of the *Pectoralis major*, frozen in LN_2, and stored at -80 °C until ^{1}H-NMR analysis.

LAB ANALYSIS

For metabolomics analyses of samples collected in S3E1 and S3E2, an ^{1}H-NMR solution with D_2O, containing TSP 10 mmol/L and NaN_3 2 mmol/L, was created. Phosphate buffer 1 M was used to achieve a pH of 7.00 ± 0.02, while TSP was used as a reference for NMR chemical-shift and NaN_3 avoided bacterial proliferation. Cecal content samples were prepared by vortex mixing 80 mg of each sample with 1 mL of bi-distilled water. Approximately 0.5 g of liver and muscle samples were homogenized (14,000 rpm; 20 s; RT) with 3 mL of a water solution of TCA 7% (w/w). All samples were centrifuged (18,630 g; 900 s; 4 °C) and 0.7 mL of supernatant were mixed with 0.1 mL of the ^{1}H-NMR solution. The pH of liver and muscle samples was further adjusted to 7.00 ± 0.02 with drops of NaOH 9 N and 1 N as needed. All samples were centrifuged again at the abovementioned conditions. The ^{1}H-NMR spectra were registered (600.13 MHz; 298 K) with an AVANCE™ III spectrometer (Bruker, Milan, Italy) equipped with TopSpin software v3.5 (Bruker). The signals from broad resonances due to large molecules were suppressed with CPMG-filter (400 echoes with a τ of 400 μs and a 180° pulse of 24 μs, for a total filter of 330 ms), while the residual signal of water was suppressed

by means of presaturation. This was done employing the *cpmgpr1d* sequence, part of the standard pulse sequence library. Each spectrum was acquired summing up 256 transients constituted by 32,000 data points encompassing a window of 7,184 Hz, separated by a relaxation delay of 5 s. The ^{1}H-NMR spectra were phase-adjusted in TopSpin v3.5 (Bruker) and then exported to ASCII format by means of the built-in script *convbin2asc*. Spectra were processed with R (R Core Team, 2020) through home-made scripts. Signal assignment was performed comparing their chemical shift and multiplicity with the Human Metabolome Database (Wishart et al., 2007) and Chenomx software library v10 (Chenomx Inc., Edmonton, Canada), by means of Chenomx software routines. For all samples, the absolute concentration of molecules was performed with the median water dilution, assessed via probabilistic quotient normalization (Dieterle et al., 2006). TSP was used as an internal standard. Differences in water content between samples from the same matrix were considered through probabilistic quotient normalization. The concentration of each molecule was obtained from the area of one of its signals, calculated by the global spectra deconvolution algorithm implemented in MestReNova software v14.2.0-26256 (Mestrelab research S.L., Santiago De Compostela, Spain), by considering a limit of quantification of 5. This was done after applying a baseline adjustment by Whittaker Smoother procedure and a line broadening of 0.3.

Moving to microbiota analyses of the cecal content, DNA extraction was performed adopting a bead-beating procedure and using the QIAmp® DNA Stool Mini Kit (Qiagen, Milan, Italy) as detailed in the work by De Cesare et al. (2017). Total DNA was fragmented and tagged with sequencing indexes and adapters using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, U.S.). Shotgun metagenomic sequencing was performed with NextSeq500 (Illumina) 2×149 bp in paired-end mode. Filtering of low-quality reads and sequence adapters trimming of raw reads were conducted using the tool *AdapterRemoval*. The microbial community composition was evaluated with the bioinformatic tool MetaphlAn3 (Truong et al., 2015) at the phylum, class, order, family, genus, and species level. For S3E1, alpha diversity was computed adopting the Shannon index implemented in *vegan* R-bioconductor package (Oksanen et al., 2020). This package was also employed to calculate the Bray-Curtis beta distance. Beta values were transformed with the Classical multidimensional scaling function 'cmdscale' to perform the PCoA analysis. For S3E2, alpha diversity indices (i.e., observed, Shannon, Simpson, and

Inverse Simpson) and Bray-Curtis beta distance were calculated with the R package *phyloseq* (McMurdie and Holmes, 2013).

DATA ANALYSIS

For S3E1, a one-way ANOVA with blocks was used to evaluate the effect of diet (i.e., the group factor) on growth performance, using the replicate as the experimental unit. Mortality rate data were transformed using the arcsine transformation before being analyzed with inferential statistics. Carcass and cut-up yields data were not statistically analyzed because they were recorded on a group basis without considering the replicates at the slaughterhouse. Count data of WS, WB, and SM were analyzed with Pearson's chi-squared test using the sampled animal as the experimental unit. Count data were also arranged in 2 by 2 contingency tables aligning the levels of the group factor (i.e., CON1 and ARG1) and having binarily aggregated scores of a myopathy in columns (i.e., "presence" as a sum of counts of score 1 and score 2, while "absence" as score 0 counts). The incidence risk ratio was computed on these tables with *epiR* package (Stevenson et al., 2021) of R (R Core Team, 2020). The risk of developing a myopathy was calculated as incidence risk ratio minus 1 and expressed in percentage. A confidence interval of 95% and Pearson's chi-squared test were used to test the incidence risk ratio significance. Regarding the analysis of metabolomics data, a two-tailed Student's *t*-test with group as the independent variable and the sampled bird as the experimental unit was carried out. Metabolomics data deviating from normality in Shapiro-Wilk test were subjected to Box-Cox transformation (Box and Cox, 1964). Concerning the analysis of cecal microbiota data, the relative frequency of abundance was computed and a two-sided Welch's *t*-test was applied to reveal statistically significant differences between groups. Alpha diversity (Shannon index) data were statistically analyzed with a two-tailed Student's *t*-test. A representative boxplot for the genus level is shown in the result section below. These analyses were carried out using R (R Core Team, 2020). *P*-values less than 0.05 were considered significant, while those ranging between 0.05 and 0.1 were considered tendencies.

For S3E2, the effect of diet (i.e., the group factor) on growth performance of the starter and grower phases was assessed with a two-way blocked ANOVA without interaction, considering the room as a fixed factor and using the replicate as the experimental unit. However, the interactive effect between the

factors group and room was tested through a two-way blocked ANOVA with interaction for the data of the finisher phase. Tukey's HSD post-hoc test was used if needed. Mortality rate data were handled as before. Rectal temperature data grouped by the day of collection were analyzed through a three-way mixed ANOVA, a type of repeated-measures ANOVA that includes between-subject factors (i.e., group and room) and within-subject factors (i.e., time point). After verifying that there was no statistically significant three-way interaction, rectal temperature data were grouped by the factors group and room to run paired *t*-tests with Bonferroni adjustment between time points. Regarding the analysis of metabolomics data, a two-way ANOVA with interaction between group and room and the sampled bird as the experimental unit was carried out. Tukey's HSD post-hoc test was used where appropriate. Metabolomics data deviating from normality in Shapiro-Wilk test were transformed with Box-Cox transformation (Box and Cox, 1964). The effects of group, room, and their interaction on alpha diversity measures and beta diversity distance matrix were tested with a two-way ANOVA and a two-way PERMANOVA [*adonis2* function implemented in the R *vegan* package (Oksanen et al., 2020)], respectively. Descriptive statistics of relative abundance data at the phylum level are reported in the result section below, while differential abundance testing and other in-depth analyses on microbiota data from S3E2 are being performed. All these analyses were carried out using R (R Core Team, 2020) and considering 0.05 as the significance threshold. *P*-values ranging between 0.05 and 0.1 were considered tendencies.

6.4 Results

For S3E1, **Table 11** presents the growth performance of groups CON1 and ARG1 in the four-phase feeding trial. At placement, chicks had an average weight of 40 g. At the end of the starter phase, group ARG1 showed lower FCR than group CON1 (1.345 *vs.* 1.303; $P < 0.05$), whereas the other performance traits showed no differences between groups. Likewise, ARG1 birds had the lowest FCR in the first grower phase (1.533 *vs.* 1.470; $P < 0.05$). No differences between groups were detected in the second grower phase. At the end of the finisher phase, group ARG1 reached greater BW than group CON1 (3,778 g *vs.* 3,937 g; $P < 0.001$). Cumulatively (0-49 d), DWG was higher ($P < 0.001$) and FCR was lower ($P < 0.05$) for ARG1 birds (76.15 g and 1.808 *vs.* 79.46 g and 1.732 for CON1 and ARG1,

respectively). However, the two groups had essentially the same cumulative FI, as also shown in **Figure 35**.

Processing data of S3E1 showed that carcass and breast yields – the latter calculated as percentage of the eviscerated carcass weight – were similar between groups (i.e., 73.5 and 33.4% *vs.* 73.6 and 33.7%, for CON1 and ARG1, respectively) and in line with the goals set by the breeding company for the broiler line used (Aviagen, 2022b). Regarding breast muscle myopathies, the incidence of WS and WB was related to the group factor ($P < 0.001$ and $P < 0.05$ for WS and WB, respectively), with ARG1 birds exhibiting a higher incidence of mild WS and severe WS and WB than CON1 birds (**Figure 36a-b**). However, the incidence risk ratio analysis revealed that the dietary supplementation of *L*-arginine had a significant effect only on the onset of WS: ARG1 birds were 1.32 (95% confidence interval of 1.15 to 1.51) times more likely to develop WS than CON1 birds; in other words, feeding broilers the arginine-supplemented diet significantly increased by 32% the relative risk of developing WS. On the other hand, SM did not show a significant association with the group factor (**Figure 36c**).

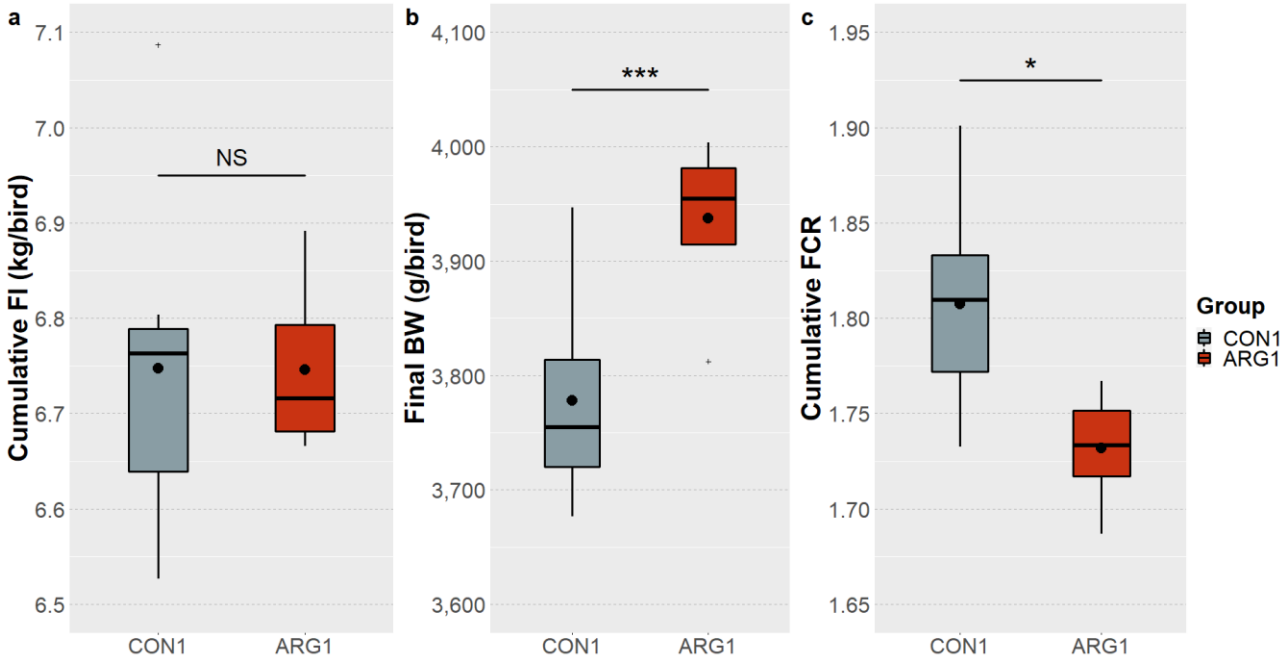
Metabolomics analyses of S3E1 identified 60, 71, and 78 metabolites in plasma, liver, and cecal content samples, respectively. **Table 12** shows the metabolites with different concentrations between CON1 and ARG1 birds in those samples. In plasma, ARG1 birds had a significantly lower concentration of 2-oxoglutarate, glutamine, and methanol, while that of fumarate and mannose showed a comparable but not significant trend. Furthermore, concentrations of arginine, betaine, and histidine were significantly greater for ARG1 birds, with those of acetate and creatine having a similar tendency toward significance. In the liver, the concentration of glutathione showed a decreasing trend in ARG1 birds, while that of aspartate, creatine, leucine, phenylalanine, and threonine varied in the opposite way. Moreover, the concentration of methionine sulfoxide was significantly higher in the liver of ARG1 birds. In the ceca, ARG1 birds showed a significantly lower concentration of leucine and an almost significant increase in thymine concentration than CON1 birds.

Table 11 – S3E1 | Growth performance of groups CON1 and ARG1 at the end of each feeding phase and in the entire trial (0-49 d)

Dependent variable	Group[†]		SE	*P*-value
	CON1	ARG1		
Chick weight (g/bird)	40.17	40.01	0.34	0.409
Starter (0-9 d)				
BW (g/bird)	214.9	218.9	6.77	0.315
DWG[§] (g/bird/d)	19.42	19.88	0.74	0.293
DFI[§] (g/bird/d)	26.12	25.89	0.85	0.631
FI[§] (kg/bird)	0.235	0.233	0.01	0.631
FCR[§]	1.345	1.303	0.03	**0.044**
Mortality %	0.00	0.00	0.00	/
Grower I (10-21 d)				
BW (g/bird)	893.2	917.6	27.63	0.149
DWG[§] (g/bird/d)	56.52	58.22	1.94	0.152
DFI[§] (g/bird/d)	86.61	85.56	1.25	0.169
FI[§] (kg/bird)	1.039	1.027	0.02	0.169
FCR[§]	1.533	1.470	0.04	**0.018**
Mortality (%)	0.00	0.00	0.00	/
Grower II (22-35 d)				
BW (g/bird)	2,262	2,339	89.06	0.157
DWG[§] (g/bird/d)	97.79	101.5	4.75	0.190
DFI[§] (g/bird/d)	169.2	168.5	1.98	0.542
FI[§] (kg/bird)	2.369	2.359	0.03	0.542
FCR[§]	1.736	1.661	0.11	0.238
Mortality (%)	0.32	0.00	0.59	0.356
Finisher (36-49 d)				
BW (g/bird)	3,778	3,937	49.26	< **0.001**
DWG[§] (g/bird/d)	108.0	114.0	7.22	0.172
DFI[§] (g/bird/d)	221.8	223.4	5.27	0.585
FI[§] (kg/bird)	3.105	3.127	0.07	0.585
FCR[§]	2.063	1.961	0.12	0.168
Mortality (%)	0.32	0.64	0.59	0.356
Entire trial (0-49 d)				
BW (g/bird)	3,778	3,937	49.26	< **0.001**
DWG[§] (g/bird/d)	76.15	79.46	0.97	< **0.001**
DFI[§] (g/bird/d)	137.6	137.6	1.88	0.994
FI[§] (kg/bird)	6.748	6.746	0.09	0.978
FCR[§]	1.808	1.732	0.04	**0.012**
Mortality (%)	0.64	0.64	0.06	1.000

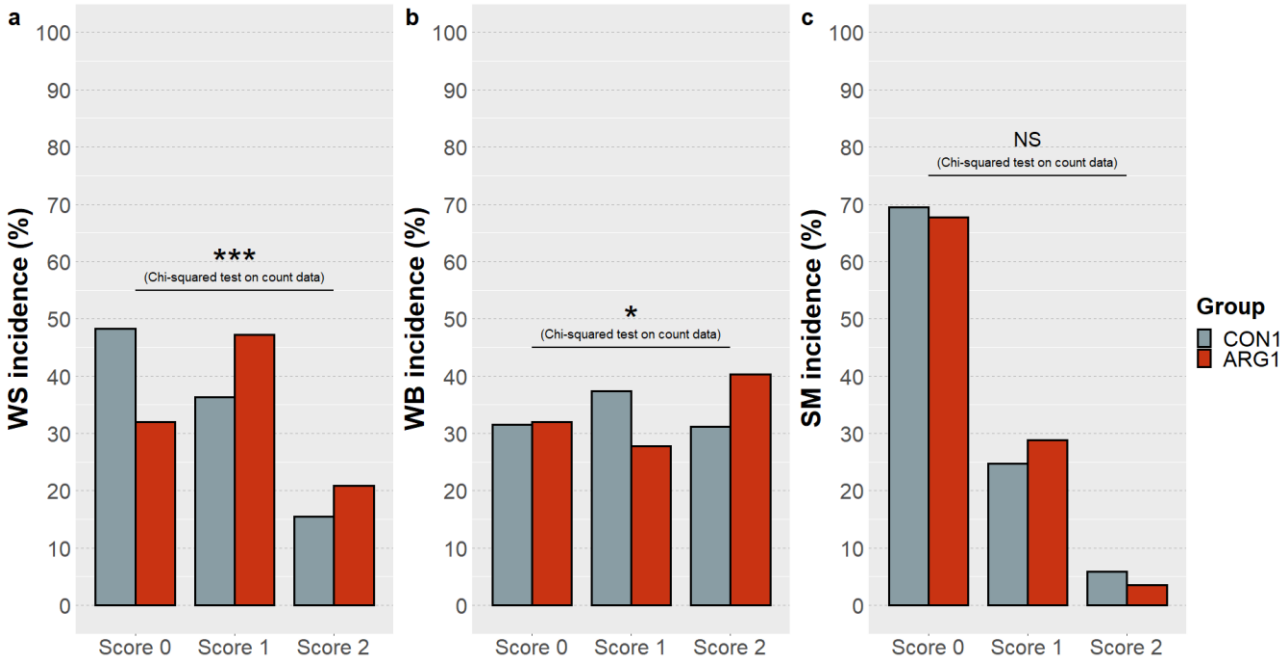
[†] Means of 7 replicate pens/group.
[§] Corrected for mortality.
Note: P-values less than 0.05 are in bold.

Figure 35 – S3E1 | Cumulative FI (**a**), final BW (**b**), and cumulative FCR (**c**) of groups CON1 and ARG1 in the entire trial (0-49 d)



Note: Means of 7 replicate pens/group are the black dots inside the boxes. NS, not significant. *, $P < 0.05$; ***, $P < 0.001$.

Figure 36 – S3E1 | Incidence and severity of WS (**a**), WB (**b**), and SM (**c**) of CON1 and ARG1 birds at D49



Note: Score 0, normal; score 1, mild myopathy; score 2, severe myopathy. *, $P < 0.05$; ***, $P < 0.001$. NS, not significant.

Table 12 – S3E1 | Metabolites showing different concentrations (mmol/L) in plasma, liver, and cecal content of CON1 and ARG1 birds at D49

Metabolite	Group[†]		SE	*P*-value	Change[§]
	CON1	ARG1			
Plasma					
2-Oxoglutarate	9.64E-02	7.67E-02	1.61E-02	**0.004**	↓
Acetate	4.37E-02	5.94E-02	2.23E-02	0.079	↑
Arginine	3.25E-01	4.09E-01	1.38E-02	**0.018**	↑
Betaine	6.06E-01	6.90E-01	8.62E-02	**0.023**	↑
Creatine	7.67E-02	9.58E-02	8.96E-02	0.060	↑
Fumarate	1.39E-02	1.22E-02	2.83E-02	0.057	↓
Glutamine	1.46E+00	1.32E+00	2.22E-03	**0.043**	↓
Histidine	9.97E-02	1.17E-01	1.77E-01	**0.009**	↑
Mannose	4.30E-02	3.95E-02	1.56E-02	0.066	↓
Methanol	5.35E-02	4.19E-02	4.67E-03	**0.026**	↓
Liver					
Aspartate	5.02E-03	5.81E-03	1.14E-03	0.078	↑
Creatine	3.60E-04	5.40E-04	2.44E-04	0.056	↑
Glutathione	1.50E-04	6.00E-05	1.09E-04	0.064	↓
Leucine	1.58E-03	2.00E-03	5.82E-04	0.066	↑
Methionine sulfoxide	2.00E-05	3.00E-05	7.05E-06	**0.034**	↑
Phenylalanine	7.90E-04	1.02E-03	3.03E-04	0.062	↑
Threonine	1.77E-03	2.12E-03	5.41E-04	0.094	↑
Cecal content					
Leucine	2.47E-03	1.79E-03	7.53E-04	**0.017**	↓
Thymine	4.80E-04	6.50E-04	2.29E-04	0.051	↑

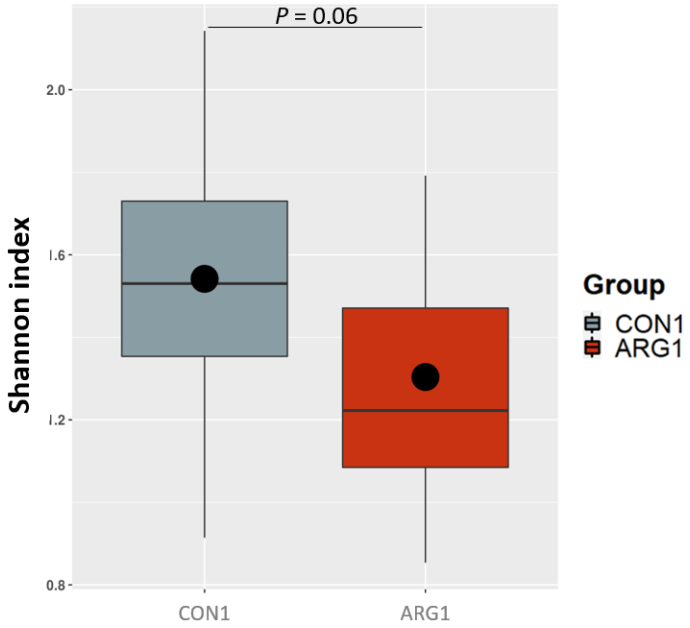
[†] Means of 14 birds/group.
[§] Ratio of the mean of group CON1 over the mean of group ARG1. The arrows indicate: ↑, ratio < 1; ↓, ratio > 1.
Note: P-values less than 0.05 are in bold.

Regarding the results of the cecal microbiota analysis of S3E1[29], alpha diversity of group ARG1 tended to be lower than that of group CON1 at almost all taxonomic levels, except for the species (data not shown). At the genus level (**Figure 37**), group ARG1 had an average alpha diversity of 1.3 while group CON1 of 1.5 ($P = 0.06$). Beta diversity analysis did not cluster the samples according to groups (data not shown). **Table 13** shows the bacteria that were differently abundant in the cecal content collected from CON1 and ARG1 birds at D49. At the phylum level, the relative abundance of Proteobacteria was significantly lower in group ARG1 than group CON1 ($P < 0.05$), while Firmicutes had a similar trend toward significance. Bacteroidetes, in contrast, were overrepresented in ARG1 compared to CON1 birds ($P < 0.05$). At the class level, Bacteroidia were more abundant in ARG1 than

[29] The metagenomes have been uploaded to the NCBI repository (BioProject entitled "PRJNA884508" available at https://dataview.ncbi.nlm.nih.gov/object/PRJNA884508?reviewer=t7u6lk5fa366vfuc3qam3p701e).

CON1 birds, as were Coriobacteriia – a class of the phylum Actinomycetota – whereas the relative abundance of Gammaproteobacteria, unclassified Firmicutes, and Clostridia changed in the opposite way ($P < 0.05$). The differences in relative abundance detected at the order level reflected those at the class level: Bacteroidales and Eggerthellales (the latter belonging to the class Coriobacteriia) were more abundant, while Enterobacterales (members of the class Gammaproteobacteria), unclassified Firmicutes, and Clostridiales were less abundant in ARG1 than CON1 birds ($P < 0.05$). Regarding the bacterial families, the relative abundance of Eggerthellaceae was higher in group ARG1 than group CON1 ($P < 0.05$), whereas the relative abundance of Enterobacteriaceae and unclassified Firmicutes was significantly lower in ARG1 than CON1 birds ($P < 0.05$). As for bacterial genera, ARG1 birds showed a greater abundance of *Gordonibacter* ($P < 0.05$) and a lower abundance of *Escherichia*, unclassified Firmicutes, and *Flavonifractor* ($0.06 \leq P \leq 0.02$). Lastly, bacterial species whose relative abundance differed between groups were *Gordonibacter pamelaeae* and *Lactobacillus salivarius* (significantly more abundant in group ARG1; $P < 0.05$), as well as *Escherichia coli*, *Firmicutes bacterium* CAG 94, and *Lachnoclostridium* An131 (less abundant in group ARG1 with a *P*-value ranging between 0.09 and 0.03).

Figure 37 – S3E1 | Shannon index of the cecal content of CON1 and ARG1 birds at D49



Note: Means of 14 birds/group are the black dots inside the boxes.

Turning now to S3E2, the growth performance results are shown in **Table 14**. Chicks did not show a significantly different weight between groups at placement (i.e., ~43 g). Before HS was applied, ARG2 birds tended to be heavier than CON2 birds at the end of the starter phase (i.e., BW of 437.9 and 450.4 g for CON2 and ARG2, respectively; $P = 0.09$). In contrast, FCR of group ARG2 was significantly lower than that of group CON2 (1.344 *vs.* 1.270; $P = 0.02$). Similarly, at the end of the grower phase, ARG2 birds showed greater BW and lower cumulative FCR than CON2 birds (1,424 g and 1.646 *vs.* and 1,471 g and 1.553 for CON2 and ARG2, respectively; $P < 0.05$). The difference in DWG, however, approached but did not achieve significance (75.16 g *vs.*77.95 g; $P = 0.08$). In the finisher phase, neither the effect of group nor that of the interaction between group and room was significant ($P > 0.05$). The exposure to HS, however, decreased ($P \leq 0.01$) BW, DWG, DFI, and (cumulative) FI, while increased mortality regardless of group.

Figure 38 shows the rectal temperature of representative birds of groups CON2 and ARG2 on the first and eighth day of the cyclic HS period at 9:00 AM and 6:00 PM. The raw data ranged between 40.4 and 44.0 °C. The mixed ANOVA used to analyze the data grouped by the day of collection did not reveal a significant interaction between the three factors (i.e., group, room, and time point; $P > 0.05$). Moreover, the effect of group was not significant, while those of room, time point, and their interaction were highly significant ($P < 0.001$). Pairwise comparisons of time points indicated that the increase in rectal temperature from 9:00 AM to 6:00 PM was greatly significant ($P < 0.001$) in either room, on both the measurement days, and irrespective of the group.

Table 13 – S3E1 | Bacteria showing different relative abundance (%) in the cecal content of CON1 and ARG1 birds at D49

Bacteria	Group[†]		*P*-value	Change[§]
	CON1	ARG1		
Phylum				
Proteobacteria	1.70	0.14	**0.018**	↓
Bacteroidetes	72.3	80.9	**0.041**	↑
Firmicutes	19.0	14.6	0.087	↓
Class				
Coriobacteriia	0.19	0.42	**0.010**	↑
Gammaproteobacteria	1.70	0.14	**0.018**	↓
Firmicutes (unclassified)	4.80	2.58	**0.041**	↓
Bacteroidia	72.3	80.9	**0.041**	↑
Clostridia	13.5	9.35	**0.043**	↓
Order				
Eggerthellales	0.19	0.42	**0.010**	↑
Enterobacterales	1.70	0.14	**0.018**	↓
Firmicutes (unclassified)	4.80	2.58	**0.041**	↓
Bacteroidales	72.3	80.9	**0.041**	↑
Clostridiales	13.5	9.35	**0.043**	↓
Family				
Eggerthellaceae	0.19	0.42	**0.010**	↑
Enterobacteriaceae	1.70	0.14	**0.018**	↓
Firmicutes (unclassified)	4.80	2.58	**0.041**	↓
Genus				
Gordonibacter	0.19	0.42	**0.010**	↑
Escherichia	1.70	0.14	**0.018**	↓
Firmicutes (unclassified)	4.80	2.58	**0.041**	↓
Flavonifractor	1.57	0.81	0.059	↓
Species				
Gordonibacter pamelaeae	0.19	0.42	**0.010**	↑
Escherichia coli	1.70	0.14	**0.018**	↓
Lactobacillus salivarius	0.01	0.06	**0.030**	↑
Firmicutes bacterium CAG 94	4.80	2.58	**0.041**	↓
Lachnoclostridium An131	0.33	0.13	0.091	↓

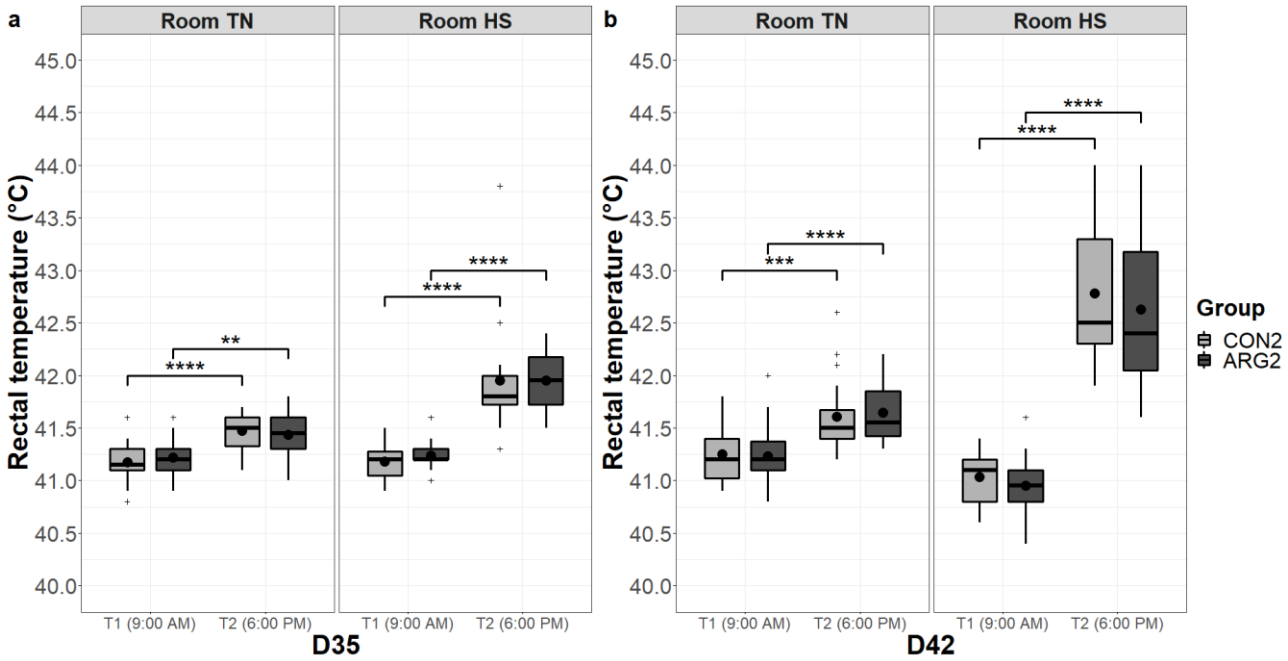
[†] Means of 14 birds/group.
[§] Ratio of the mean of group CON1 over the mean of group ARG1. The arrows indicate: ↑, ratio < 1; ↓, ratio > 1.
Note: P-values less than 0.05 are in bold.

Table 14 – S3E2 | Growth performance of groups CON2 and ARG2 in the three-phase feeding trial

Feeding phase	Factor	Dependent variable								
		Chick weight (g/bird)	BW (g/bird)	DWG[§] (g/bird/d)	DFI[§] (g/bird/d)	FI[§] (kg/bird)	Cum. FI[§, ¶] (kg/bird)	FCR[§]	Cum. FCR[§, ¶]	Mortality (%)
Starter[†] (0-14 d)	**Group**									
	CON2	43.15	437.9	28.20	37.94	0.531	/	1.344	/	0.00
	ARG2	43.60	450.4	29.06	36.91	0.517	/	1.270	/	0.00
	SE	1.07	16.69	1.22	2.28	0.03	/	0.06	/	0.00
	***P*-value**	0.327	0.094	0.113	0.292	0.292	/	**0.015**	/	/
Grower[†] (15-27 d)	**Group**									
	CON2	/	1,424	75.16	133.1	1.730	2.261	1.772	1.646	0.83
	ARG2	/	1,471	77.95	130.0	1.690	2.207	1.670	1.553	0.00
	SE	/	51.59	3.53	9.77	0.13	0.12	0.15	0.10	0.07
	***P*-value**	/	**0.049**	0.079	0.459	0.459	0.295	0.112	**0.042**	0.339
Finisher[‡] (28-44 d; cyclic HS from D35 onwards)	**Group**									
	CON2	/	3,051	96.51	196.0	3.332	5.592	2.033	1.852	5.00
	ARG2	/	3,138	98.21	188.5	3.205	5.412	1.927	1.751	0.83
	Room									
	TN	/	3,222	105.1	208.3	3.541	5.743	1.987	1.811	0.00
	HS	/	2,967	89.64	176.3	2.996	5.261	1.973	1.792	5.83
	Group × Room									
	CON2-TN	/	3,188	104.1	217.4	3.696	5.939	2.096	1.894	0.00
	ARG2-TN	/	3,257	106.1	199.1	3.385	5.546	1.878	1.728	0.00
	CON2-HS	/	2,914	88.93	174.5	2.967	5.246	1.970	1.810	10.00
	ARG2-HS	/	3,019	90.35	178.0	3.026	5.277	1.975	1.775	1.67
	SE	/	122.0	4.78	15.47	0.26	0.37	0.20	0.15	0.14
	***P*-value**									
	Group	/	0.111	0.404	0.266	0.266	0.259	0.229	0.122	0.179
	Room	/	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.010**	0.866	0.759	**0.039**
	Group × Room	/	0.732	0.889	0.115	0.115	0.191	0.208	0.297	0.179

[†] n = 12 replicate pens/group.
[‡] n = 6 replicate pens/group/room. In "room HS", temperature was raised daily to ~34 °C from 9:00 AM to 6:00 PM from D35 to D43.
[§] Corrected for mortality.
[¶] Computed for the period consisting of a feeding phase and its previous one/ones. Cum., cumulative.
Note: P-values less than 0.05 are in bold.

Figure 38 – S3E2 | Rectal temperature of CON2 and ARG2 birds in the two rooms on the first (**a**) and eighth (**b**) day of the cyclic HS period at two time points



Note: The rectal temperature of 18 labeled birds/group/room was measured on the first (D35) and the eighth (D42) day of the cyclic HS period at two time points, namely T1 (9:00 AM) and T2 (6:00 PM). Group means are the black dots inside the boxes. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Moving to the results of metabolomics analyses of S3E2, the concentration of several metabolites was found to be affected by the study factors group and room in plasma, liver, and *Pectoralis major*, as shown in the tables provided in **Annex 4**. The data of these tables have been compared and summarized in **Table 15** bearing in mind the aims of S3E2 and focusing on potentially biologically relevant metabolites. Group ARG2 had a higher concentration of arginine in plasma, liver ($P < 0.001$), and *P. major* ($P = 0.07$), creatine in plasma, liver, and *P. major* ($P < 0.05$), methionine in liver ($P = 0.08$), glycerol and lactate in *P. major* ($P < 0.05$), as well as a lower concentration of alanine in plasma ($P < 0.05$) and *P. major* ($P = 0.08$), AMP in liver and *P. major* ($P < 0.05$), glutamine in plasma ($P = 0.01$), liver ($P = 0.06$) and *P. major* ($P = 0.1$), glutathione in liver and *P. major* ($P \leq 0.01$), and glucose-1-phosphate in *P. major* ($P = 0.07$). The exposure to HS increased the concentration of glucose in plasma ($P < 0.05$), phenylalanine in plasma and liver ($P < 0.05$), glutamate ($P < 0.05$), (iso)leucine ($P = 0.07$), threonine ($P = 0.05$), uracil and uridine ($P < 0.05$) in liver, AMP ($P < 0.05$), anserine, beta-alanine, carnosine ($P \leq 0.001$), and choline ($P = 0.01$) in *P. major*, while decreased that of aspartate, betaine, carnosine, glycerol, and methionine in plasma ($P < 0.05$), succinate in plasma and liver ($P < 0.05$),

glycine ($P < 0.01$) and tyrosine ($P \leq 0.09$) in plasma and *P. major*, UDP in liver and *P. major* ($P < 0.01$), alanine, fumarate and N,N-Dimethylglycine in plasma, liver and *P. major* ($P \leq 0.05$), and (iso)leucine, threonine, and valine in *P. major* ($P \leq 0.002$). On the other hand, the interactive effect of group and room tended to be or was significant on the concentration of a few metabolites, such as carnosine and formate in plasma, sarcosine in plasma and liver, ethanolamine and propylene glycol in liver, and isoleucine and threonine in *P. major*.

Regarding the cecal microbiota[30] analyses of S3E2, it can be seen from the data in **Table 16** that group ARG2 showed reduced observed diversity ($P < 0.05$) compared to group CON2, while HS increased this alpha index ($P = 0.09$), as well as the Shannon ($P < 0.01$) and Inverse Simpson ($P = 0.05$). No significant interaction between group and room was found for alpha diversity indices. Beta diversity was also affected by group ($P = 0.001$) and room ($P = 0.07$), but not by their interaction ($P = 0.260$). Despite being preliminary results based on descriptive statistics, the differences in relative abundance of important cecal bacterial phyla for poultry are worth presenting. The data reported in **Table 17** suggest that ARG2 birds may have harbored less Actinobacteria and Proteobacteria while more Bacteroidetes, as well as less Firmicutes only under TN conditions. HS seemed to reduce the abundance of Bacteroidetes and increase that of Firmicutes. In addition, the exposure to HS may have increased the abundance of Proteobacteria only for CON2 birds.

[30] The metagenomes have been uploaded to the NCBI repository (BioProject entitled "PRJNA928248" available at https://dataview.ncbi.nlm.nih.gov/object/PRJNA928248?reviewer=i1mh70qbpbnn002pmrcvr62leu).

Table 15 – S3E2 | Biologically relevant metabolites whose concentration was affected (*P*-values of 0.1 maximum) by either arginine supplementation, HS exposure or both (see **Annex 4** for more information)

Metabolite	Arginine supplementation[§]			HS exposure[§]		
	Plasma	Liver	*P. major*	Plasma	Liver	*P. major*
Alanine	↓	-	↓	↓	↓	↓
AMP	-	↓	↓	-	-	↑
Anserine	-	-	-	-	-	↑
Arginine	↑	↑	↑	-	-	-
Aspartate	-	-	-	↓	-	-
Beta-alanine	-	-	-	-	-	↑
Betaine	-	-	-	↓	-	-
Carnosine	-	-	-	↓	-	↑
Choline	-	-	-	-	-	↑
Creatine	↑	↑	↑	-	-	-
Fumarate	-	-	-	↓	↓	↓
Glucose	-	-	-	↑	-	-
Glucose-1-phosphate	-	-	↓	-	-	-
Glutamate	-	-	-	-	↑	-
Glutamine	↓	↓	↓	-	-	-
Glutathione	-	↓	↓	-	-	-
Glycerol	-	-	↑	↓	-	-
Glycine	-	-	-	↓	-	↓
Isoleucine	-	-	-	-	↑	↓
Lactate	-	-	↑	-	-	-
Leucine	-	-	-	-	↑	↓
Methionine	-	↑	-	↓	-	-
N,N-Dimethylglycine	-	-	-	↓	↓	↓
Phenylalanine	-	-	-	↑	↑	-
Succinate	-	-	-	↓	↓	-
Threonine	-	-	-	-	↑	↓
Tyrosine	-	-	-	↓	-	↓
UDP	-	-	-	-	↓	↓
Uracil	-	-	-	-	↑	-
Uridine	-	-	-	-	↑	-
Valine	-	-	-	-	-	↓

[§] Compared with concentrations measured in the samples collected from birds fed the control diet or reared under TN conditions, the symbols indicate: ↑, increased; ↓, decreased; -, not affected.

Table 16 – S3E2 | Alpha diversity indices of the cecal content of CON2 and ARG2 birds in the two rooms at D44

Factor	Alpha diversity index			
	Observed	**Shannon**	**Simpson**	**Inverse Simpson**
Group				
CON2	189	3.65	0.94	19.1
ARG2	180	3.60	0.95	19.3
Room				
TN	181	3.56	0.94	17.9
HS	189	3.70	0.95	20.5
Group × Room				
CON2-TN	184	3.57	0.94	17.5
ARG2-TN	177	3.54	0.94	18.3
CON2-HS	194	3.72	0.95	20.6
ARG2-HS	183	3.66	0.95	20.3
SE	15.64	0.17	0.02	4.51
***P*-value**				
Group	**0.049**	0.403	0.802	0.855
Room	0.085	**0.007**	0.100	0.052
Group × Room	0.589	0.698	0.589	0.667

Note: n = 12 birds/group/room. *P*-values less than 0.05 are in bold.

Table 17 – S3E2 | Relative abundances (%) of important bacterial phyla in the cecal content of CON2 and ARG2 birds in the two rooms at D44

Phylum	Group		Room		Group × Room			
	CON2	**ARG2**	**TN**	**HS**	**CON2-TN**	**ARG2-TN**	**CON2-HS**	**ARG2-HS**
Actinobacteria	1.5 ± 1.2	0.8 ± 0.5	1.2 ± 0.7	1.1 ± 1.2	1.4 ± 0.7	0.9 ± 0.6	1.7 ± 1.5	0.6 ± 0.2
Bacteroidetes	6.8 ± 3.8	7.6 ± 4.5	8.3 ± 3.9	6.2 ± 4.2	8.5 ± 3.6	8.5 ± 4.3	5.6 ± 3.7	6.8 ± 4.7
Firmicutes	89.3 ± 5.0	88.0 ± 7.0	86.7 ± 6.4	90.6 ± 5.0	88.1 ± 4.6	85.4 ± 7.9	90.5 ± 5.3	90.7 ± 4.9
Proteobacteria	1.8 ± 0.9	1.5 ± 0.7	1.6 ± 0.6	1.7 ± 1.0	1.6 ± 0.5	1.6 ± 0.7	1.9 ± 1.2	1.4 ± 0.6

Note: n = 12 birds/group/room. Data are means ± SD.

6.5 Discussion

The first aim of S3 was to determine how dietary arginine supplementation affects growth performance of broilers reared under TN or HS conditions. The significant reduction in FCR shown by birds that received diets supplemented with arginine – up to D21 (-4.1%) and in the entire 49-day trial (-4.2%) of S3E1, as well as until D27 (-5.7%) of the TN period of S3E2 – is in line with the results obtained by Zampiga et al. (2018). Moreover, the substantial increase in cumulative DWG (+4.4%) and final BW (+4.2%) found in S3E1, coupled with that in BW at D27 (+3.3%) observed in S3E2 before the application of HS, corroborates the data from other research groups (Basoo et al., 2012; Xu et al., 2018a; Sirathonpong et al., 2019; Liu et al., 2019). The results of the present study confirm therefore that

feeding diets containing arginine levels above the recommended specifications leads to an improvement in performance when broilers are grown in TN environments. Nevertheless, Kidd et al. (2001) reported an inconsistent response of BWG to arginine levels exceeding those suggested by the NRC, while some investigators did not detect any improvement in BW by supplementing broiler diets with arginine (Fernandes et al., 2009; Fouad et al., 2013). Additional research may therefore be necessary to validate the positive effects of arginine supplementation on BWG of broilers raised in optimal thermal conditions.

Unsurprisingly, cyclic exposure to HS in S3E2 impaired growth performance and increased body temperature and mortality, as discussed earlier (see section **2.4** "**Raising Chickens in the "Hothouse Earth": The Effects of Heat Stress**"). Arginine supplementation, however, did not mitigate the deterioration in performance or prevent environmentally induced hyperthermia as birds responded similarly to the thermal stress irrespective of the diet they were fed on. While performance and body temperature results are reliable because they are based on a fair number of replicates and rectal temperature measurements, respectively, speculations about mortality data must be made cautiously as before (see chapter **4** "**STUDY #1: EFFECTS OF A SYNBIOTIC ON BROILERS EXPOSED TO THERMONEUTRAL AND HEAT STRESS CONDITIONS**"). Despite being scarce, available data suggest that arginine supplementation could help broilers counteract HS (Hassan et al., 2021; Teyssier et al., 2022a). The results of S3E2, however, seem to deny that broilers subjected to HS can benefit from this nutritional strategy, especially in terms of growth performance. A likely explanation for this contradictory finding is that the favorable effects of arginine may have been blunted by the significant reduction in FI caused by HS, which resulted in the ingestion of an insufficient amount of dietary arginine to foster or preserve performance. It is worth noting, however, that the reduction in FI was not of the same magnitude for heat-stressed control (-19.7%) and supplemented (-10.7%) birds compared to their TN counterparts. Testing greater levels of dietary arginine to offset the FI loss might therefore be advantageous if further investigations on arginine supplementation to heat-stressed broilers are to be undertaken.

