Gut function and related parameters in densely stocked broiler chickens: Responses to a novel *Bacillus* probiotic mixture

Zamile Mahlangu

220032998

orcid.org/ 0009-0003-3572-2740

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Supervisor:	Prof V Mlambo
Co-Supervisor(s):	Prof C.M. Mnisi [North-West University]
	Dr G. Moonsamy [Council for Scientific and Industrial Research]
	Prof O.E Fayemi [North-West University]

School of Agricultural Sciences

Faculty of Agriculture and Natural Sciences

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UNIVERSITY OF MPUMALANGA

DECLARATION

I, Zamile Mahlangu, confirm that this MSc dissertation has solely been submitted to the University of Mpumalanga. This study was completed under the supervision of Prof. V. Mlambo, Prof. C.M. Mnisi, Prof. O.E. Fayemi, and Dr G. Moonsamy. Sources of information and other material have been fully acknowledged.

Student: Zamile Mahlangu (220032998)

Signature:  Date: 08 November 2025

Main supervisor: Prof. V. Mlambo

Signature:  Date: 06 November 2025

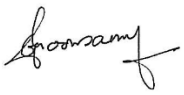
Co-Supervisor: Prof. C.M. Mnisi

Signature:  Date: 06 November 2025

Co-Supervisor: Prof. O.E. Fayemi

Signature:  Date: 07 November 2025

Co-Supervisor: Dr G. Moonsamy

Signature:  Date: 07 November 2025

GENERAL ABSTRACT

Poultry meat is one of the most affordable sources of animal protein and other micronutrients, whose demand is driven by the increasing human population. In response, producers have intensified poultry production systems to meet this demand. Antibiotic growth promoters (AGP) have been extensively used to control disease outbreaks and increase productivity in the poultry industry. However, their use has raised concerns regarding human and environmental health. Therefore, it is important to investigate appropriate dosages and the effectiveness of alternative nutritional strategies, such as probiotics, to replace AGP in poultry feed formulations. Accordingly, this study evaluated the impact of incremental dosages of a *Bacillus subtilis* probiotic mixture as a replacement for AGP in densely stocked broiler chickens. The probiotic mixture contained *B. subtilis* strains CPB 003, CPB 029, HP 1.6, and D 014 in equal proportions, resulting in a final concentration of 1×10^9 cfu/g of product. The study was split into two experimental chapters, 3 and 4. Chapter 3 reports the effects of *B. subtilis* probiotic mixture on growth performance, haemato-clinical biochemistry, carcass traits, gastrointestinal tract (GIT) and accessory GIT visceral morphometry, and meat quality. In Chapter 4, the effects of these probiotics on gut histomorphology and the phylogenetic composition of the gut microbiota of broiler chickens are reported. The feeding trial was conducted at Rooigrond farm in the North West province, South Africa. In a completely randomized design, 900 one-day-old Ross 308 broiler chicks (43.21±0.372 g live weights) were distributed evenly into 36 pens (0.75 m^2 each; 13 birds per pen), to which six experimental diets were randomly assigned. The experimental diets, provided from the starter through to the finisher phase, comprised: **1.** a basal broiler diet without AGP or *B. subtilis* probiotic mixture (PRB0); **2.** the basal diet supplemented with 0.5% (w/w) zinc bacitracin (POSC); and four versions of the basal diet supplemented with *B. subtilis* probiotic mixture at 0.3 (PRB3), 0.4 (PRB4), 0.5 (PRB5), or 0.6% (PRB6). Feed intake and body weight gain were measured weekly, blood was collected

two days prior to slaughter for haemato-biochemical analyses, and meat quality attributes and nutrient digestibility were measured post-slaughter. A quadratic effect was noted in the feed intake during the starter phase. The 0.5% inclusion rate improved body weight gain and feed efficiency, whereas the 0.3% increased feed intake and reduced body weight gain. Diets supplemented with *B. subtilis* mixture significantly improved ($p < 0.05$) digestibility of organic matter, neutral detergent fibre, and gross energy, while a negative quadratic effect was noted on crude protein digestibility. Changes in haemato-biochemical parameters, including increased neutrophils and white blood cells, indicate reduced stress and inflammation in broiler chickens fed diets containing 0.3%, 0.4%, and 0.5% of *B. subtilis* probiotic blend. Carcass traits were statistically unaffected ($p > 0.05$). The relative duodenal weight and jejunal length were significantly different ($p < 0.05$), with PRB0 and POSC promoting the highest weight, and PRB6 promoting the longest jejunum. In Chapter 4, significant differences ($p < 0.05$) were observed in jejunal villi absorption surface area and PERMANOVA on microbial diversity among the dietary treatments. However, no dietary effects were noted on the alpha diversity of caecal microbiota. Taxonomic profiling of the caecal digesta revealed a microbial community structure dominated at the phylum taxonomic rank by *Verrucomicrobiota*, *Bacillota*, and *Asgardarchaeota*. At the genus rank, *Akkermansia*, SOKP01, *Exiguobacterium* A, and *Rhizomicrobium* emerged as the most abundant across dietary treatments. Beneficial microbes were most dominant in caecal digesta from chickens fed PRB5, indicating a healthy gut, which may explain the improved performance metrics reported in Chapter 3. In contrast, broiler chickens on the PRB6 diet carried more unclassified and scientifically unknown gut microbial populations not found in other diets, which may have contributed to the poor performance reported in Chapter 3. The results indicate that the *B. subtilis* probiotic mixture enhanced growth performance and nutrient digestibility, alleviated stress and inflammation, improved jejunal absorptive surface area, and promoted beneficial gut microbes in broiler chickens reared

under high stocking density conditions. Therefore, it is recommended that the *B. subtilis* probiotic mixture be supplemented in broiler diets at a concentration of 0.5% (w/w).

Keywords: Antibiotic growth promoters; *Bacillus subtilis*; stocking density; growth; blood parameters; gut function.

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DEDICATION

I dedicate this dissertation to myself and my son, Zanokuhle Mahlangu. This work stands as a testament to my determination and resilience, a reminder that, despite every challenge, I persevered, conquered, and successfully completed this journey.

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LIST OF ABBREVIATIONS/ACRONYMS

AGP	Antibiotic growth promoters
ANOVA	Analysis of variance
BWG	Body weight gain
CL	Cooking loss
CSIR	Council for Scientific and Industrial Research
DL	Drip loss
FCR	Feed conversion ratio
FI	Feed intake
GIT	Gastrointestinal tract
GLM	General linear model
NSP	Non-starch polysaccharides
PCoA	Principal Coordinates Analysis
PERMANOVA	Permutational Multivariate Analysis of Variance
SAPA	South African Poultry Association
SCFA	Short-chain fatty acids
VASA	Villi absorptive surface area
VFA	Volatile fatty acids
WHC	Water holding capacity

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1 CHAPTER 1: GENERAL INTRODUCTION

1.1 Background

Broiler chicken production is a key agricultural subsector globally, providing an affordable source of dietary protein to support the needs of an expanding human population. According to the South African Poultry Association (2021) and Idowu *et al*. (2021), broiler chicken production in South Africa is a major contributor to the agricultural sector, accounting for 19.6% of the total gross value and 40% of animal products. It also provides direct and indirect jobs to over 110 000 individuals across its value chain and related sectors (Nkukwana, 2018; Idowu *et al*., 2021). However, local production is not sufficient to meet demand. Due to high production costs and increased demand for poultry products, producers have increased broiler chicken production by raising stocking density. High stocking density causes stress in chickens, which negatively impacts the gastrointestinal tract (GIT), resulting in poor growth and productivity (Son *et al*., 2022). As a result, antibiotic growth promoters (AGP) have been widely used to control diseases and oxidative stress associated with high-density rearing conditions, while also enhancing feed efficiency, growth rates, and the overall health of broiler chickens (Altaf *et al*., 2019).

1.2 Problem statement

Several concerns have been reported regarding the use of AGP in broiler chicken production, leading to their restriction in many countries (Krysiak *et al*., 2021). These include the emergence of antibiotic-resistant microorganisms and the presence of antibiotic residues in poultry products, which affect both human and environmental health (Aminudin *et al*., 2025; Mangweni & Teele, 2025). According to the Food and Agriculture Organization (2024), antimicrobial resistance is a leading cause of death globally, where over 1.27 million people

die due to drug-resistant bacteria. This may be associated with the routine use of antibiotics, including their application as antibiotic growth promoters in animals and for treating bacterial infections in humans (such as penicillin), which promotes the emergence of antibiotic-resistant bacteria (Marshall & Levy, 2011; FAO, 2024). Additionally, resistant bacteria secrete enzymes, such as β-lactamases, that neutralize or inactivate penicillins and other antibiotics (Rahman *et al*., 2018). Moreover, the overuse of AGP in poultry farming results in the accumulation of antibiotic residues in poultry products, posing risks to human health (Izah *et al*., 2025). These health concerns include allergic reactions and dysbiosis in the human gut microbiome, which can negatively impact immune and digestive function, and overall health (Abd El-Hack *et al*., 2022). When AGP are excreted into the environment, they contaminate agroecosystems through practices such as applying manure containing antibiotic residues as fertilizer on agricultural lands and using wastewater for irrigation (Polianciuc *et al*., 2020). As a result, most countries have banned the use of AGP on food-producing animals, except developing countries such as South Africa, where antibiotics are still commonly used to maximise productivity and profitability, particularly in intensive production systems (Chowdhury *et al*., 2022). According to Nduku *et al*. (2025), poultry reared in intensive production systems with high stocking densities are often susceptible to stress-induced diseases. Consequently, antibiotics are used to reduce disease outbreaks and mortality while maintaining flock health (Selaledi *et al*., 2020). Hence, a ban on the use of AGP in developing countries without effective alternatives could have negative implications on food security and decrease profitability for poultry producers (Laxminarayan *et al*., 2015; Hedman *et al*., 2020; Tabashsum *et al*., 2023).

Additionally, organic poultry production is very challenging due to the high incidence of disease, while most consumers increasingly prefer broiler chicken products that do not contain antibiotics or artificial growth stimulants (Cervantes, 2015). Thus, it is important to evaluate

and optimize sustainable alternatives to AGP, such as probiotics, to improve gut health, growth performance, meat quality, and safety in broiler chickens reared under densely stocked conditions. However, incorrect doses of the *B*. *subtilis* probiotic mixture can negatively impact broiler production; for example, overdosages may result in economic losses by incurring additional costs without delivering corresponding improvements in performance or health, ultimately reducing overall production efficiency. On the other hand, underdosing may not yield noticeable benefits, making the probiotic supplementation ineffective in improving the health or productivity of the chickens. Furthermore, there is a scarcity of research on the efficacy of this novel *B*. *subtilis* probiotic mixture as a potential alternative to AGP in poultry production under both normal and densely stocked conditions.

1.3 Justification

Probiotics have become increasingly popular as feed additives in poultry production, especially following bans or restrictions on in-feed AGP in some regions and countries, including Europe, the United States of America, and Indonesia (Van *et al*., 2020; Untari *et al*., 2017; Etienne *et al*., 2025). These direct-fed microbials improve host health by colonizing the GIT, leading to a balanced microbiota and better regulation of the host's immune system (Abd El-Hack *et al*., 2020). Probiotics enhance overall health and performance in poultry as alternatives to AGP (Callaway *et al*., 2021; Shini & Bryden, 2021). They modulate the gut microbiota – a complex community essential for food digestion, nutrient absorption, and immune support (Hu *et al*., 2017). Probiotics maintain a balanced gut microbiota by inhibiting pathogenic bacteria, such as *Escherichia coli* and *Salmonella*, while promoting the proliferation of beneficial bacteria, including *Lactobacillus* and *Bifidobacterium* (Jha *et al*., 2020). These beneficial microbes have the potential to replace AGP by outcompeting pathogenic bacteria, enhancing the host's immune system, and producing substances that inhibit the growth of pathogenic microbes

(Latif *et al*., 2023; Halder *et al*., 2025). Additionally, probiotics reduce the effects of heat stress at the cellular level by producing heat shock proteins, which protect cells and enhance their tolerance and adaptation to heat stress (Sugiharto *et al*., 2017). This effect is particularly important under high stocking conditions in poultry farming.

According to Sandvang *et al*. (2021), *Bacillus* probiotics, known for their ability to form spores, adapt well to manufacturing processes and harsh gut conditions. Combining different *Bacillus* probiotic strains may enhance their overall benefits. When adopting *Bacillus* probiotics as a substitute for AGP, it is crucial to take a holistic approach in determining the appropriate supplementation dosage, ensuring it supports optimal bird health and performance while avoiding potential adverse effects from insufficient or excessive intake. This study aims to reduce the negative impacts of densely stocked conditions in broiler chicken production while improving productivity through the benefits of probiotics. As consumers increasingly prefer poultry products free of antibiotics and artificial growth stimulants (Ramlucken *et al*., 2020), probiotics can help achieve these objectives. Assessing the impact and identifying optimal dosage of a *B. subtilis* probiotic blend in densely stocked broiler chickens could provide evidence on its suitability as a viable alternative to in-feed AGP. Establishing the appropriate supplementation dose is crucial to minimize the risks of over- or under-dosing, thereby ensuring both effectiveness and cost efficiency in the production of densely stocked Ross 308 broiler chickens.

1.4 Aim of the Study

To determine the gut function, growth performance, nutrient digestibility, haemato-clinical biochemistry, and meat quality parameters in densely stocked broiler chickens in response to a novel *Bacillus subtilis* probiotic mixture.

1.5 Specific objective

To investigate the effects of replacing in-feed zinc bacitracin with incremental doses of a four-strain mixture of novel *Bacillus subtilis* probiotic candidates on growth performance, nutrient digestibility, haemato-clinical biochemistry, jejunal histomorphology, phylogenetic composition of caecal microbiota, and meat quality in densely stocked Ross 308 broiler chickens.

1.6 Research hypothesis

Dietary replacement of zinc bacitracin with a blend of novel *Bacillus subtilis* probiotic candidates would maintain growth performance, nutrient digestibility, haemato-clinical biochemistry, jejunal histomorphology, phylogenetic composition of caecal microbiota, and meat quality in densely stocked Ross 308 broiler chickens.

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2 CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

As the global human population steadily increases, demand for nutrient-rich animal-source foods and products is also growing (Jafarzadeh *et al*., 2024). Poultry farming plays a significant role in supplying affordable animal protein for human consumption, particularly for consumers in developing countries (Mottet & Tempio, 2017). Chicken meat is a rich source of protein and contains essential minerals, including sodium, phosphorus, magnesium, and calcium, which are crucial for various bodily functions, such as nerve impulse conduction, bone metabolic health, and muscle contraction (Kralik *et al*., 2018). Intensive poultry farming increased by 1.3% in 2021 in an endeavour to meet the increasing demand for an affordable source of animal-derived protein for human consumption (Gržinić *et al*., 2023). The observed increase in poultry farming is based on the use of improved housing facilities in commercial systems, which provide controlled environmental conditions to manage humidity, lighting, temperature, and ventilation (Baracho *et al*., 2018). Enhanced housing facilities are designed to optimize space utilization and control environmental conditions, thereby maximizing production. In these housing environments, broiler chickens are fed carefully formulated diets rich in highly digestible nutrients to meet specified nutritional needs (Ravindran, 2013). Broiler chicken intensive production includes deep litter systems, in which litter material, such as clean, dry, and friable sawdust, is placed on the floor in a closed facility to provide a comfortable environment for the chickens. In contrast, environmental conditions are rarely controlled in small-scale broiler chicken production systems due to limited availability of funds that restrict investment into the construction of up-market housing facilities, as well as providing the necessary farm animal welfare enhancements (Ramukhithi *et al*., 2023). However, intensive production is necessary to meet demands and maximize profits. However, such broiler chicken

production conditions are characterised by high stocking densities, which induce high levels of stress, especially thermal stress (Adjei-Mensah *et al*., 2024). In poultry production, stress has been shown to compromise the gut health of chickens, leading to poor nutrient digestion and absorption, reduced bird productivity, weakened immune defence, and overall enterprise profitability (Sumanu *et al*., 2022). Traditionally, stress-induced dysbiosis and its associated negative consequences under high stocking density have been mitigated by deploying in-feed AGP as a feed supplement (Guardia *et al*., 2011). However, this practice has led to increased antimicrobial resistance and the presence of drug residues in poultry products (Tran *et al*., 2023). Therefore, eco-friendly feed additives and supplements, such as probiotics, should be explored as alternatives to AGP, especially given the impact of higher stocking density on broiler chicken health and welfare.

2.2 Impacts of high stocking density in broiler chicken production

Stocking density is a crucial management factor on the farm, as it affects bird welfare, production, and the enterprise's profitability (Shynkaruk *et al*., 2023). In poultry production, stocking densities vary by country or rearing system (Son *et al*., 2022). In South Africa, the recommended stocking density for free-range broiler production is 15 birds/m2, and for non-free-range broiler production systems, 10-12 birds/m^2 (SAPA, 2012). This implies that anything above these recommendations is considered high stocking density. High stocking density is often used to maximize profitability by increasing the number of chickens within a fixed stocking area (Son *et al*., 2022). Indeed, it is beneficial to producers, as they can earn more profit and meet their demands by using lower-than-recommended space requirements. However, high stocking density, especially in hot environments, has adverse effects on the productivity and welfare of broiler chickens.

Heat stress is a significant environmental challenge impacting poultry production globally (Lara & Rostagno, 2013). Chickens are vulnerable to high temperatures because they are unable to regulate their body temperature effectively due to limited sweat glands and dense feather cover (Fathi *et al*., 2022). Heat stress has a complex impact on poultry, as it can result in a 20 to 40% reduction in feed consumption and body weight gain, increase free radical concentrations, leading to oxidative stress, and diminish the quality of poultry meat and eggs (Mao *et al*., 2024). It can negatively affect the immune system, productivity, well-being, and overall health of poultry (Rostagno, 2020; Madkour *et al*., 2024). High ambient temperatures impair nutrient digestion and absorption and disrupt intestinal structure, increasing the likelihood of toxins and harmful antigens entering the bloodstream (Krysiak *et al*., 2021). Additionally, broiler chickens reared at high stocking density become less active, suffer from footpad dermatitis (reduced mobility), and are more susceptible to other infectious diseases (Shynkaruk *et al*., 2023). Hence, it is essential for poultry producers to implement mitigatory strategies to counteract the negative effects of high stocking densities, for example, fortifying broiler chicken feeds with probiotics positively impacts gut health.