Parenthetically, the macroscopic analysis of breast fillets collected in S3E1 showed that supplemental dietary arginine increased by 32% the risk of WS onset. This outcome is contrary to

previous studies reporting no significant effects of arginine supplementation on the occurrence of WS, or even its mitigation (Christensen et al., 2015; Zampiga et al., 2018, 2019; Bodle et al., 2018). However, unlike the considerable improvement in growth rate and BW observed here, arginine supplementations tested in the studies just cited did not exert a positive effect on BWG. Previous research has established that breast muscle abnormalities of fast-growing, high meat-yielding broilers are deeply related to the extraordinary growth potential of these birds: the higher the growth performance, the greater the risk of myopathy development (Petracci et al., 2019; Soglia et al., 2021; Bordignon et al., 2022). It is therefore very likely that, in S3E1, the significant growth promotion mediated by arginine exacerbated WS rather than the supplemental dietary arginine *per se*.

The second aim of S3 was to explore the impacts of dietary arginine supplementation on metabolism and intestinal microbiota of broilers kept in TN or HS environments. In both experiments, the plasma of arginine-supplemented birds showed a significantly higher concentration of arginine than that of birds fed the control diets. This supports again the results reported by Zampiga et al. (2018), as well as by Kidd et al. (2001) and researchers working with piglets (Kim et al., 2004) and rats (Holecek and Sispera, 2016). In addition to plasma levels of arginine, dietary arginine supplementation increased the hepatic and muscular (*P. major*) ones in ARG2 birds compared to their control counterparts. Therefore, it is conceivable that feeding broilers diets formulated with arginine above the typically recommended doses is an effective way to increase the bioavailability of dietary arginine. This can be of paramount importance for animals incapable of synthesizing arginine *de novo*, such as chickens (Ball et al., 2007). As supplementing dietary arginine has also been shown to stimulate secretion of GH, IGF-1, and insulin in broilers (Xu et al., 2018a; Sirathonpong et al., 2019) and piglets (Kim et al., 2004), high concentrations of arginine found in ARG1 and ARG2 birds may have indirectly boosted the anabolic pathways regulated by these potent hormones (Barbul, 1986; Collier et al., 2005; Zajac et al., 2010; Oh et al., 2017).

Intake and availability of dietary arginine have been demonstrated to influence creatine levels in different parts of the chicken's body (Keshavarz and Fuller, 1971a; b; Chamruspollert et al., 2002). Creatine is mainly produced by the liver and is subsequently transported to target tissues with the bloodstream (Walker, 1960; Oviedo-Rondón and Córdova-Noboa, 2020). Here, metabolomics analyses

revealed greater concentrations of hepatic and circulating creatine for ARG1 and ARG2 birds, as well as higher levels of creatine in *P. major* of ARG2 birds. The latter result confirms the observation of Chamruspollert et al. (2002) that breast muscles of arginine-supplemented broilers showed increased creatine content. Extensive research has shown that supplementing creatine – or its precursor, guanidinoacetate – considerably improves growth performance and breast meat yield of broilers (Ringel et al., 2007; Oviedo-Rondón and Córdova-Noboa, 2020; Portocarero and Braun, 2021). Consequently, a greater availability of creatine may have supported growth and lean tissue accretion in birds that received arginine supplementation. However, this hypothesis is to be confirmed by experimental data because the evaluation of lean tissue yield was beyond the scope of the present study.

Group ARG1 showed higher plasma concentrations of histidine – confirming the study by Zampiga et al. (2018) – and betaine than group CON1. Histidine is an essential AA for chickens (Leeson and Summers, 2001). Plasmatic levels of histidine have been shown to be positively correlated with the weight of *P. major* (Baéza et al., 2015), while feeding broilers a histidine-deficient diet resulted in impaired growth, reduced breast meat yield, and a complete carnosine depletion and significant decrease in anserine in pectoral muscle (Kai et al., 2015). In contrast, supplementation of dietary histidine increased breast muscle content of histidine-containing dipeptides, viz. carnosine and anserine, thereby improving the quality and antioxidant defenses of chicken meat (Kai et al., 2015; Qi et al., 2021; Lackner et al., 2021). Future research is warranted to elucidate the causes and effects of the increased plasma level of histidine found in arginine-supplemented broilers of S3E1. As for betaine, several lines of evidence suggest that it enhances the health, performance, carcass composition, and meat quality of poultry (Metzler-Zebeli et al., 2009). Thanks to its osmoregulatory function, betaine can also mitigate the effects of HS (Ratriyanto and Mosenthin, 2018). Furthermore, acting as a methyl group donor, betaine was found to contribute to and promote the biosynthesis of creatine in the liver of broilers (Zhan et al., 2006). The increased availability of betaine is therefore another plausible reason for the increased hepatic content of creatine and enhanced performance shown by ARG1 birds compared with their control counterparts. On the other hand, arginine supplementation did not change the concentrations of histidine or betaine in ARG2 birds. Nonetheless, it is still worth talking about the histidine-containing dipeptides and betaine as their levels were affected by the thermal stress applied in S3E2. Specifically,

HS increased concentrations of carnosine and anserine in *P. major*, which accords with earlier observations (Zampiga et al., 2021b), while reduced those of carnosine and betaine in plasma. Furthermore, HS raised the levels of beta-alanine and choline in *P. major*. Carnosine consists of histidine and beta-alanine, while anserine is formed by the methylation of carnosine. These two dipeptides have been shown to have powerful antioxidant activity and several biological functions, such as pH buffering, metal-ion chelation, complexing of dangerous carbonyl compounds, and anticross-linking effects on proteins, thereby protecting cells against stress and ischemia [reviewed in Lackner et al. (2021)]. Choline, however, is an important provider of methyl groups and its metabolism is intimately related to that of betaine (a product of choline oxidation) and methionine. Choline is a multifunctional molecule that, among other things, constitutes many phospholipids (e.g., phosphatidylcholine) that maintain integrity and functions of cell membranes (Simon, 1999). Taken together, these data and those from the study by Zampiga et al. (2021b) further support the idea that accumulating endogenous antioxidant and protective molecules in specific tissues, such as the pectoral muscle, is an essential part of the adaptive response to HS in chickens.

Besides arginine, the liver of arginine-supplemented birds was enriched in leucine, methionine sulfoxide, phenylalanine, and threonine in S3E1, as well as methionine in S3E2. These AAs, along with histidine mentioned before, are all indispensable for chickens (Leeson and Summers, 2001). It is intriguing to link the increased hepatic levels (plasmatic for histidine) of essential AAs to potentially better intestinal digestion and absorption of dietary protein and purified AAs by arginine-supplemented birds, such as crystalline arginine, methionine, and threonine included in the diets used in the present study. Arginine supplementation has been shown to improve intestinal health, integrity, and function (Liu et al., 2008b; Coburn et al., 2012; Zhang et al., 2017, 2018) and to increase the jejunal villus height to crypt depth ratio in broilers (Laika and Jahanian, 2017) and the jejunal and ileal villus height in intra-uterine growth retarded piglets (Wang et al., 2012). The villus height to crypt depth ratio is commonly used to assess gut health in chickens (Ducatelle et al., 2018), while villi extend the nutrient absorption area *per se* (Koutsos and Arias, 2006). So, it can be supposed that improved intestinal conditions and desirable changes in gut morphology may have been behind an elevated efficiency of AA uptake in birds fed diets supplemented with arginine. Since nutrient absorption primarily occurs in the jejunum and

AAs are not assimilated through the large intestine epithelium (Svihus, 2014; Beaumont and Blachier, 2020), the fact that group CON1 showed increased – probably unabsorbed – level of leucine in the cecal content suggests that the small intestine uptake of leucine might have been higher for group ARG1, further supporting the aforesaid hypothesis. Additional investigations (e.g., digestibility studies) focused on this topic are therefore suggested.

Weirdly, arginine supplementation resulted in reduced levels of the conditionally essential AA glutamine in the blood (in both S3E1 and S3E2), liver, and *P. major* (in S3E2). Glutamine has been shown to be extremely important in supporting GIT development and function and promoting gut health (Bortoluzzi et al., 2018, 2020). Its dietary supplementation has been found to alleviate the adverse effects of enteric challenges in broilers, such as those of NE, coccidiosis, and *Salmonella* infection (Xue et al., 2018; Oxford and Selvaraj, 2019; Wu et al., 2022), as well as the impacts of HS on intestinal barrier integrity in mice [reviewed in Bortoluzzi et al. (2018)]. In their comprehensive review article, Coster et al. (2004) pointed out that most of dietary glutamine and a quarter of glutamine in the blood are used by enterocytes and intestinal immune cells as a key nutrient to obtain nitrogen and energy. Accordingly, there is a real competition between the gut and extraintestinal tissues for glutamine. These authors also reported that almost all plasma glutamine is derived from the pool of free glutamine within skeletal muscle, the largest reservoir and most important site for the synthesis of this AA. In the liver, however, glutamine is utilized as a substrate for gluconeogenesis and the synthesis of urea (in mammals), acute phase proteins, and glutathione. The reasons for the reduction in glutamine levels observed in the present study remain to be defined, but it can be hypothesized that the intestinal mucosa of arginine-supplemented birds had an increased demand for glutamine, thereby draining arterial glutamine to fuel the accelerated metabolism associated with improved growth rates. To validate this, it is necessary to analyze the metabolic profile of the intestinal epithelium. If this hypothesis is confirmed in future studies, it might be worth re-evaluating glutamine requirements for broilers fed arginine-enriched diets.

Except for arginine, methionine, and glutamine, arginine supplementation did not significantly influence hepatic levels of other essential, conditionally essential, or non-essential AAs in S3E2. The exposure to HS, however, modified the concentrations of many of them. HS decreased the levels of (iso)leucine, threonine, and valine in *P. major*, glycine and tyrosine in plasma and *P. major*, and

aspartate and methionine in plasma, whereas increased the levels of glutamate, (iso)leucine, and threonine in liver, as well as those of phenylalanine in plasma and liver. These results corroborate what has been discussed in detail earlier regarding the alteration of protein metabolism induced by HS in chickens (see section **2.4** "**Raising Chickens in the "Hothouse Earth": The Effects of Heat Stress**", subsection "**LIPID AND PROTEIN METABOLISM**"). Curiously, both arginine supplementation and HS consistently reduced the concentration of another non-essential AA, that is alanine, in all tissues analyzed in S3E2, apart from the liver when considering the effect of arginine supplementation. Similarly, HS decreased, in all tissues, the levels of N,N-Dimethylglycine, a betaine derivative involved in choline metabolism and synthesis of the antioxidant tripeptide glutathione by serving as a source of glycine (Simon, 1999; Kalmar et al., 2010). Zampiga et al. (2021b) found a comparable reduction in N,N-Dimethylglycine in breast muscle of broilers reared under HS conditions. It has been reported that N,N-Dimethylglycine has free-radical scavenging properties (Hariganesh and Prathiba, 2010) and its use as a feed additive has been shown to produce positive effects on the health and performance of broilers (Kalmar et al., 2010, 2011; EFSA, 2011; Prola et al., 2013). The results from S3E2, particularly the drop in N,N-Dimethylglycine levels caused by HS, and their potential implications for metabolism, growth, and responses to HS and oxidative stress would merit further in-depth investigations in broilers.

Oxidation-related molecules have a prominent role in the present discussion, which is not surprising considering the effects arginine and HS have been shown to have on oxidative stress (Akbarian et al., 2016; Wu et al., 2021). Arginine supplementation reduced the level of glutathione in the liver in both experiments, as well as in *P. major* in S3E2. Glutathione, composed of glutamate/glutamine, cysteine, and glycine, is very important for the antioxidant defense system, metabolism of nutrients, and regulation of cellular activities. Being the precursor of glutamate, arginine considerably influences the biosynthesis and levels of glutathione (Castro and Kim, 2020). Like creatine, glutathione is mainly produced and provided by the liver (Wu et al., 2004). Enkvetchakul et al. (1995) described an age- and body weight-dependent increase in hepatic and blood levels of glutathione in broilers. Since control and supplemented birds were the same age at the time of sampling, one would have expected a higher glutathione level in the liver of heavier chickens, particularly those belonging to group ARG1. However, the outcome opposite to expectations raises the question of why arginine

supplementation may have produced a reduction in glutathione levels. It is difficult to answer this query, especially in view of the dietary arginine-supported increase in glutathione peroxidase activity observed by Duan et al. (2015) in broiler breeder hens and their offspring. Another unanswered question is: can the aforementioned increased concentration of methionine sulfoxide, the major product of methionine oxidation (Lee and Gladyshev, 2011), represent an indicator for hepatic oxidative stress due to greater and faster growth of ARG1 birds than their control counterparts?

Energy-related molecules are also worth discussing. AMP was reduced in liver and *P. major* by arginine supplementation and was increased in *P. major* by HS in S3E2. This nucleotide plays an important role in many cellular metabolic processes, such as regulation of energy homeostasis by modulating the activity of the enzyme AMP-activated kinase (**AMPK**). Recognized as the master energy sensor for cells (Hardie, 2003; Hardie et al., 2006), AMPK senses energy levels by detecting modifications in the AMP to ATP ratio (Xiao et al., 2011; Chen et al., 2013). Under energy depletion (i.e., increased levels of AMP and decreased levels of ATP), AMP binds to γ subunits of AMPK leading to the activation of this kinase that results in promotion of catabolic pathways and inhibition of anabolic pathways to generate ATP (Stein et al., 2000). Considering this, the data of S3E2 suggest that the liver and *P. major* of arginine-supplemented birds probably were in a good energy balance (low AMP levels), while *P. major* of heat-stressed birds suffered from energy depletion (high AMP levels). According to Baumgard and Rhoads (2013), negative energy balance and catabolic states are two of the most distinctive features of HS conditions. It is therefore intriguing that arginine supplementation may have been able to partially counterbalance the adverse effects of HS on energy homeostasis of broilers by increasing creatine levels and attenuating the increase in AMP levels, particularly in pectoral muscle. However, the significant reduction in the levels of fumarate in all tissues analyzed in S3E2 and of succinate and UDP in plasma and liver supports the hypothesis that birds exposed to HS had a suboptimal energy balance. Succinate and fumarate are two consecutive intermediates in the citric acid cycle and, as such, their reduced availability can inhibit this central metabolic pathway, interfering with cellular bioenergetic processes. On the other hand, UDP is important in glycogenesis because it is combined with glucose to form UDP-glucose units that can be polymerized to glycogen chains. As UDP levels were reduced by HS, it can be hypothesized that glycogenesis, a chief anabolic pathway occurring

in liver and muscles, was hampered in heat-stressed birds. In contrast, glycogenolysis and gluconeogenesis may have been promoted to increase hepatic glucose production [reviewed in Rhoads et al. (2013)], potentially resulting in the increased level of plasma glucose observed in heat-stressed birds.

Turning now to the results of cecal microbiota analyses, arginine supplementation reduced alpha diversity in S3E1 (Shannon index) and S3E2 (observed diversity). This is contrary to Singh et al. (2019) who measured an increase in Shannon index of colonic specimens collected from mice given high dietary arginine compared to their low-dose and control counterparts. It is reasonable to attribute this discrepancy to the different animal species (i.e., chicken *vs.* mouse) and intestinal sections digesta was collected from (i.e., cecum *vs.* colon). Cecal samples of ARG1 birds also showed decreased relative abundance of Firmicutes (e.g., Clostridia) and increased relative abundance of Bacteroidetes (e.g., Bacteroidia). Even though they are based on descriptive statistics, the results of S3E2 also indicate that arginine supplementation favored Bacteroidetes to the detriment of Firmicutes, particularly in the ceca of supplemented birds raised under TN conditions. A comparable reduction in alpha diversity and a similar variation in the abundances of Firmicutes and Bacteroidetes for high-performing broilers treated with a feed-grade muramidase were found in experiment S2E1 (see chapter **5** "**STUDY #2: EFFECTS OF A MURAMIDASE ON BROILERS EXPOSED TO THERMONEUTRAL AND HEAT STRESS CONDITIONS**"). Although it is important to take into consideration the differences between S2E1 and the experiments conducted in the present study, these findings somewhat contradict the widely known positive association between microbial diversity or relative abundance of Firmicutes (especially useful Clostridia) and broiler health and performance (Torok et al., 2011; Yeoman and White, 2014; Stanley et al., 2016). Remarkably, Singh et al. (2019) also found increased prevalence of Bacteroidetes in the colon of the aforementioned mice fed on a high-arginine diet. Thus, these topics are worth investigating further in chickens. In S3E2, however, the exposure to HS increased alpha diversity indices (i.e., observed diversity, Shannon, and Inverse Simpson), which reflects the changes in ileal alpha diversity reported by Wang et al. (2018a), affected beta diversity as in the studies by Shi et al. (2019), Liu et al. (2020), and Goel et al. (2022), and seemed to reduce the abundance of Bacteroidetes and

increase that of Firmicutes, which could also be in agreement with the last three studies mentioned above if ongoing analyses confirm the descriptive data of S3E2.

Despite the reduction in Firmicutes, ARG1 birds showed increased relative abundance of *Lactobacillus salivarius*, which has extensively been studied for its probiotic effects and has frequently been used as a feed additive to improve the health and performance of livestock and poultry (FAO, 2016; Chaves et al., 2017). It is likely therefore that the increase in *L. salivarius* abundance was a positive effect arginine supplementation had on the microbiota. Arginine supplementation also reduced Proteobacteria in S3E1 and affected them in the same way in S3E2 too, as descriptive data of the latter experiment indicate. Within this phylum, the relative abundance of *E. coli* was found to be lower in ARG1 than CON1 birds. This is consistent with the results of a murine model in which arginine supplementation was tested as a potential therapy for *E. coli* infection (Liu et al., 2017). The authors of the study just cited suggested that arginine supplementation attenuated *E. coli* infection through positive regulation of intestinal innate immunity. Moreover, Liu et al. (2008) showed that arginine supplementation alleviated gut mucosal injury in weaned piglets challenged with LPS from *E. coli*, ascribing this beneficial outcome to a possible immunomodulatory effect of arginine. Likewise, Zhang et al. (2017, 2018) demonstrated that arginine supplementation mitigated intestinal damage in *C. perfringens*-infected broilers through enhancement of mucosal barrier and immune function, increase in nitric oxide production, and restoration of a normal microbiota. It would therefore be worth delving into the potentially desirable effects of arginine supplementation on intestinal immune function of broilers. In S3E2, however, not only arginine supplementation, but also HS exposure seemed to affect the phylum Proteobacteria; these results, while preliminary, suggest that HS may have increased the abundance of Proteobacteria for CON2 birds. This outcome can be compared with that of Shi et al. (2019) who found that the abundance of cecal Proteobacteria was significantly increased in broilers exposed to HS.

In conclusion, the microbiota results obtained in S3E1 collectively indicate that arginine supplementation may have induced beneficial changes in the intestinal ecosystem, with possible beneficial implications for gut health, systemic health, and growth performance of broilers raised in a TN environment. On the other hand, the significant change in alpha and beta diversities and the preliminary results of taxonomic analysis found in S3E2 confirm that HS affects the microbiota of

broilers (see section **2.4** "**Raising Chickens in the "Hothouse Earth": The Effects of Heat Stress**", subsection "**GASTROINTESTINAL MICROBIOTA**"). However, as suggested by Singh et al. (2019), further work – especially under HS conditions – is needed to clarify how and to what extent arginine supplementation influences the microbiota and its relationship with the host (i.e., direct or indirect effects?).

7 STUDY #4: EFFECTS OF HEAT STRESS ON HYPOTHALAMIC FEEDING-RELATED NEUROPEPTIDES, OXYGEN HOMEOSTASIS, AND INFLAMMATORY STATE IN RED JUNGLE FOWL AND THREE BROILER LINES

Note: The study presented in this chapter was conducted by the PhD candidate during a six-month research period at Dr. Sami Dridi's lab of Avian Molecular Genetics and Endocrinology, Center of Excellence for Poultry Science, University of Arkansas, AR, U.S. The results of experiments S4E1 and S4E2 have been published. For more information, please refer to **Annex 1**.

7.1 Highlights

- This study aimed to investigate the effects of heat stress on hypothalamic expression of (an)orexigenic neuropeptides and molecules associated with oxygen homeostasis and inflammation in different genetically selected broiler lines and their ancestor, the red jungle fowl.
- A stable gene expression of (an)orexigenic neuropeptides was found in the four chicken populations.
- Hypothalamic integration of circadian information, acclimation of birds to long-lasting heat stress, steady hypothalamic pathways unaffected by evolution or selection, focus on mRNA abundance, and analysis of the entire hypothalamus may have attenuated the differences in neuropeptide expression.
- Heat stress modulated the expression of genes associated with hypoxia and oxygen homeostasis, as well as their up- and down-stream mediators.
- Responses to hypoxia and heat stress are likely related to feed intake regulation in chickens, which warrants further in-depth investigation.

7.2 Background and Aims

As extensively discussed in the paragraphs devoted to HS (see section **2.4** "**Raising Chickens in the "Hothouse Earth": The Effects of Heat Stress**"), the exceptional improvements in broiler performance stemming from genetic selection have come at a price. Indeed, high-performing broilers have been shown to be much less able to endure high environmental temperatures and HS than their

predecessors due to a higher metabolic rate and poor development of the cardiovascular and respiratory systems. Nonetheless, modern broilers still adopt evolutionary conserved strategies to deal with HS, such as reducing FI and maximizing sensible heat loss through a peripheral vasodilation. Despite being survival strategies, these responses considerably affect growth performance and homeostasis of heat-stressed broilers. The negative effects of reduced FI on growth performance are quite predictable and well-known, but the underlying molecular mechanisms of this reaction are not. On the other hand, diverting blood to the body periphery alters blood pressure and results in vasoconstriction of internal organs, such as the GIT and brain (Hales, 1973; Borges et al., 2007; Lambert, 2009; Bain et al., 2014), causing a hypoxia-like state that forces the organism to implement relevant metabolic adjustments.

The hypothalamus, a major center regulating FI, feeding behavior, and energy homeostasis (Simpson et al., 2008; Waterson and Horvath, 2015; Timper and Brüning, 2017; Luquet et al., 2019) mainly through numerous (an)orexigenic neuropeptides (Arora and Anubhuti, 2006; Furuse et al., 2007; Tachibana and Tsutsui, 2016; Delgado et al., 2017), may potentially be involved in the physiological response to HS leading to reduced feed ingestion. Investigating the hypothalamic control of metabolism with advanced molecular techniques, scientists have unearthed intriguing links between food intake regulation and oxygen homeostasis. The bridge between these apparently unconnected processes is the hypoxia-inducible factor (**HIF**) signaling in the hypothalamus. HIF proteins are nuclear transcription factors whose canonical function is the control of adaptation to hypoxia (Nakazawa et al., 2016), environment-induced hyperthermia (Rhoads et al., 2013), oxidative stress, and inflammation (Greer et al., 2012; Gaspar and Velloso, 2018). However, Zhang et al. (2011) demonstrated that the inhibition of HIF in proopiomelanocortin (**POMC**) neurons of the arcuate nucleus[31] impairs hypothalamic glucose sensing and causes hyperphagia and increased fat deposition in mice. In the same study, AMPK and mTOR signaling pathways were also found to converge on HIF (Zhang et al., 2011). AMPK, the master energy sensor of cells (Hardie, 2003; Hardie et al., 2006), has been shown to regulate hypothalamic glucose sensing and feeding behavior (Minokoshi et al., 2004; Claret et al., 2007), while the

[31] The arcuate nucleus, equivalent to the avian infundibular nucleus, is highly sensitive to circulating levels of glucose due to its proximity to the median eminence and a lower restriction of the blood-brain barrier than in other hypothalamic sites (Du et al., 2021).

hypothalamic mTOR cascade has been demonstrated to integrate numerous nutrient and hormone signals to control the energy balance of the body (Cota et al., 2006). Interestingly, heat shock proteins (**HSP**), nuclear factor κB (**NF-κB**), and IL-6 have all been shown to be downstream mediators of HIF (Baird et al., 2006; Bandarra et al., 2015; Fujii et al., 2020) and to regulate food intake and energy homeostasis in mammals (Shi et al., 2013; Hosoi et al., 2016; López-Ferreras et al., 2021).

It has been hypothesized that hypothalamic neuropeptidergic regulation of FI and the central HIF signaling pathways potentially related to it might differently respond to HS in unselected chickens and broiler genotypes from diverse stages of the breeding era. Therefore, S4 was undertaken to determine the expression profile of (S4E1) feeding-related hypothalamic neuropeptides (**FRHN**) and (S4E2) the hypothalamic HIF complex as well as its upstream regulators (i.e., PI3K, AMPK, and mTOR) and downstream mediators (i.e., HSPs, NF-κB, and IL-6) in heat-stressed red jungle fowl and three different genetically selected broiler lines.

7.3 Materials and Methods

EXPERIMENTAL DESIGN, HUSBANDRY CONDITIONS, AND SAMPLE COLLECTION

Hypothalamic samples analyzed in S4 had been collected during a study previously conducted at Dridi's lab (Abdelli et al., 2021). The four chicken populations used in that trial are maintained and hatched in the poultry facilities of the University of Arkansas (Fayetteville, AR, U.S.). In addition to the red jungle fowl (**JF**; Wall and Anthony, 1995), the slow-growing Athens Canadian Random Bred (**ACRB**) represented the broiler typically reared in the 1950s (Collins et al., 2016), the moderate-growing 1995 random bred (**95RB**) was the representative broiler of the 1990s (Harford et al., 2014), and the fast-growing modern random bred (**MRB**) served as the modern high-performing broiler. After hatching, 600 male chicks (150 birds/population) were placed in 12 environmentally controlled chambers divided into 2 floor pens of the same size (25 birds of the same population/pen). The trial lasted 56 d.

A 2×4 factorial design with environmental temperature and population as main factors and the pen as the experimental unit was adopted. Pens were provided with feeders, drinkers, and wood shavings as bedding material. Birds were fed and watered *ad libitum*. Commercial starter (0-28 d) and finisher

(29-56 d) broiler diets were used. The artificial photoperiod was 23L:1D during the first 7 d, while 20L:4D for the remainder of the trial. The environmental temperature was gradually decreased in all chambers as follows: 32 °C (0-3 d), 31 °C (4-6 d), 29 °C (7-10 d), 27 °C (11-14 d), and 25 °C (15-28 d). From D29 to D56, half of the chambers were constantly kept at TN conditions (25 °C), while in the other half the birds were subjected to cyclic HS (36 °C from 9:00 AM to 6:00 PM).

At D56, 2 birds per pen (i.e., 6 birds/environmental temperature/population) were randomly chosen and euthanized via cervical dislocation. Hypothalamic samples were collected and treated according to the procedures illustrated by Piekarski et al. (2016). Briefly, the brain was extracted from the skull and immersed in 2-methylbutane (Sigma, St. Louis, MO, U.S.) in dry ice for 60 s to provide the firmness needed for dissection of the hypothalamus. This was done following the stereotaxic atlas of the chick brain published by Kuenzel and Masson (1988). Brains were placed on a cold metal plate with their ventral side visible. A forward cut was performed at the corticoseptomesencephalic tract (otherwise known as the septopalliomesencephalic tract), while a posterior cut was also performed at the third oculomotor nerve. Two cuts were done bilaterally 2 mm from the brain midline. Lastly, a 5 mm cut from the brain base was performed dorsally to collect the whole hypothalamus. Hypothalamic samples were stored at -80 °C until analysis.

LAB ANALYSIS

For S4E1 and S4E2, RNA isolation, reverse transcription, and real-time quantitative PCR were carried out as previously reported by Dridi's group (Greene et al., 2019b; a, 2020). Total RNA was isolated from hypothalamic sample shares with Trizol (Life Technologies, Carlsbad, CA, U.S.) according to the manufacturer's instructions. Purity and concentrations of RNA were evaluated through Take3 micro-volume plate and Synergy HT multimode microplate reader (BioTek, Winooski, VT, U.S.). One sample belonging to group JF-HS was omitted due to poor RNA quality. RNA samples were RQ1 RNase-free DNase treated (Promega, Madison, WI, U.S.), and 1 µg of RNA was reverse transcribed with qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, U.S.). The reverse transcription reaction was performed at 42 °C for 30 min, followed by an incubation of 5 min at 85 °C. Real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR System) was performed

in a total reaction of 12.5 µL with 2.5 µl of cDNA, 0.5 µL of forward and reverse primers, and SYBR Green Master Mix (ThermoFisher Scientific, Rockford, IL, U.S.). The chicken-specific oligonucleotide primers used are listed in **Annex 5**. The qPCR cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of a two-step amplification program (95 °C for 15 s and 58 °C for 60 s). The relative expression of target genes was computed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008) with 18S rRNA gene as the housekeeping gene and group JF-TN as the calibrator.

For S4E2, protein isolation and Western Blot analysis of hypothalamic samples were performed following the procedures detailed in the article by Lassiter et al. (2015). Hypothalamic samples shares were homogenized in lysis buffer (10 mmol/L Tris base, pH 7.4; 150 mmol/L NaCl; 1 mmol/L EDTA; 0.1% Triton X-100; 0.5% Nonidet P-40; protease and phosphatase inhibitors) using glass beads and a Bullet Blender Storm (NextAdvance, Averill Park, NY, U.S.). Total protein concentration was assessed with Synergy HTX (BioTek) and a Bradford assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.). Proteins (80 µg) were run on 4-12% Novex Bis–Tris gels (Life Technologies) and then transferred to polyvinylidene difluoride membranes. Membranes were blocked using TBS with 5% non-fat milk and Tween 20 at RT for 1 h. Membranes were washed with TBS and incubated with primary antibodies (1:500-1:1,000) at 4 °C overnight. The primary antibodies used were rabbit polyclonal anti-phospho AMPKα1/2^{Thr172} (#2531, Cell Signaling Technology, Danvers, MA, U.S.), anti-AMPKα1/2 (#2795, Cell Signaling Technology), anti-HSP90 (#PA5-17610, Pierce Thermo Scientific, Rockford, IL, U.S.), goat polyclonal anti-HSP60 (#sc-1052, Santa Cruz Biotechnology, Dallas, TX, U.S.), mouse monoclonal anti-HSP70 (#MAI-91159, Pierce Thermo Scientific) and rabbit anti-GAPDH (Santa Cruz Biotechnology). After a wash with TBS, HRP-conjugated secondary antibodies (1:5,000) were added to 5% non-fat milk in TBS and Tween 20 and incubated with the membranes at RT for 1 h. Protein signals were visualized through chemiluminescence with Super ECL (ABP BioSciences, Beltsville, MD, U.S.) and images were captured using FluorChem M MultiFluor System (ProteinSimple, Santa Clara, CA, U.S.). Image acquisition and analysis were performed with AlphaView software v3.4.0 (ProteinSimple). Relative protein levels are presented as the ratio of phospho protein/Pan protein or total protein/GAPDH.

DATA ANALYSIS

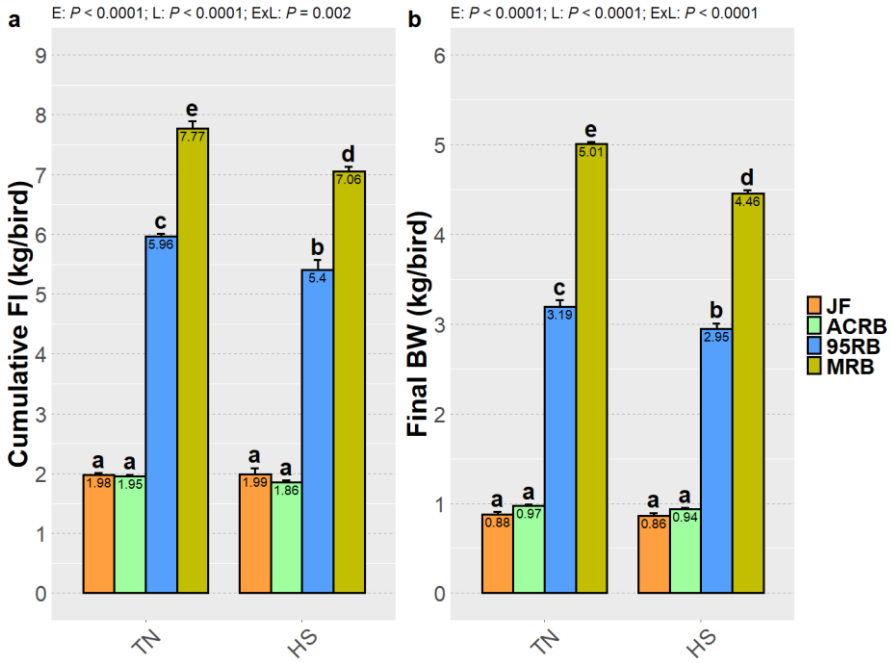
Molecular data of S4E1 and S4E2 were analyzed with a two-way ANOVA testing the effects of environmental temperature, population, and their interaction, with the sampled animal as the experimental unit. Means were separated using Tukey's HSD post-hoc test where appropriate. Differences were considered significant at a $P < 0.05$. These analyses were performed with R (R Core Team, 2020) and Graph Pad Prism v6.00 (Graph Pad Software, La Jolla, CA, U.S.).

7.4 Results

For the benefit of readers, growth performances recorded by Abdelli et al. (2021) in the trial in which the samples analyzed in S4 were collected have been summarized in **Figure 39**. Obviously, cumulative FI is the most relevant parameter due to the aims of S4. MRB showed the highest cumulative FI followed by 95RB regardless of the environmental temperature; the exposure to HS significantly decreased cumulative FI for 95RB and MRB compared to their TN counterparts; HS did not affect cumulative FI for JF and ACRB.

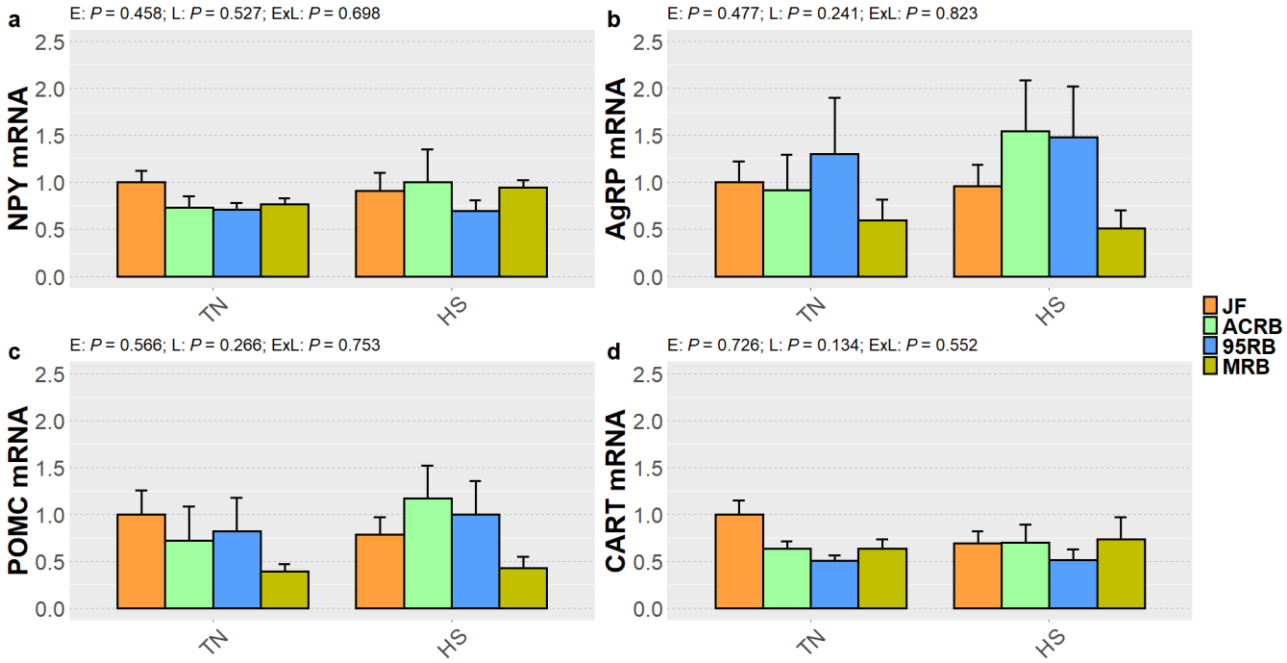
At the molecular level, neither environmental temperature nor population significantly influenced mRNA expression of neuropeptide Y (**NPY**), agouti-related peptide (**AgRP**), POMC, and cocaine and amphetamine regulated transcript (**CART**) (**Figure 40**). Similarly, mRNA expression of the five melanocortin receptors (**MCR**) was not affected in a significant fashion, even though MC1R exhibited a decreasing population-dependent trend from JF to MRB under both TN and HS conditions ($P = 0.09$) (**Figure 41**). The expression of orexin (**ORX**), its two receptors (**ORXR**), and corticotropin releasing hormone (**CRH**) genes also appeared to be unaffected by environmental temperature and population (**Figure 42**).

Figure 39 | Cumulative FI (**a**) and final BW (**b**) recorded in the 56-day trial in which the samples analyzed in S4 were collected



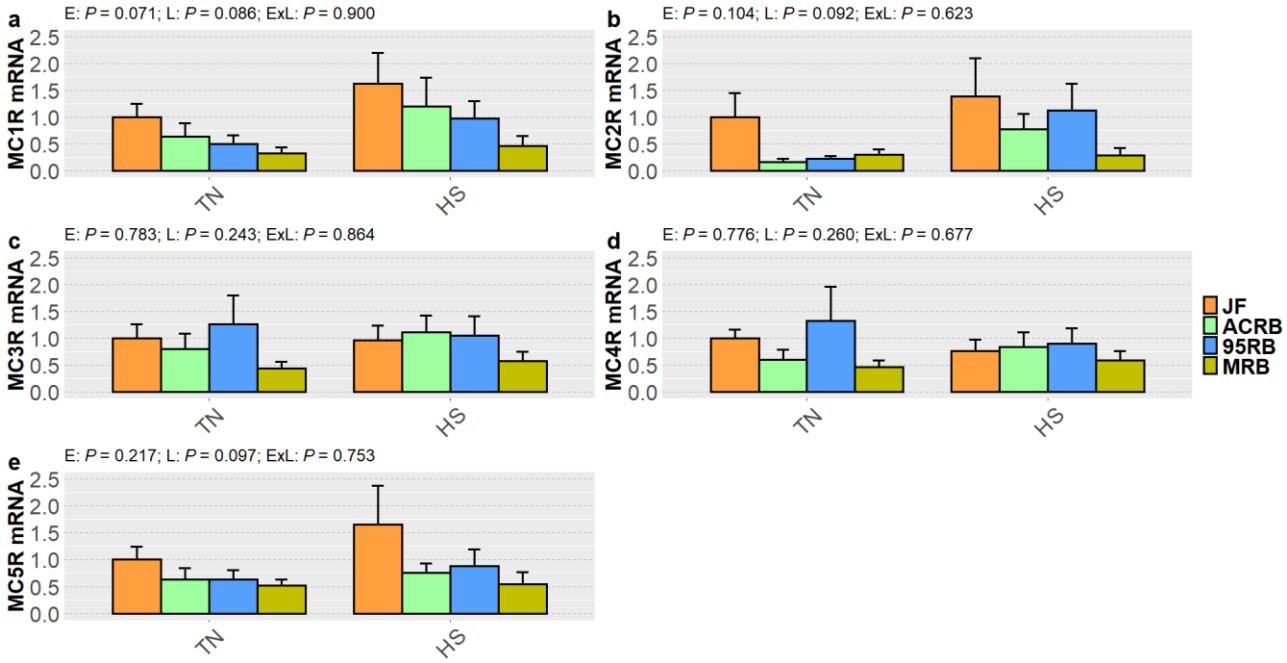
Note: Means of 3 replicate pens/environmental temperature (E)/population (L) are the colored bars. Means with distinct letters are significantly different ($P < 0.05$). Error bars represent SE.
Data source: Abdelli et al. (2021).

Figure 40 – S4E1 | Hypothalamic mRNA expression of NPY (**a**), AgRP (**b**), POMC (**c**), and CART (**d**) in JF, ACRB, 95RB, and MRB under TN and HS conditions at D56



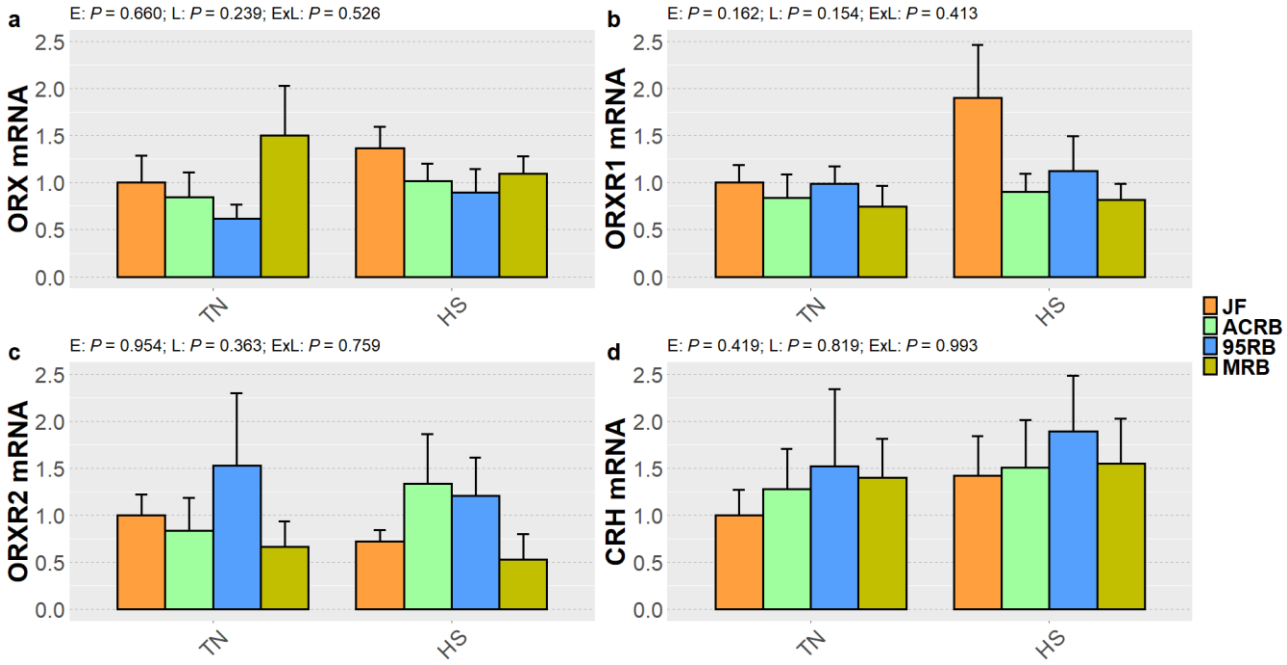
Note: Means of 6 birds/environmental temperature (E)/population (L) are the colored bars. Error bars represent SE.

Figure 41 – S4E1 | Hypothalamic mRNA expression of the MCR system in JF, ACRB, 95RB, and MRB under TN and HS conditions at D56



Note: Means of 6 birds/environmental temperature (E)/population (L) are the colored bars. Error bars represent SE.

Figure 42 – S4E1 | Hypothalamic mRNA expression of ORX system (**a-c**) and CRH (**d**) in JF, ACRB, 95RB, and MRB under TN and HS conditions at D56

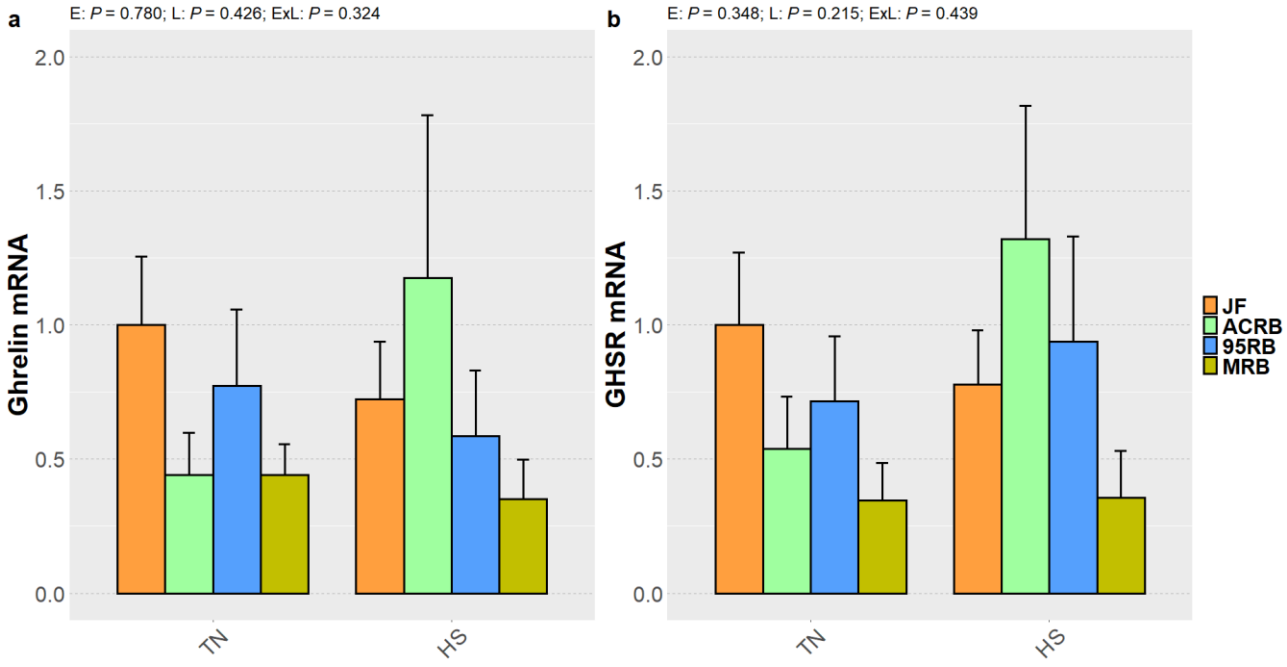


Note: Means of 6 birds/environmental temperature (E)/population (L) are the colored bars. Error bars represent SE.

Likewise, no significant differences were found for mRNA expression of ghrelin and its receptor (**GHSR**) (**Figure 43**). While the expression of leptin receptor (**Ob-R**) and adiponectin receptors

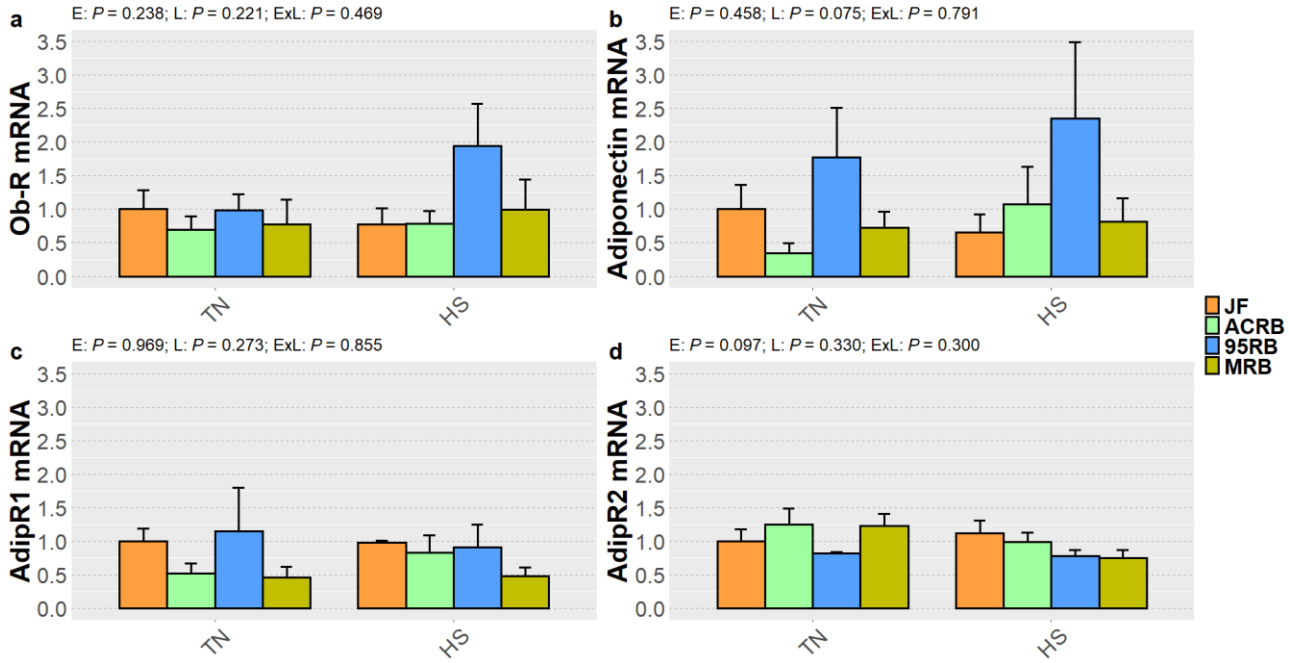
(**AdipR**) remained unchanged, that of adiponectin showed a trend toward significance with 95RB exhibiting the highest mRNA level irrespective of the environmental temperature (P = 0.08) (**Figure 44**). Although the environmental temperature did not affect mRNA expression of visfatin and neurosecretory proteins GL and GM (**NPGL** and **NPGM**, respectively), JF showed a greater mRNA abundance of visfatin than ACRB under TN conditions (P < 0.05) (**Figure 45**).

Figure 43 – S4E1 | Hypothalamic mRNA expression of the ghrelin system in JF, ACRB, 95RB, and MRB under TN and HS conditions at D56



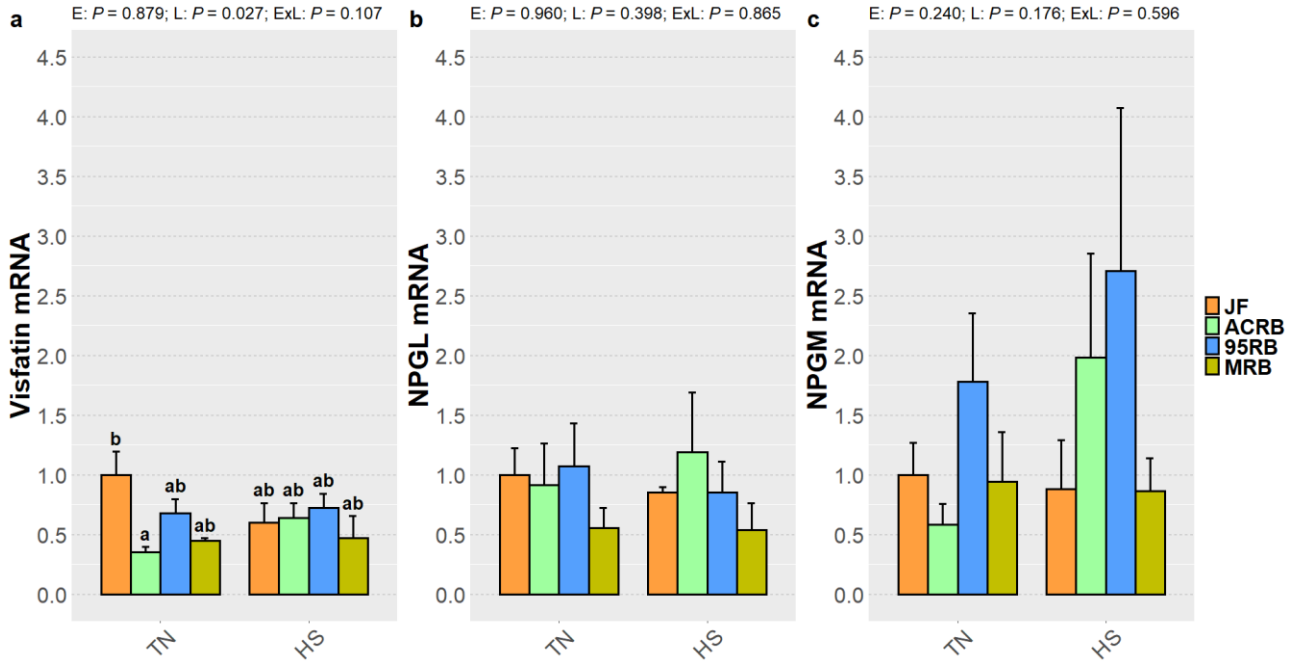
Note: Means of 6 birds/environmental temperature (E)/population (L) are the colored bars. Error bars represent SE.

Figure 44 – S4E1 | Hypothalamic mRNA expression of Ob-R (**a**) and the adiponectin system (**b-d**) in JF, ACRB, 95RB, and MRB under TN and HS conditions at D56



Note: Means of 6 birds/environmental temperature (E)/population (L) are the colored bars. Error bars represent SE.

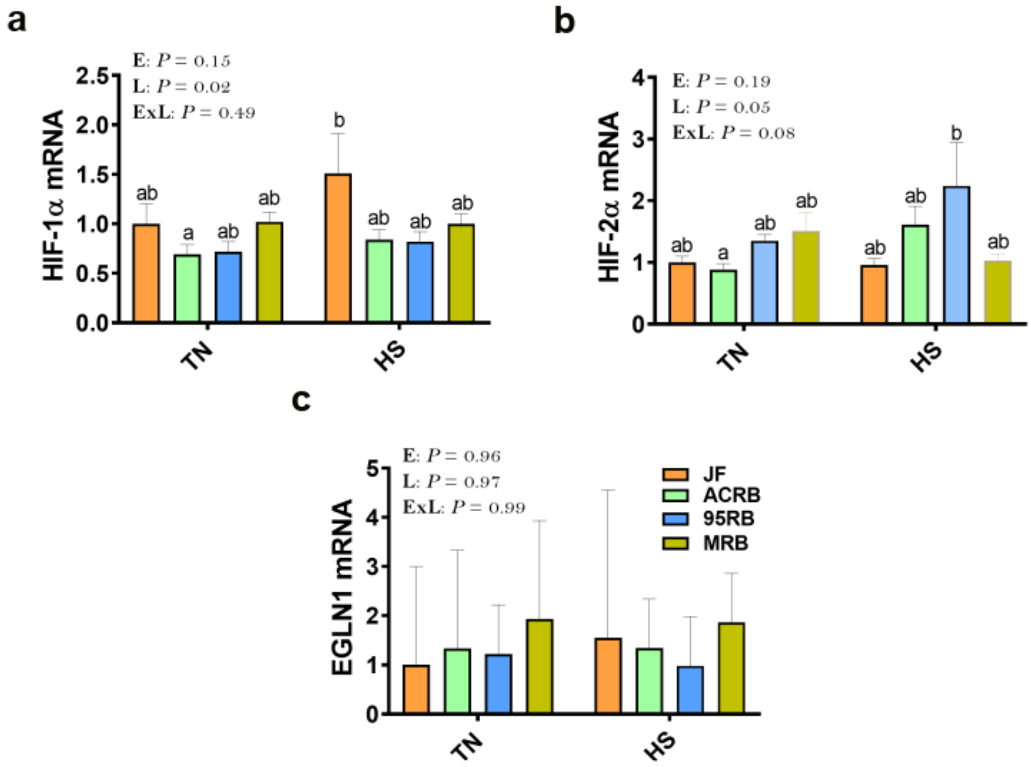
Figure 45 – S4E1 | Hypothalamic mRNA expression of visfatin (**a**), NPGL (**b**), and NPGM (**c**) in JF, ACRB, 95RB, and MRB under TN and HS conditions at D56



Note: Means of 6 birds/environmental temperature (E)/population (L) are the colored bars. Means with distinct letters are significantly different ($P < 0.05$). Error bars represent SE.

Moving to the results of S4E2, HS did not affect hypothalamic expression of HIF-1α, HIF-2α, and hypoxia-inducible factor prolyl hydroxylase 2 (**EGLN1**). However, there was a population effect (P = 0.02) for HIF-1α with higher levels in JF compared to broiler lines, especially under HS conditions. Hypothalamic expression of HIF-2α and EGLN1 did not differ between the populations under both environmental conditions (**Figure 46**).

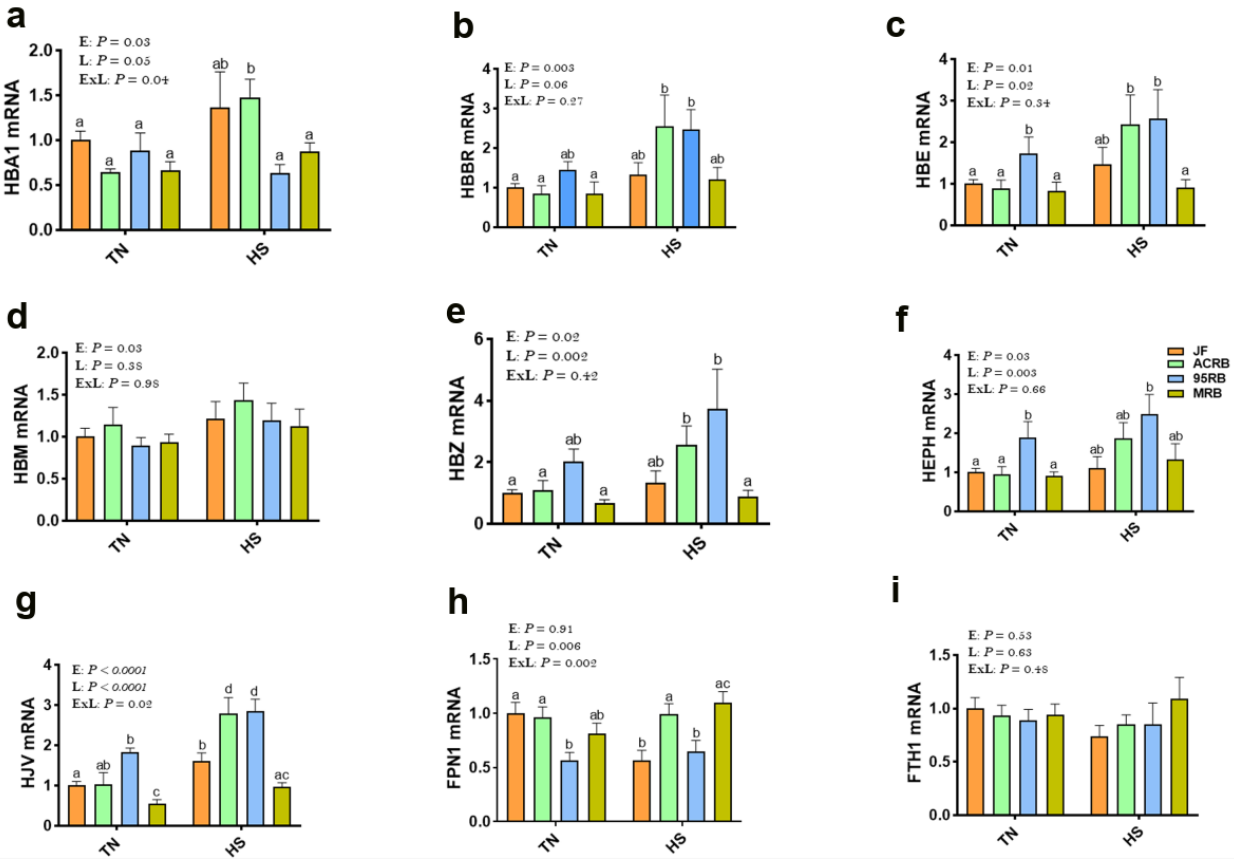
Figure 46 – S4E2 | Hypothalamic mRNA expression of HIF complex in JF, ACRB, 95RB, and MRB under TN and HS conditions at D56



Note: Means of 6 birds/environmental temperature (E)/population (L) are the colored bars. Means with distinct letters are significantly different (P < 0.05). Error bars represent SE.

For oxygen homeostasis-associated genes, there was a significant population effect for **HBA1**, **HBE**, **HBZ**, **HEPH**, **HJV**, and **FPN1** (see "**ABBREVIATIONS**" for the meaning of these acronyms). The 95RB line exhibited an elevated mRNA abundance of HBE, HEPH, and HJV under both environmental conditions and HBZ under HS conditions. ACRB birds subjected to HS showed an up-regulation of mRNA expression of HBA1, HBBR, HBE, HBZ, and HJV, while JF and 95RB of HJV compared to their TN counterparts. However, HS down-regulated mRNA expression of FPN1 in JF (**Figure 47**).

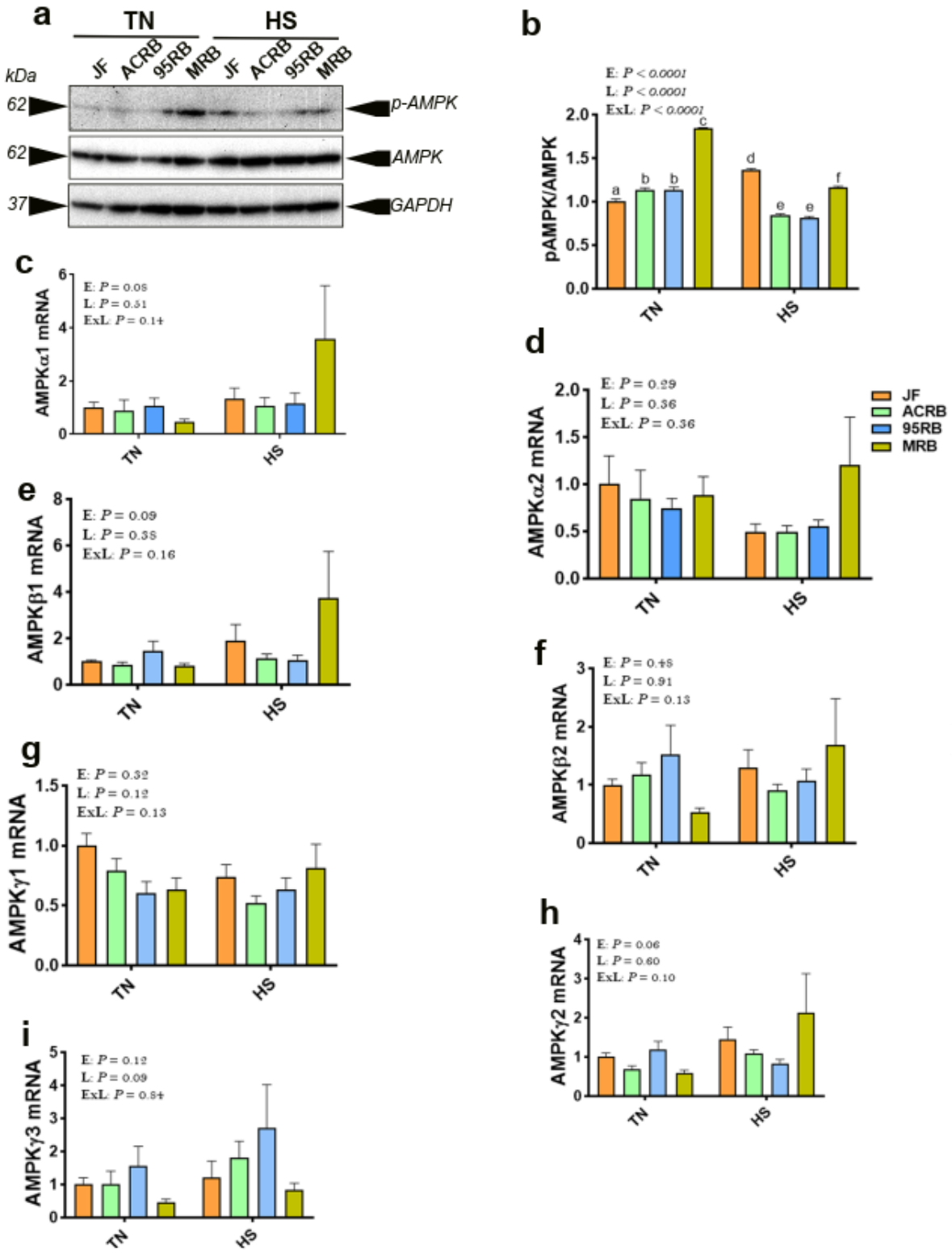
Figure 47 – S4E2 | Hypothalamic mRNA expression of oxygen homeostasis-associated genes in JF, ACRB, 95RB, and MRB under TN and HS conditions at D56



Note: Means of 6 birds/environmental temperature (E)/population (L) are the colored bars. Means with distinct letters are significantly different ($P < 0.05$). Error bars represent SE.

HS exposure increased the phosphorylated levels of AMPKα1/2^{Thr172} in JF hypothalamus, but reduced it in the three broiler lines compared to TN conditions. Under TN conditions, MRB exhibited higher levels of hypothalamic p-AMPKα1/2^{Thr172}, followed by 95RB, ACRB, and JF. Under HS conditions, however, JF hypothalamus contained elevated levels of p-AMPKα1/2^{Thr172}, followed by MRB, ACRB, and 95RB, which resulted in a significant interaction of environmental temperature and population ($P < 0.0001$). mRNA expression of AMPKα1, AMPKα2, AMPKβ1, AMPKβ2, AMPKγ1, AMPKγ2, and AMPKγ3 did not differ between the populations studied under both environmental conditions (**Figure 48**).

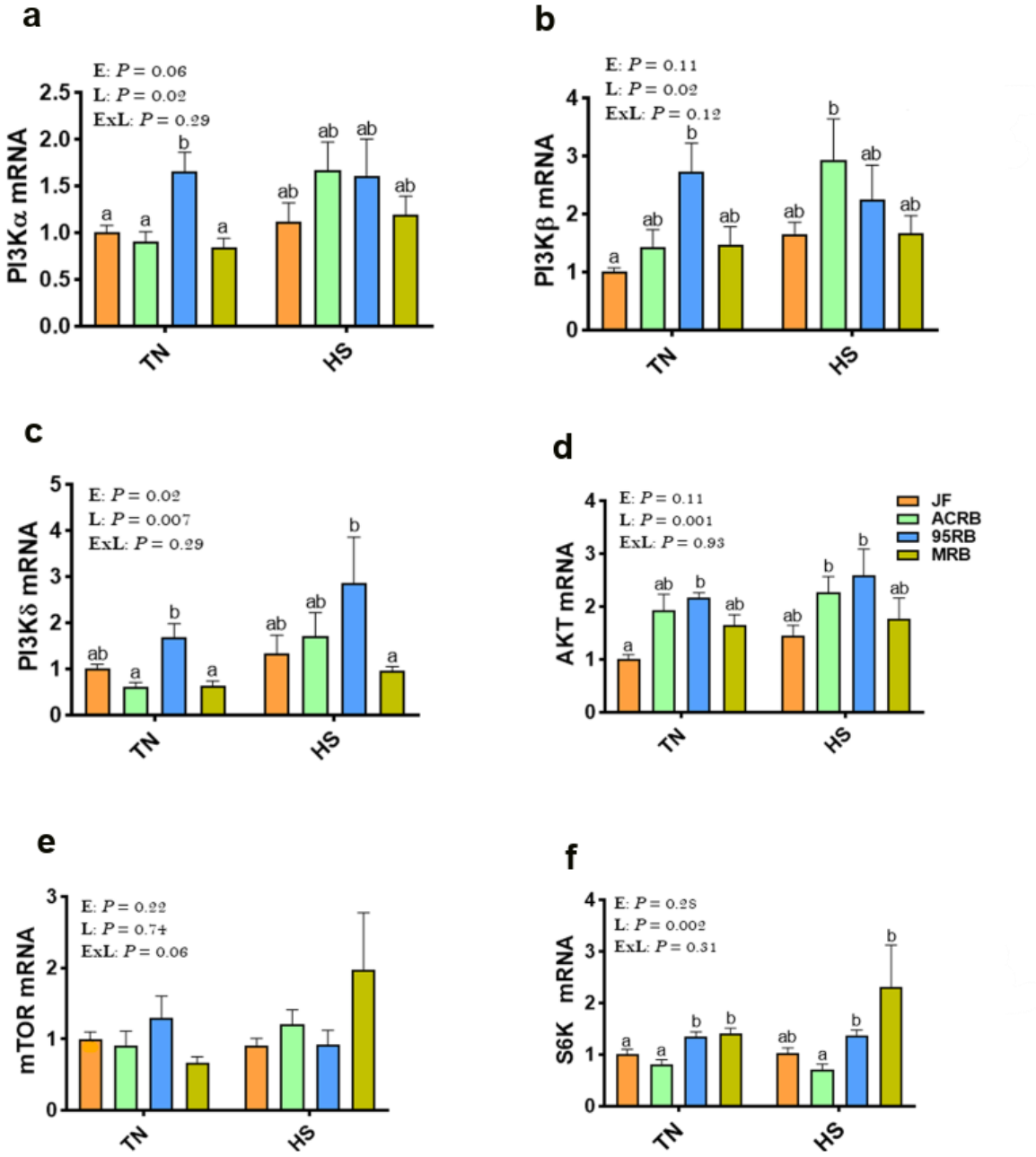
Figure 48 – S4E2 | Hypothalamic expression of AMPK pathway in JF, ACRB, 95RB, and MRB under TN and HS conditions at D56



Note: Means of 6 birds/environmental temperature (E)/population (L) are the colored bars. Means with distinct letters are significantly different ($P < 0.05$). Error bars represent SE. One representative blot is shown in figure **a**.

Analyzing the upstream regulators of HIF, qPCR data showed that HS did not affect the expression of protein kinase B (**Akt**), mTOR, and S6K. Under TN conditions, 95RB exhibited higher mRNA levels of PI3Kα and PI3Kδ than the other populations. Hypothalamic expression of S6K gene was found to be up-regulated in 95RB and MRB compared to JF and ACRB under both environmental conditions (**Figure 49**).

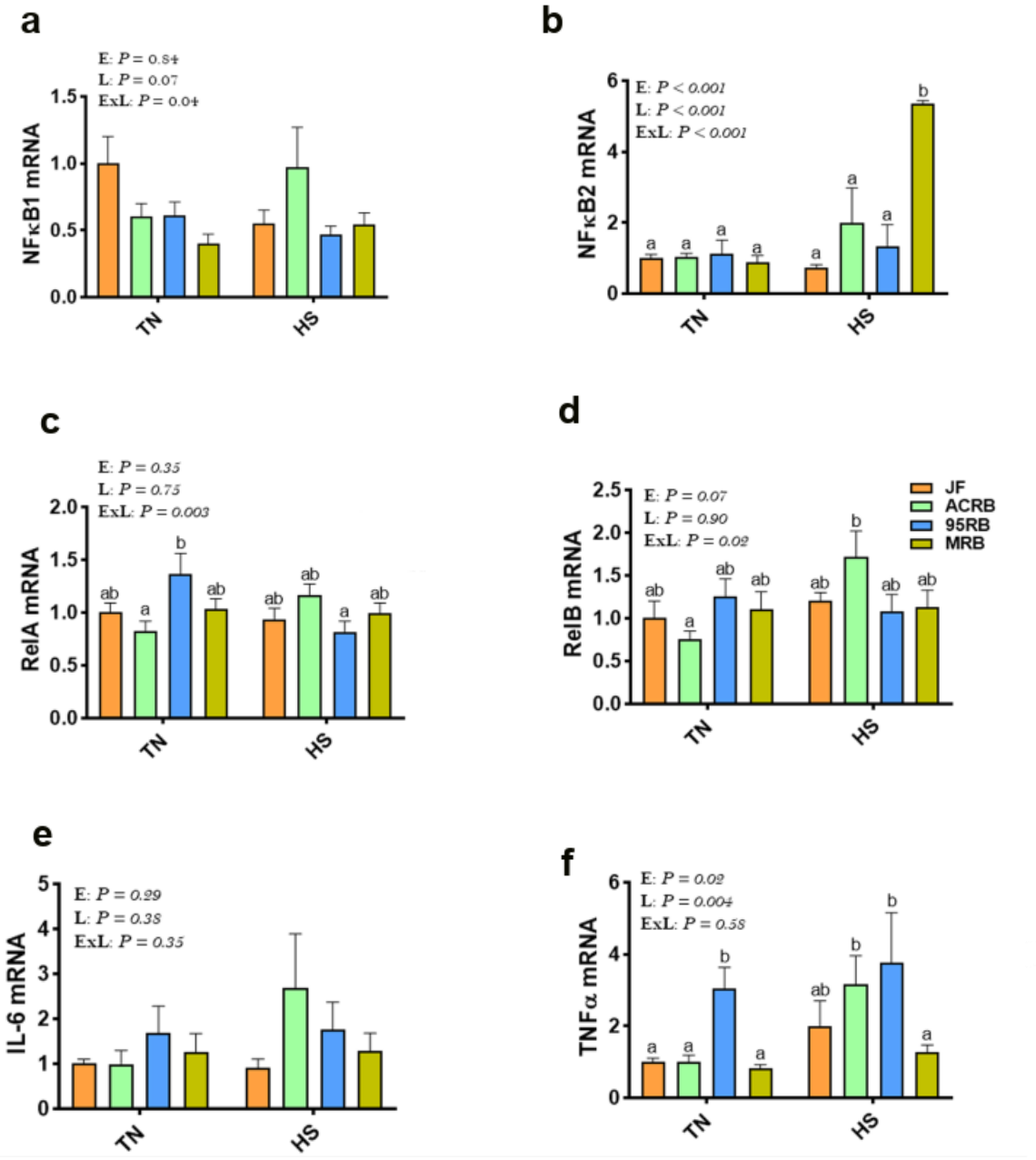
Figure 49 – S4E2 | Hypothalamic mRNA expression of PI3K-mTOR-S6K pathway in JF, ACRB, 95RB, and MRB under TN and HS conditions at D56



Note: Means of 6 birds/environmental temperature (E)/population (L) are the colored bars. Means with distinct letters are significantly different ($P < 0.05$). Error bars represent SE.

HS up-regulated gene expression of NF-κB2 in MRB and RelB and TNFα in ACRB, but it down-regulated that of RelA in 95RB compared to their TN counterparts. Under TN conditions, 95RB showed greater levels of RelA and TNFα than the other populations. Under HS conditions, MRB manifested elevated mRNA abundance of NF-κB2, while ACRB and 95RB of TNFα. mRNA expression of NF-κB1 and IL-6 did not differ between the populations under both environmental conditions (**Figure 50**).

Figure 50 – S4E2 | Hypothalamic mRNA expression of NF-κB pathway in JF, ACRB, 95RB, and MRB under TN and HS conditions at D56



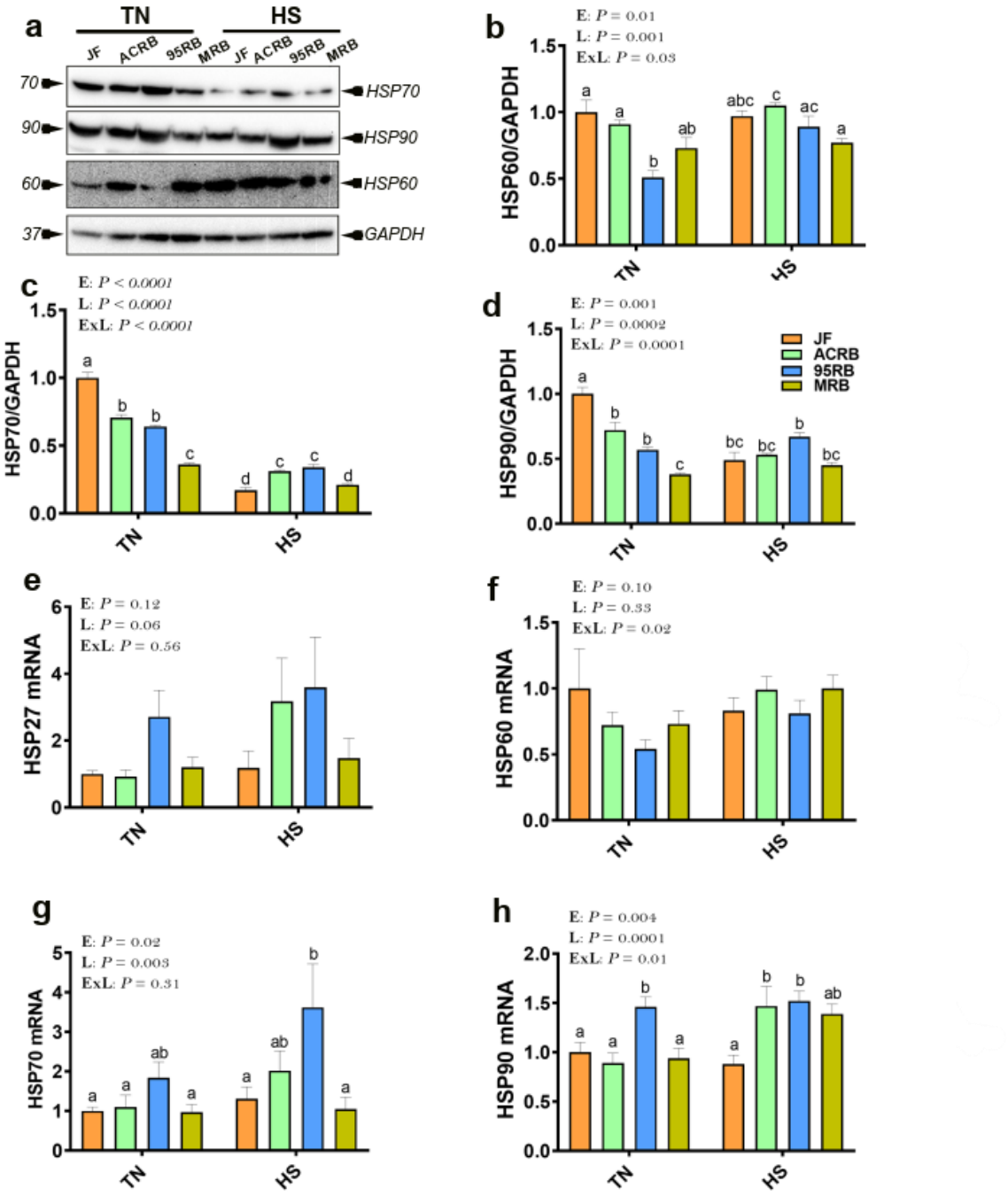
Note: Means of 6 birds/environmental temperature (E)/population (L) are the colored bars. Means with distinct letters are significantly different ($P < 0.05$). Error bars represent SE.

The immunoblot analysis showed that HS increased the levels of HSP60 protein in ACRB and 95RB, and reduced that of HSP70 protein in all birds compared to their TN counterparts. Protein level of HSP90 was reduced by HS only in JF. At the mRNA level, HS up-regulated the expression of HSP90 in ACRB, but it did not elicit any change in HSP27, HSP60, and HSP70. Under HS conditions, HSP90 mRNA level was higher in broiler lines than in JF. Under TN conditions, however, JF generally exhibited the highest protein level of HSPs (**Figure 51**).

7.5 Discussion

As was pointed out in the introductory chapter (see section **1.1** "**The Broiler Industry: Nothing Short of a Success Story**"), genetic selection has produced broilers that can efficiently convert feed into meat, with tremendously high growth rates at early market ages. Growth performance results reported by Dridi's lab in the work of Abdelli et al. (2021) are consistent with the extraordinary improvements attributable to selection because MRB line considerably outperformed the other chicken populations studied. Abdelli et al. (2021) have also confirmed that hyperselected broilers (i.e., 95RB and MRB) are very sensitive to elevated ambient temperatures since they exhibited, under HS conditions, marked reductions in FI compared to their slow-growing predecessors (i.e., ACRB) and ancestors (i.e., JF). So, although they have been reshaped by genetic selection, modern broilers still rely on an evolutionary preserved strategy to face HS, namely reducing feed consumption to decrease metabolic heat production. Interestingly, FI is tightly controlled by the hypothalamic centers of hunger and satiety (Simpson et al., 2008; Waterson and Horvath, 2015; Timper and Brüning, 2017; Luquet et al., 2019) with FRHN (Arora and Anubhuti, 2006; Furuse et al., 2007; Tachibana and Tsutsui, 2016; Delgado et al., 2017). In addition, hypothalamic FI regulation and oxygen/hypoxia-sensing have been shown to be interconnected (Virtue and Vidal-Puig, 2011). An attempt was then made to determine the expression profile of FRHN and hypothalamic pathways associated with hypoxia and oxygen homeostasis in the abovementioned four chicken populations (i.e., JF, ACRB, 95RB, and MRB) reared under HS conditions.

Figure 51 – S4E2 | Hypothalamic expression of HSPs in JF, ACRB, 95RB, and MRB under TN and HS conditions at D56



Note: Means of 6 birds/environmental temperature (E)/population (L) are the colored bars. Means with distinct letters are significantly different ($P < 0.05$). Error bars represent SE. One representative blot is shown in figure **a**.

The most renowned FRHN are the orexigenic NPY and AgRP and the anorexigenic POMC and CART (Delgado et al., 2017; Gaspar and Velloso, 2018). In S4E1, no variation was detected for gene expression of these major FRHN, confirming the results of Beckford et al. (2020) at least for mRNA level of POMC in the hypothalamus of heat-stressed broilers. It can therefore be assumed that these

major FRHN were not linked to FI differences between the chicken populations studied here. POMC-derived ligands, named melanocortins, bind to five MCR receptors to play their anorexigenic role (Tachibana and Tsutsui, 2016). MC1R showed a population-dependent trend under both environmental conditions, with a gradually decreasing mRNA abundance across the four populations. It is interesting to note that Blankenship et al. (2015) have reported an overexpression of MCRs in male quails selected for low feed efficiency. Further research on the hypothalamic melanocortin system and its different expression in poor- and high-performing poultry is therefore recommended.

The ORX system was also unaffected. While mammalian orexins have been shown to stimulate appetite, the effect of orexins in chickens is still unclear and a matter of scientific debate (Furuse et al., 1999). Nonetheless, ORX system was found to be central in muscular and hepatic energy metabolism in avian species (Lassiter et al., 2015; Greene et al., 2020). CRH, widely known for its involvement in stress response (Weninger et al., 1999), has been shown to possess a substantial anorectic effect in chickens (Furuse et al., 1997). Tachibana and Tsutsui (2016) posited that CRH is a downstream mediator of anorexigenic pathways in the chicken brain. The thermal stressor applied by Abdelli et al. (2021) was expected to increase hypothalamic expression of CRH, with a consequent CRH-mediated role in FI depression shown by heat-stressed 95RB and MRB. However, no differences were found in mRNA level of CRH, supporting again the study by Beckford et al. (2020) on broilers exposed to HS.

Some neuropeptides can play divergent roles depending on the animal class (Volkoff et al., 2005; Luquet et al., 2019). Ghrelin, a peripheral hormone mainly released by the gastric mucosa (Arora and Anubhuti, 2006), is an example of these discrepancies (Tachibana and Tsutsui, 2016). Unlike mammals and many fishes, ghrelin has been shown to suppress FI in chickens (Furuse et al., 2001; Tachibana and Tsutsui, 2016) and quails (Shousha et al., 2005). Saito et al. (2005) have observed that the intracerebroventricular injection of ghrelin induced an activation of the hypothalamic-pituitary-adrenal axis mediated by CRH resulting in corticosterone secretion and, eventually, hyperactivity and anorexia in chicks. These researchers also confirmed earlier findings demonstrating that ghrelin is expressed in the chicken brain (Kaiya et al., 2002). Moreover, at the hypothalamic and pituitary level, ghrelin has been shown to bind to GHSR, a G-protein coupled receptor that modulates GH release (Wren et al., 2000). Here, hypothalamic mRNA expression of ghrelin and GHSR showed no significant differences

due to environmental temperature. The receptor of leptin, Ob-R, is important in the control of feeding in chickens because Lei et al. (2018) have demonstrated that the administration of anti-Ob-R antibodies promotes FI in growing birds. Examining the targets of leptin in the chicken hypothalamus, Dridi et al. (2005) have also revealed that leptin down-regulates the expression of its own receptor. In S4E1, however, the differences in Ob-R mRNA level between the populations were not significant under either environmental condition.

The adipose tissue has been shown to produce peptides involved in the regulation of feeding behavior and energy homeostasis, such as adiponectin and visfatin. In addition to adipocytes, however, adiponectin has been shown to be expressed by several chicken tissues (Maddineni et al., 2005) and its two receptors (i.e., AdipR) have been found in the hypothalamus (Kadowaki et al., 2008). Appetite stimulation, longer-term fat modulation, starvation signaling properties, and antagonism to leptin associated with the adiponectin system have thoroughly been reviewed by Kadowaki et al. (2008). The observed tendency of 95RB to overexpress adiponectin irrespective of the environmental temperature is difficult to interpret and further investigations are required to clarify the role of the adiponectin system in hypothalamic modulation of FI in chickens. Visfatin, the other adipokine mentioned before, has been shown to have a wide array of physiological and pathophysiological functions (Dahl et al., 2012). In contrast to mammals, visfatin stimulates appetite in chickens (Cline et al., 2008). Furthermore, Ons et al. (2010) have demonstrated that visfatin is ubiquitously expressed in chickens and is interconnected with numerous regulatory factors of energy balance. Surprisingly, the difference in visfatin expression between JF and ACRB seems to not be consistent with their feeding behavior as cumulative FI was essentially the same for all the populations studied.

According to Parker and Bloom (2012), the involvement of several FRHN in feeding regulation has already been verified, but many others are still under study or have not been identified yet. For instance, two novel neuropeptides, called NPGL and NPGM, have recently been discovered in the chicken hypothalamus: NPGL is orexigenic, whereas NPGM exerts an anorectic activity (Ukena et al., 2014; Shikano et al., 2017, 2018). Although the results of S4E1 do not allow to establish a potential connection between these FRHN and the FI differences measured by Abdelli et al. (2021), additional studies on NPGL and NPGM in chickens are strongly recommended.

There are several possible explanations for the stability of FRHN gene expression found in S4E1. The first speculation is based on the hypothalamic integration of circadian information (Bechtold and Loudon, 2013; Delgado et al., 2017): the fact that birds had been fed before sampling and sacrificed at different times for unavoidable operational requirements may have affected the hypothalamus, resulting in a flattening of FRHN expression. A second reason may be related to the persistence of the thermal stress: birds might have gradually acclimatized to prolonged exposure to high ambient temperatures, thereby accommodating their physiological and hypothalamic responses to chronic HS (Sykes and Fataftah, 1986; Yahav, 2009; Collier et al., 2019). Thirdly, the uniform gene expression of some FRHN across the four chicken populations may be partly explained by steady hypothalamic pathways unaffected by evolution or genetic selection. Fourthly, only mRNA abundance was measured in S4E1, while it is plausible that protein levels may have been influenced in a different way. Lastly, the use of the entire hypothalamus might have masked mRNA expression in specific hypothalamic nuclei, affecting the results of the analysis. Overall, S4E1 suggests that different central pathways other than FRHN may have potentially been associated to the sizable FI differences between the chicken populations studied. The formulation of this hypothesis was the starting point of S4E2, the results of which are discussed below.

Although HS did not affect the expression of the HIF complex (i.e., HIF-1α, HIF-2α, and EGNL1), hypothalamic mRNA abundance of HIF-1α was higher in JF than in the three broiler lines, particularly under HS conditions. HIF-1 is a dimeric transcription factor complex (Wang et al., 1995b) that increases vascularization in hypoxic areas, thereby playing an essential role in homeostasis and response to low oxygen levels (Carmeliet et al., 1998; Semenza, 2009). As HS has been shown to induce hypoxia in internal organs, including the brain (Bain et al., 2014), and JF birds were found to be more thermotolerant than modern broilers (Soleimani et al., 2011), the data of S4E2 suggest that increased gene expression of hypothalamic HIF-1α might improve central O_2 transport capacity and enhance hypoxia tolerance in JF. In support of this hypothesis, Tabler et al. (2020a) have shown that HS increased core body temperature of broilers but not that of JF, indicating that the latter have better ability to withstand high ambient temperatures. Furthermore, modern broilers are heavier than JF and a negative association between body weight/size and heat tolerance has been well documented in chickens (Wilson

et al., 1975b; Sandercock et al., 2006). Although it is generally accepted that the O_2 transport pathway of birds exhibits unique characteristics that support energetic activity and aerobic metabolism during hypoxia (Monge and Leon-Velarde, 1991), modern broilers have high O_2 requirements because of their strenuous digestion, high metabolism, and fast growth, without a concomitant development of the efficiency of the cardiovascular and respiratory systems (Reeves et al., 1991; Olkowski and Classen, 1998). In fact, elegant studies have shown that fast-growing broilers have thicker respiratory membrane, lower O_2 transfer efficiency, and lower hemoglobin oxygenation capability (Reeves et al., 1991; Julian and Mirsalimi, 1992). Moreover, a relationship between heat tolerance and oxygen homeostasis in chickens has been reported (Wilson et al., 1975a). Whilst further mechanistic studies are warranted, the results of S4E2 indicate that hypothalamic HIF-1α may be involved in the regulation of FI as well as hypoxia- and thermo-tolerance in chickens.

The acclimatization and adaptive erythropoietic response to hypoxia generally involve an increase in hematocrit and hemoglobin concentrations (Kanstrup and Ekblom, 1984; Monge and Leon-Velarde, 1991; Schuler et al., 2010). However, qPCR analysis of S4E2 did not show a significant alteration in hypothalamic expression of oxygen homeostasis-associated genes, except for a significant down-regulation of FPN1 in heat-stressed JF, up-regulation of HJV in JF, ACRB, and 95RB under HS conditions, and a significant increase in HBA1, HBBR, HBE, and HBZ in heat-stressed ACRB. This apparent inconsistency is unexpected and does not support the abovementioned adaptive response to hypoxia, at least for JF. So, although the aforesaid conclusion is valid, it must be considered cautiously and supported by further evidence for several reasons. First, only gene expression was measured, whereas hemoglobin saturation and its oxygen-binding properties were not, which is a limitation for S4E2. Second, the roles of hypothalamic oxygen homeostasis-associated genes in FI regulation are still unknown in chickens. As caloric restriction has been shown to regulate FPN1 expression (Wei et al., 2014; Luo et al., 2018) and iron has been reported to control FI in mammals (Gao et al., 2015), it is therefore reasonable to assume that a dysregulation of FPN1 in heat-stressed JF might have played a role in HS adaptation and FI control. Nevertheless, further in-depth investigations are required.