2.3 Broiler chickens' gut health

Gut health is crucial in broiler chicken production, as it directly impacts chicken performance and health (Zhu *et al*., 2021). In farmed animals, including poultry, the health of the GIT is a critical determinant of feed intake, nutrient digestion, and the efficiency of nutrient absorption (Ducatelle *et al*., 2018). A healthy gut promotes feed intake, nutrient digestion, and absorption, key drivers of improved bird productivity and performance. Gut health refers to a stable condition in which the gut microbiome and the intestinal tract maintain a balanced, mutually beneficial relationship, ensuring that farmed animal/bird health and productive performance are not compromised by intestinal dysfunction (Celi *et al*., 2017). This description includes

critical determinants of gut function, such as diet, gut microbiota, gut integrity, immune function, and nutrient digestion and absorption (Celi *et al*., 2019). A nutritionally balanced diet provides nutrients that support a balanced gut microbial ecosystem and metabolite production, which are key to maintaining and improving intestinal health.

2.3.1 Key physiological markers of gut health

Gut integrity is important; it facilitates nutrient absorption and prevents bacterial invasion of the gut mucosa and the entry of bacterial products from the gut lumen (Oni & Oke, 2025). The gut-associated lymphoid tissue (GALT), a component of the immune system, closely interacts with the gut microbiota, impacting gut integrity, modulating inflammation, and preserving gut and overall animal health (Song *et al*., 2022). The interconnection of the gut mucosal barrier, GALT, and the gut immune system modulates nutrient digestion and uptake, resulting in optimal growth performance in broiler chickens.

2.3.1.1 Diet

Diet composition can modulate gut health by influencing the composition and metabolic activity of gut microbiota (Choct, 2009). Some dietary components, for example, fibre, inhibit the growth of pathogenic bacteria but promote the proliferation of beneficial species (Yao *et al*., 2023). For instance, adding dietary fibres to poultry feed has been linked to an increase in beneficial *Bifidobacterium* species known to produce short-chain fatty acids (SCFAs), including butyrate, a crucial metabolite that promotes gut health and has anti-inflammatory effects (Ali *et al*., 2022). Furthermore, accelerated SCFA production in the gut has been shown to enhance gut integrity and function by upregulating tight junction protein expression, thereby protecting against increased intestinal permeability (Liu *et al*., 2021a).

Poultry diets containing soluble non-starch polysaccharides (NSP) are fermented by gut microbiota, particularly in the ileum (Choct, 2009). This fermentation increases the production

of volatile fatty acids (VFAs). However, high VFA concentrations reduce the digestion and absorption of starch, the most important energy source in poultry (Choct, 2009). This reduction occurs because excessive VFA production destroys the integrity of the intestinal mucosal lining by increasing its thickness (Bedford & Apajalahti, 2022). Thus, the supplementation of enzymes such as β-glucanase plays a crucial role in improving gut health by breaking down NSP, preventing the overproduction of VFA, and promoting nutrient utilization efficiency (Adeola & Cowieson, 2011). In contrast, Jha & Mishra (2021) reported that the inclusion of insoluble fibre at moderate levels can enhance the development of digestive organs in poultry, particularly gizzard activity. It may also promote the release of digestive enzymes and bile salts and positively influence the composition of intestinal microflora. These effects improve nutrient uptake and growth efficiency, ultimately supporting satiety and overall health in the animals. This suggests that the fibre content of poultry feed should be optimal to enable chickens to gain benefits while avoiding the negative effects of excessive dietary fibre.

2.3.1.2 Gut microbiota

Gut microbiota in poultry refers to the density and diversity of GIT-resident microorganisms: bacteria, viruses, and fungi, but with a preponderance of bacteria in the different sections of the chicken GIT (Wei *et al.*, 2013). The gastrointestinal microbiome plays an integral role in nutrient digestion and absorption, pathogen exclusion, and the development and enhancement of the immune system (Shang *et al*., 2018). Digestion is facilitated by Bifidobacteria's ability to break down complex carbohydrates and fibre into simpler, absorbable compounds that cannot be digested by the host (Apajalahti & Vienola, 2016). The gut microbiota achieves pathogen exclusion by forming a protective barrier as they adhere to the enterocyte epithelial cells, thereby reducing the opportunity for pathogenic bacteria to colonize (Shang *et al*., 2018). Rubio (2019) stated that microbial populations in the chicken gut play a crucial role in the development of the immune system, especially after hatching, as they are born with a limited

number of immune cells that produce immunoglobulins. Therefore, the gut microbiota helps stimulate these immune cells to optimize antibody production, enabling the host to fight disease (Rubio, 2019). Furthermore, gut microbial communities synthesize vitamins (B and K) that are imperative for growth and immune function (Shang *et al*., 2018).

Several factors can influence gut microbiota composition and function, including diet and the type of production system. Diet is a major factor in shaping gut microbial composition, as undigested or unabsorbed dietary components serve as substrates for intestinal bacterial growth (Pan & Yu, 2014). Certain diet components can favour pathogenic bacteria while suppressing beneficial microbes, and vice versa. For instance, diets high in indigestible, water-soluble non-starch polysaccharides encourage the growth of *Clostridium perfringens* and increase the risk of necrotic enteritis in young chicks (Immerseel *et al*., 2004). In contrast, diets low in non-starch polysaccharides, such as corn-based diets, do not have this effect (Jia *et al*., 2009; Pan & Yu, 2014). Indigestible fibre promotes the growth of bacteria that break down fibre (Tejeda & Kim, 2021). These bacteria ferment fibre to produce butyrate, propionate, and acetate, the SCFAs which help maintain gut integrity and support immune function (Liu *et al*., 2021a; Ali *et al*., 2022). Diet has a significant effect on gut microbiota; thus, nutritional interventions should be designed to enhance gut conditions that support a balanced relationship between the host and its microbiota, preserving gut structure and function and preventing disruptions (Celi *et al*., 2017).

The microbiome composition in chickens may be altered by changes in farming practices and environments, which can impact their natural immunity (Aruwa *et al*., 2021). For instance, chickens reared in a free-range system encounter a greater variety of microbes than those raised in an indoor system. Outdoor poultry are exposed to microbes from soil, plants, and other animals, which can confer benefits or pose risks, depending on whether they harbor beneficial or pathogenic microbes (Mak *et al*., 2022). The type of production system can also influence

the composition of the microbiota, as Kers *et al*. (2018) found higher numbers of C. perfringens in the ileum and cecum on organic farms than on conventional farms. The researchers proposed that the observed difference could be attributed to the use of the antimicrobial drug salinomycin, which is applied as a coccidiostat in conventional feed. This drug possesses antibiotic properties that can alter the composition of the intestinal microbiota, potentially disrupting the balance of beneficial and harmful microbes (Kers *et al*., 2018). With weakened gut integrity, pathogen proliferation escalates, leading to reduced health and productivity. Therefore, maintaining an optimal gut microbiota is crucial for mitigating the negative effects associated with its impairment. Hygiene within flocks is also a crucial factor that must be thoroughly managed to reduce the growth of the pathogenic *Salmonella* (Tariq *et al*., 2022).

2.3.1.3 Gut barrier integrity

Gut integrity refers to the health and functionality of the GIT, primarily the maintenance of a strong gut mucosal barrier that protects against pathogens and their toxic metabolites, and efficient nutrient digestion and absorption (Kocot *et al*., 2022). The intestinal mucosal barrier serves as a crucial barrier between commensal bacteria and the host, facilitating a symbiotic relationship (Hiippala *et al*., 2018). This barrier is supported by physical defenses, including epithelial cells coated with a mucus layer (Gieryńska et al., 2022), host defense antimicrobial peptides (AMPs), secretory immunoglobulin A (sIgA), and immune cells such as macrophages (Hiippala *et al*., 2018). Epithelial cells play a vital role in maintaining gut health by strengthening and preserving gut integrity. The barrier is made of different cell populations, including enterocytes, Paneth cells, and goblet cells, which contribute to gut (Pelaseyed *et al*., 2014).

Enterocytes form microvilli on their surfaces, which significantly increase the intestinal surface area, thereby enhancing nutrient uptake efficiency (Gieryńska *et al*., 2022). Goblet cells secrete mucus, which protects epithelial cells from pathogens and toxins, and Paneth cells produce

antimicrobial molecules that prevent the proliferation of pathogens and help control the gut microbiota (Iftekhar & Siga, 2021). Epithelial cells constantly communicate with the gut microbiota, which is important for maintaining barrier function. Through this interaction, they detect the presence of microbes, which, upon detection, cause them (epithelial cells) to secrete cytokines and chemokines, which either stimulate an immune response or promote tolerance, thus modulating inflammation and regulating immune activity (Okumura & Takeda, 2017; Paone & Cani, 2020). The constant renewal of intestinal epithelial cells from stem cells maintains the integrity of the intestinal barrier by repairing damage to the intestinal epithelium (Gieryńska *et al*., 2022).

Gut neuroendocrine cells regulate immune signals by releasing neuropeptides that regulate immune response through communication with macrophages, T-lymphocytes, and dendritic cells; cellular components of the immune system which defend the body against pathogens (Gonzalez-Rey *et al*., 2010). The gut-associated lymphoid tissue (GALT) beneath the epithelium secretes immunoglobulin A (IgA), a primary antibody that neutralises toxins and viruses, thus preventing invasion and damage to the gut, and also mediates host-microbe symbiosis by facilitating mucosal colonization of bacteria (Hiippal *et al*., 2018). Attachments of beneficial bacteria to the mucus layer act as the first line of defense against pathogenic microbes, preventing them from invading the gut lining (Jandi *et al*., 2024).

In addition to maintaining gut integrity, tight junctions (TJs) serve as a physical barrier that closes off the space between adjacent epithelial cells, thereby preventing harmful metabolites and toxins from passing from the intestinal lumen into the bloodstream (Citi *et al*., 2024). Tight junctions regulate intestinal barrier permeability by blocking the passage of larger, potentially harmful substances while allowing the selective passage of ions and nutrients (Gieryńska *et al*., 2022). This selective permeability is crucial for protecting underlying tissues from microbial invasion and facilitating nutrient absorption (Chen *et al*., 2015; Ducatelle *et al*., 2023). The

integrity of TJ can be weakened when the gut experiences inflammation due to injury or stress, leading to changes in the structure or function of the proteins that comprise TJs. Consequently, increased permeability of the intestinal barrier (leaky gut) occurs because the junctions become less effective at sealing the spaces between adjacent cells (Awad *et al*., 2017), allowing the translocation of bacteria and antigens, which may potentially contribute to gastrointestinal disorders such as necrotic enteritis (Fathima *et al*., 2022a). Furthermore, beneficial food components, such as glutamine and polyphenols, strengthen the barrier, whereas harmful components, such as saturated fats, increase permeability (Hiippala *et al*., 2018). Maintenance of gut integrity promotes physiological processes that significantly contribute to gut health. The loss of intestinal integrity and function leads to poor nutrient absorption, reduced performance, bacterial translocation, contamination of meat and eggs, increased susceptibility to bacterial infections, and, in some cases, death (Adedokun & Olojede, 2019) translating to poor enterprise profitability. Therefore, the bird's ability to digest and absorb nutrients depends on the structure and functional integrity of the gut.

2.3.1.4 Digestion and absorption of nutrients

The fundamental function of the digestive system is the digestion and absorption of nutrients (Celi *et al*., 2017). When feed is ingested, digestion begins physically in the oral cavity and continues in the gut, where stomach acid and enzymes in different sections of the GIT further break it down. Physical and chemical (enzyme catalysed) digestion of feed converts macromolecules into absorbable micromolecules (Celi *et al*., 2019). Efficient digestion of feed and nutrient absorption are imperative for nutrient availability in animals (Bakke *et al*., 2010). When these processes are impaired, malnutrition sets in (Brown, 2024), and undigested nutrients are fermented by microbes resident in the colon or cecum, potentially leading to overproduction of VFAs and triggering the production of wet excreta (Ravindran, 2013).

To address compromised nutrient digestion and absorption, exogenous feed enzymes have been deployed as a feeding technology to improve digestion, as they can hydrolyze feed substrates that are not or only partially digested by endogenous enzymes (Anderson *et al*., 2023). The use of exogenous digestive enzymes is more commonly applied in young animals~~,~~ since the GIT is not fully developed to produce and secrete enough quantities of endogenous digestive enzymes (Ravindran, 2013; Celi *et al*., 2017). Exogenous digestive enzymes, through their hydrolytic activities, enhance nutrient availability to animals by a multiplicity of mechanisms (Bedford, 2018). One of their mechanisms is to reduce thickness in cereal (oat and barley)-based diets, which improves effective nutrient digestion and absorption (Saki *et al*., 2010). The cell walls surrounding starch and protein in the endosperm of barley and wheat used in poultry feeds are intact; however, after exposure to exogenous enzymes, these cell walls are disrupted by the time the digesta reaches the proximal small intestine. Bedford (2018) stated that the breakdown of cell walls may allow endogenous amylase and protease to access nutrients that were previously encapsulated in the endosperm.

Among the many mechanisms is the (i) shifting of the site of digestion of complex feed components to the anterior part of the intestine, which reduces food availability for microbes in the posterior part and controls the proliferation of potentially harmful bacteria while promoting a more balanced microbiome and (ii) converting inert fibrous materials into fermentable oligosaccharides, which lowers intestinal pH through the production of SCFAs. The low intestinal pH retards the proliferation of harmful bacteria and creates a healthier intestinal environment (Chowdhury *et al*., 2025). Additionally, fermentable oligosaccharides and SCFAs produced during fermentation stimulate the proliferation of enterocytes, which helps maintain a healthy intestinal wall and gut barrier. In addition to the aforementioned mechanisms, the inclusion of exogenous fibre-digesting enzymes in poultry diets with a high content of complex fibres promotes feed digestion and enhances nutrient absorption, resulting

in improved gut health and broiler performance (Alabi *et al*., 2019). When probiotics are used to fortify feeds, they mediate increased host endogenous digestive secretion resulting in enhanced feed digestion, nutrient uptake, and utilisation (Celi *et al*., 2017), suggesting probiotic-induced improved gut health. These probiotic benefits are closely linked to the alterations in the composition of the chicken gut microbiota.

2.3.2 Microbial composition of the chicken gut

Microbiota composition of the chicken gut is regarded as a vital 'organ' that plays an integral role in sustaining host health since feed digestion is closely connected to gut microbiota. Gut microbiota composition affects feed digestibility and nutrient absorption, as well as overall productive performance in broiler chickens (Diaz-Carrasco *et al*., 2019). The microbial community in the chicken gut is dominated by bacterial species within the phyla Firmicutes, Bacteroidetes, Proteobacteria, and *Actinobacteria* (Bindari & Gerber, 2022). *Lactobacillus* species dominate and are found in all the GIT organs of the chicken (Table 2.1). In the crop, the primary bacteria belong to the Firmicutes group, including facultative anaerobic species of *Lactobacillus*, among which *Lactobacillus reuteri*, *Lactobacillus salivarius*, and *Lactobacillus crispatus* are the most prevalent (Saxena *et al*., 2016; Al Hakeem *et al*., 2023). The production of SCFAs lowers the pH of digesta in the digestive tract, which restricts the growth of pathogenic bacteria (Rehman *et al*., 2020; Peng & Biswas, 2017), which is a desirable outcome.

Table 2.1: Some microbial species found in the GIT organs of chickens

GIT sections	Available microbes
Crop	*Lactobacillus* species *(L. reuteri*, *L. salivarius* and *L. crispatus)*; *Klebsiella* species *(K. pneumonia* and *K. ozaenae)*; *Escherichia species (E. coli* and *E. ferusonii)*; *Enterobacter aerogenes*; *Eubacterium* spp.; *Pseudomonas aeruginosa*; *Micrococus leteus*; *Staphylococcus lentus* and *Bacillus*.
Gizzard	*Lactobacillus* spp.; *Enterococci*; *Enterobacteria* and *Coliforms*.
Duodenum	*Lactobacillus*, Corynebacterium, Bacteroides, E. coli, and *Shigella*.
Jejunum	*Lactobacillus*; *Enterococcus,* and *Bifidobacterium*
Ileum	*Lactobacillus* spp.; *Clostridiaceae* and *Enterococcus* spp.
Ceca	*Lactobacillus*; *Blautia*; *Faecalibacterium*; *Heliobacterium*; *Oscillibacter*; *Clostridium* spp. *Eubacterium*; *Bacteroides* spp.; *Bifidobacterium* spp.; *Actinobacteria* and *Proteobacteria.*

Source: Adapted from Rychlik, 2020

The gizzard hosts *Lactobacillus* species, *Enterococci*, *Enterobacteria*, and coliforms predominantly (Shang *et al*., 2018). Fermentation and production of SCFAs in the pro-ventriculus and ventriculus (gizzard) are restricted due to the increased acidity (Qaisrani *et al*., 2014; Shang *et al*., 2018). In chicks, the duodenum, the first segment of the small intestine, hosts *Enterobacteriaceae* or *Clostridiaceae*, but in adult chickens, *Lactobacillus*, *Corynebacterium*, *Bacteroides*, *E. coli*, and *Shigella* are the dominant species (Xiao *et al*., 2017; Al Hakeem *et al*., 2023). The jejunum, the major segment of the small intestine where lipid digestion and absorption occur, is characterized by a minimal bacterial phylum, comprising *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* (Zhang *et al*., 2022; Al Hakeem *et al*., 2023). Along with *Clostridiaceae*, *Streptococcus*, and *Enterococcus* *Lactobacillus* is the primary species in the ileum (Al Hakeem *et al*., 2023). In the chicken gut, the cecum has the highest bacterial density and diversity compared to other GIT compartments.

Caecal bacteria primarily consist of phylum Firmicutes, followed by Bacteroidetes and Actinobacteria (Oakley *et al*., 2013; Shang *et al*., 2018). This is due to its digestive role and the associated high digesta residence time in it (Clavijo & Flórez, 2018). Primary bacterial groups identified within the Firmicutes phylum include *Lactobacillus*, *Blautia*, *Clostridium*, and *Eubacterium. Faecalibacterium*, *Heliobacterium*, *Oscillibacter*, *Oscillospira*, and *Peptococus* (Lu *et al.*, 2003; Wang *et al*., 2019). In comparison, the cloaca and excreta are populated by *Bacillus*, *Clostridium*, *Eubacterium*, *Faecalibacterium*, *Lactobacillus*, and *Ruminococcus*, but the relative composition fluctuates due to the cyclic nature of the temporally emptying of different GIT compartments (Lim *et al*., 2015).