In addition to its role in oxygen sensing, HIF regulates many biological processes, including inflammation (Cramer et al., 2003; Corcoran and O'Neill, 2016), glucose metabolism (Fulda and

Debatin, 2007; Ochiai et al., 2011) – both of which being reported to be affected by HS (Lee et al., 2015; Kimball et al., 2018) – and FI (Buchanan and Johnson, 2007; Dwarkasing et al., 2016). In S4E2, there was an up-regulation of hypothalamic NF-κB2 caused by HS only in MRB, RelB and TNFα in ACRB, and a down-regulation of RelA in 95RB. The activation of NF-κB in the hypothalamus has been shown to recruit pro-inflammatory microglia and promote appetite and overeating in mammals (Zhang et al., 2008; Li et al., 2012), suggesting that NF-κB2 might have influenced FI regulation in MRB. However, this effect seems to be only during HS as hypothalamic expression of NK-κB2 was not affected under TN conditions. NF-κB has been shown to be regulated by HS (Kretz-Remy et al., 2001) and to induce the expression of various pro-inflammatory genes, such as TNFα and IL-6 (Wang et al., 2014), which have been reported to affect feeding behavior (Romanatto et al., 2007; Buchanan and Johnson, 2007; Mishra et al., 2019). The lack of hypothalamic modulation of TNFα and IL6 indicates that this cytokine pathway was not involved. It is important to note, again, that only the measurement of gene expression was performed and not that of proteins (the motor elements and workhorse of cells) due to the lack of cross-reactive antibodies.

To gain further insights, hypothalamic expression of AMPK and mTOR pathways – the energy and nutrient sensor, respectively (Hardie et al., 2012; Sabatini, 2017) – was assessed. These two pathways have been shown to modulate HIF expression and feeding behavior (Minokoshi et al., 2004; Cota et al., 2006; Zhang et al., 2011). AMPK is a phylogenetically conserved serine/threonine heterotrimeric kinase, consisting of catalytic alpha subunits (i.e., α1 and α2), and regulatory beta (i.e., β1 and β2) and gamma subunits (i.e., γ1, γ2, and γ3), which senses energy levels by detecting elevation in the AMP to ATP ratio (Xiao et al., 2011; Chen et al., 2013). Under energy depletion, AMPK is fully activated through binding AMP to γ subunits and induction of Thr172 phosphorylation in α subunits, thereby promoting catabolic pathways to restore energy balance (Stein et al., 2000). Increased levels of hypothalamic p-AMPK^{Thr172} in MRB compared to the other chicken populations under TN conditions is not surprising as it indicates that modern broilers had greater energy demand due to higher growth rates than their predecessors and wild ancestor (Tickle et al., 2018). However, its inconsistent regulation under HS conditions (i.e., increase in JF and decrease in broiler lines) is intriguing, particularly as HS reduced FI in 95RB and MRB but not in JF (Abdelli et al., 2021). This suggests that central AMPK is probably

not involved in FI regulation in chickens. Interestingly, intracerebroventricular administration of AICAR (an AMP analog) or Compound C (an AMPK inhibitor) altered FI in chickens independently of AMPK activation (Xu et al., 2011). mTOR is also an evolutionary conserved and multi-tasking serine/threonine kinase that regulates various cellular processes in response to growth factor stimulation, nutrient, energy or oxygen availability (Laplante and Sabatini, 2012). S6K is one of the best characterized downstream targets of mTOR. Although several genes of the mTOR pathway were not affected, S6K gene expression was higher in 95RB and MRB broilers under both environmental conditions. In combination with findings from previous studies on rodents (Cota et al., 2006) and *Drosophila* (Wu et al., 2005), these results suggest that central S6K may stimulate FI and protein synthesis in chickens in an HS-independent manner.

Hypothalamic expression of HSPs, molecular chaperones involved in the regulation of cellular homeostasis, stress response, and FI via the JAK2/STAT3 signaling pathway (Hosoi et al., 2016), was also profiled in S4E2. Surprisingly, HS decreased the protein level of HSP70 in all chicken populations and HSP90 in JF, but it increased that of HSP60 in ACRB and 95RB. Although further studies are needed, these data indicate that central HSPs were probably regulated in a population-dependent manner on one hand, while, on the other hand, the unexpected down-regulation of HSP70 by heat load might have been associated with stress acclimation of birds.

In summary, S4 is the first study assessing the impacts of HS on FRHN and showing modulation of hypothalamic expression of hypoxia (HIF complex)- and oxygen-associated molecules, as well as their up- and down-stream mediators, by HS in three different genetically selected broilers and their ancestor. The results indicate that hypoxia and HS responses are probably connected to FI regulation in chickens, which deserves further in-depth investigation.

8 CONCLUSIONS AND FUTURE PERSPECTIVES

What follows is not merely the conclusion of this dissertation; it is the end of a thrilling journey. And, as in every journey, the end is bittersweet because you are happy, probably proud of yourself, for having finally achieved the goal, but also sad because you know you are concluding a chapter in your life. I must admit that the intense emotions I am feeling as I type make it difficult to take stock of the past three years. But I will do my best.

It has been exciting to carry out this PhD project at a time of great challenges for the broiler industry. Two important issues have emerged from the studies consulted while reviewing the literature. First, "ABF label claims" have literally taken off and gained popularity because of the real and growing threat of antibiotic resistance, of which consumers are increasingly aware. However, reduced feed efficiency, impaired gut health, and increased pathogen load and risk of enteric infection outbreaks are just some of the downsides of banning the use of AGPs in poultry and livestock husbandry. Accordingly, animal-food supply chains have been forced to reorganize their production processes to stay in business, such as raising on-farm biosecurity levels and finding reliable alternatives to AGPs to improve animal health and performance. Second, global warming has made HS a serious problem no longer limited to warm countries. The effects of HS on chickens are complex and include alterations in behavior (e.g., lethargy, reduced FI, and panting), metabolism (e.g., catabolic status, fat accumulation, and reduced skeletal muscle accretion), homeostasis (e.g., alkalosis, hormonal imbalance, immunodeficiency, inflammation, and oxidative stress) and gut functionality and health (e.g., compromised digestion and absorption, enteritis, paracellular barrier failure, and dysbiosis), which ultimately lead to decreased health and performance and deterioration in product quality. Therefore, a reasonable approach to minimize the loss of efficiency and multifaceted perturbations caused by feeding AGP-free diets and HS might be to use multitarget nutritional strategies that provide both GI and systemic benefits.

Those studied here were found to significantly improve the growth performance of ABF broilers raised under optimal thermal conditions following the breeding company's guidelines. In summary: the combined application of a synbiotic preparation sprayed on the chicks' feathering at the hatchery and administered through the feed during the growth cycle reduced cumulative FCR by 2.9%; a novel feed-

grade muramidase exhibited a dose-dependent effect, with the highest inclusion level tested (i.e., 450 g/ton feed) substantially increasing BW (+4.7%) and improving feed efficiency (i.e., cumulative FCR decreased by 2.5%) at market age compared to the control diet; supplementation of dietary arginine to increase the total arginine to lysine ratio to 1.20, that is 15.4% higher than that recommended by the NRC and around 11% greater than that reported in the nutrition specifications released by the breeding company, considerably enhanced final BW (+4.2%) and cumulative FCR (-4.2%). In addition to being all statistically significant, these results are very encouraging and offer a glimmer of hope that easily applicable strategies in commercial settings may be provided to broiler nutritionists to address the disadvantages of AGP removal.

Despite being very different, the three nutritional strategies were linked to modifications in the GI microbiota, a pillar of gut health and an "additional organ" that actively participates in host physiology. Curiously, the synbiotic increased the abundance of Firmicutes and reduced that of Bacteroidetes, while muramidase and arginine supplementations produced the opposite result. Although it is not the intention of this dissertation to debate on the potential effects of the Firmicutes to Bacteroidetes ratio on animal health, it is interesting to point out the apparent contradiction in these results. In fact, the synbiotic study corroborates the widely held view that broiler microbiotas with increased Firmicutes to Bacteroidetes ratios can promote energy collection from the diet and then make it available to the birds (especially in the form of butyrate), thereby improving their feed efficiency. The other two studies, however, challenge this theory as high-performing birds had a reduced abundance of Firmicutes (many of which were recognized as butyrate producers) and an increased abundance of Bacteroidetes. It could therefore be worth rethinking the role these bacterial phyla and their relationship play in the intestinal ecosystem of broilers and, more importantly, the influence they may have on extraintestinal metabolic pathways, such as a fascinating Bacteroidetes-mediated propionate supply for hepatic gluconeogenesis. In contrast, the increased abundance of Lactobacillaceae/Lactobacilli associated with all three strategies examined and the decreased abundance of (food-borne) pathogens found in the studies on muramidase and arginine (e.g., *C. perfringens*, *C. botulinum*, *L. monocytogenes*, and *E. coli*), further support the idea that broilers benefit from Lactobacillaceae/Lactobacilli, whereas the expansion of undesirable GI bacteria is detrimental to animal health and performance, as well as

potentially risky to food safety. The feed additive industry should therefore keep on investing in R&D of those microbiota modulators and feed-grade AAs that might favor, either directly or indirectly, beneficial GI microbial clusters at the expense of the harmful ones to help broilers grow more efficiently and better withstand environmental stressors.

Another important finding was that muramidase and arginine supplementations seemed to positively affect the metabolic profile of different tissues, particularly the levels of molecules related to energy metabolism (e.g., pyruvate, 2-oxoglutarate, glucose, creatine, and AMP) and oxidative stress (e.g., hypoxanthine). Considering the significant performance improvement these two nutritional strategies produced, it can be suggested that "metabolic enhancers", namely feed additives and ingredients that can favorably modulate the metabolism of crucial organs and tissues, such as the liver and skeletal muscle, should be given more consideration in broiler feed formulation. Arginine supplementation also increased the levels of essential AAs (e.g., arginine, histidine, methionine, leucine, phenylalanine, and threonine), suggesting that this nutritional strategy may have promoted intestinal absorption of AAs. In future investigations, a greater focus on the intestinal epithelium (e.g., by assessing the mucosal morphology and profiling the expression of nutrient transporters and inflammatory molecules) might allow useful data to be generated to validate the hypothesis of improved gut health and function proposed in the arginine study. Ironically, reduced glutamine levels were also found to be related to arginine supplementation. Despite being conditionally essential for chickens, this AA has been shown to play a pivotal role in development, function, and health of the GIT. Once again, more attention to the intestinal epithelium (e.g., analysis of its aminogram and metabolic profile?) is recommended to test the hypothesis of increased glutamine requirements of the intestinal mucosa in arginine-supplemented birds. Interestingly, confirmation of this hypothesis might make it necessary to recalculate glutamine specifications when formulating broiler diets high in arginine.

HS, the other major question addressed in this dissertation, alters chicken physiology as summarized above. It has conclusively been shown that modern fast-growing broilers are less thermotolerant and more susceptible to HS than slow-growing lines and their predecessors. Nevertheless, the molecular mechanisms underlying the response to HS are not fully understood. To try to learn more about it, I decided to go to the Center of Excellence for Poultry Science, University of

Arkansas, AR, U.S., where I joined Dr. Sami Dridi's lab for a six-month research period mainly devoted to exploring the hypothalamic response to HS in three broiler lines from diverse stages of genetic selection and in the red jungle fowl, the ancestor of domestic chickens. The gist of the results of this overseas investigation is that FRHN do not seem to be behind the significant differences in FI between the chicken populations studied, while hypoxia and HS responses appear to be associated with FI regulation. A natural progression of this work could be to analyze other key organs and tissues for metabolism, such as again the liver and skeletal muscle, to determine whether and how they react to HS in chickens that differ in selection intensity and performance potential. For instance, insights into hepatic *de novo* lipogenesis and anabolic and catabolic signaling in pectoral muscle (e.g., insulin and glucocorticoid receptors, IGF-I/Akt/mTOR pathway, S6K, PI3K/Akt pathway, FAS, and ubiquitin-proteasome pathway), as well as their regulation by nutrients and feed additives, in differently-performing, heat-stressed broilers could be a research idea to consider. Anyway, several questions remain to be answered to develop a full picture of the response to HS in chickens with the aim of conceiving appropriate solutions to mitigate its adverse effects on their health and performance.

Speaking of this, I had already started working on HS mitigation strategies before going to the U.S., particularly testing the synbiotic and muramidase in small-scale trials. The HS experiment with the synbiotic was too important to me because it was preparatory to learning how to conduct HS trials with broilers. Its data, however, indicate that the synbiotic failed to reduce the negative effects of HS on growth performance. Similarly, muramidase did not attenuate the reduction in performance caused by HS. Frankly, I expected this dietary enzyme to show at least some semblance of HS mitigation activity following the considerable performance improvement observed in the previous feeding trial under TN conditions. Nonetheless, I would recommend not giving up testing muramidases to fight HS in broilers since these enzymes, besides promoting growth performance, have been shown to positively modulate the microbiota and exert anti-inflammatory and immunomodulatory activities that make them promising, perhaps underestimated, allies against HS.

In the muramidase study, comparing the performance recorded in the experiment run under optimal thermal conditions with that of the HS experiment showed that heat-stressed birds consumed about a quarter less feed per day. Given the well-known limiting effect of high environmental

temperatures on feeding, it is quite unsurprising that HS was found to reduce FI in a similar manner in the study on the synbiotic and that on arginine supplementation, the latter conducted after returning from the U.S. These findings could lead to the introduction of a concept that may seem trivial on the surface: "anorexia-induced neutralization". Simply put, the decrease in FI due to HS not only results in inadequate consumption of nutrients, but also of the anti-HS products added to the diet. For instance, it is likely that anorexia caused by HS neutralized the efficacy of supplemental arginine rather than the HS reaction itself. Therefore, as the efficacy of these products can potentially be dampened by FI reduction, the use of extra doses compared with those recommended under TN conditions might be an option worthy of investigation.

Although the HS experiment on arginine supplementation did not produce the hoped-for outcome, namely an attenuation of HS mediated by arginine, it offered valuable insights into the physiological perturbations occurring in heat-stressed broilers. Metabolomics analysis confirmed that HS profoundly changes protein metabolism, promoting the degradation of skeletal muscle protein to supply the liver with AAs to be "burned" for energy. Consistently with the literature, HS was also found to increase concentrations of antioxidant and protective molecules (e.g., histidine-containing dipeptides, beta-alanine, and choline) in pectoral muscle, whose tissue is known to be particularly sensitive to oxidative stress. Meat scientists should therefore be involved in future studies to address these intricate processes from multiple vantage points to succeed in developing effective nutritional approaches to enhance protein metabolism and antioxidant defenses in broilers under HS. It is, however, inspiring that with the increasing availability of feed-grade AAs and their mixtures, antioxidant compounds or compounds that promote endogenous synthesis of antioxidants, as well as with the re-evaluation of maybe overlooked products, there is no shortage of starting points from which to begin investigating.

Well, this PhD project has been an amazing adventure both scientifically and personally. Honestly, I am already nostalgic for the wonderful memories of this experience and I wish these conclusions were endless to push back the finale. There would be a lot to be said about the phenomenal mentors I was given the opportunity to learn from, the elite scientists I met, the outstanding and talented friends I worked alongside, and the achievements and falls I had. Unfortunately, I must wrap this up so as not to bore readers. Somebody may say it is time to celebrate, but it is not. When I get to the top of a

mountain, I cannot enjoy the view until I find a bigger mountain to climb. So, let's scan the horizon in search of it!

ANNEXES

Annex 1 | Papers authored and co-authored by the PhD candidate

- **Brugaletta, G.**, L. Laghi, M. Zampiga, C. Oliveri, V. Indio, R. Piscitelli, S. Pignata, M. Petracci, A. De Cesare, F. Sirri. 2023. Metabolic and microbiota response to arginine supplementation and cyclic heat stress in broiler chickens. Front. Physiol. 14:537. https://doi.org/10.3389/fphys.2023.1155324.
- **Brugaletta, G.**, M. Zampiga, L. Laghi, V. Indio, C. Oliveri, A. De Cesare, F. Sirri. 2023. Feeding broiler chickens with arginine above recommended levels: Effects on growth performance, metabolism, and intestinal microbiota. J. Anim. Sci. Biotechnol. 14:33. https://doi.org/10.1186/s40104-023-00839-y.
- Zampiga, M., **G. Brugaletta**, F. Ceccaroni, A. Bonaldo, S. Pignata, F. Sirri. 2023. Performance response of broiler chickens fed diets containing dehydrated microalgae meal as partial replacement for soybean until 22 days of age. Anim. Feed Sci. Technol. 297:115573. https://doi.org/10.1016/j.anifeedsci.2023.115573.
- Teyssier, J.-R., **G. Brugaletta**, F. Sirri, S. Dridi, and S. J. Rochell. 2022. A review of heat stress in chickens. Part II: Insights into protein and energy utilization and feeding. Front. Physiol. 13:1521. https://doi.org/10.3389/fphys.2022.943612.
- **Brugaletta, G.**, J.-R. Teyssier, S. J. Rochell, S. Dridi, and F. Sirri. 2022. A review of heat stress in chickens. Part I: Insights into physiology and gut health. Front. Physiol. 13:1535. https://doi.org/10.3389/fphys.2022.934381.
- Dridi, J. S., E. S. Greene, C. W. Maynard, **G. Brugaletta**, A. Ramser, C. J. Christopher, S. R. Campagna, H. F. Castro, and S. Dridi. 2022. Duodenal metabolic profile changes in heat-stressed broilers. Animals 12:1337. https://doi.org/10.3390/ani12111337.
- **Brugaletta, G.**, E. Greene, A. Ramser, C. W. Maynard, T. W. Tabler, F. Sirri, N. B. Anthony, S. Orlowski, and S. Dridi. 2022. Effect of cyclic heat stress on hypothalamic oxygen homeostasis and inflammatory state in the Jungle Fowl and three broiler-based research lines. Front. Vet. Sci. 9:905225. https://doi.org/10.3389/fvets.2022.905225.

- **Brugaletta, G.**, A. De Cesare, L. Laghi, G. Manfreda, M. Zampiga, C. Oliveri, E. Pérez-Calvo, G. Litta, S. Lolli, and F. Sirri. 2022. A multi-omics approach to elucidate the mechanisms of action of a dietary muramidase administered to broiler chickens. Sci. Rep. 12:5559. https://doi.org/10.1038/s41598-022-09546-6.
- **Brugaletta, G.**, E. Greene, T. Tabler, S. Orlowski, F. Sirri, and S. Dridi. 2021. Effect of cyclic heat stress on feeding-related hypothalamic neuropeptides of three broiler populations and their ancestor Jungle Fowl. Front. Physiol. 12:2383. https://doi.org/10.3389/fphys.2021.809341.
- **Brugaletta, G.**, D. Luise, A. De Cesare, M. Zampiga, L. Laghi, P. Trevisi, G. Manfreda, and F. Sirri. 2020. Insights into the mode of action of tannin-based feed additives in broiler chickens: Looking for connections with the plasma metabolome and cecal microbiota. Ital. J. Anim. Sci. 19:1349. https://doi.org/10.1080/1828051X.2020.1842813.
- **Brugaletta, G.**, A. De Cesare, M. Zampiga, L. Laghi, C. Oliveri, C. Zhu, G. Manfreda, B. Syed, L. Valenzuela, and F. Sirri. 2020. Effects of alternative administration programs of a synbiotic supplement on broiler performance, foot pad dermatitis, cecal microbiota, and blood metabolites. Animals 10:522. https://doi.org/10.3390/ani10030522.

Annex 2 | Posters and oral presentations the PhD candidate has given at scientific conferences

- **Brugaletta G.**, M. Zampiga, L. Laghi, A. De Cesare, G. Manfreda, and F. Sirri. Effects of dietary arginine supplementation on growth performance, metabolism, and intestinal microbiome of broiler chickens. Poster. 2022 World's Poultry Congress, Paris, France. August 7-11, 2022.
- **Brugaletta G.**, M. Zampiga, L. Laghi, A. De Cesare, G. Manfreda, C. Zhu, G. Litta, I. Eising, S. Lolli, and F. Sirri. Feeding broiler chickens a dietary muramidase: Enlightening the performance improvement through a multi-omics approach. Poster. 2022 World's Poultry Congress, Paris, France. August 7-11, 2022.
- **Brugaletta, G.**, E. Greene, T. Tabler, S. Orlowski, F. Sirri, and S. Dridi. Effect of cyclic heat stress on gut barrier integrity, growth performance, and feeding-related hypothalamic neuropeptides of three broiler lines and their ancestor. Oral presentation. 2022 International Poultry Scientific Forum, Atlanta, GA, U.S. January 24-25, 2022.
- **Brugaletta, G.**, E. Greene, T. Tabler, S. Orlowski, F. Sirri, and S. Dridi. Effect of cyclic heat stress on the hypothalamic oxygen homeostasis and inflammatory state in different broiler populations and their ancestor. Poster. 2022 International Poultry Scientific Forum, Atlanta, GA, U.S. January 24-25, 2022.
- **Brugaletta, G.**, L. Laghi, M. Zampiga, B. Syed, L. Valenzuela, and F. Sirri. Multi-omics approach to assess the effects of a dual mode synbiotic supplementation on gut health and performance of broiler chickens. Poster. 24th Congress of the Italian Animal Science and Production Association, Padova, Italy. September 21-24, 2021.

Annex 3 | Formula and composition of the basal diets used in studies #1-3 of the PhD project

- **Study #1**
  - **Basal diet used in experiment S1E1**

Ingredient (g/100 g feed)	Feeding phase		
	Starter (0-14 d)	**Grower (15-28 d)**	**Finisher (29-42 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 meal	3.00	3.00	3.00
Corn gluten meal	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
Sodium chloride	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
L-Threonine	0.15	0.14	0.14
Non-starch polysaccharides-degrading enzyme	0.08	0.08	0.08
Phytase	0.10	0.10	0.10
Vitamin and mineral premix[†]	0.50	0.50	0.50
Calculated composition			
Dry matter (%)	88.57	88.65	88.64
Crude protein (%)	22.70	21.49	19.74
Total lipid (%)	7.06	8.24	9.74
Crude fiber (%)	3.08	3.04	3.07
Ash (%)	5.85	5.17	4.49
Total lysine (%)	1.38	1.29	1.21
Total methionine + cysteine (%)	1.01	0.92	0.82
Calcium (%)	0.91	0.80	0.59
Phosphorus (%)	0.63	0.57	0.46
Metabolizable energy (kcal/kg)	3,076	3,168	3,264

[†] The premix provides the following per kg of feed: 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 B_{12} 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.

- **Basal diet used in experiment S1E2**

Ingredient (g/100 g feed)	Feeding phase		
	Starter (0-15 d)	**Grower (16-29 d)**	**Finisher (30-41 d)**
Corn	35.76	38.89	38.54
Wheat	14.98	14.98	19.99
Soybean meal	21.89	17.30	11.70
Wheat bran	2.00	2.00	2.00
Pea	3.00	3.00	3.00
Roasted soybean	9.99	14.99	14.99
Sunflower meal	3.00	3.00	3.00
Corn gluten meal	3.00	0.00	0.00
Soybean oil	2.49	2.76	4.04
Calcium carbonate	0.39	0.55	0.96
Dicalcium phosphate	1.09	0.56	0.11
Sodium bicarbonate	0.07	0.07	0.17
Sodium chloride	0.35	0.30	0.24
Choline chloride	0.10	0.10	0.10
Lysine sulphate	0.56	0.37	0.34
DL-Methionine	0.29	0.29	0.25
L-Threonine	0.12	0.09	0.07
Non-starch polysaccharides-degrading enzyme	0.05	0.05	0.05
Phytase	0.20	0.20	0.15
Amino acid mix (arginine, valine)	0.07	0.05	0.05
Mycotoxin binder	0.10	0.00	0.00
Vitamin and mineral premix[†]	0.50	0.45	0.25
Calculated composition			
Dry matter (%)	88.54	88.50	88.74
Crude protein (%)	23.21	21.12	19.02
Total lipid (%)	6.27	7.53	8.80
Crude fiber (%)	3.10	3.18	3.05
Ash (%)	5.10	4.66	4.35
Total lysine (%)	1.40	1.26	1.10
Total methionine + cysteine (%)	1.05	0.97	0.87
Calcium (%)	0.72	0.64	0.61
Phosphorus (%)	0.60	0.50	0.40
Metabolizable energy (kcal/kg)	3,011	3,101	3,227

[†] The premix provides the following per kg of feed: 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 B_{12} 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.

- **Study #2**

  - **Basal diet used in experiment S2E1**

Ingredient (g/100 g feed)	Feeding phase			
	Starter (0-9 d)	**Grower I (10-21 d)**	**Grower II (22-28 d)**	**Finisher (29-42 d)**
Corn	44.10	42.40	44.70	45.45
Wheat	10.10	15.00	15.00	15.00
Soybean meal	15.80	18.90	14.00	10.80
Pea	3.00	3.00	3.00	4.00
Fermented soybean meal	10.00	0.00	0.00	0.00
Roasted soybean	5.60	12.66	15.00	15.00
Sunflower meal	3.00	3.00	3.00	4.00
Corn gluten meal	3.00	0.00	0.00	0.00
Soybean oil	1.85	1.95	2.29	2.84
Calcium carbonate	0.42	0.50	0.68	0.77
Dicalcium phosphate	1.07	0.63	0.37	0.22
Sodium bicarbonate	0.00	0.00	0.12	0.28
Sodium chloride	0.33	0.31	0.24	0.17
Choline chloride	0.10	0.10	0.05	0.00
Lysine sulphate	0.52	0.47	0.41	0.38
DL-Methionine	0.22	0.13	0.22	0.10
Methionine hydroxy analogue	0.00	0.15	0.00	0.14
L-Threonine	0.14	0.15	0.13	0.11
Non-starch polysaccharides-degrading enzyme	0.05	0.05	0.05	0.05
Phytase	0.20	0.15	0.15	0.15
Natural pigments	0.00	0.00	0.24	0.24
Vitamin and mineral premix[†]	0.50	0.45	0.35	0.30
Calculated composition				
Dry matter (%)	88.46	88.02	88.11	88.09
Crude protein (%)	22.88	20.31	19.11	18.08
Total lipid (%)	4.99	6.37	7.24	7.81
Crude fiber (%)	3.07	3.23	3.20	3.33
Ash (%)	5.04	4.59	4.42	4.35
Total lysine (%)	1.26	1.15	1.05	0.97
Total methionine + cysteine (%)	0.91	0.84	0.78	0.74
Calcium (%)	0.72	0.61	0.59	0.58
Phosphorus (%)	0.57	0.49	0.43	0.40
Metabolizable energy (kcal/kg)	3,020	3,097	3,172	3,222

[†] The premix provides the following per kg of feed: 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 B_{12} 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.

- **Basal diet used in experiment S2E2**

Ingredient (g/100 g feed)	Feeding phase		
	Starter (0-14 d)	**Grower (15-27 d)**	**Finisher (28-42 d)**
Corn	25.70	32.47	22.76
Wheat	24.98	24.98	29.98
Sorghum	0.00	0.00	10.00
Soybean meal	22.72	15.42	10.18
Pea	3.00	3.00	3.00
Roasted soybean	9.99	14.99	14.99
Sunflower meal	3.00	3.00	2.00
Corn gluten meal	3.00	0.00	0.00
Soybean oil	3.09	2.68	4.01
Calcium carbonate	0.37	0.54	0.91
Dicalcium phosphate	1.34	0.40	0.00
Sodium bicarbonate	0.06	0.07	0.10
Sodium chloride	0.36	0.30	0.29
Choline chloride	0.10	0.10	0.06
Lysine sulphate	0.66	0.56	0.52
DL-Methionine	0.32	0.13	0.07
Methionine hydroxy analogue	0.00	0.24	0.27
L-Threonine	0.19	0.15	0.13
Non-starch polysaccharides-degrading enzyme	0.05	0.05	0.05
Phytase	0.20	0.20	0.15
Emulsifier	0.05	0.05	0.05
Amino acid mix (arginine, valine, isoleucine)	0.22	0.24	0.22
Mycotoxin binder	0.10	0.00	0.00
Vitamin and mineral premix[†]	0.50	0.45	0.25
Calculated composition			
Dry matter (%)	88.62	88.35	88.37
Crude protein (%)	23.43	20.44	18.29
Total lipid (%)	6.59	7.23	8.53
Crude fiber (%)	3.01	3.03	2.85
Ash (%)	5.32	4.42	4.07
Total lysine (%)	1.46	1.30	1.14
Total methionine + cysteine (%)	1.09	0.98	0.88
Calcium (%)	0.80	0.61	0.58
Phosphorus (%)	0.63	0.45	0.35
Metabolizable energy (kcal/kg)	3,072	3,162	3,298

[†] The premix provides the following per kg of feed: 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 B_{12} 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.

- **Study #3**

  - **Basal diet used in experiment S3E1**

Ingredient (g/100 g feed)	Feeding phase			
	Starter (0-9 d)	**Grower I (10-21 d)**	**Grower II (22-35 d)**	**Finisher (36-49 d)**
Corn	37.60	44.88	34.99	25.00
White corn	0.00	0.00	5.00	5.00
Wheat	12.49	12.51	15.83	22.29
Sorghum	0.00	0.00	5.00	10.00
Pea	3.00	3.00	3.00	3.00
Soybean meal	25.04	17.10	13.26	11.36
Roasted soybean	10.00	15.00	15.00	15.00
Sunflower meal	2.00	2.00	2.00	2.00
Corn gluten meal	3.00	0.00	0.00	0.00
Soybean oil	2.36	2.01	2.69	3.47
Calcium carbonate	0.32	0.56	0.73	0.93
Dicalcium phosphate	1.48	0.54	0.29	0.00
Sodium bicarbonate	0.06	0.07	0.15	0.15
Sodium chloride	0.36	0.31	0.25	0.25
Choline chloride	0.10	0.10	0.05	0.06
Lysine sulphate	0.58	0.56	0.54	0.49
DL-Methionine	0.31	0.13	0.10	0.05
Methionine hydroxy analogue	0.00	0.24	0.24	0.28
L-Threonine	0.21	0.17	0.14	0.12
Amino acid mix (arginine, valine, isoleucine)	0.20	0.20	0.19	0.13
Non-starch polysaccharides-degrading enzyme	0.05	0.05	0.05	0.05
Phytase	0.08	0.08	0.08	0.08
Emulsifier	0.05	0.05	0.05	0.05
Mycotoxin binder	0.10	0.00	0.00	0.00
Vitamin and mineral premix[†]	0.50	0.45	0.36	0.25
Composition				
Dry matter[§] (%)	88.50	88.42	88.30	88.30
Crude protein[§] (%)	24.02	20.78	19.52	19.13
Total lipid[§] (%)	6.05	6.73	7.47	8.12
Crude fiber[§] (%)	2.90	2.91	2.84	2.84
Ash[§] (%)	5.31	4.48	4.22	4.07
Total lysine[§] (%)	1.47	1.31	1.23	1.16
Total arginine[§] (%)	1.59	1.42	1.32	1.25
Total arginine : lysine[§]	1.08	1.08	1.07	1.08
Total methionine + cysteine[§] (%)	1.10	0.99	0.93	0.89
Calcium (%)	0.76	0.60	0.57	0.55
Phosphorus (%)	0.65	0.47	0.41	0.36
Metabolizable energy (kcal/kg)	3,050	3,152	3,225	3,275

[†] The premix provides the following per kg of feed: vitamin A (retinyl acetate), 12,500 IU; vitamin D3, 5,000 IU (i.e., cholecalciferol, 3,500 IU + 25-OH D3, 1,500 IU); vitamin E (*DL*-α-tocopheryl acetate), 125 mg; vitamin K (menadione sodium bisulfite), 6.75 mg; riboflavin, 9.0 mg; pantothenic acid, 22.0 mg; niacin, 75 mg; pyridoxine, 5 mg; folic acid, 3.0 mg; biotin, 0.35 mg; thiamine, 4.0 mg; vitamin B_{12}, 50 µg; Mn, 100 mg; Zn, 102 mg; Fe, 30 mg; Cu, 15 mg; I, 2.0 mg; Se, 0.35 mg.
[§] Analyzed values.

- **Basal diet used in experiment S3E2**

Ingredient (g/100 g feed)	Feeding phase		
	Starter (0-14 d)	**Grower (15-27 d)**	**Finisher (28-44 d)**
Corn	29.86	32.49	15.03
White corn	0.00	0.00	6.00
Wheat	24.97	24.97	39.95
Pea	3.00	3.00	3.00
Soybean meal	15.89	15.17	8.68
Roasted soybean	9.99	14.99	16.99
Potato protein meal	3.51	0.00	0.00
Sunflower meal	3.00	3.00	3.00
Corn gluten meal	3.00	0.00	0.00
Soybean oil	2.27	2.83	4.47
Calcium carbonate	0.19	0.56	0.87
Dicalcium phosphate	1.54	0.48	0.00
Sodium bicarbonate	0.05	0.00	0.05
Sodium chloride	0.31	0.33	0.27
Choline chloride	0.10	0.10	0.06
Lysine sulphate	0.67	0.63	0.54
DL-Methionine	0.30	0.33	0.29
L-Threonine	0.14	0.18	0.14
Amino acid mix (arginine, valine, isoleucine)	0.35	0.24	0.17
Non-starch polysaccharides-degrading enzyme	0.05	0.05	0.05
Phytase	0.20	0.20	0.20
Mycotoxin binder	0.10	0.00	0.00
Vitamin and mineral premix[†]	0.50	0.45	0.25
Composition			
Dry matter[§] (%)	88.24	88.28	88.52
Crude protein[§] (%)	22.88	20.06	18.36
Total lipid[§] (%)	5.89	7.38	9.18
Crude fiber[§] (%)	3.05	3.18	3.11
Ash[§] (%)	5.00	4.47	4.06
Total lysine[§] (%)	1.47	1.31	1.15
Total arginine[§] (%)	1.50	1.38	1.23
Total arginine : lysine[§]	1.05	1.07	1.09
Total methionine + cysteine[§] (%)	1.08	0.99	0.88
Calcium (%)	0.78	0.65	0.59
Phosphorus (%)	0.63	0.46	0.36
Metabolizable energy (kcal/kg)	3,072	3,146	3,296

[†] The premix provides the following per kg of feed: vitamin A (retinyl acetate), 12,500 IU; vitamin D3, 5,000 IU (i.e., cholecalciferol, 3,500 IU + 25-OH D3, 1,500 IU); vitamin E (*DL*-α-tocopheryl acetate), 125 mg; vitamin K (menadione sodium bisulfite), 6.75 mg; riboflavin, 9.0 mg; pantothenic acid, 22.0 mg; niacin, 75 mg; pyridoxine, 5 mg; folic acid, 3.0 mg; biotin, 0.35 mg; thiamine, 4.0 mg; vitamin B_{12}, 50 μg; Mn, 100 mg; Zn, 102 mg; Fe, 30 mg; Cu, 15 mg; I, 2.0 mg; Se, 0.35 mg.
[§] Analyzed values.

Annex 4 | Metabolites showing different concentrations (mmol/L) in plasma, liver, and *Pectoralis major* in experiment S3E2 of study #3 of the PhD project

- **Plasma – S3E2**

Metabolite	Group		Room		Group × Room				SE	*P*-value		
	CON2	ARG2	TN	HS	CON2-TN	ARG2-TN	CON2-HS	ARG2-HS		Group	Room	Group × Room
2,3-Butanediol	4.25E-02	3.96E-02	3.37E-02	4.91E-02	3.47E-02	3.27E-02	5.11E-02	4.70E-02	2.16E-02	0.591	**0.021**	0.991
3-Hydroxyisobutyrate	1.56E-02	1.60E-02	1.60E-02	1.56E-02	1.68E-02	1.52E-02	1.42E-02	1.70E-02	4.32E-03	0.729	0.745	0.092
3-Methyl-2-oxovalerate	8.23E-03	7.97E-03	6.89E-03	9.41E-03	7.45E-03	6.32E-03	9.07E-03	9.76E-03	3.93E-03	0.863	0.087	0.227
Acetone	2.27E-02	2.13E-02	1.99E-02	2.43E-02	2.05E-02	1.94E-02	2.51E-02	2.35E-02	7.73E-03	0.559	0.098	0.983
Alanine	1.03E+00	8.95E-01	1.08E+00	8.36E-01	1.18E+00	9.78E-01	8.68E-01	8.05E-01	1.94E-01	**0.022**	**0.000**	0.227
Arabinose	7.52E-02	7.41E-02	7.10E-02	7.86E-02	7.21E-02	6.99E-02	7.86E-02	7.86E-02	1.28E-02	0.765	0.051	0.769
Arginine	2.02E-01	3.31E-01	2.86E-01	2.46E-01	2.24E-01	3.48E-01	1.79E-01	3.13E-01	1.03E-01	**0.000**	0.195	0.862
Aspartate	1.15E-01	1.09E-01	1.26E-01	9.73E-02	1.33E-01	1.19E-01	9.54E-02	9.93E-02	4.13E-02	0.830	**0.044**	0.607
Betaine	8.55E-01	8.69E-01	9.07E-01	8.13E-01	9.07E-01	9.08E-01	7.99E-01	8.27E-01	1.46E-01	0.599	**0.048**	0.968
Carnosine	5.41E-02	4.72E-02	5.90E-02	4.15E-02	6.71E-02	5.08E-02	3.98E-02	4.33E-02	2.00E-02	0.378	**0.008**	0.095
Creatine	8.48E-02	1.10E-01	1.06E-01	8.77E-02	1.02E-01	1.10E-01	6.61E-02	1.09E-01	6.11E-02	**0.032**	0.074	0.395
Ethanol	7.33E-02	5.08E-02	3.43E-02	9.24E-02	3.94E-02	2.92E-02	1.10E-01	7.43E-02	6.26E-02	0.180	**0.000**	0.754
Formate	9.53E-02	1.02E-01	7.12E-02	1.29E-01	6.31E-02	7.92E-02	1.30E-01	1.27E-01	4.14E-02	0.130	**0.000**	0.068
Fumarate	8.65E-03	8.08E-03	9.23E-03	7.43E-03	9.36E-03	9.09E-03	7.87E-03	6.98E-03	2.77E-03	0.490	**0.033**	0.707
Glucose	1.42E+01	1.44E+01	1.34E+01	1.53E+01	1.32E+01	1.36E+01	1.54E+01	1.52E+01	1.62E+00	0.688	**0.000**	0.528
Glutamine	1.46E+00	1.27E+00	1.42E+00	1.31E+00	1.48E+00	1.36E+00	1.43E+00	1.18E+00	2.33E-01	**0.010**	0.102	0.385
Glycerol	1.40E-01	1.36E-01	1.62E-01	1.11E-01	1.72E-01	1.53E-01	1.05E-01	1.17E-01	5.50E-02	0.903	**0.001**	0.481
Glycine	7.13E-01	6.77E-01	7.43E-01	6.42E-01	7.80E-01	7.07E-01	6.40E-01	6.45E-01	1.13E-01	0.290	**0.004**	0.248
Mannose	5.67E-02	5.61E-02	4.29E-02	7.12E-02	4.42E-02	4.15E-02	7.03E-02	7.21E-02	2.17E-02	0.552	**0.000**	0.306
Methionine	1.34E-01	1.37E-01	1.54E-01	1.16E-01	1.52E-01	1.56E-01	1.15E-01	1.17E-01	3.63E-02	0.790	**0.001**	0.913
N,N-Dimethylglycine	1.19E-01	1.12E-01	1.20E-01	1.10E-01	1.23E-01	1.18E-01	1.14E-01	1.05E-01	1.80E-02	0.190	0.052	0.755
Phenylalanine	1.32E-01	1.34E-01	1.27E-01	1.39E-01	1.24E-01	1.29E-01	1.40E-01	1.39E-01	1.72E-02	0.678	**0.019**	0.553
Pyroglutamate	8.72E-02	8.19E-02	7.94E-02	9.01E-02	8.04E-02	7.85E-02	9.46E-02	8.56E-02	2.38E-02	0.602	**0.009**	0.233
Sarcosine	2.92E-02	2.66E-02	3.00E-02	2.56E-02	3.36E-02 a	2.64E-02 b	2.44E-02 b	2.67E-02 b	5.05E-03	0.085	**0.004**	**0.003**
Serine	8.16E-01	8.01E-01	7.55E-01	8.66E-01	7.41E-01	7.70E-01	8.97E-01	8.36E-01	1.35E-01	0.715	**0.008**	0.262
Succinate	5.73E-02	5.46E-02	6.20E-02	4.93E-02	6.39E-02	6.00E-02	5.00E-02	4.87E-02	1.60E-02	0.749	**0.013**	0.740
Threonine	8.50E-01	7.70E-01	7.75E-01	8.48E-01	8.46E-01	7.04E-01	8.54E-01	8.43E-01	1.51E-01	0.081	0.108	0.148
Tyrosine	1.65E-01	1.56E-01	1.72E-01	1.49E-01	1.68E-01	1.75E-01	1.62E-01	1.36E-01	4.47E-02	0.514	0.090	0.214

Note: n = 12 birds/group/room. *P*-values less than 0.05 are in bold. Means that fall under the interaction between group and room and show distinct letters are significantly different ($P < 0.05$).

- **Liver – S3E2**

Metabolite	Group		Room		Group × Room				SE	*P*-value		
	CON2	ARG2	TN	HS	CON2-TN	ARG2-TN	CON2-HS	ARG2-HS		Group	Room	Group × Room
1-Methylhistidine	5.26E-04	5.40E-04	4.77E-04	5.96E-04	4.73E-04	4.81E-04	5.91E-04	6.00E-04	1.30E-04	0.717	**0.004**	1.000
1,3-Dihydroxyacetone	7.65E-05	5.96E-05	6.52E-05	7.04E-05	7.66E-05	5.38E-05	7.64E-05	6.54E-05	2.30E-05	**0.043**	0.202	0.366
3-Hydroxybutyrate	9.17E-04	7.28E-04	9.42E-04	6.84E-04	1.01E-03	8.74E-04	8.07E-04	5.81E-04	2.87E-04	**0.011**	**0.006**	0.403
4-Aminobutyrate	4.87E-04	5.76E-04	5.37E-04	5.29E-04	4.90E-04	5.85E-04	4.84E-04	5.67E-04	1.48E-04	0.056	0.753	0.994
Adenine	6.78E-04	5.11E-04	5.70E-04	6.14E-04	6.34E-04	5.05E-04	7.30E-04	5.17E-04	2.53E-04	0.052	0.435	0.506
Alanine	5.14E-03	4.60E-03	5.26E-03	4.42E-03	5.64E-03	4.88E-03	4.55E-03	4.32E-03	1.12E-03	0.106	**0.018**	0.427
AMP	2.86E-03	2.32E-03	2.68E-03	2.46E-03	2.98E-03	2.39E-03	2.71E-03	2.26E-03	4.84E-04	**0.001**	0.175	0.619
Arginine	9.16E-04	1.29E-03	1.07E-03	1.16E-03	9.25E-04	1.21E-03	9.05E-04	1.37E-03	3.25E-04	**0.000**	0.467	0.377
Creatine	7.81E-04	1.05E-03	9.99E-04	8.31E-04	7.64E-04	1.23E-03	8.02E-04	8.55E-04	6.83E-04	**0.019**	**0.033**	0.544
Dimethylamine	2.00E-04	2.37E-04	2.29E-04	2.09E-04	2.15E-04	2.43E-04	1.83E-04	2.30E-04	5.10E-05	**0.019**	0.155	0.538
Ethanol	1.20E-02	1.99E-02	1.48E-02	1.75E-02	1.09E-02	1.87E-02	1.32E-02	2.10E-02	1.23E-02	**0.029**	0.854	0.952
Ethanolamine	7.33E-04	8.89E-04	7.82E-04	8.49E-04	5.71E-04	9.93E-04	9.27E-04	7.85E-04	5.09E-04	0.148	0.466	0.058
Fumarate	1.08E-03	1.09E-03	1.16E-03	9.94E-04	1.13E-03	1.20E-03	1.02E-03	9.75E-04	2.19E-04	0.967	**0.011**	0.380
Glutamate	6.83E-03	6.51E-03	6.11E-03	7.27E-03	6.14E-03	6.08E-03	7.66E-03	6.94E-03	1.31E-03	0.412	**0.004**	0.408
Glutamine	7.16E-03	5.84E-03	5.31E-03	7.74E-03	5.70E-03	4.91E-03	8.90E-03	6.77E-03	2.09E-03	0.063	**0.000**	0.363
Glutathione	2.16E-03	1.76E-03	2.20E-03	1.68E-03	2.45E-03	1.94E-03	1.81E-03	1.57E-03	4.88E-04	**0.010**	**0.001**	0.481
Hypoxanthine	3.09E-04	2.36E-04	2.66E-04	2.76E-04	2.84E-04	2.48E-04	3.38E-04	2.25E-04	1.12E-04	0.078	0.631	0.221
Isoleucine	3.89E-04	4.27E-04	3.73E-04	4.49E-04	3.72E-04	3.74E-04	4.10E-04	4.81E-04	1.33E-04	0.335	0.065	0.384
Isopropanol	9.71E-05	1.65E-04	1.17E-04	1.50E-04	8.44E-05	1.49E-04	1.12E-04	1.81E-04	9.21E-05	**0.007**	0.490	0.945
Leucine	9.79E-04	1.13E-03	9.58E-04	1.17E-03	9.40E-04	9.75E-04	1.02E-03	1.28E-03	3.67E-04	0.172	0.070	0.309
Methionine	3.86E-04	4.60E-04	4.06E-04	4.45E-04	3.86E-04	4.26E-04	3.86E-04	4.95E-04	1.42E-04	0.083	0.392	0.410
myo-Inositol	3.57E-02	3.21E-02	3.56E-02	3.19E-02	3.75E-02	3.37E-02	3.35E-02	3.05E-02	6.15E-03	0.056	0.053	0.815
N,N-Dimethylglycine	1.27E-04	1.12E-04	1.33E-04	1.05E-04	1.37E-04	1.29E-04	1.16E-04	9.57E-05	3.11E-05	0.055	**0.003**	0.323
NAD+	6.10E-04	4.46E-04	5.00E-04	5.51E-04	5.61E-04	4.39E-04	6.69E-04	4.53E-04	2.18E-04	**0.014**	0.364	0.466
Pantothenate	1.05E-04	8.52E-05	1.02E-04	8.63E-05	1.09E-04	9.48E-05	9.91E-05	7.57E-05	2.34E-05	**0.007**	**0.037**	0.537
Phenylalanine	5.78E-04	6.72E-04	5.61E-04	7.00E-04	5.46E-04	5.75E-04	6.17E-04	7.69E-04	2.20E-04	0.119	**0.041**	0.422
Propylene glycol	9.69E-05	8.23E-05	8.60E-05	9.28E-05	8.30E-05 b	8.91E-05 ab	1.14E-04 a	7.55E-05 b	2.17E-05	0.053	0.398	**0.001**
Pyroglutamate	3.14E-04	3.06E-04	2.50E-04	3.75E-04	2.40E-04	2.60E-04	4.02E-04	3.52E-04	1.22E-04	0.834	**0.001**	0.328
Sarcosine	1.14E-04	9.42E-05	1.22E-04	8.38E-05	1.42E-04	1.02E-04	8.12E-05	8.59E-05	3.79E-05	0.099	**0.001**	0.095
Succinate	3.01E-03	2.92E-03	3.16E-03	2.75E-03	3.16E-03	3.16E-03	2.83E-03	2.69E-03	5.98E-04	0.626	**0.029**	0.687
Threonine	1.75E-03	1.84E-03	1.64E-03	1.96E-03	1.68E-03	1.61E-03	1.84E-03	2.07E-03	5.41E-04	0.591	0.054	0.361
UDP	2.25E-04	1.96E-04	2.35E-04	1.82E-04	2.51E-04	2.19E-04	1.94E-04	1.73E-04	3.17E-05	**0.003**	**0.000**	0.582
Uracil	1.31E-04	1.67E-04	1.26E-04	1.77E-04	1.15E-04	1.36E-04	1.51E-04	1.98E-04	5.81E-05	**0.026**	**0.005**	0.830
Uridine	4.93E-04	5.00E-04	4.62E-04	5.35E-04	4.74E-04	4.51E-04	5.16E-04	5.50E-04	1.05E-04	0.821	**0.025**	0.373

Note: n = 12 birds/group/room. *P*-values less than 0.05 are in bold. Means that fall under the interaction between group and room and show distinct letters are significantly different ($P < 0.05$).

- ***Pectoralis major* – S3E2**

Metabolite	Group		Room		Group × Room				SE	*P*-value		
	CON2	ARG2	TN	HS	CON2-TN	ARG2-TN	CON2-HS	ARG2-HS		Group	Room	Group × Room
2-Aminobutyrate	9.22E-05	9.31E-05	1.13E-04	7.26E-05	1.11E-04	1.14E-04	7.30E-05	7.21E-05	5.45E-05	0.600	**0.003**	0.744
2-Oxoglutarate	7.58E-04	5.69E-04	4.52E-04	8.75E-04	5.22E-04	3.82E-04	9.94E-04	7.57E-04	4.56E-04	0.172	**0.002**	0.947
2,3-Butanediol	5.79E-05	6.28E-05	5.50E-05	6.57E-05	4.87E-05	6.13E-05	6.71E-05	6.43E-05	1.95E-05	0.388	0.065	0.178
3-Hydroxyisovalerate	1.74E-05	1.96E-05	1.76E-05	1.94E-05	1.63E-05	1.90E-05	1.85E-05	2.03E-05	3.21E-06	**0.022**	0.063	0.621
4-Aminobutyrate	1.19E-04	8.97E-05	9.48E-05	1.14E-04	1.09E-04	8.07E-05	1.29E-04	9.88E-05	3.93E-05	**0.013**	0.098	0.927
4-Hydroxyphenylacetate	3.09E-04	1.85E-04	2.37E-04	2.57E-04	2.88E-04	1.86E-04	3.29E-04	1.85E-04	1.38E-04	**0.004**	0.779	0.844
Acetate	2.17E-04	3.15E-04	2.67E-04	2.66E-04	2.15E-04	3.18E-04	2.19E-04	3.12E-04	6.78E-05	**0.000**	0.961	0.803
Acetone	1.85E-05	1.59E-05	1.61E-05	1.83E-05	1.71E-05	1.52E-05	2.00E-05	1.66E-05	5.09E-06	0.083	0.152	0.619
Alanine	3.03E-03	2.68E-03	3.27E-03	2.44E-03	3.58E-03	2.96E-03	2.47E-03	2.40E-03	6.76E-04	0.083	**0.000**	0.161
AMP	2.96E-04	2.36E-04	2.35E-04	2.97E-04	2.50E-04	2.21E-04	3.42E-04	2.52E-04	9.21E-05	**0.030**	**0.025**	0.261
Anserine	2.51E-02	2.70E-02	2.30E-02	2.91E-02	2.33E-02	2.27E-02	2.69E-02	3.12E-02	5.81E-03	0.454	**0.001**	0.142
Arginine	1.92E-04	2.17E-04	2.08E-04	2.01E-04	1.93E-04	2.24E-04	1.91E-04	2.11E-04	7.72E-05	0.068	0.423	0.919
Asparagine	6.93E-04	5.48E-04	7.22E-04	5.20E-04	8.14E-04	6.29E-04	5.72E-04	4.67E-04	2.49E-04	0.050	**0.007**	0.584
Beta-Alanine	5.41E-03	4.45E-03	3.72E-03	6.14E-03	3.71E-03	3.72E-03	7.10E-03	5.17E-03	2.25E-03	0.147	**0.001**	0.141
Carnosine	4.19E-04	4.00E-04	3.22E-04	4.96E-04	3.36E-04	3.08E-04	5.01E-04	4.92E-04	1.37E-04	0.641	**0.000**	0.815
Choline	2.25E-04	2.21E-04	1.92E-04	2.54E-04	1.95E-04	1.89E-04	2.55E-04	2.54E-04	8.43E-05	0.890	**0.014**	0.918
Creatine	4.93E-02	5.89E-02	4.87E-02	5.95E-02	4.47E-02	5.28E-02	5.40E-02	6.50E-02	9.83E-03	**0.002**	**0.000**	0.621
Dimethyl sulfone	1.72E-04	1.96E-04	1.67E-04	2.01E-04	1.54E-04	1.81E-04	1.91E-04	2.10E-04	3.20E-05	**0.016**	**0.001**	0.693
Ethanolamine	9.43E-04	9.24E-04	1.08E-03	7.85E-04	1.12E-03	1.05E-03	7.71E-04	8.00E-04	5.10E-04	0.896	0.050	0.746
Formate	1.50E-04	1.73E-04	1.62E-04	1.61E-04	1.47E-04	1.76E-04	1.52E-04	1.70E-04	4.29E-05	0.068	0.957	0.691
Fumarate	7.07E-05	6.89E-05	7.71E-05	6.24E-05	7.81E-05	7.61E-05	6.33E-05	6.16E-05	2.39E-05	0.792	**0.039**	0.984
Glucose-1-phosphate	4.53E-04	2.51E-03	2.51E-03	4.58E-04	4.86E-04	4.53E-03	4.19E-04	4.97E-04	6.91E-03	0.070	0.125	0.998
Glutamine	3.20E-03	2.78E-03	3.43E-03	2.55E-03	3.68E-03	3.19E-03	2.72E-03	2.38E-03	8.59E-04	0.099	**0.001**	0.769
Glutathione	5.52E-04	3.84E-04	4.67E-04	4.69E-04	5.95E-04	3.40E-04	5.09E-04	4.29E-04	1.97E-04	**0.002**	0.568	0.216
Glycerol	5.31E-04	7.96E-04	6.90E-04	6.37E-04	5.19E-04	8.61E-04	5.42E-04	7.31E-04	3.84E-04	**0.016**	0.783	0.351
Glycine	4.56E-03	4.31E-03	5.48E-03	3.38E-03	5.69E-03	5.28E-03	3.43E-03	3.34E-03	1.73E-03	0.859	**0.000**	0.865
Isoleucine	8.13E-05	8.45E-05	9.94E-05	6.64E-05	1.03E-04	9.54E-05	5.93E-05	7.36E-05	2.97E-05	0.359	**0.000**	0.059
Lactate	9.50E-02	1.08E-01	9.57E-02	1.08E-01	9.01E-02	1.01E-01	9.99E-02	1.15E-01	2.09E-02	**0.031**	0.067	0.802
Leucine	1.41E-04	1.52E-04	1.71E-04	1.22E-04	1.67E-04	1.75E-04	1.15E-04	1.28E-04	5.13E-05	0.531	**0.001**	0.665
Malonate	4.83E-04	6.09E-04	5.07E-04	5.85E-04	4.58E-04	5.55E-04	5.07E-04	6.63E-04	1.33E-04	**0.002**	**0.047**	0.441
Methionine sulfoxide	6.26E-05	1.42E-04	7.97E-05	1.25E-04	5.77E-05	1.02E-04	6.75E-05	1.83E-04	1.08E-04	**0.010**	0.208	0.701
N,N-Dimethylglycine	1.05E-03	9.96E-04	1.12E-03	9.29E-04	1.10E-03	1.14E-03	1.00E-03	8.54E-04	2.77E-04	0.491	**0.021**	0.244
N2-Acetyllysine	5.39E-04	8.83E-04	5.40E-04	8.82E-04	4.02E-04	6.78E-04	6.77E-04	1.09E-03	5.89E-04	0.068	0.056	0.440
NAD	8.47E-04	8.12E-04	7.35E-04	9.24E-04	7.46E-04	7.25E-04	9.48E-04	9.00E-04	2.04E-04	0.653	**0.002**	0.867
Niacinamide	1.08E-04	2.46E-04	2.02E-04	1.52E-04	1.12E-04	2.92E-04	1.04E-04	2.00E-04	2.78E-04	0.094	0.536	0.602
Proline	7.75E-04	1.31E-03	7.89E-04	1.29E-03	5.07E-04	1.07E-03	1.04E-03	1.55E-03	1.15E-03	**0.043**	**0.036**	0.563
Serine	1.38E-03	1.27E-03	1.40E-03	1.25E-03	1.44E-03	1.36E-03	1.32E-03	1.19E-03	4.31E-04	0.089	0.288	0.713
Threonine	8.03E-04	7.08E-04	8.56E-04	6.54E-04	9.81E-04	7.31E-04	6.24E-04	6.84E-04	2.88E-04	0.260	**0.020**	0.069
Tyrosine	4.19E-04	3.94E-04	4.65E-04	3.47E-04	4.76E-04	4.54E-04	3.61E-04	3.33E-04	1.23E-04	0.489	**0.002**	0.932
UDP	1.57E-04	6.43E-04	6.62E-04	1.38E-04	1.85E-04	1.14E-03	1.30E-04	1.46E-04	1.60E-03	0.084	**0.003**	0.298
Valine	1.86E-04	2.02E-04	2.26E-04	1.61E-04	2.19E-04	2.33E-04	1.52E-04	1.70E-04	5.40E-05	0.311	**0.000**	0.909

Note: n = 12 birds/group/room. *P*-values less than 0.05 are in bold.

Annex 5 | Chicken-specific qPCR oligonucleotide primers used in study #4 of the PhD project

Gene	Accession number[†]	Primer sequence[§] (5′ → 3′)	Size (bp)
NPY	NM_205473	F, CATGCAGGGCACCATGAG R, CAGCGACAAGGCGAAAGTC	55
AgRP	AB029443	F, GCGGGAGCTTTCACAGAACA R, CGACAGGATTGACCCCAAAA	58
POMC	AB019555	F, GCCAGACCCCGCTGATG R, CTTGTAGGCGCTTTTGACGAT	56
CART	KC249966	F, GCTGGAGAAGCTGAAGAGCAA R, GGCACCTGCCCGAACTT	60
ORX	AB056748	F, CCAGGAGCACGCTGAGAAG R, CCCATCTCAGTAAAAGCTCTTTGC	67
ORXR1	AB110634	F, TGCGCTACCTCTGGAAGGA R, GCGATCAGCGCCCATTC	58
ORXR2	XM_004945362	F, AAGTGCTGAAGCAACCATTGC R, AAGGCCACACTCTCCCTTCTG	61
CRH	NM_001123031	F, TCAGCACCAGAGCCATCACA R, GCTCTATAAAAATAAAGAGGTGACATCAGA	74
Ghrelin	AY303688	F, CACTCCTGCTCACATACAAGTTCA R, TCATATGTACACCTGTGGCAGAAA	75
GHSR	NM_204394	F, GCACAAATCGGCAAGGAAA R, GTGACATCTCCCAGCAAATCC	61
MC1R	NM_001031462	F, GCTCTGCCTCATTGGCTTCT R, TGCCAGCGCGAACATGT	76
MC2R	NM_001031515	F, GCTGTTGGGCCCCCTTT R, AAGGGTTGTGTGGGCAAAAC	60
MC3R	AB017137	F, GCCTCCCTTTACGTTCACATGT R, GCTGCGATGCGCTTCAC	59
MC4R	NM_001031514	F, CCTCGGGAGGCTGCTATGA R, GATGCCCAGAGTCACAAACACTT	62
MC5R	NM_001031015	F, GCCCTGCGTTACCACAACAT R, CCAAATGCATGCAATGATAAGC	63
Ob-R	NM_204323	F, GCAAGACCCTCTCCCTTATCTCT R, TCTGTGAAAGCATCATCCTGATCT	70
Adiponectin	AY786316	F, ATGGACAAAAGGGAGACAAAGG R, TCCAGCACCCATATACCCAAA	64
AdipR1	NM_001031027	F, CCGGGCAAATTCGACATC R, CCACCACGAGCACATGGA	58
AdipR2	NM_001007854	F, TTGCCACTCGGAAGGTGTTT R, AGTGCAATGCCAGAATAATCCA	60
Visfatin	NM_001030728	F, CCGGTAGCTGATCCAAACAAA R, CCAGCAGGTGTCCTATGCAA	65
NPGL	AB909129	F, CCCTCAGTGCTGGGAATCC R, AGAAATGCGAGGCTTCCTCAT	61
NPGM	XM_040665724.1	F, CACGGGCTGGTGGAAATG R, ATGAAGTCCCAGAGAGCAATGAC	65
HIF-1α	NM_204297	F, AACACACCATGATATGTTCACGAAA R, CCCAGACGTAGCCACCTTGT	83
HIF-2α	NM_204807.3	F, CCAGTGCGTTCTCCCAACAT R, GCCTCGTTGCCCCAAAC	66
EGLN1	XM_015284393	F, CGCCGCAACCCTCATG R, AATACCACACTGTTATTGCGTACCTT	64
HBA1	NM_001004376	F, TCCATGCTTCCCTGGACAA R, GTACTTGGCGGTCAGCACAGT	59
HBBR	NM_001004390	F, CCGAGGAGAAGCAGCTCATC R, TTCGGCACCGCATTCC	65
HBE	NM_001081704	F, TCCTGCCTGCCAATTTGC R, CAGAGCATGAGCCACAACGT	55
HBM	NM_001004375	F, GAGCAACCTGCATGCCTACA R, GCGACAACAGCTTGAAATTGAC	59
HBZ	NM_001004374	F, TGCCGTGACCACCATCTG	56

		R, CCAGCCCAATGGACTCAATC	
HEPH	XM_420165	F, GGACTGGAATTATGCTCCAACAG R, CCTTTAGGCTACGTGTGATGCTT	68
HJV	XM_025143560	F, GCTCCGGATCACCAAAGCT R, AGCGGAACGTCTTCTCGTAGTC	61
FPN1	BM486402	F, CGCATAAGGCTAGCGCTTTC R, GTGTTGCCTTCCCCGACTT	62
FTH1	NM_205086	F, CCACGAGGAGCGTGAACAT R, TCCACCCCTCTGGTTTTGC	58
AMPK-α1	NM_001039603	F, CCACCCCTGTACCGGAAATA R, GGAAGCGAGTGCCAGAGTTC	68
AMPK-α2	NM_001039605	F, GCGGAGAGAATCTGCTGGAA R, TGTAAGCATGGACGTGTTGAAGA	62
AMPK-β1	NM_001039912	F, TTGGCAGCAGGATCTGGAA R, AAGACTGTTGGTCGAGCTTGAGT	60
AMPK-β2	NM_001044662	F, TGTGACCCGGCCCTACTG R, GCGTAGAGGTGATTGAGCATGA	56
AMPK-γ1	NM_001034827	F, CAAGCCGTTGGTCTGCATCT R, GGGAGGAGACGGCATCAA	56
AMPK-γ2	NM_001278142	F, TGCCATGCCATTCTTGGA R, CCACCTTGCGAGAAGCATTT	62
AMPK-γ3	NM_001031258	F, CCCAAGCCACGCTTCCTA R, ACGGAAGGTGCCGAC ACA-3′	57
PI3Kα	NM_001004410	F, GCCATCTTACTCCAGGCGTATC R, GAGGGACTTGGCTGTAGCTTCTC	70
PI3Kβ	NM_001031311.1	F, TGCCTCCTGCCGTGACA R, TCAGCACCGATCTGTGAATCC	61
PI3Kδ	NM_001012696.2	F, TGACCAATATCCACAAGCTTTGG R, GCCACGTCTTCATGCTTGTTC	69
Akt	AF039943	F, TTCAACGGTGATCTTTTGACTGA R, CGGGAATGTCTCTTGGTGGAT	64
mTOR	XM_417614	F, CATGTCAGGCACTGTGTCTATTCTC R, CTTTCGCCCTTGTTTCTTCACT	77
S6K	NM_001109771	F, GTCAGACATCACTTGGGTAGAGAAAG R, ACGCCCTCGCCCTTGT	60
NF-κB1	NM_205134.2	F, CAGTCAACGCAGGACCTAAAGA R, TGTGACGTGAAGTATTCCAAGGTT	65
NF-κB2	NM_204413.2	F, AGATCTCGCGGATGGACAAG R, CTCAATGTCATCTTTCTGCACCTT	92
IL-6	NM_204628.2	F, GCTTCGACGAGGAGAAATGC R, GGTAGGTCTGAAAGGCGAACAG	63
TNF-α	NM_204267.2	F, CGTTTGGGAGTGGGCTTTAA R, GCTGATGGCAGAGGCAGAA	61
RelA	NM_001396038.1	F, CGCTGCGTGCACAGTTTC R, CTTCCAGTTCCCGTTTCTTCAC	61
RelB	XM_003643092.5	F, CCACGGCGCTAATAATTTGC R, GAAGGGCATTGCATGCATT	60
HSP60	NM_001012916	F, CGCAGACATGCTCCGTTTG R, TCTGGACACCGGCCTGAT	55
HSP70	JO2579	F, GGGAGAGGGTTGGGCTAGAG R, TTGCCTCCTGCCCAATCA	55
HSP90	NM_001109785.1	F, TGACCTTGTCAACAATCTTGGTACTAT R, CCTGCAGTGCTTCCATGAAA	68
HSP27	XM_001231557	F, TTGAAGGCTGGCTCCTGATC R, AAGCCATGCTCATCCATCCT	58
18S	AF173612	F, TCCCCTCCCGTTACTTGGAT R, GCGCTCGTCGGCATGTA	60

† GenBank.
§ F, forward; R, reverse.

ABBREVIATIONS

(s)IgA, (secretory) immunoglobulin A

^{1}H-NMR, proton nuclear magnetic resonance

95RB, 1995 random bred population in study #4

AA, amino acid

ABF, antibiotic-free

ACRB, Athens Canadian Random Bred population in study #4

AdipR1/2, adiponectin receptors 1/2

AGP, antibiotic growth promoter

AgRP, agouti-related peptide

Akt, protein kinase B

AMP, antimicrobial defense peptide

AMPK, AMP-activated kinase

ARG, supplemented group in study #3

BCO, bacterial chondronecrosis with osteomyelitis

BW(G), body weight (gain)

CART, cocaine and amphetamine regulated transcript

CE, competitive exclusion

CON, control group in studies #1-3

CRH, corticotropin releasing hormone

DFI, daily feed intake

DWG, daily weight gain

EGLN1, hypoxia-inducible factor prolyl hydroxylase 2

FAS, fatty acid synthase

FCR, feed conversion ratio

FI, feed intake

FITC-D, fluorescein isothiocyanate-dextran

FOS, fructo-oligosaccharides

FPD, footpad dermatitis

FPN1, ferroportin 1

FRHN, feeding-related hypothalamic neuropeptides

FTH1, ferritin heavy chain 1

GALT, gut-associated lymphoid tissue

GH(S)R, growth hormone (secretagogue) receptor

GH, growth hormone

GI(T), gastrointestinal (tract)

HBA1, hemoglobin subunit alpha 1

HBBR, hemoglobin beta subunit rho

HBE, hemoglobin subunit epsilon

HBM, hemoglobin subunit mu

HBZ, hemoglobin subunit zeta

HEPH, hephaestin

HIF, hypoxia-inducible factor

HJV, hemojuvelin

HSP, heat shock protein

IBD, inflammatory bowel disease

IEC, intestinal epithelial cell

IFA, in-feed antibiotic

IFN-γ, interferon gamma

IGF-1, insulin-like growth factor 1

IL-1β/6, interleukin 1 beta/6

JF, red jungle fowl population in study #4

KEGG, Kyoto Encyclopedia of Genes and Genome

LAB, lactic acid bacteria

LPS, lipopolysaccharide

MAFbx, muscle atrophy F-box

MC1/2/3/4/5R, melanocortin receptor 1/2/3/4/5

MLCK, myosin light chain kinase

MRB, modern random bred population in study #4

mTOR, mechanistic target of rapamycin

MUH, high-dose supplemented group in study #2

MUL, low-dose supplemented group in study #2

NAG, N-acetylglucosamine

NAM, N-acetylmuramic acid

NE, necrotic enteritis

NEFA, non-esterified fatty acids

NF-κB, nuclear factor κB

NPGL, neurosecretory protein GL

NPGM, neurosecretory protein GM

NPY, neuropeptide Y

NRC, National Research Council

NSP, non-starch polysaccharides

Ob-R, leptin receptor

ORX, orexin

ORXR1/2, orexin receptor 1/2

PAMP, pathogen-associated molecular pattern

PGN, peptidoglycan

PI3K, phosphatidylinositol 3-kinase

POMC, proopiomelanocortin

PRR, pattern recognition receptor

RH, relative humidity

RNS, reactive nitrogen species

ROS, reactive oxygen

S6K, ribosomal protein S6 kinase beta-1

SCFA, short-chain fatty acid

SE, standard error

SM, spaghetti meat

SnE1/2, experiment #1/2 of study #*n* of the PhD project

SYN, supplemented group in study #1

T3, triiodothyronine

T4, thyroxine

TEER, transepithelial electrical resistance

TJ, tight junction

TLR, Toll-like receptor

TN(Z), thermoneutral (zone)

TNF-α, tumor necrosis factor alpha

WB, woody breast

WS, white striping

ZO, zonula occludens

LIST OF FIGURES

REFERENCES

Abasht, B., M. F. Mutryn, R. D. Michalek, and W. R. Lee. 2016. Oxidative Stress and Metabolic Perturbations in Wooden Breast Disorder in Chickens. PLoS One 11:e0153750.

Abd El-Hack, M. E., M. T. El-Saadony, M. E. Shafi, S. Y. A. Qattan, G. E. Batiha, A. F. Khafaga, A. E. Abdel-Moneim, and M. Alagawany. 2020. Probiotics in poultry feed: A comprehensive review. J. Anim. Physiol. Anim. Nutr. 104:1835–1850.

Abdel-Latif, M. A., A. H. El-Far, A. R. Elbestawy, R. Ghanem, S. A. Mousa, and H. S. Abd El-Hamid. 2017. Exogenous dietary lysozyme improves the growth performance and gut microbiota in broiler chickens targeting the antioxidant and non-specific immunity mRNA expression. PLoS One 12:e0185153.

Abdelli, N., A. Ramser, E. S. Greene, L. Beer, T. W. Tabler, S. K. Orlowski, J. F. Pérez, D. Solà-Oriol, N. B. Anthony, and S. Dridi. 2021. Effects of Cyclic Chronic Heat Stress on the Expression of Nutrient Transporters in the Jejunum of Modern Broilers and Their Ancestor Wild Jungle Fowl. Front. Physiol. 0:1732.

Abrams, G. D., H. Bauer, and H. Sprinz. 1963. Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. A comparison of germ-free and conventional mice. Lab. Investig. 12:355–364.

Abreu, M. T. 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat. Rev. Immunol. 10:131–144.

Ain Baziz, H., P. A. Geraert, J. C. F. Padilha, and S. Guillaumin. 1996. Chronic heat exposure enhances fat deposition and modifies muscle and fat partition in broiler carcasses. Poult. Sci. 75:505–513.

Akbarian, A., J. Michiels, J. Degroote, M. Majdeddin, A. Golian, and S. De Smet. 2016. Association between heat stress and oxidative stress in poultry; mitochondrial dysfunction and dietary interventions with phytochemicals. J. Anim. Sci. Biotechnol. 7:37.

Alexander, P., C. Brown, A. Arneth, J. Finnigan, and M. D. A. Rounsevell. 2016. Human appropriation of land for food: The role of diet. Glob. Environ. Chang. 41:88–98.

Alhenaky, A., A. Abdelqader, M. Abuajamieh, and A.-R. Al-Fataftah. 2017. The effect of heat stress on intestinal integrity and Salmonella invasion in broiler birds. J. Therm. Biol. 70:9–14.

Amann, R. I., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143–69.

Anderson, J. M., and C. M. Van Itallie. 2009. Physiology and Function of the Tight Junction. Cold Spring Harb. Perspect. Biol. 1:a002584–a002584.

de Andrade, A. N., J. C. Rogler, W. R. Featherston, and C. W. Alliston. 1977. Interrelationships Between Diet and Elevated Temperatures (Cyclic and Constant) on Egg Production and Shell Quality. Poult. Sci. 56:1178–1188.

Annison, E. F., K. J. Hill, and R. Kenworthy. 1968. Volatile fatty acids in the digestive tract of the fowl. Br. J. Nutr. 22:207–216.

Apajalahti, J., A. Kettunen, and H. Graham. 2004. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. Worlds. Poult. Sci. J. 60:223–232.

Applegate, T. J., and C. Troche. 2014. Influence of probiotics on intestinal structure and barrier functionality of poultry.Pages 51–70 in Probiotics in Poultry Production: Concept and Applications. H. A. Abdelrahman, W., Mohnl, M., eds. 5M Publishing Ltd.

Arnold, A., O. L. Kline, C. A. Elvehjem, and E. B. Hart. 1936. Further Studies On The Growth Factor Required By Chicksç The Essential Nature of Arginine. J. Biol. Chem. 116:699–709.

Arora, S., and Anubhuti. 2006. Role of neuropeptides in appetite regulation and obesity – A review. Neuropeptides 40:375–401.

Atuma, C., V. Strugala, A. Allen, and L. Holm. 2001. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am. J. Physiol. Liver Physiol. 280:G922–G929.

Aviagen. 2018. ROSS BROILER: Management Handbook. Available at https://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-BroilerHandbook2018-EN.pdf.

Aviagen. 2022a. ROSS BROILER: Nutrition Specifications. Available at https://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-BroilerNutritionSpecifications2022-EN.pdf.

Aviagen. 2022b. ROSS 308 and ROSS 308 FF Performance Objectives. Available at http://eu.aviagen.com/tech-center/download/1339/Ross308-308FF-BroilerPO2019-EN.pdf.

Awad, W., C. Hess, and M. Hess. 2017. Enteric Pathogens and Their Toxin-Induced Disruption of the Intestinal Barrier through Alteration of Tight Junctions in Chickens. Toxins 9:60.

Azad, M. A. K., M. Kikusato, T. Maekawa, H. Shirakawa, and M. Toyomizu. 2010. Metabolic characteristics and oxidative damage to skeletal muscle in broiler chickens exposed to chronic heat stress. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 155:401–406.

Bäckhed, F., R. E. Ley, J. L. Sonnenburg, D. A. Peterson, and J. I. Gordon. 2005. Host-bacterial mutualism in the human intestine. Science 307:1915–1920.

Baéza, E., M. Jégou, F. Gondret, J. Lalande-Martin, I. Tea, E. Le Bihan-Duval, C. Berri, A. Collin, S. Métayer-Coustard, I. Louveau, S. Lagarrigue, and M. J. Duclos. 2015. Pertinent plasma indicators of the ability of chickens to synthesize and store lipids. J. Anim. Sci. 93:107–116.

Baier, J., M. Gänsbauer, C. Giessler, H. Arnold, M. Muske, U. Schleicher, S. Lukassen, A. Ekici, M. Rauh, C. Daniel, A. Ha rtmann, B. Schmid, P. Tripal, K. Dettmer, P. J. Oefner, R. Atreya, S. Wirtz, C. Bogdan, and J. Mattner. 2020. Arginase impedes the resolution of colitis by altering the microbiome and metabolome. J. Clin. Invest. 130:5703–5720.

Bain, A. R., S. A. Morrison, and P. N. Ainslie. 2014. Cerebral oxygenation and hyperthermia. Front. Physiol. 5 MAR:92.

Baird, N. A., D. W. Turnbull, and E. A. Johnson. 2006. Induction of the Heat Shock Pathway during Hypoxia Requires Regulation of Heat Shock Factor by Hypoxia-inducible Factor-1. J. Biol. Chem. 281:38675–38681.

Baldi, G., F. Soglia, and M. Petracci. 2020. Current Status of Poultry Meat Abnormalities. Meat Muscle Biol. 4:4–5.

Baldwin, S., R. J. Hughes, T. T. H. Van, R. J. Moore, and D. Stanley. 2018. At-hatch administration of probiotic to chickens can introduce beneficial changes in gut microbiota. PLoS One 13:e0194825.

Bali, V., P. S. Panesar, M. B. Bera, and R. Panesar. 2015. Fructo-oligosaccharides: Production, Purification and Potential Applications. Crit. Rev. Food Sci. Nutr. 55:1475–1490.

Ball, R. O., K. L. Urschel, and P. B. Pencharz. 2007. Nutritional Consequences of Interspecies Differences in Arginine and Lysine Metabolism. J. Nutr. 137:1626S-1641S.

Balnave, D., and J. Brake. 2002. Re-evaluation of the classical dietary arginine:lysine interaction for modern poultry diets: a review. Worlds. Poult. Sci. J. 58:275–289.

Bandarra, D., J. Biddlestone, S. Mudie, H. A. J. Müller, and S. Rocha. 2015. HIF-1α restricts NF-κB-dependent gene expression to control innate immunity signals. DMM Dis. Model. Mech. 8:169–181.

Barbul, A. 1986. Arginine: Biochemistry, Physiology, and Therapeutic Implications. J. Parenter. Enter. Nutr. 10:227–238.

Basoo, H., F. Khajali, E. Asadi Khoshoui, M. Faraji, and R. F. Wideman. 2012. Re-evaluation of Arginine Requirements for Broilers Exposed to Hypobaric Condition during the 3-to 6-week Period. J. Poult. Sci. 49:303–307.

Baumgard, L. H., and R. P. Rhoads. 2013. Effects of Heat Stress on Postabsorptive Metabolism and Energetics. Annu. Rev. Anim. Biosci. 1:311–337.

Beauclercq, S., L. Nadal-Desbarats, C. Hennequet-Antier, A. Collin, S. Tesseraud, M. Bourin, E. Le Bihan-Duval, and C. Berri. 2016. Serum and Muscle Metabolomics for the Prediction of Ultimate pH, a Key Factor for Chicken-Meat Quality. J. Proteome Res. 15:1168–1178.

Beaumont, M., and F. Blachier. 2020. Amino Acids in Intestinal Physiology and Health.Pages 1–20 in Amino Acids in Nutrition and Health: Amino acids in systems function and health. Wu, G., ed. Springer Nature Switzerland AG.

Bechtold, D. A., and A. S. I. Loudon. 2013. Hypothalamic clocks and rhythms in feeding behaviour. Trends Neurosci. 36:74–82.

Beckford, R. C., L. E. Ellestad, M. Proszkowiec-Weglarz, L. Farley, K. Brady, R. Angel, H.-C. Liu, and T. E. Porter. 2020. Effects of heat stress on performance, blood chemistry, and hypothalamic and pituitary mRNA expression in broiler chickens. Poult. Sci. 99:6317–6325.

Bedford, M. 2000. Removal of antibiotic growth promoters from poultry diets: implications and strategies to minimise subsequent problems. Worlds. Poult. Sci. J. 56:347–365.

Bedford, M. R., and A. J. Cowieson. 2012. Exogenous enzymes and their effects on intestinal microbiology. Anim. Feed Sci. Technol. 173:76–85.

Belhadj Slimen, I., T. Najar, A. Ghram, and M. Abdrrabba. 2016. Heat stress effects on livestock: molecular, cellular and metabolic aspects, a review. J. Anim. Physiol. Anim. Nutr. 100:401–412.

Belkaid, Y., and T. W. Hand. 2014. Role of the Microbiota in Immunity and Inflammation. Cell 157:121–141.

Di Bella, J. M., Y. Bao, G. B. Gloor, J. P. Burton, and G. Reid. 2013. High throughput sequencing methods and analysis for microbiome research. J. Microbiol. Methods 95:401–414.

Bennett, C. E., R. Thomas, M. Williams, J. Zalasiewicz, M. Edgeworth, H. Miller, B. Coles, A. Foster, E. J. Burton, and U. Marume. 2018. The broiler chicken as a signal of a human reconfigured biosphere. R. Soc. Open Sci. 5:180325.

Bester, B. H., and S. H. Lombard. 1990. Influence of lysozyme on selected bacteria associated with gouda cheese. J. Food Prot. 53:306–311.

Bezner Kerr, R., T. Hasegawa, R. Lasco, I. Bhatt, D. Deryng, A. Farrell, H. Gurney-Smith, H. Ju, S. Lluch-Cota, F. Meza, G. Nelson, H. Neufeldt, and P. Thornton. 2022. Food, Fibre, and Other Ecosystem Products.in Climate Change 2022: Impacts, Adaptation, and Vulnerability. Contribution of Working Group II to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. Pörtner, H.-O., Roberts, D.C., Tignor, M., Poloczanska, E.S., Mintenbeck, K., Alegría, A., Craig, M., Langsdorf, S., Löschke, S., Möller, V., Okem, A., Rama, B., eds. Cambridge University Press.

Bhutta, B., F. Alghoula, and I. Berim. 2022. Hypoxia. StatPearls Available at https://pubmed.ncbi.nlm.nih.gov/29493941/.

Bigot, K., M. Taouis, and S. Tesseraud. 2003. Refeeding and Insulin Regulate S6K1 Activity in Chicken Skeletal Muscles. J. Nutr. 133:369–373.

Billman, G. E. 2020. Homeostasis: The Underappreciated and Far Too Often Ignored Central Organizing Principle of Physiology. Front. Physiol. 11:200.

Bindari, Y. R., and P. F. Gerber. 2022. Centennial Review: Factors affecting the chicken gastrointestinal microbial composition and their association with gut health and productive performance. Poult. Sci. 101:101612.

Bindels, L. B., N. M. Delzenne, P. D. Cani, and J. Walter. 2015. Opinion: Toward a more comprehensive concept for prebiotics. Nat. Rev. Gastroenterol. Hepatol. 12:303–310.

Bischoff, S. C., G. Barbara, W. Buurman, T. Ockhuizen, J.-D. Schulzke, M. Serino, H. Tilg, A. Watson, and J. M. Wells. 2014. Intestinal permeability – a new target for disease prevention and therapy. BMC Gastroenterol. 14:189.

Bjerrum, L., R. M. Engberg, B. B. Jensen, T. Leser, K. Finster, and K. Pedersen. 2004. Investigation of the intestinal microflora of broilers.in Proc. AFAC Workshop, Alternatives to feed antibiotics and anticoccidials in the pig and poultry meat production. Aarhus, Denmark.

Blajman, J. E., L. S. Frizzo, M. V. Zbrun, D. M. Astesana, M. L. Fusari, L. P. Soto, M. R. Rosmini, and M. L. Signorini. 2014. Probiotics and broiler growth performance: a meta-analysis of randomised controlled trials. Br. Poult. Sci. 55:483–494.

Blankenship, L. C., J. S. Bailey, N. A. Cox, N. J. Stern, R. Brewer, and O. Williams. 1993. Two-step mucosal competitive exclusion flora treatment to diminish salmonellae in commercial broiler chickens. Poult. Sci. 72:1667–1672.

Blankenship, K., A. Gilley, A. Piekarski, S. Orlowski, E. Greene, W. Bottje, N. Anthony, and S. Dridi. 2015. Differential expression of feeding-related hypothalamic neuropeptides in the first generation of quails divergently selected for low or high feed efficiency. Neuropeptides 58:31–40.

Bligh, J., and K. G. Johnson. 1973. Glossary of terms for thermal physiology. J. Appl. Physiol. 35:941–961.

Bobek, S., A. Sechman, E. Wieczorek, D. Wronska-Fortuna, K. Koziec, and J. Niezgoda. 1997. Reverse 3,3′,5′-Triiodothyronine (rT 3) Enhances Hyperglycemic and Lipemic Effects of Heat-Stress in Chickens. Horm. Metab. Res. 29:252–254.

Bocci, V. 1992. The Neglected Organ: Bacterial Flora Has a Crucial Immunostimulatory Role. Perspect. Biol. Med. 35:251–260.

Bodle, B. C., C. Alvarado, R. B. Shirley, Y. Mercier, and J. T. Lee. 2018. Evaluation of different dietary alterations in their ability to mitigate the incidence and severity of woody breast and white striping in commercial male broilers. Poult. Sci. 97:3298–3310.

Boland, M. J., A. N. Rae, J. M. Vereijken, M. P. M. Meuwissen, A. R. H. Fischer, M. A. J. S. van Boekel, S. M. Rutherfurd, H. Gruppen, P. J. Moughan, and W. H. Hendriks. 2013. The future supply of animal-derived protein for human consumption. Trends Food Sci. Technol. 29:62–73.

Borda-Molina, D., J. Seifert, and A. Camarinha-Silva. 2018. Current Perspectives of the Chicken Gastrointestinal Tract and Its Microbiome. Comput. Struct. Biotechnol. J. 16:131–139.

Borda-Molina, D., M. Vital, V. Sommerfeld, M. Rodehutscord, and A. Camarinha-Silva. 2016. Insights into

Broilers' Gut Microbiota Fed with Phosphorus, Calcium, and Phytase Supplemented Diets. Front. Microbiol. 7.

Bordignon, F., G. Xiccato, M. Boskovic Cabrol, M. Birolo, and A. Trocino. 2022. Factors Affecting Breast Myopathies in Broiler Chickens and Quality of Defective Meat: A Meta-Analysis. Front. Physiol. 13:1322.

Borges, S. A., A. V. F. Da Silva, and A. Maiorka. 2007. Acid-base balance in broilers. Worlds. Poult. Sci. J. 63:73–81.

Bortoluzzi, C., J. I. M. Fernandes, K. Doranalli, and T. J. Applegate. 2020. Effects of dietary amino acids in ameliorating intestinal function during enteric challenges in broiler chickens. Anim. Feed Sci. Technol. 262:114383.

Bortoluzzi, C., S. J. Rochell, and T. J. Applegate. 2018. Threonine, arginine, and glutamine: Influences on intestinal physiology, immunology, and microbiology in broilers. Poult. Sci. 97:937–945.

Boussaid-Om Ezzine, S., N. Everaert, S. Métayer-Coustard, N. Rideau, C. Berri, R. Joubert, S. Temim, A. Collin, and S. Tesseraud. 2010. Effects of heat exposure on Akt/S6K1 signaling and expression of genes related to protein and energy metabolism in chicken (Gallus gallus) pectoralis major muscle. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 157:281–287.

Box, G. E. P., and D. R. Cox. 1964. An Analysis of Transformations. J. R. Stat. Soc. Ser. B 26:211–243.

Brackenbury, J. ., and P. Avery. 1980. Energy consumption and ventilatory mechanisms in the exercising fowl. Comp. Biochem. Physiol. Part A Physiol. 66:439–445.

Brisbin, J. T., J. Gong, and S. Sharif. 2008. Interactions between commensal bacteria and the gut-associated immune system of the chicken. Anim. Heal. Res. Rev. 9:101–110.

Brook, I. 2017. Anaerobic Bacteria.Pages 1628-1644.e2 in Infectious Diseases. Cohen, J., Powderly, W.G., Opal, S.M., eds. 4th ed. Elsevier, Amsterdam, The Netherlands.

Broom, L. J., and M. H. Kogut. 2018a. The role of the gut microbiome in shaping the immune system of chickens. Vet. Immunol. Immunopathol. 204:44–51.

Broom, L. J., and M. H. Kogut. 2018b. Gut immunity: Its development and reasons and opportunities for modulation in monogastric production animals. Anim. Heal. Res. Rev. 19:46–52.

Broom, L. J., and M. H. Kogut. 2018c. Inflammation: Friend or foe for animal production? Poult. Sci. 97:510–514.

Brugaletta, G., J.-R. Teyssier, S. J. Rochell, S. Dridi, and F. Sirri. 2022. A review of heat stress in chickens. Part I: Insights into physiology and gut health. Front. Physiol. 13:1535.

Brumfield, K. D., A. Huq, R. R. Colwell, J. L. Olds, and M. B. Leddy. 2020. Microbial resolution of whole genome shotgun and 16S amplicon metagenomic sequencing using publicly available NEON data. PLoS One 15:e0228899.

Buchanan, J. B., and R. W. Johnson. 2007. Regulation of Food Intake by Inflammatory Cytokines in the Brain. Neuroendocrinology 86:183–190.

Burkholder, K. M., K. L. Thompson, M. E. Einstein, T. J. Applegate, and J. A. Patterson. 2008. Influence of Stressors on Normal Intestinal Microbiota, Intestinal Morphology, and Susceptibility to Salmonella Enteritidis Colonization in Broilers. Poult. Sci. 87:1734–1741.

Cahaner, A., and F. Leenstra. 1992. Effects of high temperature on growth and efficiency of male and female broilers from lines selected for high weight gain, favorable feed conversion, and high or low fat content.

Poult. Sci. 71:1237–1250.

Callewaert, L., and C. W. Michiels. 2010. Lysozymes in the animal kingdom. J. Biosci. 35:127–160.

CarbonBrief. 2022. Guest post: What have been the most extreme heatwaves in history? Available at https://www.carbonbrief.org/guest-post-what-have-been-the-most-extreme-heatwaves-in-history/.

Carmeliet, P., Y. Dor, J.-M. Herbert, D. Fukumura, K. Brusselmans, M. Dewerchin, M. Neeman, F. Bono, R. Abramovitch, P. Maxwell, C. J. Koch, P. Ratcliffe, L. Moons, R. K. Jain, D. Collen, and E. Keshet. 1998. Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 394:485–490.

Cason, J. A., N. A. Cox, and J. S. Bailey. 1994. Transmission of Salmonella typhimurium during Hatching of Broiler Chicks. Avian Dis. 38:583.

Castanon, J. I. R. 2007. History of the Use of Antibiotic as Growth Promoters in European Poultry Feeds. Poult. Sci. 86:2466–2471.

Casteleyn, C., M. Doom, E. Lambrechts, W. van den Broeck, P. Simoens, and P. Cornillie. 2010. Locations of gut-associated lymphoid tissue in the 3-month-old chicken: A review. Avian Pathol. 39:143–150.

Castro, F. L. de S., and W. K. Kim. 2020. Secondary Functions of Arginine and Sulfur Amino Acids in Poultry Health: Review. Animals 10:2106.

Celi, P., A. J. Cowieson, F. Fru-Nji, R. E. Steinert, A.-M. Kluenter, and V. Verlhac. 2017. Gastrointestinal functionality in animal nutrition and health: New opportunities for sustainable animal production. Anim. Feed Sci. Technol. 234:88–100.

De Cesare, A., F. Sirri, G. Manfreda, P. Moniaci, A. Giardini, M. Zampiga, and A. Meluzzi. 2017. Effect of dietary supplementation with Lactobacillus acidophilus D2/CSL (CECT 4529) on caecum microbioma and productive performance in broiler chickens. PLoS One 12:1–21.

Chamruspollert, M., G. M. Pesti, and R. I. Bakalli. 2002. Dietary interrelationships among arginine, methionine, and lysine in young broiler chicks. Br. J. Nutr. 88:655–660.

Chauhan, S. S., V. P. Rashamol, M. Bagath, V. Sejian, and F. R. Dunshea. 2021. Impacts of heat stress on immune responses and oxidative stress in farm animals and nutritional strategies for amelioration. Int. J. Biometeorol. 65:1231–1244.

Chaves, B. D., M. M. Brashears, and K. K. Nightingale. 2017. Applications and safety considerations of Lactobacillus salivarius as a probiotic in animal and human health. J. Appl. Microbiol. 123:18–28.

Chen, M., N. J. Stern, J. S. Bailey, and N. A. Cox. 1998. Administering mucosal competitive exclusion flora for control of salmonellae. J. Appl. Poult. Res. 7:384–391.