2.3.3 Role of gut microbiota in growth promotion

Interactions between the microbiota and the host are crucial for the host's growth, development, and health (Chen *et al*., 2022). The microbial community in the digestive system of chickens plays a significant role in maintaining intestinal health. It achieves this by influencing the host's physiological functions required for intestinal microbiota balance, as imbalanced microbiota correlates with poor growth and performance (Naeem *et al*., 2018). This is primarily achieved by competitively excluding harmful microorganisms and pathogens, thereby preventing their colonization and promoting a healthy gut (Paul *et al*., 2020). Consequently, this reduces the energy expenditure that birds typically invest in maintaining their immune system's activity against these threats (Jadhav *et al*., 2015), thereby allowing them to channel the energy towards growth promotion. Digestive enzymes released by the gut-resident microbiota are critical for further digestion and the release of nutrients from consumed feed, and this microbiota-assisted digestion increases nutrient availability to the broiler chicken, facilitating enhanced growth performance.

2.3.4 Nexus between gut health, nutrient absorption, and immune function

Maintaining optimal gut health is crucial for effective feed digestion and nutrient absorption. A healthy gut maintains optimal luminal temperature, pH, and osmotic pressure, which are critical to efficient GIT function (Celi *et al*., 2017). Optimal GIT conditions facilitate the growth of beneficial gut microbes whose ecosystem and metabolic activities aid with the digestion of complex feed that cannot be digested by endogenous mammalian digestive enzymes. Synergy between endogenous host-derived and exogenous microbiota-derived digestive enzymes increases the efficiency of feed digestion, thereby enhancing nutrient absorption (Rehman *et al*., 2020). In an unhealthy gut, poor digestion and absorption of nutrients occurs and the maldigested feed in the gut triggers a shift in microbiota composition, creating dysbiosis, a condition where the balance between harmful and beneficial microbes in the gut is lost (Fathima *et al*., 2022b). This results in poor digestion and, subsequently, poor growth performance. Changes in diet, stress induced by temperature fluctuations, overcrowding, overuse or inappropriate use of antibiotics, and infectious agents such as Salmonella (Teirlynck et al., 2009) drive dysbiosis. Preventing the proliferation of pathogenic bacteria is crucial for maintaining a healthy gut and promoting a balance between beneficial microbes (Kumar *et al*., 2025). Ducatelle *et al*. (2023) stated that a healthy gut ensures that epithelial cells secrete sufficient digestive enzymes, for example, aminopeptidases, which hydrolyse nutrients to absorbable monomers, facilitating the final step in preparing nutrients for absorption. Furthermore, effective nutrient digestion and absorption in a healthy gut have been shown to improve immune function in broiler chickens (Azizi *et al*., 2026).

Natural intestinal defenses that fight invading pathogens and may damage the intestinal mucosa include a protective mucus layer, lysozymes, immunoglobulin A (IgA), and cathelicidins, which are host defense peptides (Ducatelle *et al*., 2023). Toll-like receptors mediate sensitisation of the body to pathogens, resulting in the stimulation and upregulation of defense

mechanisms to combat them (Faghfouri *et al*., 2020). However, in an unhealthy intestinal mucosa, natural defenses are suppressed, such that insults, for example, pathogens and/or stress, fail to elicit optimal natural defenses, compromising bird health and productivity. In such a state, dietary fortification with probiotics may help support endogenous mechanisms that combat pathogens and stress (Yu *et al*., 2022).

2.4 Probiotics in poultry nutrition

2.4.1 Description and functions of probiotics

Probiotics are beneficial live micro-organisms, primarily bacteria and yeasts, added to animal feed as supplements (Krysiak *et al*., 2021). They are direct-fed microbes that, in the poultry GIT, promote the proliferation of desirable microbiota and enhance gut health (Jha *et al*., 2020). These probiotics balance gut microbiota populations, resulting in improved gut health, enhanced feed digestion, and nutrient absorption, which concomitantly manifest as improved bird welfare, growth, and productivity (Abd El-Hack *et al*., 2023). According to Fathima *et al*. (2022a), probiotics enhance the nutritional value of feed by fermenting indigestible carbohydrates in the ileum and colon, producing SCFAs. The generated SCFAs are metabolised by the host for energy.

Furthermore, probiotics also stimulate and accelerate the synthesis and release of host-endogenous digestive enzymes, thereby increasing feed and nutrient digestion and absorption (Neveling & Dicks, 2021). The probiotics have also been shown to mediate the restoration of broiler chicken intestinal integrity, as evidenced by healthy villi and an increase in beneficial and desirable gut bacteria (Salim *et al*., 2013; He *et al*., 2019; Xu *et al*., 2022). Abd El-Hack *et al*. (2023) reported that supplemental probiotics improve gut immune function and cytokine production, thereby enhancing gut mucosal protection against pathogens and their toxic metabolites.

2.4.2 Commonly used probiotics in poultry

Either single- or multiple-strain probiotic microbial species in the genera *Lactobacillus*, *Streptococcus*, *Bacillus*, *Enterococcus*, *Pediococcus*, *Aspergillus*, and *Saccharomyces* can be used as a feeding technology in poultry (Ritzi *et al*., 2016; Arczewska-Wlosek *et al*., 2022). However, most research has focused on *Bacillus* strains due to their spore-forming ability, which enables them to survive feed processing conditions and harsh GIT conditions (Golnari *et al*., 2024). The *B*. *subtilis* and *B*. *licheniformis* are the most investigated; for example, their effects, either individually or in combination, were evaluated in broiler chickens infected with *C. perfringens* and *Salmonella* (Abudabos *et al*., 2020; Liu *et al*., 2021b). Probiotics are typically administered to poultry early in life to maximize the benefits they confer, such as increased immune system development and improved overall health (Song *et al*., 2022). Dietary fortification of poultry chick feeds or inclusion in drinking water are convenient ways to administer probiotics to newly hatched chicks (Fathima *et al*., 2022a). Besides inclusion in feed or drinking water, probiotic species can be injected into the egg amniotic fluid, administered *in ovo*, or into the embryo during incubation (Roto *et al*., 2016). This route allows for early colonisation by the beneficial bacteria (Roto *et al*., 2016).

2.4.3 Probiotics: mechanisms of action

The mechanisms by which probiotics exert their effects in poultry are not yet fully understood. However, suggested mechanisms include the release of antimicrobial compounds, competing with pathogens for attachment to gut mucosal surfaces, enhancing intestinal mucosal barrier integrity, and upregulating the enteric immune system response (Broom & Kogut, 2018; Jha *et al*., 2020; Krysiak *et al*., 2021). Indeed, some probiotics have been reported to secrete antibacterial organic acids, lactocidin, hydrogen peroxide, and acidophillin, which inhibit the proliferation of pathogenic bacteria (Monika *et al*., 2021). The organic acids acetic, lactic, and

propionic acid kill harmful microbes by inducing proton-mediated disruption of bacterial cell membranes (Ibrahim *et al*., 2021). Additionally, by lowering gut pH, these organic acids create an unfavourable environment that inhibits the survival of pathogenic bacteria (Al-Khalaifah, 2018). A lower gut pH promotes the growth of acidophilic bacteria, including Bacillus probiotics, and also prevents the proliferation of gut pathogens, such as *Salmonella*, *C. perfringens*, and *E. coli* (Ogbuewu *et al*., 2022).

Probiotics upregulate immune system activity in poultry, resulting in enhanced IgA production and increased macrophage activity (Jadhav *et al*., 2015; Monika *et al*., 2021). By mediating competitive exclusion, probiotics outcompete pathogenic bacteria by utilizing the nutrients pathogens require for reproduction and by binding to intestinal cells, where pathogens typically attach (Jadhav *et al*., 2015; Ibrahim *et al*., 2021). The probiotics *Bacillus subtilis* and *Bacillus amyloliquefaciens*, when used to fortify broiler chicken diets, have been shown to enhance poultry health and productivity by controlling pathogens through the production of antimicrobial compounds that suppress and inhibit the growth of pathogenic gut bacteria (Park *et al*., 2020). Furthermore, by modulating the birds' immune system, these probiotics reduce intestinal inflammation, strengthen the gut barrier, improve nutrient absorption, and increase feed efficiency (Wang *et al*., 2021b) by mediating improvements in intestinal mucosal integrity and intestinal villi structure. The latter increases the surface area for nutrient absorption, resulting in increased growth and better bird growth performance (Park *et al*., 2020; Wang *et al*., 2021a). Probiotics compete with pathogenic bacteria for receptor-binding sites and nutrients, thus restricting the survival of undesirable bacteria in the gut (Plaza-Diaz *et al*., 2019; Latif *et al*., 2023). Through the mechanisms discussed, probiotics can potentially improve broiler chicken gut health, particularly for flocks reared under high stocking density and in hot tropical environments, conditions that induce stress. In addition to promoting farmed poultry gut health, probiotics also improve bird growth performance.

2.5 Probiotics: growth-promoting effects in poultry

Average daily feed intake (ADFI), body weight gain (BWG), and feed conversion ratio (FCR) are indices that correlate with broiler chicken growth performance (Shabani *et al*., 2012). These indices of performance are interrelated: when FI is high, BWG typically increases and FCR decreases, which is desirable for optimal growth in poultry production (Hwa *et al*., 2023). These metrics are strongly influenced by diet composition as determined by the quantities of different ingredients used to formulate a diet. For instance, the effects of probiotic supplementation may vary based on the formulation and level of dietary inclusion. When probiotics are supplemented at very high dietary inclusion levels, this may lead to diet dilution, in which feed nutrient density is reduced because probiotics substitute for a portion of the feed ingredients without providing essential nutritional value (Rezaei *et al*., 2006; Kuleile, 2024). Such a reduction in available nutrients can diminish the potential benefits of probiotic supplementation. Several studies have reported inconsistent results regarding the effects of in-feed probiotics on the growth performance of broiler chickens, with some showing improvements in ADFI, BWG, and FCR (Qorbanpour *et al*., 2018; Jha *et al*., 2020; Yu *et al*., 2022) as shown in Table 2.2 below.