Chen, J., G. Tellez, J. D. Richards, and J. Escobar. 2015. Identification of Potential Biomarkers for Gut Barrier Failure in Broiler Chickens. Front. Vet. Sci. 2:14.

Chen, S., J. Wang, D. Peng, G. Li, J. Chen, and X. Gu. 2018. Exposure to heat-stress environment affects the physiology, circulation levels of cytokines, and microbiome in dairy cows. Sci. Rep. 8:1–11.

Chen, L., F.-J. Xin, J. Wang, J. Hu, Y.-Y. Zhang, S. Wan, L.-S. Cao, C. Lu, P. Li, S. F. Yan, D. Neumann, U. Schlattner, B. Xia, Z.-X. Wang, and J.-W. Wu. 2013. Conserved regulatory elements in AMPK. Nature 498:E8–E10.

Cheng, H.-Y., M.-X. Ning, D.-K. Chen, and W.-T. Ma. 2019. Interactions Between the Gut Microbiota and the Host Innate Immune Response Against Pathogens. Front. Immunol. 10:607.

Chichlowski, M., J. Croom, B. W. McBride, G. B. Havenstein, and M. D. Koci. 2007. Metabolic and physiological impact of probiotics or direct-fed-microbials on poultry: A brief review of current knowledge. Int. J. Poult. Sci. 6:694–704.

Choct, M. 2009. Managing gut health through nutrition. Br. Poult. Sci. 50:9–15.

Christensen, K., C. Owens, and M. T. Kidd. 2015. The effect of added l-arginine on the severity of woody breast and white striping in male broilers.Page 147 in Poultry Science Association 104th Annual Meeting. Louisville, Kentucky.

Claret, M., M. A. Smith, R. L. Batterham, C. Selman, A. I. Choudhury, L. G. D. Fryer, M. Clements, H. Al-Qassab, H. Heffron, A. W. Xu, J. R. Speakman, G. S. Barsh, B. Viollet, S. Vaulont, M. L. J. Ashford, D. Carling, and D. J. Withers. 2007. AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons. J. Clin. Invest. 117:2325–2336.

Clavijo, V., and M. J. V. Flórez. 2018. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. Poult. Sci. 97:1006–1021.

Cline, M. A., W. Nandar, B. C. Prall, C. N. Bowden, and D. M. Denbow. 2008. Central visfatin causes orexigenic effects in chicks. Behav. Brain Res. 186:293–297.

Coburn, L. A., X. Gong, K. Singh, M. Asim, B. P. Scull, M. M. Allaman, C. S. Williams, M. J. Rosen, M. K. Washington, D. P. Barry, M. B. Piazuelo, R. A. Casero, R. Chaturvedi, Z. Zhao, and K. T. Wilson. 2012. L-arginine Supplementation Improves Responses to Injury and Inflammation in Dextran Sulfate Sodium Colitis. PLoS One 7:e33546.

Colgrave, M. L., S. Dominik, A. B. Tobin, R. Stockmann, C. Simon, C. A. Howitt, D. P. Belobrajdic, C. Paull, and T. Vanhercke. 2021. Perspectives on Future Protein Production. J. Agric. Food Chem. 69:15076–15083.

Collier, R. J., L. H. Baumgard, R. B. Zimbelman, and Y. Xiao. 2019. Heat stress: physiology of acclimation and adaptation. Anim. Front. 9:12–19.

Collier, S. R., D. P. Casey, and J. A. Kanaley. 2005. Growth hormone responses to varying doses of oral arginine. Growth Horm. IGF Res. 15:136–139.

Collier, C. T., J. D. van der Klis, B. Deplancke, D. B. Anderson, and H. R. Gaskins. 2003. Effects of Tylosin on Bacterial Mucolysis, Clostridium perfringens Colonization, and Intestinal Barrier Function in a Chick Model of Necrotic Enteritis. Antimicrob. Agents Chemother. 47:3311–3317.

Collins, K. E., H. L. Marks, S. E. Aggrey, M. P. Lacy, and J. L. Wilson. 2016. History of the Athens Canadian Random Bred and the Athens Random Bred control populations. Poult. Sci. 95:997–1004.

Corcoran, S. E., and L. A. J. O'Neill. 2016. HIF1α and metabolic reprogramming in inflammation. J. Clin. Invest. 126:3699–3707.

Coster, J., R. McCauley, and J. Hall. 2004. Glutamine: metabolism and application in nutrition support. Asia Pac. J. Clin. Nutr. 13:25–31.

Cota, D., K. Proulx, K. A. Blake Smith, S. C. Kozma, G. Thomas, S. C. Woods, and R. J. Seeley. 2006. Hypothalamic mTOR signaling regulates food intake. Science 312:927–930.

Coufal, C. D., C. Chavez, P. R. Niemeyer, and J. B. Carey. 2006. Measurement of broiler litter production rates and nutrient content using recycled litter. Poult. Sci. 85:398–403.

Cox, N. A., J. S. Bailey, L. C. Blankenship, and R. P. Gildersleeve. 1992. Research note: in ovo administration of a competitive exclusion culture treatment to broiler embryos. Poult. Sci. 71:1781–1784.

Cramer, T., Y. Yamanishi, B. E. Clausen, I. Förster, R. Pawlinski, N. Mackman, V. H. Haase, R. Jaenisch, M. Corr, V. Nizet, G. S. Firestein, H.-P. Gerber, N. Ferrara, and R. S. Johnson. 2003. HIF-1α Is Essential for Myeloid Cell-Mediated Inflammation. Cell 112:645–657.

Cressman, M. D., Z. Yu, M. C. Nelson, S. J. Moeller, M. S. Lilburn, and H. N. Zerby. 2010. Interrelations between the Microbiotas in the Litter and in the Intestines of Commercial Broiler Chickens. Appl. Environ. Microbiol. 76:6572–6582.

Cryan, J. F., K. J. O'riordan, C. S. M. Cowan, K. V. Sandhu, T. F. S. Bastiaanssen, M. Boehme, M. G. Codagnone, S. Cussotto, C. Fulling, A. V. Golubeva, K. E. Guzzetta, M. Jaggar, C. M. Long-Smith, J. M. Lyte, J. A. Martin, A. Molinero-Perez, G. Moloney, E. Morelli, E. Morillas, R. O'connor, J. S. Cruz-Pereira, V. L. Peterson, K. Rea, N. L. Ritz, E. Sherwin, S. Spichak, E. M. Teichman, M. van de Wouw, A. P. Ventura-Silva, S. E. Wallace-Fitzsimons, N. Hyland, G. Clarke, and T. G. Dinan. 2019. The microbiota-gut-brain axis. Physiol. Rev. 99:1877–2013.

Cummings, J. H., and G. T. Macfarlane. 1991. The control and consequences of bacterial fermentation in the human colon. J. Appl. Bacteriol. 70:443–459.

Czepiel, J., M. Dróżdż, H. Pituch, E. J. Kuijper, W. Perucki, A. Mielimonka, S. Goldman, D. Wultańska, A. Garlicki, and G. Biesiada. 2019. Clostridium difficile infection: review. Eur. J. Clin. Microbiol. Infect. Dis. 38:1211–1221.

Dahl, T. B., S. Holm, P. Aukrust, and B. Halvorsen. 2012. Visfatin/NAMPT: A Multifaceted Molecule with Diverse Roles in Physiology and Pathophysiology. Annu. Rev. Nutr. 32:229–243.

Dale, N. M., and H. L. Fuller. 1980. Effect of Diet Composition on Feed Intake and Growth of Chicks Under Heat Stress: II. Constant vs. Cycling Temperatures. Poult. Sci. 59:1434–1441.

Danzeisen, J. L., H. B. Kim, R. E. Isaacson, Z. J. Tu, and T. J. Johnson. 2011. Modulations of the Chicken Cecal Microbiome and Metagenome in Response to Anticoccidial and Growth Promoter Treatment. PLoS One 6:e27949.

Debrah, M., and A. R. Feingold. 2012. Anaerobic Cocci.Pages 988-990.e1 in Principles and Practice of Pediatric Infectious Diseases. 4th ed. Elsevier.

Decuypere, E., and J. Buyse. 1988. Thyroid hormones, corticosterone, growth hormone and somatomedins in avian species: general effects and possible implications in fattening.Pages 295–312 in Leanness in Domestic Birds. Leclercq, B., Whitehead, C.C., eds. Butterworth-Heinemann.

Delacour, D., J. Salomon, S. Robine, and D. Louvard. 2016. Plasticity of the brush border — the yin and yang of intestinal homeostasis. Nat. Rev. Gastroenterol. Hepatol. 13:161–174.

Delgado, M. J., J. M. Cerdá-Reverter, and J. L. Soengas. 2017. Hypothalamic Integration of Metabolic, Endocrine, and Circadian Signals in Fish: Involvement in the Control of Food Intake. Front. Neurosci. 11:354.

Delzenne, N. M., and P. D. Cani. 2011. Interaction between obesity and the gut microbiota: relevance in nutrition. Annu. Rev. Nutr. 31:15–31.

Denbow, D. M. 2015. Gastrointestinal Anatomy and Physiology.Pages 337–366 in Sturkie's Avian Physiology: Sixth Edition. Scanes, C., ed. 6th ed. Academic Press.

Dibner, J. J., and J. D. Richards. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poult. Sci. 84:634–643.

Dibner, J. J., J. D. Richards, and C. D. Knight. 2008. Microbial Imprinting in Gut Development and Health. J.

Appl. Poult. Res. 17:174–188.

Dieterle, F., A. Ross, G. Schlotterbeck, and H. Senn. 2006. Probabilistic Quotient Normalization as Robust Method to Account for Dilution of Complex Biological Mixtures. Application in 1 H NMR Metabonomics. Anal. Chem. 78:4281–4290.

Ding, J., R. Dai, L. Yang, C. He, K. Xu, S. Liu, W. Zhao, L. Xiao, L. Luo, Y. Zhang, and H. Meng. 2017. Inheritance and establishment of gut microbiota in chickens. Front. Microbiol. 8.

Dobson, A., P. D. Cotter, R. Paul Ross, and C. Hill. 2012. Bacteriocin production: A probiotic trait? Appl. Environ. Microbiol. 78:1–6.

Dokladny, K., M. N. Zuhl, and P. L. Moseley. 2016. Intestinal epithelial barrier function and tight junction proteins with heat and exercise. J. Appl. Physiol. 120:692–701.

Donaldson, E. E., D. Stanley, R. J. Hughes, and R. J. Moore. 2017. The time-course of broiler intestinal microbiota development after administration of cecal contents to incubating eggs. PeerJ 5:e3587.

Dong, H., H. Lin, H. C. Jiao, Z. G. Song, J. P. Zhao, and K. . Jiang. 2007. Altered development and protein metabolism in skeletal muscles of broiler chickens (Gallus gallus domesticus) by corticosterone. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 147:189–195.

Donia, M. S., and M. A. Fischbach. 2015. Small molecules from the human microbiota. Science (80-.). 349.

Drew, M. D., N. A. Syed, B. G. Goldade, B. Laarveld, and A. G. Van Kessel. 2004. Effects of Dietary Protein Source and Level on Intestinal Populations of Clostridium perfringens in Broiler Chickens. Poult. Sci. 83:414–420.

Dridi, S. 2017. Molecular mechanisms involved in the regulation of energy homeostasis and fat metabolism in chickens. USDA Res. Propos. Available at https://portal.nifa.usda.gov/web/crisprojectpages/1000846-molecular-mechanisms-involved-in-the-regulation-of-energy-homeostasis-and-fat-metabolism-in-chickens.html.

Dridi, S., Q. Swennen, E. Decuypere, and J. Buyse. 2005. Mode of leptin action in chicken hypothalamus. Brain Res. 1047:214–223.

Du, D., Y. Zhang, C. Zhu, H. Chen, and J. Sun. 2021. Metabolic Regulation of Hypoxia-Inducible Factors in Hypothalamus. Front. Endocrinol. 12:128.

Duan, X., F. Li, S. Mou, J. Feng, P. Liu, and L. Xu. 2015. Effects of dietary L-arginine on laying performance and anti-oxidant capacity of broiler breeder hens, eggs, and offspring during the late laying period. Poult. Sci. 94:2938–2943.

Ducatelle, R., V. Eeckhaut, F. Haesebrouck, and F. Van Immerseel. 2015. A review on prebiotics and probiotics for the control of dysbiosis: present status and future perspectives. Animal 9:43–48.

Ducatelle, R., E. Goossens, F. De Meyer, V. Eeckhaut, G. Antonissen, F. Haesebrouck, and F. Van Immerseel. 2018. Biomarkers for monitoring intestinal health in poultry: present status and future perspectives. Vet. Res. 49:43.

Duchêne, S., E. Audouin, C. Berri, J. Dupont, and S. Tesseraud. 2008. Tissue-specific regulation of S6K1 by insulin in chickens divergently selected for growth. Gen. Comp. Endocrinol. 156:190–198.

Duffy, J. 2020. Character of the Day: The Delaware Housewife Who Invented the Modern Chicken Industry. Secrets East. Shore Available at https://www.secretsoftheeasternshore.com/character-cecile-steele/.

Duke, G. E. 1986. Alimentary Canal: Anatomy, Regulation of Feeding, and Motility.Pages 289–302 in Avian

Physiology. Sturkie, P.D., ed. 4th ed. Springer New York, New York, NY.

Dumonceaux, T. J., J. E. Hill, S. M. Hemmingsen, and A. G. Van Kessel. 2006. Characterization of Intestinal Microbiota and Response to Dietary Virginiamycin Supplementation in the Broiler Chicken. Appl. Environ. Microbiol. 72:2815–2823.

Duncan, S. H., G. E. Lobley, G. Holtrop, J. Ince, A. M. Johnstone, P. Louis, and H. J. Flint. 2008. Human colonic microbiota associated with diet, obesity and weight loss. Int. J. Obes. 32:1720–1724.

Dupont, J., S. Métayer-Coustard, B. Ji, C. Ramé, C. Gespach, B. Voy, and J. Simon. 2012. Characterization of major elements of insulin signaling cascade in chicken adipose tissue: Apparent insulin refractoriness. Gen. Comp. Endocrinol. 176:86–93.

Dupont, J., N. Rideau, and J. Simon. 2015. Endocrine Pancreas.Pages 613–631 in Sturkie's Avian Physiology. Scanes, C.G., ed. 6th ed. Academic Press, London, UK.

Dwarkasing, J. ., D. L. Marks, R. . Witkamp, and K. van Norren. 2016. Hypothalamic inflammation and food intake regulation during chronic illness. Peptides 77:60–66.

EFSA/ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control). 2021. The European Union One Health 2019 Zoonoses Report. EFSA J. 19.

EFSA. 2011. Scientific Opinion on the safety and efficacy of Taminizer D (dimethylglycine sodium salt) as a feed additive for chickens for fattening. EFSA J. 9:1950.

Ekstrand, C., T. E. Carpenter, I. Andersson, and B. Algers. 1998. Prevalence and control of foot-pad dermatitis in broilers in Sweden. Br. Poult. Sci.:318–324.

EL-Deep, M. H., K. A. Amber, Y. Z. Eid, S. T. Alrashood, H. A. Khan, M. S. Sakr, and M. A. O. Dawood. 2020. The Influence of Dietary Chicken Egg Lysozyme on the Growth Performance, Blood Health, and Resistance Against Escherichia coli in the Growing Rabbits' Cecum. Front. Vet. Sci. 7:579576.

Engberg, R. M., M. S. Hedemann, and B. B. Jensen. 2002. The influence of grinding and pelleting of feed on the microbial composition and activity in the digestive tract of broiler chickens. Br. Poult. Sci. 43:569–579.

Engberg, R. M., M. S. Hedemann, T. D. Leser, and B. B. Jensen. 2000. Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. Poult. Sci. 79:1311–1319.

Engberg, R. M., M. S. Hedemann, S. Steenfeldt, and B. B. Jensen. 2004. Influence of Whole Wheat and Xylanase on Broiler Performance and Microbial Composition and Activity in the Digestive Tract. Poult. Sci. 83:925–938.

Enkvetchakul, B., N. B. Anthony, and W. G. Bottje. 1995. Liver and Blood Glutathione in Male Broiler Chickens, Turkeys, and Quail. Poult. Sci. 74:885–889.

Epstein, Y., and R. Yanovich. 2019. Heatstroke. N. Engl. J. Med. 380:2449–2459.

Everaert, N., Q. Swennen, S. M. Coustard, H. Willemsen, C. Careghi, J. Buyse, V. Bruggeman, E. Decuypere, and S. Tesseraud. 2010. The effect of the protein level in a pre-starter diet on the post-hatch performance and activation of ribosomal protein S6 kinase in muscle of neonatal broilers. Br. J. Nutr. 103:206–211.

Ezaki, T., Y. Kawamura, N. Li, Z. Y. Li, L. Zhao, and S. E. Shu. 2001. Proposal of the genera Anaerococcus gen. nov., Peptoniphilus gen. nov. and Gallicola gen. nov for members of the genus Peptostreptococcus. Int. J. Syst. Evol. Microbiol. 51:1521–1528.

Ezaki, T., N. Li, Y. Hashimoto, H. Miura, and H. Yamamoto. 1994. 16S ribosomal DNA sequences of anaerobic cocci and proposal of Ruminococcus hansenii comb. nov. and Ruminococcus productus comb. nov. Int. J.

Syst. Bacteriol. 44:130–136.

FAO (Food and Agriculture Organization of the United Nations). 2016. Probiotics in animal nutrition – Production, impact and regulation. FAO Anim. Prod. Heal. Pap. No. 179.

FAO/WHO (Food and Agriculture Organization and World Health Organization of the United Nations). 2001. Joint expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria.

Farag, M. R., and M. Alagawany. 2018. Physiological alterations of poultry to the high environmental temperature. J. Therm. Biol. 76:101–106.

Farquhar, M. G., and G. E. Palade. 1963. Junctional complexes in various epithelia. J. Cell Biol. 17:375–412.

Fernandes, J. I. M., A. E. Murakami, E. N. Martins, M. I. Sakamoto, and E. R. M. Garcia. 2009. Effect of arginine on the development of the pectoralis muscle and the diameter and the protein:deoxyribonucleic acid rate of its skeletal myofibers in broilers. Poult. Sci. 88:1399–1406.

Flees, J., H. Rajaei-Sharifabadi, E. Greene, L. Beer, B. M. Hargis, L. Ellestad, T. Porter, A. Donoghue, W. G. Bottje, and S. Dridi. 2017. Effect of Morinda citrifolia (Noni)-Enriched Diet on Hepatic Heat Shock Protein and Lipid Metabolism-Related Genes in Heat Stressed Broiler Chickens. Front. Physiol. 8:919.

Forder, R. E. A., G. S. Howarth, D. R. Tivey, and R. J. Hughes. 2007. Bacterial Modulation of Small Intestinal Goblet Cells and Mucin Composition During Early Posthatch Development of Poultry. Poult. Sci. 86:2396–2403.

Fouad, A. M., H. K. El-Senousey, X. J. Yang, and J. H. Yao. 2013. Dietary L-arginine supplementation reduces abdominal fat content by modulating lipid metabolism in broiler chickens. Animal 7:1239–1245.

Fouquet, S., V. H. Lugo-Martínez, A. M. Faussat, F. Renaud, P. Cardot, J. Chambaz, M. Pinçon-Raymond, and S. Thenet. 2004. Early loss of E-cadherin from cell-cell contacts is involved in the onset of anoikis in enterocytes. J. Biol. Chem. 279:43061–43069.

France, M. M., and J. R. Turner. 2017. The mucosal barrier at a glance. J. Cell Sci. 130:307–314.

Frederiksen, C. Ø., M. T. Cohn, L. K. Skov, E. G. W. Schmidt, K. M. Schnorr, S. Buskov, M. Leppänen, I. Maasilta, E. Perez-Calvo, R. Lopez-Ulibarri, and M. Klausen. 2021. A muramidase from Acremonium alcalophilum hydrolyse peptidoglycan found in the gastrointestinal tract of broiler chickens. J. Ind. Microbiol. Biotechnol. 48:8.

Fritz, J. H. 2013. Arginine Cools the Inflamed Gut. Infect. Immun. 81:3500–3502.

Fujii, M., N. Kawashima, K. Tazawa, K. Hashimoto, K. Nara, S. Noda, M. Kuramoto, S. Orikasa, S. Nagai, and T. Okiji. 2020. HIF1α inhibits LPS-mediated induction of IL-6 synthesis via SOCS3-dependent CEBPβ suppression in human dental pulp cells. Biochem. Biophys. Res. Commun. 522:308–314.

Fukunaga, T., M. Sasaki, Y. Araki, T. Okamoto, T. Yasuoka, T. Tsujikawa, Y. Fujiyama, and T. Bamba. 2003. Effects of the soluble fibre pectin on intestinal cell proliferation, fecal short chain fatty acid production and microbial population. Digestion 67:42–49.

Fulda, S., and K.-M. Debatin. 2007. HIF-1-Regulated Glucose Metabolism: A Key to Apoptosis Resistance? Cell Cycle 6:790–792.

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66:365–378.

Furukawa, K., M. Toyomizu, and M. Kikusato. 2021. Possible role of corticosterone in proteolysis, glycolytic, and amino acid metabolism in primary cultured avian myotubes incubated at high-temperature conditions.

Domest. Anim. Endocrinol. 76:106608.

Furuse, M., R. Ando, T. Bungo, M. Shimojo, and Y. Masuda. 1999. Intracerebroventricular injection of orexins does not stimulate food intake in neonatal chicks. Br. Poult. Sci. 40:698–700.

Furuse, M., M. Matsumoto, N. Saito, K. Sugahara, and S. Hasegawa. 1997. The central corticotropin-releasing factor and glucagon-like peptide-1 in food intake of the neonatal chick. Eur. J. Pharmacol. 339:211–213.

Furuse, M., T. Tachibana, A. Ohgushi, R. Ando, T. Yoshimatsu, and D. M. Denbow. 2001. Intracerebroventricular injection of ghrelin and growth hormone releasing factor inhibits food intake in neonatal chicks. Neurosci. Lett. 301:123–126.

Furuse, M., H. Yamane, S. Tomonaga, Y. Tsuneyoshi, and D. M. Denbow. 2007. Neuropeptidergic Regulation of Food Intake in the Neonatal Chick: A Review. J. Poult. Sci. 44:349–356.

Gabriel, I., M. Lessire, S. Mallet, and J. F. Guillot. 2006. Microflora of the digestive tract: Critical factors and consequences for poultry. Worlds. Poult. Sci. J. 62:499–511.

Gadde, U., W. H. Kim, S. T. Oh, and H. S. Lillehoj. 2017. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. Anim. Heal. Res. Rev. 18:26–45.

Gaggìa, F., P. Mattarelli, and B. Biavati. 2010. Probiotics and prebiotics in animal feeding for safe food production. Int. J. Food Microbiol. 141:S15–S28.

Gao, Y., Z. Li, J. Scott Gabrielsen, J. A. Simcox, S. H. Lee, D. Jones, B. Cooksey, G. Stoddard, W. T. Cefalu, and D. A. McClain. 2015. Adipocyte iron regulates leptin and food intake. J. Clin. Invest. 125:3681–3691.

Gardiner, B. J., A. Y. Tai, D. Kotsanas, M. J. Francis, S. A. Roberts, S. A. Ballard, R. K. Junckerstorff, and T. M. Kormana. 2015. Clinical and Microbiological Characteristics of Eggerthella lenta Bacteremia. J. Clin. Microbiol. 53:626–635.

Gaskins, H. R., C. T. Collier, and D. B. Anderson. 2002. Antibiotics as growth promotants: Mode of action. Anim. Biotechnol. 13:29–42.

Gaspar, J. M., and L. A. Velloso. 2018. Hypoxia Inducible Factor as a Central Regulator of Metabolism – Implications for the Development of Obesity. Front. Neurosci. 12:813.

Geraert, P. A., J. C. F. Padilha, and S. Guillaumin. 1996a. Metabolic and endocrine changes induced by chronic heat exposure in broiler chickens: growth performance, body composition and energy retention. Br. J. Nutr. 75:195–204.

Geraert, P. A., J. C. F. Padilha, and S. Guillaumin. 1996b. Metabolic and endocrine changes induced by chronic heate xposure in broiler chickens: biological and endocrinological variables. Br. J. Nutr. 75:205–216.

Gibson, G. R., and M. B. Roberfroid. 1995. Dietary Modulation of the Human Colonic Microbiota: Introducing the Concept of Prebiotics. J. Nutr. 125:1401–1412.

Gilani, S., P. V. Chrystal, and R. Barekatain. 2021. Current experimental models, assessment and dietary modulations of intestinal permeability in broiler chickens. Anim. Nutr. 7:801–811.

Goel, A., C. M. Ncho, B.-J. Kim, C.-M. Jeong, V. Gupta, J.-Y. Jung, S.-Y. Ha, J.-K. Yang, and Y.-H. Choi. 2022. Dietary shredded steam-exploded pine particle supplementation as a strategy to mitigate chronic cyclic heat stress by modulating gut microbiota in broilers. Sci. Rep. 12:19704.

Gogoi, S., G. Kolluri, J. S. Tyagi, G. Marappan, K. Manickam, and R. Narayan. 2021. Impact of heat stress on broilers with varying body weights: Elucidating their interactive role through physiological signatures. J. Therm. Biol. 97:102840.

Gomez-Osorio, L. M., V. Yepes-Medina, A. Ballou, M. Parini, and R. Angel. 2021. Short and Medium Chain Fatty Acids and Their Derivatives as a Natural Strategy in the Control of Necrotic Enteritis and Microbial Homeostasis in Broiler Chickens. Front. Vet. Sci. 8:1528.

Gong, M., D. Anderson, B. Rathgeber, and J. MacIsaac. 2017. The effect of dietary lysozyme with EDTA on growth performance and intestinal microbiota of broiler chickens in each period of the growth cycle. J. Appl. Poult. Res. 26:1–8.

Gong, J., R. J. Forster, H. Yu, J. R. Chambers, P. M. Sabour, R. Wheatcroft, and S. Chen. 2002. Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen. FEMS Microbiol. Lett. 208:1–7.

Gong, J., W. Si, R. J. Forster, R. Huang, H. Yu, Y. Yin, C. Yang, and Y. Han. 2007. 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: From crops to ceca. FEMS Microbiol. Ecol. 59:147–157.

Gong, J., H. Yu, T. Liu, J. J. Gill, J. R. Chambers, R. Wheatcroft, and P. M. Sabour. 2008. Effects of zinc bacitracin, bird age and access to range on bacterial microbiota in the ileum and caeca of broiler chickens. J. Appl. Microbiol. 104:1372–1382.

González-Mariscal, L., M. Quirós, and M. Díaz-Coránguez. 2011. ZO Proteins and Redox-Dependent Processes. Antioxid. Redox Signal. 15:1235–1253.

Gonzalez-Rivas, P. A., S. S. Chauhan, M. Ha, N. Fegan, F. R. Dunshea, and R. D. Warner. 2020. Effects of heat stress on animal physiology, metabolism, and meat quality: A review. Meat Sci. 162:108025.

Goodarzi Boroojeni, F., K. Männer, J. Rieger, E. Pérez Calvo, and J. Zentek. 2019. Evaluation of a microbial muramidase supplementation on growth performance, apparent ileal digestibility, and intestinal histology of broiler chickens. Poult. Sci. 98:2080–2086.

Goren, E., W. A. de Jong, P. Doornenbal, J. P. Koopman, and H. M. Kennis. 1984. Protection of chicks against salmonella infection induced by spray application of intestinal microflora in the hatchery. Vet. Q. 6:73–79.

Gous, R. M., and T. R. Morris. 2005. Nutritional interventions in alleviating the effects of high temperatures in broiler production. Worlds. Poult. Sci. J. 61:463–475.

Greene, E., J. Flees, S. Dadgar, B. Mallmann, S. Orlowski, A. Dhamad, S. Rochell, M. Kidd, C. Laurendon, H. Whitfield, C. Brearley, N. Rajaram, C. Walk, and S. Dridi. 2019a. Quantum Blue Reduces the Severity of Woody Breast Myopathy via Modulation of Oxygen Homeostasis-Related Genes in Broiler Chickens. Front. Physiol. 10:1251.

Greene, E., J. Flees, A. Dhamad, A. Alrubaye, S. Hennigan, J. Pleimann, M. Smeltzer, S. Murray, J. Kugel, J. Goodrich, A. Robertson, R. Wideman, D. Rhoads, and S. Dridi. 2019b. Double-Stranded RNA Is a Novel Molecular Target in Osteomyelitis Pathogenesis. Am. J. Pathol. 189:2077–2089.

Greene, E. S., M. Zampiga, F. Sirri, T. Ohkubo, and S. Dridi. 2020. Orexin system is expressed in avian liver and regulates hepatic lipogenesis via ERK1/2 activation. Sci. Rep. 10:19191.

Greer, S. N., J. L. Metcalf, Y. Wang, and M. Ohh. 2012. The updated biology of hypoxia-inducible factor. EMBO J. 31:2448–2460.

Gribar, S. C., W. M. Richardson, C. P. Sodhi, and D. J. Hackam. 2008. No Longer an Innocent Bystander: Epithelial Toll-Like Receptor Signaling in the Development of Mucosal Inflammation. Mol. Med. 14:645–659.

Guardia, S., B. Konsak, S. Combes, F. Levenez, L. Cauquil, J.-F. Guillot, C. Moreau-Vauzelle, M. Lessire, H. Juin, and I. Gabriel. 2011. Effects of stocking density on the growth performance and digestive microbiota of broiler chickens. Poult. Sci. 90:1878–1889.

Guarner, F., and G. J. Schaafsma. 1998. Probiotics. Int. J. Food Microbiol. 39:237–8.

El Hage, R., E. Hernandez-Sanabria, M. Calatayud Arroyo, R. Props, and T. Van de Wiele. 2019. Propionate-Producing Consortium Restores Antibiotic-Induced Dysbiosis in a Dynamic in vitro Model of the Human Intestinal Microbial Ecosystem. Front. Microbiol. 10.

Hales, J. R. S. 1973. Effects of Exposure to Hot Environments on the Regional Distribution of Blood Flow and on Cardiorespiratory Function in Sheep. Pflugers Arch. 344:133–148.

Hall, D. M., K. R. Baumgardner, T. D. Oberley, and C. V. Gisolfi. 1999. Splanchnic tissues undergo hypoxic stress during whole body hyperthermia. Am. J. Physiol. - Gastrointest. Liver Physiol. 276.

Hall, D. M., G. R. Buettner, L. W. Oberley, L. Xu, R. D. Matthes, and C. V. Gisolfi. 2001. Mechanisms of circulatory and intestinal barrier dysfunction during whole body hyperthermia. Am. J. Physiol. - Hear. Circ. Physiol. 280:H509–H521.

Han, G. G., E. B. Kim, J. Lee, J.-Y. Lee, G. Jin, J. Park, C.-S. Huh, I.-K. Kwon, D. Y. Kil, Y.-J. Choi, and C. Kong. 2016. Relationship between the microbiota in different sections of the gastrointestinal tract, and the body weight of broiler chickens. Springerplus 5:911.

Hardie, D. G. 2003. Minireview: The AMP-Activated Protein Kinase Cascade: The Key Sensor of Cellular Energy Status. Endocrinology 144:5179–5183.

Hardie, D. G., S. A. Hawley, and J. W. Scott. 2006. AMP-activated protein kinase - development of the energy sensor concept. J. Physiol. 574:7–15.

Hardie, D. G., F. A. Ross, and S. A. Hawley. 2012. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat. Rev. Mol. Cell Biol. 13:251–262.

Harford, I. D., H. O. Pavlidis, and N. B. Anthony. 2014. Divergent selection for muscle color in broilers. Poult. Sci. 93:1059–1066.

Hariganesh, K., and J. Prathiba. 2010. Effect of Dimethylglycine on Gastric Ulcers in Rats. J. Pharm. Pharmacol. 52:1519–1522.

Harris, G., R. KuoLee, and W. Chen. 2006. Role of toll-like receptors in health and diseases of gastrointestinal tract. World J. Gastroenterol. 12:2149–2160.

Hassan, F., M. A. Arshad, S. Hassan, R. M. Bilal, M. Saeed, and M. S. Rehman. 2021. Physiological role of Arginine in growth performance, gut health and immune response in broilers: a review. Worlds. Poult. Sci. J. 77:517–537.

Havenstein, G. B. 2006. Performance changes in poultry and livestock following 50 years of genetic selection. Lohmann Inf. 41:30–37.

Havenstein, G. B., P. R. Ferket, and M. A. Qureshi. 2003a. Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult. Sci. 82:1500–1508.

Havenstein, G. B., P. R. Ferket, and M. A. Qureshi. 2003b. Carcass composition and yield of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult. Sci. 82:1509–1518.

Havenstein, G. B., P. R. Ferket, S. E. Scheideler, and B. T. Larson. 1994. Growth, livability, and feed conversion of 1957 vs 1991 broilers when fed “typical” 1957 and 1991 broiler diets. Poult. Sci. 73:1785–1794.

He, J., H. Guo, W. Zheng, Y. Xue, R. Zhao, and W. Yao. 2019a. Heat stress affects fecal microbial and metabolic alterations of primiparous sows during late gestation. J. Anim. Sci. Biotechnol. 10:1–12.

He, J., Y. He, D. Pan, J. Cao, Y. Sun, and X. Zeng. 2019b. Associations of Gut Microbiota With Heat Stress-Induced Changes of Growth, Fat Deposition, Intestinal Morphology, and Antioxidant Capacity in Ducks. Front. Microbiol. 10.

He, X., Z. Lu, B. Ma, L. Zhang, J. Li, Y. Jiang, G. Zhou, and F. Gao. 2018a. Chronic Heat Stress Damages Small Intestinal Epithelium Cells Associated with the Adenosine 5′-Monophosphate-Activated Protein Kinase Pathway in Broilers. J. Agric. Food Chem. 66:7301–7309.

He, X., Z. Lu, B. Ma, L. Zhang, J. Li, Y. Jiang, G. Zhou, and F. Gao. 2018b. Effects of chronic heat exposure on growth performance, intestinal epithelial histology, appetite-related hormones and genes expression in broilers. J. Sci. Food Agric. 98:4471–4478.

He, Y., C. Maltecca, and F. Tiezzi. 2021. Potential Use of Gut Microbiota Composition as a Biomarker of Heat Stress in Monogastric Species: A Review. Animals 11:1833.

He, S., S. Zhao, S. Dai, D. Liu, and S. G. Bokhari. 2015. Effects of dietary betaine on growth performance, fat deposition and serum lipids in broilers subjected to chronic heat stress. Anim. Sci. J. 86:897–903.

Hervé, M. 2021. RVAideMemoire: Testing and Plotting Procedures for Biostatistics.

Hester, P. Y., S. A. Enneking, B. K. Haley, H. W. Cheng, M. E. Einstein, and D. A. Rubin. 2013. The effect of perch availability during pullet rearing and egg laying on musculoskeletal health of caged White Leghorn hens. Poult. Sci. 92:1972–1980.

Hillman, E. T., H. Lu, T. Yao, and C. H. Nakatsu. 2017. Microbial Ecology along the Gastrointestinal Tract. Microbes Environ. 32:300–313.

Hirakawa, R., S. Nurjanah, K. Furukawa, A. Murai, M. Kikusato, T. Nochi, and M. Toyomizu. 2020. Heat Stress Causes Immune Abnormalities via Massive Damage to Effect Proliferation and Differentiation of Lymphocytes in Broiler Chickens. Front. Vet. Sci. 7:46.

Holecek, M., and L. Sispera. 2016. Effects of Arginine Supplementation on Amino Acid Profiles in Blood and Tissues in Fed and Overnight-Fasted Rats. Nutrients 8:206.

Honda, K. 2021. Peripheral regulation of food intake in chickens: adiposity signals, satiety signals and others. Worlds. Poult. Sci. J. 77:301–312.

Hosoi, T., T. Kohda, S. Matsuzaki, M. Ishiguchi, A. Kuwamura, T. Akita, J. Tanaka, and K. Ozawa. 2016. Key role of heat shock protein 90 in leptin-induced STAT3 activation and feeding regulation. Br. J. Pharmacol. 173:2434–2445.

Howlider, M. A. R., and S. P. Rose. 1989. Rearing temperature and the meat yield of broilers. Br. Poult. Sci. 30:61–67.

Hubert, M., P. J. Rousseeuw, and K. Vanden Branden. 2005. ROBPCA: A new approach to robust principal component analysis. Technometrics 47:64–79.

Hughey, V. L., and E. A. Johnson. 1987. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. Appl. Environ. Microbiol. 53:2165–2170.

Hughey, V. L., P. A. Wilger, and E. A. Johnson. 1989. Antibacterial activity of hen egg white lysozyme against Listeria monocytogenes Scott A in foods. Appl. Environ. Microbiol. 55:631–638.

Huston, T. M., and J. L. Carmon. 1962. The Influence of High Environmental Temperature on Thyroid Size of

Domestic Fowl ,. Poult. Sci. 41:175–179.

Hutkins, R. W., J. A. Krumbeck, L. B. Bindels, P. D. Cani, G. Fahey, Y. J. Goh, B. Hamaker, E. C. Martens, D. A. Mills, R. A. Rastal, E. Vaughan, and M. E. Sanders. 2016. Prebiotics: why definitions matter. Curr. Opin. Biotechnol. 37:1–7.

Huyghebaert, G., R. Ducatelle, and F. Van Immerseel. 2011. An update on alternatives to antimicrobial growth promoters for broilers. Vet. J. 187:182–188.

Iebba, V., V. Totino, A. Gagliardi, F. Santangelo, F. Cacciotti, M. Trancassini, C. Mancini, C. Cicerone, E. Corazziari, F. Pantanella, and S. Schippa. 2016. Eubiosis and dysbiosis: The two sides of the microbiota. New Microbiol. 39:1–12.

Iizuka, M., and S. Konno. 2011. Wound healing of intestinal epithelial cells. World J. Gastroenterol. 17:2161–2171.

Ilan, Y. 2012. Leaky gut and the liver: A role for bacterial translocation in nonalcoholic steatohepatitis. World J. Gastroenterol. 18:2609.

Van Immerseel, F. 2022. Why classical broiler production yields animals with high sensitivity to intestinal inflammation and infection? In 56th Annual Meeting of Associazione Scientifica di Avicoltura (Italian branch of World's Poultry Science Association). Rimini, Italy, April 22, 2022.

Van Immerseel, F., J. I. Rood, R. J. Moore, and R. W. Titball. 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends Microbiol. 17:32–36.

Imondi, A. R., and F. H. Bird. 1966. The Turnover of Intestinal Epithelium in the Chick. Poult. Sci. 45:142–147.

Imoto, S., and S. Namioka. 1978. VFA production in the pig large intestine. J. Anim. Sci. 47:467–478.

IUPS Thermal Commission. 1987. Glossary of terms for thermal physiology. Second Edition. Pflugers Arch. 57:567–587.

Iyer, S. S., and R. S. Blumberg. 2018. Influence of the Gut Microbiome on Immune Development During Early Life.Pages 767–774 in Physiology of the Gastrointestinal Tract. Said, H.M., ed. 6th ed. Academic Press, London, UK.

Jackson, S., and G. E. Duke. 1995. Intestine fullness influences feeding behaviour and crop filling in the domestic turkey. Physiol. Behav. 58:1027–1034.

Jacobson, A., L. Lam, M. Rajendram, F. Tamburini, J. Honeycutt, T. Pham, W. Van Treuren, K. Pruss, S. R. Stabler, K. Lugo, D. M. Bouley, J. G. Vilches-Moure, M. Smith, J. L. Sonnenburg, A. S. Bhatt, K. C. Huang, and D. Monack. 2018. A Gut Commensal-Produced Metabolite Mediates Colonization Resistance to Salmonella Infection. Cell Host Microbe 24:296-307.e7.

Jeurissen, S. H. M., F. Lewis, J. D. Van Der Klis, Z. Mroz, J. M. J. Rebel, and A. A. H. M. Ter Huurne. 2002. Parameters and techniques to determine intestinal health of poultry as constituted by immunity, integrity, and functionality. Curr. Issues Intest. Microbiol. 3:1–14.

Jha, R., R. Das, S. Oak, and P. Mishra. 2020. Probiotics (Direct-Fed Microbials) in Poultry Nutrition and Their Effects on Nutrient Utilization, Growth and Laying Performance, and Gut Health: A Systematic Review. Animals 10:1863.

Jha, R., A. K. Singh, S. Yadav, J. F. D. Berrocoso, and B. Mishra. 2019. Early Nutrition Programming (in ovo and Post-hatch Feeding) as a Strategy to Modulate Gut Health of Poultry. Front. Vet. Sci. 6:1–10.

Johnson, C. H., C. Zhao, Y. Xu, and T. Mori. 2017. Timing the day: what makes bacterial clocks tick? Nat. Rev.

Microbiol. 2017 154 15:232–242.

de Jong, I. C., J. Van Riel, M. B. M. Bracke, and H. van den Brand. 2017. A'meta-analysis' of effects of post-hatch food and water deprivation on development, performance and welfare of chickens. PLoS One 12:e0189350.

Józefiak, D., A. Rutkowski, and S. . Martin. 2004. Carbohydrate fermentation in the avian ceca: a review. Anim. Feed Sci. Technol. 113:1–15.

Jukes, M. M. 1971. Transport of blood gases.in Physiology and Biochemistry of the Domestic Fowl Vol. 1. Bell, D.J., Freeman, B.M., eds. Academic Press, New York, NY.

Julian, R. J., and S. M. Mirsalimi. 1992. Blood oxygen concentration of fast-growing and slow-growing broiler chickens, and chickens with ascites from right ventricular failure. Avian Dis. 36:730–732.

Jung, C., J.-P. Hugot, and F. Barreau. 2010. Peyer's Patches: The Immune Sensors of the Intestine. Int. J. Inflam. 2010:1–12.

Jurburg, S. D., M. S. M. Brouwer, D. Ceccarelli, J. Goot, A. J. M. Jansman, and A. Bossers. 2019. Patterns of community assembly in the developing chicken microbiome reveal rapid primary succession. MicrobiologyOpen 8.

Kadowaki, T., T. Yamauchi, and N. Kubota. 2008. The physiological and pathophysiological role of adiponectin and adiponectin receptors in the peripheral tissues and CNS. FEBS Lett. 582:74–80.

Kadzere, C. T., M. R. Murphy, N. Silanikove, and E. Maltz. 2002. Heat stress in lactating dairy cows: a review. Livest. Prod. Sci. 77:59–91.

Kageyama, A., and Y. Benno. 2000. Emendation of genus Collinsella and proposal of Collinsella stercoris sp. nov. and Collinsella intestinalis sp. nov. Int. J. Syst. Evol. Microbiol. 50:1767–1774.

Kai, S., G. Watanabe, M. Kubota, M. Kadowaki, and S. Fujimura. 2015. Effect of dietary histidine on contents of carnosine and anserine in muscles of broilers. Anim. Sci. J. 86:541–546.

Kaiya, H., S. van der Geyten, M. Kojima, H. Hosoda, Y. Kitajima, M. Matsumoto, S. Geelissen, V. M. Darras, and K. Kangawa. 2002. Chicken Ghrelin: Purification, cDNA Cloning, and Biological Activity. Endocrinology 143:3454–3463.

Kalmar, I. D., A. Cools, J. Buyse, P. Roose, and G. P. J. Janssens. 2010. Dietary N,N-dimethylglycine supplementation improves nutrient digestibility and attenuates pulmonary hypertension syndrome in broilers. J. Anim. Physiol. Anim. Nutr. 94:e339–e347.

Kalmar, I. D., A. Cools, M. W. A. Verstegen, G. Huyghebaert, J. Buyse, P. Roose, and G. P. J. Janssens. 2011. Dietary supplementation with dimethylglycine affects broiler performance and plasma metabolites depending on dose and dietary fatty acid profile. J. Anim. Physiol. Anim. Nutr. 95:146–153.

Kanehisa, M., and S. Goto. 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res. 28:27–30.

Kanstrup, I. L., and B. Ekblom. 1984. Blood volume and hemoglobin concentration as determinants of maximal aerobic power. Med. Sci. Sports Exerc. 16:256–262.

Kaufman, J. D., Y. Seidler, H. R. Bailey, L. Whitacre, F. Bargo, K. Lüersen, G. Rimbach, G. M. Pighetti, I. R. Ipharraguerre, and A. G. Ríus. 2021. A postbiotic from Aspergillus oryzae attenuates the impact of heat stress in ectothermic and endothermic organisms. Sci. Rep. 11:6407.

Kayal, A., D. Stanley, A. Radovanovic, D. Horyanto, T. T. H. Van, and Y. S. Bajagai. 2022. Controlled Intestinal

Microbiota Colonisation in Broilers under the Industrial Production System. Animals 12:3296.

Keestra, A. M., M. R. de Zoete, L. I. Bouwman, M. M. Vaezirad, and J. P. M. van Putten. 2013. Unique features of chicken Toll-like receptors. Dev. Comp. Immunol. 41:316–323.

KEGG. 2022. KEGG Compound C00140: N-Acetyl-D-glucosamine; N-Acetylchitosamine; 2-Acetamido-2-deoxy-D-glucose; GlcNAc. Available at https://www.genome.jp/dbget-bin/www_bget?C00140.

Kers, J. G., F. C. Velkers, E. A. J. Fischer, G. D. A. Hermes, J. A. Stegeman, and H. Smidt. 2018. Host and Environmental Factors Affecting the Intestinal Microbiota in Chickens. Front. Microbiol. 9.

Keshavarz, K., and H. L. Fuller. 1971a. Relationship of Arginine and Methionine in the Nutrition of the Chick and the Significance of Creatine Biosynthesis in Their Interaction. J. Nutr. 101:217–222.

Keshavarz, K., and H. L. Fuller. 1971b. Relationship of Arginine and Methionine to Creatine Formation in Chicks. J. Nutr. 101:855–862.

Khajali, F., and R. F. Wideman. 2010. Dietary arginine: metabolic, environmental, immunological and physiological interrelationships. Worlds. Poult. Sci. J. 66:751–766.

Kiarie, E., L. F. Romero, and C. M. Nyachoti. 2013. The role of added feed enzymes in promoting gut health in swine and poultry. Nutr. Res. Rev. 26:71–88.

Kidd, M. T., E. D. Peebles, S. K. Whitmarsh, J. B. Yeatman, and R. F. Wideman. 2001. Growth and Immunity of Broiler Chicks as Affected by Dietary Arginine. Poult. Sci. 80:1535–1542.

Kikusato, M., and M. Toyomizu. 2013. Crucial Role of Membrane Potential in Heat Stress-Induced Overproduction of Reactive Oxygen Species in Avian Skeletal Muscle Mitochondria. PLoS One 8:e64412.

Kim, S. W., R. L. McPherson, and G. Wu. 2004. Dietary Arginine Supplementation Enhances the Growth of Milk-Fed Young Pigs. J. Nutr. 134:625–630.

Kimball, A. L., P. M. McCue, M. A. Petrie, and R. K. Shields. 2018. Whole body heat exposure modulates acute glucose metabolism. Int. J. Hyperth. 35:644–651.

Klasing, K. C. 1998. Nutritional modulation of resistance to infectious diseases. Poult. Sci. 77:1119–1125.

Kleiber, M., and J. E. Dougherty. 1934. The influence of environmental temperature on the utilization of food energy in baby chicks. J. Gen. Physiol. 17:701–726.

Klose, V. 2005. Isolation and characterisation of key-organisms from the chicken gut microflora for development of a Competitive Exclusion product. Dissertation am Interuniversitären Department für Agrarbiotechnologie Tulln der Universität für Bodenkultur Wien.

Klose, A. A., E. L. R. Stokstad, and H. J. Almquist. 1938. The Essential Nature Of Arginine In The Diet Of The Chick. J. Biol. Chem. 123:691–698.

Knarreborg, A., M. A. Simon, R. M. Engberg, B. B. Jensen, and G. W. Tannock. 2002. Effects of Dietary Fat Source and Subtherapeutic Levels of Antibiotic on the Bacterial Community in the Ileum of Broiler Chickens at Various Ages. Appl. Environ. Microbiol. 68:5918–5924.

Koch, F., U. Thom, E. Albrecht, R. Weikard, W. Nolte, B. Kuhla, and C. Kuehn. 2019. Heat stress directly impairs gut integrity and recruits distinct immune cell populations into the bovine intestine. PNAS 116:10333–10338.

Kogut, M. 2017. Gut health in poultry. CAB Rev. 12.

Kogut, M. H. 2019. The effect of microbiome modulation on the intestinal health of poultry. Anim. Feed Sci. Technol. 250:32–40.

Kogut, M. H. 2022. Role of diet-microbiota interactions in precision nutrition of the chicken: facts, gaps, and new concepts. Poult. Sci. 101:101673.

Kogut, M. H., and R. J. Arsenault. 2016. Editorial: Gut Health: The New Paradigm in Food Animal Production. Front. Vet. Sci. 3:10–13.

Kogut, M. H., X. Yin, J. Yuan, and L. Broom. 2017. Gut health in poultry. CAB Rev. Perspect. Agric. Vet. Sci. Nutr. Nat. Resour. 12.

Koh, A., F. De Vadder, P. Kovatcheva-Datchary, and F. Bäckhed. 2016. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. Cell 165:1332–1345.

Koutsos, E. A., and V. J. Arias. 2006. Intestinal Ecology: Interactions Among the Gastrointestinal Tract, Nutrition, and the Microflora. J. Appl. Poult. Res. 15:161–173.

Kretz-Remy, C., B. Munsch, and A.-P. Arrigo. 2001. NFκB-dependent Transcriptional Activation during Heat Shock Recovery. J. Biol. Chem. 276:43723–43733.

Kubena, L. F., B. D. Lott, J. W. Deaton, F. N. Reece, and J. D. May. 1972. Body Composition of Chicks as Influenced by Environmental Temperature and Selected Dietary Factors. Poult. Sci. 51:517–522.

Kuenzel, W.J., Masson, M. 1988. A Stereotaxic Atlas of the Brain of the Chick (Gallus domesticus). The Johns Hopkins University Press, Baltimore, MD.

Kunath, B. J., G. Minniti, M. Skaugen, L. H. Hagen, G. Vaaje-Kolstad, V. G. H. Eijsink, P. B. Pope, and M. Arntzen. 2019. Metaproteomics: Sample Preparation and Methodological Considerations.Pages 187–215 in Advances in Experimental Medicine and Biology.

Lackner, J., A. Albrecht, M. Mittler, A. Marx, J. Kreyenschmidt, V. Hess, and H. Sauerwein. 2021. Effect of feeding histidine and β-alanine on carnosine concentration, growth performance, and meat quality of broiler chickens. Poult. Sci. 100:101393.

Lai, H. C., and G. E. Duke. 1978. Colonic motility in domestic Turkeys. Am. J. Dig. Dis. 23:673–681.

Laika, M., and R. Jahanian. 2017. Increase in dietary arginine level could ameliorate detrimental impacts of coccidial infection in broiler chickens. Livest. Sci. 195:38–44.

Lambert, G. P. 2009. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. J. Anim. Sci. 87:E101–E108.

Lambert, G. P., C. V. Gisolfi, D. J. Berg, P. L. Moseley, L. W. Oberley, and K. C. Kregel. 2002. Selected Contribution: Hyperthermia-induced intestinal permeability and the role of oxidative and nitrosative stress. J. Appl. Physiol. 92:1750–1761.

Lan, P. T. N., M. Sakamoto, and Y. Benno. 2004. Effects of two probiotic Lactobacillus strains on jejunal and cecal microbiota of broiler chicken under acute heart stress condition as revealed by molecular analysis of 16S rRNA genes. Microbiol. Immunol. 48:917–929.

Laplante, M., and D. M. Sabatini. 2012. mTOR Signaling in Growth Control and Disease. Cell 149:274–293.

Lashner, B. A., and E. V. Loftus. 2006. True or False? The Hygiene Hypothesis for Crohn's Disease. Am. J. Gastroenterol. 101:1003–1004.

Lassiter, K., E. Greene, A. Piekarski, O. B. Faulkner, B. M. Hargis, W. Bottje, and S. Dridi. 2015. Orexin system is expressed in avian muscle cells and regulates mitochondrial dynamics. Am. J. Physiol. Integr. Comp. Physiol. 308:R173–R187.

Lawson, P. A., and S. M. Finegold. 2015. Reclassification of Ruminococcus obeum as Blautia obeum comb. nov.

Int. J. Syst. Evol. Microbiol. 65:789–793.

Lederberg, J. 2000. Infectious History. Science 288:287–293.

Lee, B. C., and V. N. Gladyshev. 2011. The biological significance of methionine sulfoxide stereochemistry. Free Radic. Biol. Med. 50:221–227.

Lee, M., J. Kovacs-Nolan, C. Yang, T. Archbold, M. Z. Fan, and Y. Mine. 2009. Hen egg lysozyme attenuates inflammation and modulates local gene expression in a porcine model of dextran sodium sulfate (DSS)-Induced Colitis. J. Agric. Food Chem. 57:2233–2240.

Lee, S.-W., T.-M. La, H.-J. Lee, I.-S. Choi, C.-S. Song, S.-Y. Park, J.-B. Lee, and S.-W. Lee. 2019. Characterization of microbial communities in the chicken oviduct and the origin of chicken embryo gut microbiota. Sci. Rep. 9:6838.

Lee, W., M. Moon, G. H. Kim, H. T. Lee, and S. M. Oh. 2015. Heat stress-induced memory impairment is associated with neuroinflammation in mice. J. Neuroinflammation 12:1–13.

Lee, J. T., S. J. Rochell, R. Kriseldi, W. K. Kim, and R. D. Mitchell. 2022. Functional properties of amino acids: Improve health status and sustainability. Poult. Sci.:102288.

Leeson, S., and J. D. Summers. 2001. Nutrition of the Chicken. 4th ed. University Books, Guelph, Ontario, Canada.

Lei, M. M., C. K. Wei, Z. Chen, S. Yosefi, H. X. Zhu, and Z. D. Shi. 2018. Anti-leptin receptor antibodies strengthen leptin biofunction in growing chickens. Gen. Comp. Endocrinol. 259:223–230.

Lenis, Y. Y., M. A. Elmetwally, J. G. Maldonado-Estrada, and F. W. Bazer. 2017. Physiological importance of polyamines. Zygote 25:244–255.

Ley, R. E. 2016. Prevotella in the gut: Choose carefully. Nat. Rev. Gastroenterol. Hepatol. 13:69.

Ley, R. E., P. J. Turnbaugh, S. Klein, and J. I. Gordon. 2006. Human gut microbes associated with obesity. Nature 444:1022–1023.

Li, J., Y. Tang, and D. Cai. 2012. IKKβ/NF-κB disrupts adult hypothalamic neural stem cells to mediate a neurodegenerative mechanism of dietary obesity and pre-diabetes. Nat. Cell Biol. 14:999–1012.

Lichtenberg, J., E. Perez Calvo, K. Madsen, T. Østergaard Lund, F. Kramer Birkved, S. van Cauwenberghe, M. Mourier, L. Wulf-Andersen, A. J. M. Jansman, and R. Lopez-Ulibarri. 2017. Safety evaluation of a novel muramidase for feed application. Regul. Toxicol. Pharmacol. 89:57–69.

Lilly, D. M., and R. H. Stillwell. 1965. Probiotics: Growth-promoting factors produced by microorganisms. Science 147:747–748.

Lin, H., E. Decuypere, and J. Buyse. 2006. Acute heat stress induces oxidative stress in broiler chickens. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 144:11–17.

Line, J. E., E. A. Svetoch, B. V Eruslanov, V. V Perelygin, E. V. Mitsevich, I. P. Mitsevich, V. P. Levchuk, O. E. Svetoch, B. S. Seal, G. R. Siragusa, and N. J. Stern. 2008. Isolation and purification of enterocin E-760 with broad antimicrobial activity against Gram-positive and Gram-negative bacteria. Antimicrob. Agents Chemother. 52:1094–1100.

Liu, C., S. M. Finegold, Y. Song, and P. A. Lawson. 2008a. Reclassification of Clostridium coccoides, Ruminococcus hansenii, Ruminococcus hydrogenotrophicus, Ruminococcus luti, Ruminococcus productus and Ruminococcus schinkii as Blautia coccoides gen. nov., comb. nov., Blautia hansenii comb. nov., Blautia hydroge. Int. J. Syst. Evol. Microbiol. 58:1896–1902.

Liu, D., Y. Guo, Z. Wang, and J. Yuan. 2010. Exogenous lysozyme influences Clostridium perfringens

colonization and intestinal barrier function in broiler chickens. Avian Pathol. 39:17–24.

Liu, Y., J. Huang, Y. Hou, H. Zhu, S. Zhao, B. Ding, Y. Yin, G. Yi, J. Shi, and W. Fan. 2008b. Dietary arginine supplementation alleviates intestinal mucosal disruption induced by Escherichia coli lipopolysaccharide in weaned pigs. Br. J. Nutr. 100:552–560.

Liu, W.-C., Z.-Y. Pan, Y. Zhao, Y. Guo, S.-J. Qiu, B. Balasubramanian, and R. Jha. 2022. Effects of Heat Stress on Production Performance, Redox Status, Intestinal Morphology and Barrier-Related Gene Expression, Cecal Microbiome, and Metabolome in Indigenous Broiler Chickens. Front. Physiol. 13:761.

Liu, G., W. Ren, J. Fang, C. A. A. Hu, G. Guan, N. A. Al-Dhabi, J. Yin, V. Duraipandiyan, S. Chen, Y. Peng, and Y. Yin. 2017. l-Glutamine and l-arginine protect against enterotoxigenic Escherichia coli infection via intestinal innate immunity in mice. Amino Acids 49:1945–1954.

Liu, S., J. Z. Tan, Y. Hu, X. Jia, M. H. Kogut, J. Yuan, and H. Zhang. 2019. Dietary l-arginine supplementation influences growth performance and B-cell secretion of immunoglobulin in broiler chickens. J. Anim. Physiol. Anim. Nutr. 103:1125–1134.

Liu, H., J. Wang, T. He, S. Becker, G. Zhang, D. Li, and X. Ma. 2018. Butyrate: A Double-Edged Sword for Health? Adv. Nutr. 9:21–29.

Liu, G., H. Zhu, T. Ma, Z. Yan, Y. Zhang, Y. Geng, Y. Zhu, and Y. Shi. 2020. Effect of chronic cyclic heat stress on the intestinal morphology, oxidative status and cecal bacterial communities in broilers. J. Therm. Biol. 91:102619.

López-Ferreras, L., F. Longo, J. E. Richard, K. Eerola, O. T. Shevchouk, M. Tuzinovic, and K. P. Skibicka. 2021. Key role for hypothalamic interleukin-6 in food-motivated behavior and body weight regulation. Psychoneuroendocrinology 131:105284.

Lu, Z., X. He, B. Ma, L. Zhang, J. Li, Y. Jiang, G. Zhou, and F. Gao. 2018. Serum metabolomics study of nutrient metabolic variations in chronic heat-stressed broilers. Br. J. Nutr. 119:771–781.

Lu, Z., X. F. He, B. B. Ma, L. Zhang, J. L. Li, Y. Jiang, G. H. Zhou, and F. Gao. 2019. Increased fat synthesis and limited apolipoprotein B cause lipid accumulation in the liver of broiler chickens exposed to chronic heat stress. Poult. Sci. 98:3695–3704.

Lu, Q., J. Wen, and H. Zhang. 2007. Effect of chronic heat exposure on fat deposition and meat quality in two genetic types of chicken. Poult. Sci. 86:1059–64.

Luo, Q. Q., Y. F. Zhou, M. Y. J. Chen, L. Liu, J. Ma, M. W. Zhang, F. L. Zhang, Y. Ke, and Z. M. Qian. 2018. Fasting up-regulates ferroportin 1 expression via a Ghrelin/GHSR/MAPK signaling pathway. J. Cell. Physiol. 233:30–37.

Luquet, S. H., H. Vaudry, and R. Granata. 2019. Editorial: Neuroendocrine Control of Feeding Behavior. Front. Endocrinol. 10:399.

Ma, T. Y., P. Nighot, and R. Al-Sadi. 2018. Tight Junctions and the Intestinal Barrier.Pages 587–639 in Physiology of the Gastrointestinal Tract. Said, H.M., ed. 6th ed. Academic Press, London, UK.

Ma, B., L. Zhang, J. Li, T. Xing, Y. Jiang, and F. Gao. 2021. Heat stress alters muscle protein and amino acid metabolism and accelerates liver gluconeogenesis for energy supply in broilers. Poult. Sci. 100:215–223.

Ma, X., S. Zhang, L. Pan, and X. Piao. 2017. Effects of lysozyme on the growth performance, nutrient digestibility, intestinal barrier and microbiota of weaned pigs fed diets containing spray-dried whole egg or albumen powder. Can. J. Anim. Sci. 97:CJAS-2016-0171.

Maddineni, S., S. Metzger, O. Ocón, G. Hendricks, and R. Ramachandran. 2005. Adiponectin Gene Is Expressed in Multiple Tissues in the Chicken: Food Deprivation Influences Adiponectin Messenger Ribonucleic Acid Expression. Endocrinology 146:4250–4256.

Madsen, K., and H. Park. 2017. Immunologic Response in the Host.Pages 233–241 in The Microbiota in Gastrointestinal Pathophysiology. Floch, M.H., Ringel, Y., Walker, W.A., eds. Academic Press, London, UK.

De Maesschalck, C., V. Eeckhaut, L. Maertens, L. De Lange, L. Marchal, C. Nezer, S. De Baere, S. Croubels, G. Daube, J. Dewulf, F. Haesebrouck, R. Ducatelle, B. Taminau, and F. Van Immerseel. 2015. Effects of Xylo-Oligosaccharides on Broiler Chicken Performance and Microbiota. Appl. Environ. Microbiol. 81:5880–5888.

Maga, E. A., P. T. Desai, B. C. Weimer, N. Dao, D. Kültz, and J. D. Murraya. 2012. Consumption of lysozyme-rich milk can alter microbial fecal populations. Appl. Environ. Microbiol. 78:6153–6160.

Magne, F., M. Gotteland, L. Gauthier, A. Zazueta, S. Pesoa, P. Navarrete, and R. Balamurugan. 2020. The Firmicutes/Bacteroidetes Ratio: A Relevant Marker of Gut Dysbiosis in Obese Patients? Nutrients 12:1474.

Mancabelli, L., C. Ferrario, C. Milani, M. Mangifesta, F. Turroni, S. Duranti, G. A. Lugli, A. Viappiani, M. C. Ossiprandi, D. van Sinderen, and M. Ventura. 2016. Insights into the biodiversity of the gut microbiota of broiler chickens. Environ. Microbiol. 18:4727–4738.

Marchaim, U., and R. G. Kulka. 1967. The non-parallel increase of amylase, chymotrypsinogen and procarboxypeptidase in the developing chick pancreas. Biochim. Biophys. Acta - Enzymol. 146:553–559.

Marchesi, J. R., and J. Ravel. 2015. The vocabulary of microbiome research: a proposal. Microbiome 3:1–3.

Marder, J., and Z. Arad. 1989. Panting and acid-base regulation in heat stressed birds. Comp. Biochem. Physiol. Part A Physiol. 94:395–400.

Marteau, P., P. Seksik, P. Lepage, and J. Dore. 2012. Cellular and Physiological Effects of Probiotics and Prebiotics. Mini-Reviews Med. Chem. 4:889–896.

Masip, L., K. Veeravalli, and G. Georgiou. 2006. The Many Faces of Glutathione in Bacteria. Antioxid. Redox Signal. 8:753–762.

Masschalck, B., and C. W. Michiels. 2003. Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. Crit. Rev. Microbiol. 29:191–214.

May, K. D., J. E. Wells, C. V. Maxwell, and W. T. Oliver. 2012. Granulated lysozyme as an alternative to antibiotics improves growth performance and small intestinal morphology of 10-day-old pigs. J. Anim. Sci. 90:1118–1125.

McCracken, V. J., and R. G. Lorenz. 2001. The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. Microreview. Cell. Microbiol. 3:1–11.

McMurdie, P. J., and S. Holmes. 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One 8:e61217.

McNab, J. M. 1973. The Avian Caeca: A Review. Worlds. Poult. Sci. J. 29:251–263.

McNabb, F. M. A., and V. M. Darras. 2015. Thyroids.Pages 535–547 in Sturkie's Avian Physiology. Scanes, C.G., ed. 6th ed. Academic Press, London, UK.

Menzies, G., and A. Fisk. 1963. Observations on the Oxyntico-Peptic Cells in the Proventricular Mucosa of Gallus Domesticus. J. Cell Sci. s3-104:207–215.

Metchnikoff, E. 1907. The prolongation of life: optimistic studies. W. Heinemann, ed. G. P. Putnam's Sons, London.

Metzler-Zebeli, B. U., M. Eklund, and R. Mosenthin. 2009. Impact of osmoregulatory and methyl donor functions of betaine on intestinal health and performance in poultry. Worlds. Poult. Sci. J. 65:419–442.

Mevissen-Verhage, E. A. E., J. H. Marcelis, M. N. De Vos, W. C. M. Harmsen-Van Amerongen, and J. Verhoef. 1987. Bifidobacterium, Bacteriodes, and Clostridium spp. in fecal samples from breast-fed and bottle-fed infants with and without iron supplement. J. Clin. Microbiol. 25:285–289.

Meyer, F., D. Paarmann, M. D'Souza, R. Olson, E. Glass, M. Kubal, T. Paczian, A. Rodriguez, R. Stevens, A. Wilke, J. Wilkening, and R. Edwards. 2008. The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics 9:386.

Milani, C., S. Duranti, F. Bottacini, E. Casey, F. Turroni, J. Mahony, C. Belzer, S. Delgado Palacio, S. Arboleya Montes, L. Mancabelli, G. A. Lugli, J. M. Rodriguez, L. Bode, W. de Vos, M. Gueimonde, A. Margolles, D. van Sinderen, and M. Ventura. 2017. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. Microbiol. Mol. Biol. Rev. 81:e00036-17.

Miles, R. D., and S. M. Bootwalla. 1991. Direct-fed microbials in animal production.Pages 117–132 in Direct-fed Microbials in Animal Production. A Review of Literature. Hutcheson, D.P., ed. National Feed Ingredient Association, West Des Moines, Iowa, USA.

Minokoshi, Y., T. Alquier, N. Furukawa, Y.-B. Kim, A. Lee, B. Xue, J. Mu, F. Foufelle, P. Ferré, M. J. Birnbaum, B. J. Stuck, and B. B. Kahn. 2004. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 428:569–574.

Mishra, D., J. E. Richard, I. Maric, B. Porteiro, M. Häring, S. Kooijman, S. Musovic, K. Eerola, L. López-Ferreras, E. Peris, K. Grycel, O. T. Shevchouk, P. Micallef, C. S. Olofsson, I. Wernstedt Asterholm, H. J. Grill, R. Nogueiras, and K. P. Skibicka. 2019. Parabrachial Interleukin-6 Reduces Body Weight and Food Intake and Increases Thermogenesis to Regulate Energy Metabolism. Cell Rep. 26:3011-3026.e5.

Mohammad, S., and C. Thiemermann. 2021. Role of Metabolic Endotoxemia in Systemic Inflammation and Potential Interventions. Front. Immunol. 11:1–16.

Mohnl, M. 2014. Probiotics - basic concept, mode of action and product development aspects.Pages 15–32 in Probiotics in Poultry Production: Concept and Applications. H. A. Abdelrahman, W., Mohnl, M., eds. 5M Publishing Ltd.

Monge, C., and F. Leon-Velarde. 1991. Physiological adaptation to high altitude: oxygen transport in mammals and birds. Physiol. Rev. 71:1135–1172.

Mookherjee, N., M. A. Anderson, H. P. Haagsman, and D. J. Davidson. 2020. Antimicrobial host defence peptides: functions and clinical potential. Nat. Rev. Drug Discov. 19:311–332.

Moore, R. 2020. Understanding gut microbiota in poultry.Pages 71–86 in Improving gut health in poultry. Ricke, S.C., ed. Burleigh Dodds Science Publishing, Sawston, Cambridge, UK.

Moore, P. R., A. Evenson, T. D. Luckey, E. Mccoy, C. A. Elvehjem, and E. B. Hart. 1946. Use of sulfasuxidine, streptothricin, and streptomycin in nutritional studies with the chick. J. Biol. Chem. 165:437–441.

Moore, W. E. C., J. L. Johnson, and L. V. Holdeman. 1976. Emendation of Bacteroidaceae and Butyrivibrio and descriptions of Desulfomonas gen. nov. and ten new species in the genera Desulfomonas, Butyrivibrio, Eubacterium, Clostridium, and Ruminococcus. Int. J. Syst. Bacteriol. 26:238–252.

Morris, S. M. 2007. Arginine Metabolism: Boundaries of Our Knowledge. J. Nutr. 137:1602S-1609S.

Mottet, A., and G. Tempio. 2017. Global poultry production: current state and future outlook and challenges. Worlds. Poult. Sci. J. 73:245–256.

Mowat, A. M. 2018. To respond or not to respond — a personal perspective of intestinal tolerance. Nat. Rev. Immunol. 18:405–415.

Mujahid, A., K. Sato, Y. Akiba, and M. Toyomizu. 2006. Acute heat stress stimulates mitochondrial superoxide production in broiler skeletal muscle, possibly via downregulation of uncoupling protein content. Poult. Sci. 85:1259–1265.

Nakazawa, M. S., B. Keith, and M. C. Simon. 2016. Oxygen availability and metabolic adaptations. Nat. Rev. Cancer 16:663–673.

Nanto-Hara, F., M. Kikusato, S. Ohwada, and M. Toyomizu. 2020. Heat Stress Directly Affects Intestinal Integrity in Broiler Chickens. J. Poult. Sci. 57:284–290.

Nardone, A., B. Ronchi, N. Lacetera, M. S. Ranieri, and U. Bernabucci. 2010. Effects of climate changes on animal production and sustainability of livestock systems. Livest. Sci. 130:57–69.

NASA. 2018. What's in a name? Weather, global warming and climate change. Available at https://web.archive.org/web/20180928145703/https://climate.nasa.gov/resources/global-warming/.

National Chicken Council. 2022. U.S. Chicken Industry History. Available at https://www.nationalchickencouncil.org/about-the-industry/history/#:~:text=Wilmer Steele of Sussex County,a capacity of 10%2C000 birds.

National Research Council. 1994. Nutrient Requirements of Poultry: Ninth Revised Edition, 1994. The National Academies Press, Washington, DC.

Neviani, E., M. Gatti, M. T. Tarelli, and R. Divizia. 1996. Lysozyme resistance of lactic acid bacteria. Latte:90–91.

Nichelmann, M., E. Baranyiová, and R. Goll; B. Tzschentke. 1986. Influence of feather cover on heat balance in laying hens (Gallus domesticus). J. Therm. Biol. 11:121–126.

Nile, C. J., C. L. Townes, G. Michailidis, B. H. Hirst, and J. Hall. 2004. Identification of chicken lysozyme g2 and its expression in the intestine. Cell. Mol. Life Sci. 61:2760–2766.

Noy, Y., and D. Sklan. 1995. Digestion and absorption in the young chick. Poult. Sci. 74:366–373.

Noy, Y., and Z. Uni. 2010. Early nutritional strategies. Worlds. Poult. Sci. J. 66:639–646.

Nurmi, E., and M. Rantala. 1973. New aspects of Salmonella infection in broiler production. Nature 241:210–211.

O'Hara, A. M., and F. Shanahan. 2006. The gut flora as a forgotten organ. EMBO Rep. 7:688–693.

O'Leary, N. A., M. W. Wright, J. R. Brister, S. Ciufo, D. Haddad, R. McVeigh, B. Rajput, B. Robbertse, B. Smith-White, D. Ako-Adjei, A. Astashyn, A. Badretdin, Y. Bao, O. Blinkova, V. Brover, V. Chetvernin, J. Choi, E. Cox, O. Ermolaeva, C. M. Farrell, T. Goldfarb, T. Gupta, D. Haft, E. Hatcher, W. Hlavina, V. S. Joardar, V. K. Kodali, W. Li, D. Maglott, P. Masterson, K. M. McGarvey, M. R. Murphy, K. O'Neill, S. Pujar, S. H. Rangwala, D. Rausch, L. D. Riddick, C. Schoch, A. Shkeda, S. S. Storz, H. Sun, F. Thibaud-Nissen, I. Tolstoy, R. E. Tully, A. R. Vatsan, C. Wallin, D. Webb, W. Wu, M. J. Landrum, A. Kimchi, T. Tatusova, M. DiCuccio, P. Kitts, T. D. Murphy, and K. D. Pruitt. 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res. 44:D733–D745.

Oakley, B. B., and M. H. Kogut. 2016. Spatial and Temporal Changes in the Broiler Chicken Cecal and Fecal

Microbiomes and Correlations of Bacterial Taxa with Cytokine Gene Expression. Front. Vet. Sci. 3.

Oakley, B. B., H. S. Lillehoj, M. H. Kogut, W. K. Kim, J. J. Maurer, A. Pedroso, M. D. Lee, S. R. Collett, T. J. Johnson, and N. A. Cox. 2014. The chicken gastrointestinal microbiome. FEMS Microbiol. Lett. 360:100–112.

Oakley, B. B., C. A. Morales, J. Line, M. E. Berrang, R. J. Meinersmann, G. E. Tillman, M. G. Wise, G. R. Siragusa, K. L. Hiett, and B. S. Seal. 2013. The Poultry-Associated Microbiome: Network Analysis and Farm-to-Fork Characterizations. PLoS One 8:e57190.

Ochiai, D., N. Goda, T. Hishiki, M. Kanai, N. Senoo-Matsuda, T. Soga, R. S. Johnson, Y. Yoshimura, and M. Suematsu. 2011. Disruption of HIF-1α in hepatocytes impairs glucose metabolism in diet-induced obesity mice. Biochem. Biophys. Res. Commun. 415:445–449.

Odom, T. W., P. C. Harrison, and W. G. Bottje. 1986. Effects of Thermal-Induced Respiratory Alkalosis on Blood Ionized Calcium Levels in the Domestic Hen. Poult. Sci. 65:570–573.

Oh, H.-S., S. K. Oh, J. S. Lee, C. Wu, and S.-J. Lee. 2017. Effects of l-arginine on growth hormone and insulin-like growth factor 1. Food Sci. Biotechnol. 26:1749–1754.

Oksanen, J., F. Guillaume Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. R. Minchin, B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stvens, E. Szoecs, and H. Wagner. 2020. vegan: Community Ecology Package. R package version 2.5-6.

Olkowski, A. A., and H. L. Classen. 1998. Progressive bradycardia, a possible factor in the pathogenesis of ascites in fast growing broiler chickens raised at low altitude. Br. Poult. Sci. 39:139–146.

Olukosi, O. A., A. J. Cowieson, and O. Adeola. 2007. Age-Related Influence of a Cocktail of Xylanase, Amylase, and Protease or Phytase Individually or in Combination in Broilers. Poult. Sci. 86:77–86.

Ons, E., A. Gertler, J. Buyse, E. Lebihan-Duval, A. Bordas, B. Goddeeris, and S. Dridi. 2010. Visfatin gene expression in chickens is sex and tissue dependent. Domest. Anim. Endocrinol. 38:63–74.

Oviedo-Rondón, E. O. 2019. Holistic view of intestinal health in poultry. Anim. Feed Sci. Technol. 250:1–8.

Oviedo-Rondón, E. O., and H. A. Córdova-Noboa. 2020. The Potential of Guanidino Acetic Acid to Reduce the Occurrence and Severity of Broiler Muscle Myopathies. Front. Physiol. 11:909.

Oxford, J. H., and R. K. Selvaraj. 2019. Effects of Glutamine Supplementation on Broiler Performance and Intestinal Immune Parameters During an Experimental Coccidiosis Infection. J. Appl. Poult. Res. 28:1279–1287.

Pan, D., and Z. Yu. 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes 5:108–119.

Panda, A. K., S. V. R. Rao, M. V. L. N. Raju, and G. S. Sunder. 2009. Effect of Butyric Acid on Performance, Gastrointestinal Tract Health and Carcass Characteristics in Broiler Chickens. Asian-Australasian J. Anim. Sci. 22:1026–1031.

Parker, J. A., and S. R. Bloom. 2012. Hypothalamic neuropeptides and the regulation of appetite. Neuropharmacology 63:18–30.

Parks, D. H., G. W. Tyson, P. Hugenholtz, and R. G. Beiko. 2014. STAMP: Statistical analysis of taxonomic and functional profiles. Bioinformatics 30:3123–3124.

Patterson, J., and K. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82:627–631.

Pearce, S. C., N. K. Gabler, J. W. Ross, J. Escobar, J. F. Patience, R. P. Rhoads, and L. H. Baumgard. 2013a. The effects of heat stress and plane of nutrition on metabolism in growing pigs. J. Anim. Sci. 91:2108–2118.

Pearce, S. C., V. Mani, R. L. Boddicker, J. S. Johnson, T. E. Weber, J. W. Ross, L. H. Baumgard, and N. K. Gabler. 2012. Heat stress reduces barrier function and alters intestinal metabolism in growing pigs. J. Anim. Sci. 90:257–259.

Pearce, S. C., V. Mani, R. L. Boddicker, J. S. Johnson, T. E. Weber, J. W. Ross, R. P. Rhoads, L. H. Baumgard, and N. K. Gabler. 2013b. Heat Stress Reduces Intestinal Barrier Integrity and Favors Intestinal Glucose Transport in Growing Pigs. PLoS One 8:e70215.

Pedroso, A. A., J. F. Menten, M. R. Lambais, A. M. C. Racanicci, F. A. Longo, and J. O. B. Sorbara. 2006. Intestinal Bacterial Community and Growth Performance of Chickens Fed Diets Containing Antibiotics. Poult. Sci. 85:747–752.

Pegg, A. E. 1986. Recent advances in the biochemistry of polyamines in eukaryotes. Biochem. J. 234:249–262.

Pertea, M., and S. L. Salzberg. 2010. Between a chicken and a grape: estimating the number of human genes. Genome Biol. 11.

Petracci, M., F. Soglia, M. Madruga, L. Carvalho, E. Ida, and M. Estévez. 2019. Wooden-Breast, White Striping, and Spaghetti Meat: Causes, Consequences and Consumer Perception of Emerging Broiler Meat Abnormalities. Compr. Rev. Food Sci. Food Saf. 18:565–583.

Piekarski, A., E. Decuypere, J. Buyse, and S. Dridi. 2016. Chenodeoxycholic acid reduces feed intake and modulates the expression of hypothalamic neuropeptides and hepatic lipogenic genes in broiler chickens. Gen. Comp. Endocrinol. 229:74–83.

Pineiro, M., N.-G. Asp, G. Reid, S. Macfarlane, L. Morelli, O. Brunser, and K. Tuohy. 2008. FAO Technical Meeting on Prebiotics. J. Clin. Gastroenterol. 42:S156–S159.

Pirgozliev, V., A. Simic, S. P. Rose, and E. Pérez Calvo. 2021. Dietary microbial muramidase improves feed efficiency, energy and nutrient availability and welfare of broilers fed commercial type diets containing exogenous enzymes. Br. Poult. Sci. 62:131–137.

Portocarero, N., and U. Braun. 2021. The physiological role of guanidinoacetic acid and its relationship with arginine in broiler chickens. Poult. Sci. 100:101203.

Pourabedin, M., and X. Zhao. 2015. Prebiotics and gut microbiota in chickens. FEMS Microbiol. Lett. 362:122.

Prola, L., J. Nery, A. Lauwaerts, C. Bianchi, L. Sterpone, M. De Marco, L. Pozzo, and A. Schiavone. 2013. Effects of N,N-dimethylglycine sodium salt on apparent digestibility, vitamin E absorption, and serum proteins in broiler chickens fed a high- or low-fat diet. Poult. Sci. 92:1221–1226.

Proszkowiec-Weglarz, M., K. B. Miska, L. E. Ellestad, L. L. Schreier, S. Kahl, N. Darwish, P. Campos, and J. Shao. 2022. Delayed access to feed early post-hatch affects the development and maturation of gastrointestinal tract microbiota in broiler chickens. BMC Microbiol. 22:206.

Pruesse, E., C. Quast, K. Knittel, B. M. Fuchs, W. Ludwig, J. Peplies, and F. O. Glöckner. 2007. SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35:7188–7196.

Qaid, M. M., and M. A. Al-Garadi. 2021. Protein and Amino Acid Metabolism in Poultry during and after Heat Stress: A Review. Animals 11:1167.

Qi, B., J. Wang, M. Hu, Y. Ma, S. Wu, G. Qi, K. Qiu, and H. Zhang. 2021. Influences of Beta-Alanine and l-

Histidine Supplementation on Growth Performance, Meat Quality, Carnosine Content, and mRNA Expression of Carnosine-Related Enzymes in Broilers. Animals 11:2265.

Quinteiro-Filho, W. M., A. Ribeiro, V. Ferraz-de-Paula, M. L. Pinheiro, M. Sakai, L. R. M. Sá, A. J. P. Ferreira, and J. Palermo-Neto. 2010. Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. Poult. Sci. 89:1905–1914.

Quinteiro-Filho, W. M., M. V. Rodrigues, A. Ribeiro, V. Ferraz-de-Paula, M. L. Pinheiro, L. R. M. Sá, A. J. P. Ferreira, and J. Palermo-Neto. 2012. Acute heat stress impairs performance parameters and induces mild intestinal enteritis in broiler chickens: Role of acute hypothalamic-pituitary-adrenal axis activation. J. Anim. Sci. 90:1986–1994.

R Core Team. 2020. R: A language and environment for statistical computing.

Ragland, S. A., and A. K. Criss. 2017. From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. PLOS Pathog. 13:e1006512.

Rahman, M., I. Fliss, and E. Biron. 2022. Insights in the Development and Uses of Alternatives to Antibiotic Growth Promoters in Poultry and Swine Production. Antibiotics 11:766.

Rahner, R. 1901. Bakteriologische mitteilungen ueber die darmbakterien der huehner. Zentbl. Bakteriol. Parasitenkd 80:239–244.

Rajaei-Sharifabadi, H., E. Greene, A. Piekarski, D. Falcon, L. Ellestad, A. Donoghue, W. Bottje, T. Porter, Y. Liang, and S. Dridi. 2017. Surface wetting strategy prevents acute heat exposure–induced alterations of hypothalamic stress– and metabolic-related genes in broiler chickens. J. Anim. Sci. 95:1132.

Rao, R. 2008. Oxidative stress-induced disruption of epithelial and endothelial tight junctions. Front. Biosci. 13:7210–7226.

Ratriyanto, A., and R. Mosenthin. 2018. Osmoregulatory function of betaine in alleviating heat stress in poultry. J. Anim. Physiol. Anim. Nutr. 102:1634–1650.

Reeves, J. T., G. Ballam, S. Hofmeister, C. Pickett, K. Morris, and A. Peacock. 1991. Improved arterial oxygenation with feed restriction in rapidly growing broiler chickens. Comp. Biochem. Physiol. Part A Physiol. 99:481–485.

Rehman, H. U., W. Vahjen, W. A. Awad, and J. Zentek. 2007. Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. Arch. Anim. Nutr. 61:319–335.

Reisinger, N., T. Steiner, S. Nitsch, G. Schatzmayr, and T. J. Applegate. 2011. Effects of a blend of essential oils on broiler performance and intestinal morphology during coccidial vaccine exposure. J. Appl. Poult. Res. 20:272–283.

Renaudeau, D., A. Collin, S. Yahav, V. de Basilio, J. L. Gourdine, and R. J. Collier. 2012. Adaptation to hot climate and strategies to alleviate heat stress in livestock production. Animal 6:707–728.

Rhoads, R. P., L. H. Baumgard, and J. K. Suagee. 2013. Metabolic priorities during heat stress with an emphasis on skeletal muscle. J. Anim. Sci. 91:2492–2503.

Rhoads, J. M., W. Chen, J. Gookin, G. Y. Wu, Q. Fu, A. T. Blikslager, R. A. Rippe, R. A. Argenzio, W. G. Cance, E. M. Weaver, and L. H. Romer. 2004. Arginine stimulates intestinal cell migration through a focal adhesion kinase dependent mechanism. Gut 53:514–522.

Rhoads, M. L., R. P. Rhoads, M. J. VanBaale, R. J. Collier, S. R. Sanders, W. J. Weber, B. A. Crooker, and L. H. Baumgard. 2009. Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production,

metabolism, and aspects of circulating somatotropin. J. Dairy Sci. 92:1986–1997.

Rhoads, J. M., and G. Wu. 2009. Glutamine, arginine, and leucine signaling in the intestine. Amino Acids 37:111–122.

Richards, S. A. 1970. Physiology of thermal panting in birds. Ann. Biol. Anim. Biochim. Biophys. 10:151–168.

Ricke, S. 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poult. Sci. 82:632–639.

Ringel, J., A. Lemme, A. Knox, J. Mc Nab, and M. S. Redshaw. 2007. Effects of graded levels of creatine and guanidino acetic acid in vegetable-based diets on performance and biochemical parameters in muscle tissue.Page 234 in 16th European Symposium on Poultry Nutrition. Strasbourg, France.

Ringseis, R., and K. Eder. 2022. Heat stress in pigs and broilers: role of gut dysbiosis in the impairment of the gut-liver axis and restoration of these effects by probiotics, prebiotics and synbiotics. J. Anim. Sci. Biotechnol. 1:1–16.

Rinttilä, T., and J. Apajalahti. 2013. Intestinal microbiota and metabolites—Implications for broiler chicken health and performance. J. Appl. Poult. Res. 22:647–658.

Rios-Covian, D., N. Salazar, M. Gueimonde, and C. G. de los Reyes-Gavilan. 2017. Shaping the Metabolism of Intestinal Bacteroides Population through Diet to Improve Human Health. Front. Microbiol. 8.

Ritchie, H. 2017. How do we reduce antibiotic resistance from livestock? Available at https://ourworldindata.org/antibiotic-resistance-from-livestock.

Ritzi, M. M., W. Abdelrahman, K. Van-Heerden, M. Mohnl, N. W. Barrett, and R. A. Dalloul. 2016. Combination of probiotics and coccidiosis vaccine enhances protection against an Eimeria challenge. Vet. Res. 47:1–8.

Rivière, A., M. Selak, D. Lantin, F. Leroy, and L. De Vuyst. 2016. Bifidobacteria and butyrate-producing colon bacteria: Importance and strategies for their stimulation in the human gut. Front. Microbiol. 7.

Roberfroid, M. B. 1998. Prebiotics and synbiotics: concepts and nutritional properties. Br. J. Nutr. 80:S197–S202.

Roberfroid, M., G. R. Gibson, L. Hoyles, A. L. McCartney, R. Rastall, I. Rowland, D. Wolvers, B. Watzl, H. Szajewska, B. Stahl, F. Guarner, F. Respondek, K. Whelan, V. Coxam, M. J. Davicco, L. Léotoing, Y. Wittrant, N. M. Delzenne, P. D. Cani, A. M. Neyrinck, and A. Meheust. 2010. Prebiotic effects: Metabolic and health benefits. Br. J. Nutr. 104.

Rodrigues, I., and M. Choct. 2018. The foregut and its manipulation via feeding practices in the chicken. Poult. Sci. 97:3188–3206.

Roediger, W. E. W. 1982. Utilization of Nutrients by Isolated Epithelial Cells of the Rat Colon. Gastroenterology 83:424–429.

Romanatto, T., M. Cesquini, M. E. Amaral, É. A. Roman, J. C. Moraes, M. A. Torsoni, A. P. Cruz-Neto, and L. A. Velloso. 2007. TNF-α acts in the hypothalamus inhibiting food intake and increasing the respiratory quotient—Effects on leptin and insulin signaling pathways. Peptides 28:1050–1058.

Rose, W. C. 1937. The Nutritive Significance of the Amino Acids and Certain Related Compounds. Science 86:298–300.

Rosenthal, M. E., A. D. Rojtman, and E. Frank. 2012. Finegoldia magna (formerly Peptostreptococcus magnus): an overlooked etiology for toxic shock syndrome? Med. Hypotheses 79:138–40.

Rostagno, M. H. 2020. Effects of heat stress on the gut health of poultry. J. Anim. Sci. 98:1–9.

Rothrock, M. J., M. L. Davis, A. Locatelli, A. Bodie, T. G. McIntosh, J. R. Donaldson, and S. C. Ricke. 2017.

Listeria Occurrence in Poultry Flocks: Detection and Potential Implications. Front. Vet. Sci. 4:125.

Round, J. L., and S. K. Mazmanian. 2009. The gut microbiota shapes intestinal immune responses during health and disease. Nat. Rev. Immunol. 9:313–323.

Rubio, L. A. 2019. Possibilities of early life programming in broiler chickens via intestinal microbiota modulation. Poult. Sci. 98:695–706.

Ruff, J., T. L. Barros, G. Tellez, J. Blankenship, H. Lester, B. D. Graham, C. A. M. Selby, C. N. Vuong, S. Dridi, E. S. Greene, X. Hernandez-Velasco, B. M. Hargis, and G. Tellez-Isaias. 2020. Research Note: Evaluation of a heat stress model to induce gastrointestinal leakage in broiler chickens. Poult. Sci. 99:1687–1692.

Sabatini, D. M. 2017. Twenty-five years of mTOR: Uncovering the link from nutrients to growth. PNAS 114:11818–11825.

Sais, M., A. C. Barroeta, P. López-Colom, M. Nofrarías, N. Majó, R. Lopez-Ulibarri, E. Pérez Calvo, and S. M. Martín-Orúe. 2020. Evaluation of dietary supplementation of a novel microbial muramidase on gastrointestinal functionality and growth performance in broiler chickens. Poult. Sci. 99:235–245.

Saito, E.-S., H. Kaiya, T. Tachibana, S. Tomonaga, D. M. Denbow, K. Kangawa, and M. Furuse. 2005. Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. Regul. Pept. 125:201–208.

Salanitro, J. P., I. G. Blake, and P. A. Muirhead. 1974. Studies on the cecal microflora of commercial broiler chickens. Appl. Microbiol. 28:439–47.

Sandercock, D. A., R. R. Hunter, M. A. Mitchell, and P. M. Hocking. 2006. Thermoregulatory capacity and muscle membrane integrity are compromised in broilers compared with layers at the same age or body weight. Br. Poult. Sci. 47:322–329.