Table 2.2: Summary of studies evaluating the impacts of probiotics on growth performance in broiler chickens.

Probiotic species	Dosage and administration route	Duration	Outcome	References
Lactobacillus plantarum B1	2×10^9 cfu/kg feed	42 days	Improved weight gain and reduced FCR	Peng *et al*., 2016
Bacillus coagulans	5×10^9 cfu/kg in the diet	47 days	Improved ADFI and weight gain	Yu *et al*., 2022
Bacillus subtilis fmbJ	4×10^{10} cfu/kg in the feed	42 days	Enhanced ADFI, average daily gain, and decreased FCR	Bai *et al*., 2017
Lactobacillus paracaseis *Lactobacillus rhamnosus*	Treatment 1(4 g/kg of feed), Treatment 2 (2 g/kg) in the diet	5 weeks	Improved BWG and FCR	Fesseha *et al.*, 2021
Lactobacillus johnsonii, *Lactobacillus crispatus*, *Lactobacillus salivarius*	1 kg/300kg of feed	35 days	No significant effects on growth FI, BWG, and FCR	Olnood *et al*., 2015

The improvement in FCR observed in broiler chickens fed diets fortified with *B*. *subtilis* is ascribed to the probiotic stimulating and boosting production of digestive enzymes that break down food to absorbable monomeric units (Bai *et al*., 2017). The improved FCR positively correlates with increased efficiency in converting absorbed nutrients into meat and reduced feed metabolic waste (Stanley *et al*., 2012). Interestingly, it has been observed that in broiler chickens administered *Lactobacillus* and *Bifidobacterium* probiotics, whether in a lyophilized (L-LAB) or liquid (S-LAB) form, these probiotics maintained health and optimal performance under heat-stress conditions (Hatipoglu *et al*., 2024). This effect was attributed to the probiotics' modulation of the chickens' gut microbiota, increasing the preponderance of beneficial bacteria within the birds' GITs. This implies that broiler chickens challenged by heat stress, for example,

when reared in humid tropical areas, can be fed probiotic-supplemented feeds to ensure optimal productivity. Despite many studies reporting positive outcomes from feeding probiotic fortified feeds, Olnood *et al*. (2015) reported that in broiler chickens reared in cages over a 6-week period, feeding broiler chicken diets fortified with *Lactobacillus* probiotic spp. did not improve the birds' FI, body weight gain, and FCR. The lack of positive effects on broiler chickens' performance indices may be due to the low probiotic dose (10^6 cfu/g) used, compared to the commercial recommendation of 108 cfu/g, resulting in limited probiotic-driven fermentation.

Due to their mediation of improved gut health and increased digestive enzyme secretion, probiotics, even under suboptimal feeding and nutrient availability, facilitate improved absorption and utilization of available nutrients, thereby compensating for dietary shortages (Kadam *et al*., 2023). This suggests that probiotics play an important role in promoting enhanced productive performance in broiler chickens, even under suboptimal dietary conditions (Kadam *et al*., 2023). This assertion is confirmed by findings of a study where broiler chickens were fed a probiotic *B*. *subtilis* fortified diet under two feeding regimens: nutrient-adequate or nutrient-deficient, resulted in significant improvement in ileal digestibility of protein, starch, and metabolized energy that led to efficient absorption contributing to optimal growth in chickens (Boroojeni *et al*., 2018). In addition to improving broiler chicken growth performance, probiotics also affect haemato-clinical indices in poultry.

2.6 Probiotics: effects on poultry haemato-clinical indices

Measurable blood components used as diagnostic tools to determine the nutritional status, immune response, and overall health of birds constitute markers referred to as blood parameters (Etim *et al*., 2014). Such "blood parameters" comprise haematological and serum biochemical

markers that serve as reliable indicators of the animals' physiological status. Changes in these markers are crucial in assessing how animals respond to different stimuli (Aikpitanyi & Egweh, 2020). Haematological indices, which are largely affected by nutritional, environmental, or pathological stimuli, are often used as markers of stress and, to some extent, provide valuable insights into the animals' immune status (Aikpitanyi & Egweh, 2020; Londok & Rompis, 2022). The haematological indices include white blood cell count, erythrocyte count, haemoglobin, and packed cell volume.

White blood cell (WBC) counts, or concentrations, are integral to chickens' immune status, as they are primarily responsible for defence against disease (Oluwagbenga & Fraley, 2023). A low WBC concentration increases the birds' susceptibility to infections, and a concentration within the normal range of 5.50-19.50 K/µL is associated with enhanced antibody production, resulting in greater disease resistance (Onunkwo *et al*., 2022). However, an abnormally high increase in WBC count is indicative of overstimulation by either excessive stress or infection (Animashahun & Omoikhoje, 2014). Furthermore, heterophils, the specialised avian equivalents of human neutrophils, respond to microbial stimuli, demonstrate chemotaxis, engulf pathogens, and release cytokines and metabolites that support immune system functionality. The heterophils mediate innate immune responses, including defence against pathogens, inflammation, wound healing, and tissue remodelling (Kogut, 2022). Red blood cells transport oxygen from the lungs to body tissues, thus are critical to tissue perfusion, and they ferry carbon dioxide produced by metabolic reactions to the lungs, where it is exhaled (Kuhn *et al*., 2017). Oxygen is transported to body tissues bound to haemoglobin (Huang *et al*., 2019a). A decrease in Hb concentration will negatively impact tissue oxygen supply.

Serum clinical biochemistry is used for two key purposes: to determine protein and amino acid requirements in farm animals and to assess the effects of interventions, including dietary effects, on the health of key organs (heart, kidney, and liver) (Hagan *et al*., 2022). The

parameters include serum lipid (cholesterol and triglycerides) and protein (albumin, total protein, and globulin) profiles, as well as liver function (aspartate aminotransferase and alanine aminotransferase). The serum lipid profile is largely affected by feed: lower-fibre diets result in higher serum cholesterol, while diets rich in crude fibre lower it (Regar *et al*., 2019). Elevated serum cholesterol promotes excessive fat accretion in broiler muscles, which decreases meat quality by inducing paler colour, greater lipid oxidation, and a less favourable fatty acid profile, thereby reducing nutritional value and consumer acceptability (Abdulla *et al*., 2015; Ding *et al*., 2023). A favourable serum lipid profile is associated with broiler chicken meat of acceptable quality and desirable for human consumption. In addition to dietary effects, the age of the chicken also significantly affects serum and muscle cholesterol concentrations, and these concentrations increase with bird age (Son *et al*., 2007). Consequently, consumers are advised to choose younger, lighter-weight broiler chickens for meat with lower cholesterol content (Alam *et al*., 2021). Broiler chicken meat, with a high concentration of triglycerides, is also a proxy for excess cholesterol and is associated with an increased risk of cardiovascular disease (Makama *et al*., 2021). The serum protein profile of broiler chickens is indicative of the birds' health status and a proxy for productive performance (Kareem *et al*., 2024); thus, determining the serum protein profile is key to detecting metabolic changes (Tóthová *et al*., 2019). Serum albumin and globulin concentrations are key indicators of inflammatory and immune responses (Karadağoğlu *et al*., 2020), where globulin upregulates the humoral immune response during infection, inflammation, or environmental stress, thereby reflecting an active immune system (Sugiharto *et al*., 2024). In contrast, serum albumin transports cytokines, thus directly impacting the body's defense mechanisms. Low albumin concentrations have been shown to weaken chickens' immune response, increasing vulnerability to infections and disease (Belinskaia *et al*., 2021).

Aminotransferases, which are resident in healthy hepatocytes, serve as reliable markers for detecting liver cell injury (Karadağoğlu *et al*., 2020). Hepatocyte damage causes these aminotransferases to leak into the bloodstream (Khanam *et al*., 2016) where activities above normal ranges of serum ALT and AST activities indicate possible liver injury (Lebednikaite *et al*., 2024).

Diet type and composition significantly affect serum clinical biochemistry of broiler chickens. Dietary components and nutrients impact metabolism, which in turn affects bird productive performance and overall health (Etim *et al*., 2014). It has been demonstrated that fortifying broiler chicken feeds with prebiotics, probiotics, and exogenous digestive enzymes affects their serum clinical biochemistry (FAO, 2016).

Various studies have reported varying results on the impact of different probiotics on serum clinical biochemistry of broiler chickens (Amad *et al*., 2013). In a study where broiler chickens were supplements with the *Lactobacillus paracasei* probiotic strain at 5.8×10^7 cfu/ml under three treatment regiments of 0 mL/day (control), 1 mL/day, 3 mL/day, and 5 mL/day administered through drinking water, the birds' haematocrit increased with an increase in the probiotic dose (Hidiyat *et al*., 2020). However, the observed haematocrit increase did not compromise the chickens' physiological status, as it remained within the normal range for chickens (22–35%) (Horhoruw & Kewilaa, 2024). A lower-than-normal hematocrit concentration is associated with anaemia and compromised tissue oxygenation (Horhoruw & Kewilaa, 2024). Higher-than-normal haematocrit increases blood viscosity, slowing blood flow in capillaries and affecting blood circulation throughout the body (Su *et al*., 2018). Hematocrit and hemoglobin concentrations within normal ranges indicate that the animals are in a physiologically healthy state (Hidiyat *et al*., 2020). In poultry, the heterophil-to-lymphocyte (H/L) ratio, which is not impacted by the intensity and duration of a stressor, is used as a biomarker of heat stress (Kim *et al*., 2021; Oluwagbenga & Fraley, 2023). A reduced H/L ratio

in broiler chickens is desirable since an increased H/L ratio indicates stress (Scanes, 2016). In Ross 308 broiler chickens, whose feed was fortified by a multi-strain probiotic, Protexin, consisting of *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *L. plantarum*, *L. rhamnosus*, *Aspergillus oryzae*, *Bifidobacterium bifidum*, *Candida pintolopesi*, *Enterococcus faecium* at 2 × 10^9 cfu/g and *Streptococcus thermophilus* at 0.10 g/kg of feed, a decreased H/L ratio was observed (Arczewsk-Wlosek *et al*., 2022). This finding suggests that the multi-strain probiotic enhanced the adaptive immune response and reduced stress concentrations in broiler chickens.