Scanes, C. G., and K. D. Christensen. 2020. Poultry science. 5th ed. Waveland Press, Inc., Long Grove, IL, USA.

Scanes, C. G., and K. Pierzchala-Koziec. 2014. Biology of the Gastrointestinal Tract in Poultry. Avian Biol. Res. 7:193–222.

Schmittgen, T. D., and K. J. Livak. 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3:1101–1108.

Schneitz, C. 2005. Competitive exclusion in poultry—30 years of research. Food Control 16:657–667.

Schuler, B., M. Arras, S. Keller, A. Rettich, C. Lundby, J. Vogel, and M. Gassmann. 2010. Optimal hematocrit for maximal exercise performance in acute and chronic erythropoietin-treated mice. PNAS A. 107:419–423.

Schwiertz, A., D. Taras, K. Schäfer, S. Beijer, N. A. Bos, C. Donus, and P. D. Hardt. 2010. Microbiota and SCFA in Lean and Overweight Healthy Subjects. Obesity 18:190–195.

Le Sciellour, M., O. Zemb, I. Hochu, J. Riquet, H. Gilbert, M. Giorgi, Y. Billon, J.-L. Gourdine, and D. Renaudeau. 2019. Effect of chronic and acute heat challenges on fecal microbiota composition, production, and thermoregulation traits in growing pigs. J. Anim. Sci. 97:3845–3858.

Searchinger, T., R. Waite, C. Hanson, J. Ranganathan, and E. Matthews. 2019. Creating a Sustainable Food Future: A Menu of Solutions to Feed Nearly 10 Billion People by 2050. World Resour. Rep. 1:1–564 Available at https://www.wri.org/research/creating-sustainable-food-future.

Semenza, G. L. 2009. Regulation of Oxygen Homeostasis by Hypoxia-Inducible Factor 1. Physiology 24:97–106.

Shapiro, S. K., and W. B. Sarles. 1949. Microorganisms in the intestinal tract of normal chickens. J. Bacteriol. 58:531–544.

Shen, L., C. R. Weber, D. R. Raleigh, D. Yu, and J. R. Turner. 2011. Tight Junction Pore and Leak Pathways: A Dynamic Duo. Annu. Rev. Physiol. 73:283–309.

Shepherd, E. M., and B. D. Fairchild. 2010. Footpad dermatitis in poultry. Poult. Sci. 89:2043–2051.

Shi, D., L. Bai, Q. Qu, S. Zhou, M. Yang, S. Guo, Q. Li, and C. Liu. 2019. Impact of gut microbiota structure in heat-stressed broilers. Poult. Sci. 98:2405–2413.

Shi, X., X. Wang, Q. Li, M. Su, E. Chew, E. T. Wong, Z. Lacza, G. K. Radda, V. Tergaonkar, and W. Han. 2013. Nuclear factor κb (NF-κB) suppresses food intake and energy expenditure in mice by directly activating the Pomc promoter. Diabetologia 56:925–936.

Shikano, K., Y. Bessho, M. Kato, E. Iwakoshi-Ukena, S. Taniuchi, M. Furumitsu, T. Tachibana, G. E. Bentley, L. J. Kriegsfeld, and K. Ukena. 2018. Localization and function of neurosecretory protein GM, a novel small secretory protein, in the chicken hypothalamus. Sci. Rep. 8:704.

Shikano, K., M. Kato, E. Iwakoshi-Ukena, M. Furumitsu, D. Matsuura, K. Masuda, T. Tachibana, G. E. Bentley, L. J. Kriegsfeld, and K. Ukena. 2017. Effects of chronic intracerebroventricular infusion of neurosecretory protein GL on body mass and food and water intake in chicks. Gen. Comp. Endocrinol. 256:37–42.

Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto. 1999. MD-2, a Molecule that Confers Lipopolysaccharide Responsiveness on Toll-like Receptor 4. J. Exp. Med. 189:1777–1782.

Shojadoost, B., A. R. Vince, and J. F. Prescott. 2012. The successful experimental induction of necrotic enteritis in chickens by Clostridium perfringens: a critical review. Vet. Res. 43:74.

Shousha, S., K. Nakahara, M. Kojima, M. Miyazato, H. Hosoda, K. Kangawa, and N. Murakami. 2005. Different effects of peripheral and central ghrelin on regulation of food intake in the Japanese quail. Gen. Comp. Endocrinol. 141:178–183.

Siegel, P. B. 2014. Evolution of the Modern Broiler and Feed Efficiency. Annu. Rev. Anim. Biosci. 2:375–385.

Siegel, P. B., J. A. Cherry, and E. A. Dunnington. 1984. Feeding behaviour and feed consumption in chickens selected for body weight. Ann. Agric. Fenn. 23:247–252.

Simon, J. 1999. Choline, betaine and methionine interactions in chickens, pigs and fish (including crustaceans). Worlds. Poult. Sci. J. 55:353–374.

Simpson, K. A., N. M. Martin, and S. R. Bloom. 2008. Hypothalamic regulation of appetite. Expert Rev. Endocrinol. Metab. 3:577–592.

Singh, K., A. P. Gobert, L. A. Coburn, D. P. Barry, M. Allaman, M. Asim, P. B. Luis, C. Schneider, G. L. Milne, H. H. Boone, M. H. Shilts, M. K. Washington, S. R. Das, M. B. Piazuelo, and K. T. Wilson. 2019. Dietary arginine regulates severity of experimental colitis and affects the colonic microbiome. Front. Cell. Infect. Microbiol. 9:66.

Singh, S. P., J. S. Jadaun, L. K. Narnoliya, and A. Pandey. 2017. Prebiotic Oligosaccharides: Special Focus on Fructooligosaccharides, Its Biosynthesis and Bioactivity. Appl. Biochem. Biotechnol. 183:613–635.

Sirathonpong, O., Y. Ruangpanit, O. Songserm, E. J. Koo, and S. Attamangkune. 2019. Determination of the optimum arginine : lysine ratio in broiler diets. Anim. Prod. Sci. 59:1705.

Sirri, F., G. Maiorano, S. Tavaniello, J. Chen, M. Petracci, and A. Meluzzi. 2016. Effect of different levels of dietary zinc, manganese, and copper from organic or inorganic sources on performance, bacterial chondronecrosis, intramuscular collagen characteristics, and occurrence of meat quality defects of broiler chickens. Poult. Sci. 95:1813–1824.

Sirri, F., S. Pignata, A. Franchini, and A. Meluzzi. 2011. Sweet chestnut tannin supplementation as a way to reduce the incidence of foot pad dermatitis of broiler chickens.in Poster session presented at: 18th European Symposium on Poultry Nutrition. Cesme-Izmir, Turkey, October 31–Novemberr 04, 2011.

Smil, V. 2002. Worldwide transformation of diets, burdens of meat production and opportunities for novel food proteins. Enzyme Microb. Technol. 30:305–311.

Smirnov, A., D. Sklan, and Z. Uni. 2004. Mucin Dynamics in the Chick Small Intestine Are Altered by Starvation. J. Nutr. 134:736–742.

Smith, A. L., C. Powers, and R. K. Beal. 2014. The Avian Enteric Immune System in Health and Disease.Pages 227–250 in Avian Immunology. Shat, K.A., Kaspers, B., Kaiser, P., eds. 2nd ed. Academic Press.

Snipe, R. M. J., A. Khoo, C. M. Kitic, P. R. Gibson, and R. J. S. Costa. 2018. The impact of exertional-heat stress on gastrointestinal integrity, gastrointestinal symptoms, systemic endotoxin and cytokine profile. Eur. J. Appl. Physiol. 118:389–400.

Soglia, F., M. Petracci, R. Davoli, and M. Zappaterra. 2021. A critical review of the mechanisms involved in the occurrence of growth-related abnormalities affecting broiler chicken breast muscles. Poult. Sci. 100:101180.

Soglia, F., A. K. Silva, L. M. Lião, L. Laghi, and M. Petracci. 2019. Effect of broiler breast abnormality and freezing on meat quality and metabolites assessed by 1 H-NMR spectroscopy. Poult. Sci. 98:7139–7150.

Sohail, M. U., A. Ijaz, M. S. Yousaf, K. Ashraf, H. Zaneb, M. Aleem, and H. Rehman. 2010. Alleviation of cyclic heat stress in broilers by dietary supplementation of mannan-oligosaccharide and lactobacillus-based probiotic: Dynamics of cortisol, thyroid hormones, cholesterol, C-reactive protein, and humoral immunity. Poult. Sci. 89:1934–1938.

Soleimani, A. F., I. Zulkifli, A. R. Omar, and A. R. Raha. 2011. Physiological responses of 3 chicken breeds to acute heat stress. Poult. Sci. 90:1435–1440.

Soliman, E. S., E. Taha, K. D. Infante, K. Laboy, M. A. Sobieh, and P. G. Reddy. 2009. Stressors Influence on Salmonella enterica Serovar Enteritidis Colonization in Broilers. Am. J. Anim. Vet. Sci. 4:42–48.

Son, J. H., and Y. Karasawa. 2001. Effects of caecal ligation and colostomy on water intake and excretion in chickens. Br. Poult. Sci. 42:130–133.

Song, J., K. Xiao, Y. L. Ke, L. F. Jiao, C. H. Hu, Q. Y. Diao, B. Shi, and X. T. Zou. 2014. Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. Poult. Sci. 93:581–588.

Souillard, R., C. Woudstra, C. Le Maréchal, M. Dia, M. H. Bayon-Auboyer, M. Chemaly, P. Fach, and S. Le Bouquin. 2014. Investigation of Clostridium botulinum in commercial poultry farms in France between 2011 and 2013. Avian Pathol. 43:458–464.

Spohn, S. N., and V. B. Young. 2018. Gastrointestinal Microbial Ecology With Perspectives on Health and Disease.Pages 737–753 in Physiology of the Gastrointestinal Tract. Said, H.M., ed. 6th ed. Academic Press, London.

Stanley, D., and Y. S. Bajagai. 2022. Feed Safety and the Development of Poultry Intestinal Microbiota. Animals 12:2890.

Stanley, D., R. Batacan, and Y. S. Bajagai. 2022. Rapid growth of antimicrobial resistance: the role of agriculture in the problem and the solutions. Appl. Microbiol. Biotechnol. 106:6953–6962.

Stanley, D., S. E. Denman, R. J. Hughes, M. S. Geier, T. M. Crowley, H. Chen, V. R. Haring, and R. J. Moore.

2012. Intestinal microbiota associated with differential feed conversion efficiency in chickens. Appl. Microbiol. Biotechnol. 96:1361–1369.

Stanley, D., M. S. Geier, R. J. Hughes, S. E. Denman, and R. J. Moore. 2013. Highly variable microbiota development in the chicken gastrointestinal tract. PLoS One 8:6–12.

Stanley, V. G., C. Gray, M. Daley, W. F. Krueger, and A. E. Sefton. 2004. An Alternative to Antibiotic-Based Drugs in Feed for Enhancing Performance of Broilers Grown on Eimeria Spp.-Infected Litter. Poult. Sci. 83:39–44.

Stanley, D., R. J. Hughes, M. S. Geier, and R. J. Moore. 2016. Bacteria within the Gastrointestinal Tract Microbiota Correlated with Improved Growth and Feed Conversion: Challenges Presented for the Identification of Performance Enhancing Probiotic Bacteria. Front. Microbiol. 7.

Stanley, D., R. J. Hughes, and R. J. Moore. 2014. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. Appl. Microbiol. Biotechnol. 98:4301–4310.

Steffen, W., J. Rockström, K. Richardson, T. M. Lenton, C. Folke, D. Liverman, C. P. Summerhayes, A. D. Barnosky, S. E. Cornell, M. Crucifix, J. F. Donges, I. Fetzer, S. J. Lade, M. Scheffer, R. Winkelmann, and H. J. Schellnhuber. 2018. Trajectories of the Earth System in the Anthropocene. PNAS 115:8252–8259.

Stein, S. C., A. Woods, N. A. Jones, M. D. Davison, and D. Cabling. 2000. The regulation of AMP-activated protein kinase by phosphorylation. Biochem. J. 345:437–443.

Stevenson, M., E. Sergeant, T. Nunes, C. Heuer, J. Marshall, J. Sanchez, R. Thornton, and J. Reiczigel. 2021. epiR: Tools for the Analysis of Epidemiological Data. R package version 2.0.19.

Sugiharto, S., T. Yudiarti, I. Isroli, E. Widiastuti, and E. Kusumanti. 2017. Dietary supplementation of probiotics in poultry exposed to heat stress – a review. Ann. Anim. Sci. 17:591–604.

Suzuki, K., R. Harasawa, Y. Yoshitake, and T. Mitsuoka. 1983. Effects of crowding and heat stress on intestinal flora, body weight gain, and feed efficiency of growing rats and chicks. Japanese J. Vet. Sci. 45:331–338.

Suzuki, K., Y. Kodama, and T. Mitsuoka. 1989. Stress and Intestinal Flora. Bifidobact. Microflora 8:23–38.

Svihus, B. 2014. Function of the digestive system. J. Appl. Poult. Res. 23:306–314.

Svihus, B., V. B. Lund, B. Borjgen, M. R. Bedford, and M. Bakken. 2013. Effect of intermittent feeding, structural components and phytase on performance and behaviour of broiler chickens. Br. Poult. Sci. 54:222–230.

Svihus, B., A. Sacranie, V. Denstadli, and M. Choct. 2010. Nutrient utilization and functionality of the anterior digestive tract caused by intermittent feeding and inclusion of whole wheat in diets for broiler chickens. Poult. Sci. 89:2617–2625.

Sykes, A. H., and A. R. A. Fataftah. 1986. Acclimatisation of the fowl to intermittent acute heat stress. Br. Poult. Sci. 27:289–300.

Tabler, T. W., E. S. Greene, S. K. Orlowski, J. Z. Hiltz, N. B. Anthony, and S. Dridi. 2020a. Intestinal Barrier Integrity in Heat-Stressed Modern Broilers and Their Ancestor Wild Jungle Fowl. Front. Vet. Sci. 7:249.

Tabler, T., Y. Liang, J. Moon, and J. Wells. 2020b. NAE Production Challenges Poultry Industry on Several Fronts. Mississippi State Univ. Ext. Serv. Available at http://extension.msstate.edu/publications/nae-production-challenges-poultry-industry-several-fronts.

Tachibana, T., and K. Tsutsui. 2016. Neuropeptide Control of Feeding Behavior in Birds and Its Difference with Mammals. Front. Neurosci. 10:485.

Tamir, H., and S. Ratner. 1963. Enzymes of arginine metabolism in chicks. Arch. Biochem. Biophys. 102:249–

258.

Tan, G.-Y., L. Yang, Y.-Q. Fu, J.-H. Feng, and M.-H. Zhang. 2010. Effects of different acute high ambient temperatures on function of hepatic mitochondrial respiration, antioxidative enzymes, and oxidative injury in broiler chickens. Poult. Sci. 89:115–122.

Tang, Y., A. Underwood, A. Gielbert, M. J. Woodward, and L. Petrovska. 2014. Metaproteomics Analysis Reveals the Adaptation Process for the Chicken Gut Microbiota. Appl. Environ. Microbiol. 80:478–485.

Tang, S., J. Yu, M. Zhang, and E. Bao. 2013. Effects of different heat stress periods on various blood and meat quality parameters in young arbor Acer broiler chickens. Can. J. Anim. Sci. 93:453–460.

Tavárez, M. A., and F. Solis de los Santos. 2016. Impact of genetics and breeding on broiler production performance: a look into the past, present, and future of the industry. Anim. Front. 6:37–41.

Tayeri, V., A. Seidavi, L. Asadpour, and C. J. C. Phillips. 2018. A comparison of the effects of antibiotics, probiotics, synbiotics and prebiotics on the performance and carcass characteristics of broilers. Vet. Res. Commun. 42:195–207.

Teeter, R. G., M. O. Smith, F. N. Owens, S. C. Arp, S. Sangiah, and J. E. Breazile. 1985. Chronic heat stress and respiratory alkalosis: occurrence and treatment in broiler chicks. Poult. Sci. 64:1060–1064.

Tellez, G., S. E. Higgins, A. M. Donoghue, and B. M. Hargis. 2006. Digestive physiology and the role of microorganisms. J. Appl. Poult. Res. 15:136–144.

Tellez, G., J. D. Latorre, V. A. Kuttappan, M. H. Kogut, A. Wolfenden, X. Hernandez-Velasco, B. M. Hargis, W. G. Bottje, L. R. Bielke, and O. B. Faulkner. 2014. Utilization of rye as energy source affects bacterial translocation, intestinal viscosity, microbiota composition, and bone mineralization in broiler chickens. Front. Genet. 5:339.

Temim, S., A.-M. Chagneau, R. Peresson, and S. Tesseraud. 2000. Chronic Heat Exposure Alters Protein Turnover of Three Different Skeletal Muscles in Finishing Broiler Chickens Fed 20 or 25% Protein Diets. J. Nutr. 130:813–819.

Teyssier, J.-R., G. Brugaletta, F. Sirri, S. Dridi, and S. J. Rochell. 2022a. A review of heat stress in chickens. Part II: Insights into protein and energy utilization and feeding. Front. Physiol. 13:1521.

Teyssier, J. R., A. Preynat, P. Cozannet, M. Briens, A. Mauromoustakos, E. S. Greene, C. M. Owens, S. Dridi, and S. J. Rochell. 2022b. Constant and cyclic chronic heat stress models differentially influence growth performance, carcass traits and meat quality of broilers. Poult. Sci. 101:101963.

Thomke, S., and K. Elwinger. 1998a. Growth promotants in feeding pigs and poultry. I. Growth and feed efficiency responses to antibiotic growth promotants. Ann. Zootech. 47:85–97.

Thomke, S., and K. Elwinger. 1998b. Growth promotants in feeding pigs and poultry. II. Mode of action of antibiotic growth promotants. Ann. Zootech. 47:153–167.

Thomke, S., and K. Elwinger. 1998c. Growth promotants in feeding pigs and poultry. III. Alternatives to antibiotic growth promotants. Ann. Zootech. 47:245–271.

Thompson, K., K. Burkholder, J. Patterson, and T. J. Applegate. 2008. Microbial Ecology Shifts in the Ileum of Broilers During Feed Withdrawal and Dietary Manipulations. Poult. Sci. 87:1624–1632.

Tickle, P. G., J. R. Hutchinson, and J. R. Codd. 2018. Energy allocation and behaviour in the growing broiler chicken. Sci. Rep. 8:4562.

Tickle, P. G., H. Paxton, J. W. Rankin, J. R. Hutchinson, and J. R. Codd. 2014. Anatomical and biomechanical

traits of broiler chickens across ontogeny. Part II. Body segment inertial properties and muscle architecture of the pelvic limb. PeerJ 2:e473.

Timbermont, L., F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2011. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathol. 40:341–347.

Timper, K., and J. C. Brüning. 2017. Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity. Dis. Model. Mech. 10:679–689.

Torok, V. A., R. J. Hughes, L. L. Mikkelsen, R. Perez-Maldonado, K. Balding, R. MacAlpine, N. J. Percy, and K. Ophel-Keller. 2011. Identification and Characterization of Potential Performance-Related Gut Microbiotas in Broiler Chickens across Various Feeding Trials. Appl. Environ. Microbiol. 77:5868–5878.

Truong, D. T., E. A. Franzosa, T. L. Tickle, M. Scholz, G. Weingart, E. Pasolli, A. Tett, C. Huttenhower, and N. Segata. 2015. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. Nat. Methods 12:902–903.

Tsiouris, V., I. Georgopoulou, C. Batzios, N. Pappaioannou, R. Ducatelle, and P. Fortomaris. 2018. Heat stress as a predisposing factor for necrotic enteritis in broiler chicks. Avian Pathol. 47:616–624.

Turner, J. R. 2009. Intestinal mucosal barrier function in health and disease. Nat. Rev. Immunol. 9:799–809.

Turner, J. R., M. M. Buschmann, I. Romero-Calvo, A. Sailer, and L. Shen. 2014. The role of molecular remodeling in differential regulation of tight junction permeability. Semin. Cell Dev. Biol. 36:204–212.

Turroni, S., P. Brigidi, A. Cavalli, and M. Candela. 2018. Microbiota–Host Transgenomic Metabolism, Bioactive Molecules from the Inside. J. Med. Chem. 61:47–61.

Tzschentke, B., M. Nichelmann, and T. Postel. 1996. Effects of ambient temperature, age and wind speed on the thermal balance of layer-strain fowls. Br. Poult. Sci. 37:501–520.

Ukena, K., E. Iwakoshi-Ukena, S. Taniuchi, Y. Bessho, S. Maejima, K. Masuda, K. Shikano, K. Kondo, M. Furumitsu, and T. Tachibana. 2014. Identification of a cDNA encoding a novel small secretory protein, neurosecretory protein GL, in the chicken hypothalamic infundibulum. Biochem. Biophys. Res. Commun. 446:298–303.

Ulluwishewa, D., R. C. Anderson, W. C. McNabb, P. J. Moughan, J. M. Wells, and N. C. Roy. 2011. Regulation of Tight Junction Permeability by Intestinal Bacteria and Dietary Components. J. Nutr. 141:769–776.

Uni, Z., and R. P. Ferket. 2004. Methods for early nutrition and their potential. Worlds. Poult. Sci. J. 60:101–111.

Uni, Z., S. Ganot, and D. Sklan. 1998a. Posthatch development of mucosal function in the broiler small intestine. Poult. Sci. 77:75–82.

Uni, Z., R. Platin, and D. Sklan. 1998b. Cell proliferation in chicken intestinal epithelium occurs both in the crypt and along the villus. J. Comp. Physiol. B 168:241–247.

Uzal, F. A., B. L. Plattner, and J. M. Hostetter. 2015. Alimentary System.Pages 1-257.e2 in Jubb, Kennedy and Palmer's Pathology of Domestic Animals. 6th ed. Elsevier, Amsterdam, The Netherlands.

Vartoukian, S. R., R. M. Palmer, and W. G. Wade. 2007. The division "Synergistes." Anaerobe 13:99–106.

Vaure, C., and Y. Liu. 2014. A Comparative Review of Toll-Like Receptor 4 Expression and Functionality in Different Animal Species. Front. Immunol. 5:316.

Vesterlund, S. 2008. Mechanisms of probiotics: production of antimicrobial substances.Pages 391–395 in Handbook of probiotics & prebiotics. Lee, Y.K., Salminen, S., eds. 2nd ed. John Wiley & Sons, Inc., New Jersey.

Victoria Sanz Fernandez, M., J. S. Johnson, M. Abuajamieh, S. K. Stoakes, J. T. Seibert, L. Cox, S. Kahl, T. H.

Elsasser, J. W. Ross, S. Clay Isom, R. P. Rhoads, and L. H. Baumgard. 2015. Effects of heat stress on carbohydrate and lipid metabolism in growing pigs. Physiol. Rep. 3:e12315.

Videnska, P., M. M. Rahman, M. Faldynova, V. Babak, M. E. Matulova, E. Prukner-Radovcic, I. Krizek, S. Smole-Mozina, J. Kovac, A. Szmolka, B. Nagy, K. Sedlar, D. Cejkova, and I. Rychlik. 2014. Characterization of Egg Laying Hen and Broiler Fecal Microbiota in Poultry Farms in Croatia, Czech Republic, Hungary and Slovenia. PLoS One 9:e110076.

Virtue, S., and A. Vidal-Puig. 2011. Nothing Iffy about HIF in the Hypothalamus. PLoS Biol. 9:e1001116.

Vital, M., A. Karch, and D. H. Pieper. 2017. Colonic Butyrate-Producing Communities in Humans: an Overview Using Omics Data. mSystems 2:e00130-17.

Volkoff, H., L. F. Canosa, S. Unniappan, J. M. Cerdá-Reverter, N. J. Bernier, S. P. Kelly, and R. E. Peter. 2005. Neuropeptides and the control of food intake in fish. Gen. Comp. Endocrinol. 142:3–19.

Walker, J. B. 1960. Metabolic Control of Creatine Biosynthesis. J. Biol. Chem. 235:2357–2361.

Walker, W. A. 2017a. Dysbiosis.Pages 227–232 in The Microbiota in Gastrointestinal Pathophysiology. Floch, M.H., Ringel, Y., Walker, W.A., eds. Academic Press, London, UK.

Walker, W. A. 2017b. The importance of appropriate initial bacterial colonization of the intestine in newborn, child, and adult health. Pediatr. Res. 82:387–395.

Wall, C. W., and N. B. Anthony. 1995. Inheritance of carcass variables when giant jungle fowl and broilers achieve a common physiological body weight. Poult. Sci. 74:231–236.

Walsh, M. C., V. Jacquier, G. Schyns, J. Claypool, I. Tamburini, B. Blokker, and J. M. Geremia. 2021. A novel microbiome metabolic modulator improves the growth performance of broiler chickens in multiple trials and modulates targeted energy and amino acid metabolic pathways in the cecal metagenome. Poult. Sci. 100:100800.

Wang, X. J., J. H. Feng, M. H. Zhang, X. M. Li, D. D. Ma, and S. S. Chang. 2018a. Effects of high ambient temperature on the community structure and composition of ileal microbiome of broilers. Poult. Sci. 97:2153–2158.

Wang, K., L. Gan, I. Lee, and L. Hood. 1995a. Isolation and characterization of the chicken trypsinogen gene family. Biochem. J. 307:471–479.

Wang, Y., E. Goossens, V. Eeckhaut, E. Pérez Calvo, R. Lopez-Ulibarri, I. Eising, M. Klausen, N. Debunne, B. De Spiegeleer, R. Ducatelle, and F. Van Immerseel. 2020a. Dietary muramidase degrades bacterial peptidoglycan to NOD-activating muramyl dipeptides and reduces duodenal inflammation in broiler chickens. Br. J. Nutr.:1–11.

Wang, Y., E. Goossens, V. Eeckhaut, E. Pérez Calvo, R. Lopez-Ulibarri, I. Eising, M. Klausen, N. Debunne, B. De Spiegeleer, R. Ducatelle, and F. Van Immerseel. 2020b. Dietary muramidase degrades bacterial peptidoglycan to NOD-activating muramyl dipeptides and reduces duodenal inflammation in broiler chickens. Br. J. Nutr.:1–11.

Wang, G. L., B. H. Jiang, E. A. Rue, and G. L. Semenza. 1995b. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. PNAS 92:5510–5514.

Wang, R., K. Li, L. Sun, H. Jiao, Y. Zhou, and H. Li. 2022. L-Arginine/nitric oxide regulates skeletal muscle development via muscle fibre-specific nitric oxide/mTOR pathway in chickens. Anim. Nutr. J.

Wang, N., H. Liang, and K. Zen. 2014. Molecular Mechanisms That Influence the Macrophage M1–M2

Polarization Balance. Front. Immunol. 5.

Wang, L., M. Lilburn, and Z. Yu. 2016. Intestinal Microbiota of Broiler Chickens As Affected by Litter Management Regimens. Front. Microbiol. 7.

Wang, M., X. Lin, H. Jiao, V. Uyanga, J. Zhao, X. Wang, H. Li, Y. Zhou, S. Sun, and H. Lin. 2020c. Mild heat stress changes the microbiota diversity in the respiratory tract and the cecum of layer-type pullets. Poult. Sci. 99:7015–7026.

Wang, L., C. Llorente, P. Hartmann, A.-M. Yang, P. Chen, and B. Schnabl. 2015. Methods to determine intestinal permeability and bacterial translocation during liver disease. J. Immunol. Methods 421:44–53.

Wang, R. R., X. J. Pan, and Z. Q. Peng. 2009. Effects of heat exposure on muscle oxidation and protein functionalities of pectoralis majors in broilers. Poult. Sci. 88:1078–1084.

Wang, W. C., F. F. Yan, J. Y. Hu, O. A. Amen, and H. W. Cheng. 2018b. Supplementation of Bacillus subtilis-based probiotic reduces heat stress-related behaviors and inflammatory response in broiler chickens. J. Anim. Sci. 96:1654–1666.

Wang, Y., L. Zhang, G. Zhou, Z. Liao, H. Ahmad, W. Liu, and T. Wang. 2012. Dietary l-arginine supplementation improves the intestinal development through increasing mucosal Akt and mammalian target of rapamycin signals in intra-uterine growth retarded piglets. Br. J. Nutr. 108:1371–1381.

Wassenaar, T. M., and K. Zimmermann. 2018. Lipopolysaccharides in food, food supplements, and probiotics: should we be worried? Eur. J. Microbiol. Immunol. 8:63–69.

Wasti, S., N. Sah, and B. Mishra. 2020. Impact of Heat Stress on Poultry Health and Performances, and Potential Mitigation Strategies. Animals 10:1266.

Waterland, R. A., and C. Garza. 1999. Potential mechanisms of metabolic imprinting that lead to chronic disease. Am. J. Clin. Nutr. 69:179–197.

Waterson, M. J., and T. L. Horvath. 2015. Neuronal Regulation of Energy Homeostasis: Beyond the Hypothalamus and Feeding. Cell Metab. 22:962–970.

Wei, S., M. Morrison, and Z. Yu. 2013. Bacterial census of poultry intestinal microbiome. Poult. Sci. 92:671–683.

Wei, S., W. Shi, M. Li, and Q. Gao. 2014. Calorie restriction down-regulates expression of the iron regulatory hormone Hepcidin in normal and d-galactose-induced aging mouse brain. Rejuvenation Res. 17:19–26.

Wellman-Labadie, O., J. Picman, and M. T. Hincke. 2007. Avian antimicrobial proteins: structure, distribution and activity. Worlds. Poult. Sci. J. 63:421–438.

Wells, J. E., E. D. Berry, N. Kalchayanand, L. A. Rempel, M. Kim, and W. T. Oliver. 2015. Effect of lysozyme or antibiotics on faecal zoonotic pathogens in nursery pigs. J. Appl. Microbiol. 118:1489–1497.

Weninger, S. C., A. J. Dunn, L. J. Muglia, P. Dikkes, K. A. Miczek, A. H. Swiergiel, C. W. Berridge, and J. A. Majzoub. 1999. Stress-induced behaviors require the corticotropin-releasing hormone (CRH) receptor, but not CRH. PNAS 96:8283–8288.

West, C. E., H. Renz, M. C. Jenmalm, A. L. Kozyrskyj, K. J. Allen, P. Vuillermin, S. L. Prescott, C. MacKay, S. Salminen, G. Wong, J. Sinn, J. Stokholm, H. Bisgaard, R. Pawankar, P. Noakes, D. Kesper, and M. Tulic. 2015. The gut microbiota and inflammatory noncommunicable diseases: Associations and potentials for gut microbiota therapies. J. Allergy Clin. Immunol. 135:3–13.

Wideman, R. F. 2016. Bacterial chondronecrosis with osteomyelitis and lameness in broilers: A review. Poult. Sci. 95:325–344.

Wielen, P. W. J. J., D. A. Keuzenkamp, L. J. A. Lipman, F. Knapen, and S. Biesterveld. 2002. Spatial and Temporal Variation of the Intestinal Bacterial Community in Commercially Raised Broiler Chickens During Growth. Microb. Ecol. 44:286–293.

Van Der Wielen, P. W. J. J., S. Biesterveld, S. Notermans, H. Hofstra, B. A. P. Urlings, and F. Van Knapen. 2000. Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. Appl. Environ. Microbiol. 66:2536–2540.

Willems, O. W., S. P. Miller, and B. J. Wood. 2013. Aspects of selection for feed efficiency in meat producing poultry. Worlds. Poult. Sci. J. 69:77–88.

Willemsen, H., M. Debonne, Q. Swennen, N. Everaert, C. Careghi, H. Han, V. Bruggeman, K. Tona, and E. Decuypere. 2010. Delay in feed access and spread of hatch: importance of early nutrition. Worlds. Poult. Sci. J. 66:177–188.

Wilson, H. R., D. W. MacLaury, T. H. Johnson, and C. D. Baird. 1975a. Relationship of Heat Tolerance and Oxygen Consumption in Chickens. Poult. Sci. 54:299–300.

Wilson, H. R., C. J. Wilcox, R. A. Voitle, C. D. Baird, and R. W. Dorminey. 1975b. Characteristics of White Leghorn Chickens Selected for Heat Tolerance. Poult. Sci. 54:126–130.

Wiseman, J., and P. C. Garnsworthy. 1999. Recent Developments in Poultry Nutrition 2. Nottingham University Press, Nottingham, UK.

Wishart, D. S., D. Tzur, C. Knox, R. Eisner, A. C. Guo, N. Young, D. Cheng, K. Jewell, D. Arndt, S. Sawhney, C. Fung, L. Nikolai, M. Lewis, M. A. Coutouly, I. Forsythe, P. Tang, S. Shrivastava, K. Jeroncic, P. Stothard, G. Amegbey, D. Block, D. D. Hau, J. Wagner, J. Miniaci, M. Clements, M. Gebremedhin, N. Guo, Y. Zhang, G. E. Duggan, G. D. MacInnis, A. M. Weljie, R. Dowlatabadi, F. Bamforth, D. Clive, R. Greiner, L. Li, T. Marrie, B. D. Sykes, H. J. Vogel, and L. Querengesser. 2007. HMDB: The human metabolome database. Nucleic Acids Res. 35.

WMO (World Meteorological Organization). 2022. WMO update: 50:50 chance of global temperature temporarily reaching 1.5°C threshold in next five years. Available at https://public.wmo.int/en/media/press-release/wmo-update-5050-chance-of-global-temperature-temporarily-reaching-15°c-threshold.

Wren, A. M., C. J. Small, H. L. Ward, K. G. Murphy, C. L. Dakin, S. Taheri, A. R. Kennedy, G. H. Roberts, D. G. A. Morgan, M. A. Ghatei, and S. R. Bloom. 2000. The Novel Hypothalamic Peptide Ghrelin Stimulates Food Intake and Growth Hormone Secretion. Endocrinology 141:4325–4328.

Wu, G. 2010. Functional Amino Acids in Growth, Reproduction, and Health. Adv. Nutr. 1:31–37.

Wu, G., F. W. Bazer, R. C. Burghardt, G. A. Johnson, S. W. Kim, D. A. Knabe, P. Li, X. Li, J. R. McKnight, M. C. Satterfield, and T. E. Spencer. 2011. Proline and hydroxyproline metabolism: implications for animal and human nutrition. Amino Acids 40:1053–1063.

Wu, G., Y.-Z. Fang, S. Yang, J. R. Lupton, and N. D. Turner. 2004. Glutathione Metabolism and Its Implications for Health. J. Nutr. 134:489–492.

Wu, G., C. J. Meininger, D. A. Knabe, F. W. Baze, and J. M. Rhoads. 2000. Arginine nutrition in development, health and disease. Curr. Opin. Clin. Nutr. Metab. Care 3:59–66.

Wu, G., C. J. Meininger, C. J. McNeal, F. W. Bazer, and J. M. Rhoads. 2021. Role of L-Arginine in Nitric Oxide Synthesis and Health in Humans.Pages 167–187 in Amino Acids in Nutrition and Health: Amino Acids in Gene Expression, Metabolic Regulation, and Exercising Performance. Wu, G., ed. Springer Nature

Switzerland AG.

Wu, G., and S. M. Morris. 1998. Arginine metabolism: Nitric oxide and beyond. Biochem. J. 336:1–17.

Wu, Q., C. Wang, J. Liao, N. Hu, B. Cheng, Y. Ma, and Y. Wang. 2022. Effects of Dietary Supplementation with Glutamine on the Immunity and Intestinal Barrier Gene Expression in Broiler Chickens Infected with Salmonella Enteritidis. Animals 12:2168.

Wu, Q., Y. Zhang, J. Xu, and P. Shen. 2005. Regulation of hunger-driven behaviors by neural ribosomal S6 kinase in Drosophila. PNAS 102:13289–13294.

Xia, Y., J. Kong, G. Zhang, X. Zhang, R. Seviour, and Y. Kong. 2019. Effects of dietary supplementation with lysozyme on the structure and function of the cecal microbiota in broiler chickens. PLoS One 14:e0216748.

Xia, M., L. Ye, Q. Hou, and Q. Yu. 2016. Effects of arginine on intestinal epithelial cell integrity and nutrient uptake. Br. J. Nutr. 116:1675–1681.

Xiao, B., M. J. Sanders, E. Underwood, R. Heath, F. V. Mayer, D. Carmena, C. Jing, P. A. Walker, J. F. Eccleston, L. F. Haire, P. Saiu, S. A. Howell, R. Aasland, S. R. Martin, D. Carling, and S. J. Gamblin. 2011. Structure of mammalian AMPK and its regulation by ADP. Nature 472:230–233.

Xing, S., X. Wang, H. Diao, M. Zhang, Y. Zhou, and J. Feng. 2019. Changes in the cecal microbiota of laying hens during heat stress is mainly associated with reduced feed intake. Poult. Sci. 98:5257–5264.

Xiong, Y., H. Yi, Q. Wu, Z. Jiang, and L. Wang. 2020. Effects of acute heat stress on intestinal microbiota in grow-finishing pigs, and associations with feed intake and serum profile. J. Appl. Microbiol. 128:840–852.

Xiong, X., J. Zhou, H. Liu, Y. Tang, B. Tan, and Y. Yin. 2019. Dietary lysozyme supplementation contributes to enhanced intestinal functions and gut microflora of piglets. Food Funct. 10:1696–1706.

Xu, Y. Q., Y. W. Guo, B. L. Shi, S. M. Yan, and X. Y. Guo. 2018a. Dietary arginine supplementation enhances the growth performance and immune status of broiler chickens. Livest. Sci. 209:8–13.

Xu, Y., X. Lai, Z. Li, X. Zhang, and Q. Luo. 2018b. Effect of chronic heat stress on some physiological and immunological parameters in different breed of broilers. Poult. Sci. 97:4073–4082.

Xu, S., J. Shi, Y. Dong, Z. Li, X. Wu, Y. Lin, L. Che, J. Li, B. Feng, Z. Fang, Y. Zhuo, J. Wang, D. Wu, Z. Ren, and Y. Shen. 2020. Fecal bacteria and metabolite responses to dietary lysozyme in a sow model from late gestation until lactation. Sci. Rep. 10:3210.

Xu, S., J. Shi, X. Shi, Y. Dong, X. Wu, Z. Li, Z. Fang, Y. Lin, L. Che, J. Li, B. Feng, J. Wang, D. Wu, and Y. Shen. 2018c. Effects of dietary supplementation with lysozyme during late gestation and lactation stage on the performance of sows and their offspring. J. Anim. Sci. 96:4768–4779.

Xu, P., P. B. Siegel, and D. M. Denbow. 2011. AICAR and Compound C regulate food intake independently of AMP-activated protein kinase in lines of chickens selected for high or low body weight. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 159:401–412.

Xu, Y., H. Yang, L. Zhang, Y. Su, D. Shi, H. Xiao, and Y. Tian. 2016. High-throughput sequencing technology to reveal the composition and function of cecal microbiota in Dagu chicken. BMC Microbiol. 16:1–9.

Xue, G. D., R. Barekatain, S. B. Wu, M. Choct, and R. A. Swick. 2018. Dietary L-glutamine supplementation improves growth performance, gut morphology, and serum biochemical indices of broiler chickens during necrotic enteritis challenge. Poult. Sci. 97:1334–1341.

Yahav, S. 2009. Alleviating heat stress in domestic fowl: different strategies. Worlds. Poult. Sci. J. 65:719–732.

Yahav, S., D. Shinder, J. Tanny, and S. Cohen. 2005. Sensible heat loss: the broiler's paradox. Worlds. Poult. Sci.

J. 61:419–434.

Yahav, S., A. Straschnow, D. Luger, D. Shinder, J. Tanny, and S. Cohen. 2004. Ventilation, sensible heat loss, broiler energy, and water balance under harsh environmental conditions. Poult. Sci. 83:253–258.

Yang, Y., P. A. Iji, and M. Choct. 2009. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. Worlds. Poult. Sci. J. 65:97–114.

Yegani, M., and D. R. Korver. 2008. Factors Affecting Intestinal Health in Poultry. Poult. Sci. 87:2052–2063.

Yeoman, C. J., N. Chia, P. Jeraldo, M. Sipos, N. D. Goldenfeld, and B. A. White. 2012. The microbiome of the chicken gastrointestinal tract. Anim. Heal. Res. Rev. 13:89–99.

Yeoman, C. J., and B. A. White. 2014. Gastrointestinal Tract Microbiota and Probiotics in Production Animals. Annu. Rev. Anim. Biosci 2:469–86.

Yuan, L., H. Lin, K. J. Jiang, H. C. Jiao, and Z. G. Song. 2008. Corticosterone administration and high-energy feed results in enhanced fat accumulation and insulin resistance in broiler chickens. Br. Poult. Sci. 49:487–95.

Yunianto, V. D., K. Hayashit, S. Kaiwda, A. Ohtsuka, and Y. Tomita. 1997. Effect of environmental temperature on muscle protein turnover and heat production in tube-fed broiler chickens. Br. J. Nutr. 77:897–909.

Yunis, R., and A. Cahaner. 1999. The effects of the naked neck (Na) and frizzle (F) genes on growth and meat yield of broilers and their interactions with ambient temperatures and potential growth rate. Poult. Sci. 78:1347–1352.

Zajac, A., S. Poprzęcki, A. Żebrowska, M. Chalimoniuk, and J. Langfort. 2010. Arginine and Ornithine Supplementation Increases Growth Hormone and Insulin-Like Growth Factor-1 Serum Levels After Heavy-Resistance Exercise in Strength-Trained Athletes. J. Strength Cond. Res. 24:1082–1090.

Zampiga, M. 2019. Application of traditional and innovative techniques to investigate productive efficiency and related molecular traits in broiler chickens. Available at http://amsdottorato.unibo.it/9002/#.

Zampiga, M., F. Calini, and F. Sirri. 2021a. Importance of feed efficiency for sustainable intensification of chicken meat production: implications and role for amino acids, feed enzymes and organic trace minerals. Worlds. Poult. Sci. J. 77:639–659.

Zampiga, M., L. Laghi, M. Petracci, C. Zhu, A. Meluzzi, S. Dridi, and F. Sirri. 2018. Effect of dietary arginine to lysine ratios on productive performance, meat quality, plasma and muscle metabolomics profile in fast-growing broiler chickens. J. Anim. Sci. Biotechnol. 9.

Zampiga, M., L. Laghi, C. Zhu, A. Cartoni Mancinelli, S. Mattioli, and F. Sirri. 2021b. Breast muscle and plasma metabolomics profile of broiler chickens exposed to chronic heat stress conditions. Animal 15:100275.

Zampiga, M., F. Soglia, M. Petracci, A. Meluzzi, and F. Sirri. 2019. Effect of different arginine-to-lysine ratios in broiler chicken diets on the occurrence of breast myopathies and meat quality attributes. Poult. Sci. 98:2691–2697.

Zhan, X. A., J. X. Li, Z. R. Xu, and R. Q. Zhao. 2006. Effects of methionine and betaine supplementation on growth performance, carcase composition and metabolism of lipids in male broilers. Br. Poult. Sci. 47:576–580.

Zhang, B., Z. Lv, H. Li, S. Guo, D. Liu, and Y. Guo. 2017. Dietary l-arginine inhibits intestinal Clostridium perfringens colonisation and attenuates intestinal mucosal injury in broiler chickens. Br. J. Nutr. 118:321–332.

Zhang, B., Z. Lv, Z. Li, W. Wang, G. Li, and Y. Guo. 2018. Dietary l-arginine Supplementation Alleviates the Intestinal Injury and Modulates the Gut Microbiota in Broiler Chickens Challenged by Clostridium perfringens. Front. Microbiol. 9:1716.

Zhang, H., G. Zhang, F. J. Gonzalez, S. Park, and D. Cai. 2011. Hypoxia-Inducible Factor Directs POMC Gene to Mediate Hypothalamic Glucose Sensing and Energy Balance Regulation. PLoS Biol. 9:e1001112.

Zhang, X., G. Zhang, H. Zhang, M. Karin, H. Bai, and D. Cai. 2008. Hypothalamic IKKβ/NF-κB and ER Stress Link Overnutrition to Energy Imbalance and Obesity. Cell 135:61–73.

Zheng, D., T. Liwinski, and E. Elinav. 2020. Interaction between microbiota and immunity in health and disease. Cell Res. 30:492–506.

Zhou, X., X. Jiang, C. Yang, B. Ma, C. Lei, C. Xu, A. Zhang, X. Yang, Q. Xiong, P. Zhang, S. Men, R. Xiang, and H. Wang. 2016. Cecal microbiota of Tibetan Chickens from five geographic regions were determined by 16S rRNA sequencing. MicrobiologyOpen 5:753–762.

Zhu, L., R. Liao, N. Wu, G. Zhu, and C. Yang. 2019. Heat stress mediates changes in fecal microbiome and functional pathways of laying hens. Appl. Microbiol. Biotechnol. 103:461–472.

Zuidhof, M. J., B. L. Schneider, V. L. Carney, D. R. Korver, and F. E. Robinson. 2014. Growth, efficiency, and yield of commercial broilers from 1957, 1978, and 2005. Poult. Sci. 93:2970–2982.

Zuo, J., M. Xu, Y. A. uwal. Abdullahi, L. Ma, Z. Zhang, and D. Feng. 2015. Constant heat stress reduces skeletal muscle protein deposition in broilers. J. Sci. Food Agric. 95:429–436.

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