The tissue (muscle) lipid profile is associated with meat quality, as it affects broiler chicken meat colour. In Ross 308 broiler chickens fed diets fortified with Protexin Compounder at 0.10 g/kg feed for 42 days, blood triglycerides and total cholesterol decreased, a proxy for the quality of broiler chicken meat (Pourakbari *et al*., 2016). Low cholesterol and triglyceride concentrations in chicken meat are beneficial to human health, as they reduce the risk of cardiovascular disease among consumers (Giampietro-Ganeco *et al*., 2020). Proteins are essential nutrients in an animal's body, where they are used for tissue synthesis and repair, and for growth and productivity (Beski *et al*., 2015). Thus, it is prudent to evaluate the effects of dietary fortification with probiotics on serum protein concentration and profile.

Dietary supplementation with complex probiotic formulations improved serum protein status in broiler chickens by increasing total protein and albumin concentrations. Inclusion of a probiotic blend containing *B. subtilis* (2 × 10^8 cfu/g), *Clostridium butyricum* (2 × 10^6 cfu/g), and *Enterococcus faecalis* (1 × 10^6 cfu/g) at 0.025% and 0.05% enhances protein utilisation, likely through suppression of pathogenic bacteria, increased protein breakdown, and improved nutrient absorption in the gut (Zou *et al*., 2022). In addition, probiotic supplementation also enhances humoral immune responses, as evidenced by increased serum and jejunal concentrations of immunoglobulins (IgA, IgY, and IgM) in Cobb 500 broiler chickens fed diets supplemented with *B. coagulans* (5 × 10^9 cfu/kg) for 42 days (Yu *et al*., 2022). This immune

improvement is likely driven by modulation of the caecal microbiota, thereby improving resistance to infection-related stress in broiler chickens (Yu *et al*., 2022).

In a study in which Ross 308 broiler chickens were fed diets fortified with Lactobacillus culture (0, 0.05, 0.10, and 0.15 mg/g of feed) at 7×10^7 cfu/g, decreased ALT and AST activities were observed in chickens fed the fortified diets (Faith, 2013). ALT and AST are transferases in hepatocytes; thus, a decrease in their plasma activities indicates reduced hepatic parenchymal cell damage, most likely due to the *Lactobacillus* probiotic-induced enhancement of antioxidant activity (Halder *et al*., 2024). An appreciation of the effects of supplements used to fortify feed, including probiotics, on haematology and serum clinical biochemistry is crucial, since derangements in haematological and clinical biochemistry can compromise broiler chickens' health and productivity. In addition to affecting haematological and biochemical parameters, probiotics modulate the poultry gut microbiota.

2.7 Probiotics: impacts on poultry gut microbiota

Poultry health is closely linked to the composition and functionality of the intestinal microbiota. The establishment and maturation of gut microbiota throughout the birds' growth cycle play a vital role in shaping the development of the intestinal lining and regulating key physiological processes that support gut balance and overall homeostasis (Diaz Carrasco *et al*., 2019). Probiotics have been shown to mediate increased proliferation of desirable gut microbiota at the expense of pathogenic species, which translates into increased nutrient digestion and absorption, decreased nutrient losses, and enhanced gut immune function (Wang *et al*., 2021b). In the gut, probiotics reduce the adhesion and colonization of disease-causing microbes and upregulate the immune activity of broiler chickens (Wang *et al*., 2021a).

The probiotic-induced suppression of harmful gut microbes is mediated by the generation of hydrogen peroxide, diacetyl, bacteriocins, and organic acids, which lower intestinal lumen pH

and slow the proliferation of gut-colonizing pathogens (Monika *et al*., 2021). *Lactobacillus salivarus* and *L. crispatus* may inhibit the colonization of pathogenic bacteria by producing hydrogen peroxide (Neveling & Dicks, 2021). *Clostridium butyricum* can produce a bacteriocin that inhibits the growth of *C. difficile* in the GIT (Yang *et al*., 2012). Dietary fortification with Lavipan (*Lactococcus lactis* IBB 500, *Carnobacterium divergens* S-1, *Lactobacillus casei* ŁOCK 0915, and *Saccharomyces cerevisae* ŁOCK 0141), a multi-probiotic, caused a reduction in the invasion of the GIT of commercially reared broiler chicken with *Campylobacter* species (Smialek *et al*., 2018). *C. butyricum* or *E. faecium,* when used to fortify broiler chicken diets, have been shown to inhibit the proliferation of pathogenic *E. coli* and *C. perfringes* (Huang *et al*., 2019b), yet promote the growth of the beneficial *Lactobacillus* and *Bifidobacterium* species (Wang *et al*., 2019), thus regulating the composition and proportion of broiler chicken caecal microflora. Accordingly, it is evident that probiotics positively mediate the balance of the gut microbiota, thereby improving gut health in poultry and broiler chickens. Although the gut microbiota plays a key role in gut health, probiotics can further enhance intestinal structure and function, underscoring the importance of examining their effects on gut morphology.

2.8 Probiotics: impacts on poultry gut histomorphology

Gut histomorphology refers to the tissue structure and arrangement within the gastrointestinal tract: it encompasses villus height, crypt depth, and the presence of goblet cells (Vatsos, 2021). This structural makeup is essential for effective feed digestion, nutrient uptake, and the overall well-being of chickens (Orr *et al*., 2021). Use of probiotics in broiler chicken feed has been shown to modulate gut histomorphological indices of the birds (Azizi *et al*., 2021; Xu *et al*., 2025; Yosi & Metzler-Zebeli, 2023). Probiotics have also been demonstrated to improve villus height and the villus height to crypt depth ratio in both non-pathogen-exposed and pathogen-exposed animals (Azizi *et al*., 2021; Xu *et al*., 2025; Yosi & Metzler-Zebeli, 2023). This was

attributed to probiotics enhancing villus height by stimulating mitotic cell division and promoting epithelial cell growth. An elevated villus length correlates well with improved digestion and nutrient absorption in the small intestine, as well as enhanced feed utilization and growth promotion (Jha *et al*., 2020).

Al-Baadani *et al*. (2016) found that probiotics had the greatest impact on crypt depth in the ileum and jejunum, especially under pathogenic stress. A reduction in crypt depth correlates with slower cell turnover, implying that probiotics protect epithelial cells from damage by invading pathogens. These findings and explanations were corroborated by a study by Awad *et al*. (2009), in which 1-day-old Ross 308 chicks were fed starter and grower feeds containing 1 kg of a probiotic Lactobacillus sp. product at a concentration of 1×10^8 cfu/kg. After a 5-week trial, a reduction in crypt depth was observed. Overall, the administration of probiotics offers a promising strategy for improving gut health by modulating gut barrier integrity, balancing the gut microbiota, and minimizing intestinal permeability-related challenges in birds through strengthened immune responses (Naeem & Bourassa, 2025). To achieve effective probiotic fortification in broiler diets, it is essential to understand the specific probiotic strains used; therefore, identifying and characterising *Bacillus* probiotic candidates is important.

2.9 *Bacillus* probiotics candidates: identification and characteristics

2.9.1 Overview and attributes of Bacillus probiotics

Bacillus strains represent a diverse group of beneficial bacteria that have gained considerable attention for their potential applications in agriculture and animal husbandry. These strains are gram-positive, spore-forming, catalase-positive, aerobic, and facultative anaerobic bacteria (Abdel-Moneim *et al*., 2020; Luise *et al*., 2022). The genus *Bacillus* is closely related to *Lactobacillus* species, which are well-known probiotics, and they belong to the same class, *Bacilli*, under the Firmicutes phylum. Luise *et al*. (2022) report a total of 2 552 recognized

Bacillus species. *Bacillus subtilis*, *Bacillus licheniformis*, *B. coagulans*, *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus cereus* are usually found in soil and are extensively used in the diets of simple non-ruminants. These *Bacillus* probiotic species have demonstrated various beneficial properties, including the production of antimicrobial molecules and enzymes (Mazanko *et al*., 2022).

Additionally, Bacillus species form spores, which enhance survival in adverse environments and account for their tolerance to heat and longer shelf life compared to other probiotics, such as *Lactobacillus* (Abdel-Moneim *et al*., 2020). They produce a variety of bioactive compounds, among many, antimicrobial peptides, enzymes, organic acids, and antioxidants, which contribute to their beneficial effects (Bilal *et al*., 2021). They have also been reported to potentially affect broiler chicken growth performance, haematological and clinical biochemistry, gut health, and meat quality (Alkhalif *et al*., 2010). The primary benefits of using *Bacillus*-based probiotics lie in their resilience, as their spores can be generated at high densities (Mazanko *et al*., 2022), heat tolerance during feed processing, and an ability to maintain viability when incorporated into probiotic formulations (Ogbuewu *et al*., 2022). Spore-forming probiotics can enhance the health and productivity of poultry by inhibiting the proliferation of disease-causing Salmonella in the gut (Liu *et al*., 2021b; Ye *et al*., 2020; Rivera-Perez *et al*., 2021; Mazanko *et al*., 2022). However, their effectiveness depends on environmental conditions, particularly those in which chickens are reared (Popova *et al*., 2024). By inhibiting the proliferation of disease-causing gut bacteria, these spore-forming probiotics promote enhanced average daily weight gain, FCR, and meat and egg quality.

Maintaining viability over a long period prolongs the shelf life of *Bacillus*-containing feed products (Ramlucken *et al*., 2020), a feature that helps feed manufacturers minimize feed losses and enables poultry farmers to store sufficient feed for their flock without compromising the probiotic viability. *Bacillus* probiotics can survive harsh conditions in the GIT, including low

pH and bile salts (Ogbuewu *et al*., 2022). Their notable survival rate across various sections of animals' GITs amplifies their effectiveness (Popov *et al*., 2024). They also have a rapid rate of growth, which enables them to competitively eliminate pathogenic gut-resident microorganisms (Jha *et al*., 2020), thus reducing the risk of diseases. Moreover, they modify the intestinal ecosystem in poultry by producing a wide range of digestive enzymes, which help with the digestion of complex feedstuffs given to chickens (Ramlucken *et al*., 2020).

Additionally, *Bacillus* probiotics are inexpensive to produce as their downstream processing is affordable, and they can be easily manufactured through conventional fermentation processes (Cutting, 2011). Probiotics from *Bacillus* strains vary in their effectiveness at enhancing broiler chickens' growth performance through a variety of mechanisms, including inhibiting pathogens, altering the microbial environment of the GIT, influencing host metabolism, and improving immune responses (Bilal *et al*., 2021). They enhance the immune system by stimulating the production of antimicrobial molecules and increasing the concentrations of secreted immunoglobulins, especially IgA, which protects the mucosal surfaces of the respiratory and digestive tracts, thereby further aiding in disease prevention (Ogbuewu *et al*., 2022). Additionally, *Bacillus* probiotics have been shown to lower serum cholesterol concentrations and prevent intestinal disorders, such as diarrhoea and lactose intolerance, thus contributing to overall health benefits (Latif *et al*., 2023).

2.9.2 Combination and dosage of Bacillus probiotic strains

A variety of probiotic strains have been extensively used, with studies reporting varying effects on broiler chicken production outcomes. Most studies have shown positive outcomes when probiotics are used as feed additives, while some have found no impact or reported negative results (Yirga, 2015; Ramlucken *et al*. 2020; Mousapoor *et al*., 2023; Mârza *et al*., 2025). These discrepancies may be due to several factors that impact probiotic efficacy, including, among

many, type and strain of bacteria, survival and stability after feed processing, method route of administration, appropriateness of dosa, the bird's diet and age as well as biosecurity measures and environmental stress (El-Jeni *et al*., 2021).

Probiotics can be mixed with other feed additives or other probiotic strains (Krysiak *et al*., 2021). It is widely recognized that combining multiple probiotic strains can yield synergistic benefits (Horyanto *et al*., 2024). However, some scholars report that combining probiotic strains does not guarantee improved effects on tested health and productive indices of broiler chickens (Lambo *et al*., 2021; McFarland, 2021). For instance, a study by Buiatte *et al*. (2023) evaluating the effects of four strains of probiotics (*B*. *subtilis, B*. *licheniformis, B*. *coagulans,* and *B*. *pumilus*) against *C*. *perfringens in vitro* showed that the probiotic mixture (2.5×10^5 cfu/ml) did not offer anti-clostridial activity for combating the growth of *C*. *perfringens*. However, when *B*. *subtilis* was applied individually, it was more effective *in vitro* at controlling *C. perfringens* than other strains. They further explained that this could be due to *B. subtilis*'s unique ability to produce the bacteriocin mersacidin, which suppresses the growth of other bacterial species, including *C*. *perfringens*. These bacteriocins form pores in the bacterial cell membrane, inhibiting cell wall formation and ultimately leading to cell death. However, the production of bacteriocins by *B*. *subtilis* may have been suppressed in the presence of other *Bacillus* strains due to competitive inhibition, potentially disrupting metabolic pathways involved in bacteriocin synthesis (Buiatte *et al*., 2023).

Another study, conducted by Ramlucken *et al*. (2020), used a 6-strain probiotic mixture containing 4 strains of *B. subtilis* (HP 1.6, D 014, CPB 011, and CPB 029) and 2 strains of *Bacillus velezensis* (CBP 020 and CPB 035), on Ross 308 broiler chickens. These chickens were challenged with *C. perfringens,* and their mash feed was supplemented with a *Bacillus*

probiotic mixture at 50 g or 100 g per ton of feed. In contrast to the studies reviewed above, they concluded that a *Bacillus* probiotic mixture improved growth performance and gut and liver health, indicating positive results in broiler chickens challenged with *C. perfringens*.

Another cause for discrepancies in study results is varying effective doses of *Bacillus* probiotics used in broiler chickens (Mousapoor *et al*., 2023). Ogbuewu *et al*. (2022) stated that the effective dose of *Bacillus* probiotics ranges from 1.0×10^6 - 8.0×10^7 cfu/g feed. Musa *et al*. (2019) examined the effects of *B*. *subtilis* B21 and *B*. *licheniformis* at a higher dose of 2×10^9 cfu/g on the performance and intestinal health of broiler chickens challenged with *C. perfringens*-induced necrotic enteritis. They reported that the probiotic groups produced higher levels of SCFAs and showed improved intestinal morphology compared with the control groups. The study suggests that both *B*. *subtilis* and *B*. *licheniformis* probiotics have effects similar to those of AGP in mitigating necrotic enteritis in birds. Abudabos *et al*. (2020) found that *Salmonella* infection significantly impaired performance; however, the addition of *B*. *subtilis* (2×10^7 cfu/g) improved FI, body weight gain, and intestinal health more effectively than *B*. *licheniformis* (1.2×10^6 cfu/g). *B*. *subtilis* was found to mitigate the negative effects of *Salmonella* infection, making it a superior treatment for enhancing growth and intestinal health in broiler chickens.

Liu *et al*. (2021b) investigated the effects of *B*. *subtilis* PB6 on broiler chickens challenged with *C*. *perfringens*, the main cause of necrotic enteritis. The probiotic strains were supplemented in feed at two dosage levels: low (4×10^7 cfu/kg) and high (6×10^7 cfu/kg). While PB6 in high dosage did not improve growth during the pre-challenge phase, it enhanced growth performance during the *C*. *perfringens* challenge phase, reduced intestinal lesions, increased tight junction gene expression, and decreased inflammation (Liu *et al*. 2021b).

Additionally, PB6 restored gut microbial balance, suggesting its potential in preventing *C. perfringens*-induced necrotic enteritis. The study also suggested that increasing the dosage improves the effectiveness of *Bacillus* probiotic strains. However, findings from the stated studies reported contradictory outcomes when different *Bacillus* probiotic species were administered at different doses. Most studies focused on the efficacy of *B. subtilis* and *B. licheniformis* strains in disease-challenged broiler chickens, primarily targeting necrotic enteritis and *Salmonella.* There are scarce studies that have been conducted to evaluate the dose-response of a four-strain *Bacillus* probiotic mixture on gut function and related parameters in broiler chickens reared under densely stocked conditions; hence, this study investigated how their supplementation affects meat quality in Ross 308 broiler chickens.

2.10 Meat quality

2.10.1 Broiler chicken meat quality: key indicators

Meat quality encompasses the fundamental characteristics of meat that determine its suitability for consumption, processing, storage, and presentation in retail settings (Anderson, 2005). As with other meats, appearance and texture are key indicators of poultry meat quality that significantly impact consumer choice and satisfaction (Fletcher, 2002). The appearance of meat as a quality determinant encompasses meat colour and the presence of defects, including bruises and haemorrhages, which ultimately influence consumer selection and overall satisfaction (Nusairat *et al*., 2022). The measurable characteristics of meat quality, including its ability to retain water, resistance to shearing, loss during cooking and dripping, acidity, longevity, collagen concentrations, protein solubility, cohesion, and ability to bind fat, are crucial for manufacturers producing value-added meat products (Toma *et al*., 2024). The colour of chicken meat is important for consumers' perceptions of meat freshness and quality (Popova, 2017). Meat colour is determined by measuring its redness (a^*), yellowness (b^*), and lightness

(*L**), and consumers prefer meat with a more pronounced redness but with lower b* as the latter is indicative of less pale meat (Jiang *et al*., 2014; Popova, 2017).

The pH significantly impacts meat tenderness, water retention, colour, juiciness, and shelf life (Mir *et al*., 2017). Broiler chicken breast meat with elevated pH exhibits a greater water-binding capacity than meat with lower pH (Mir *et al*., 2017). Broiler chicken muscles with a pH of 6.0 or higher exhibit minimal protein denaturation, low light scattering, and a translucent appearance (Mir *et al*., 2017). At pH ≤ 6.0, they undergo greater protein denaturation, leading to increased light scattering and reduced transparency (Vilian, 2019). Overall, good-quality broiler chicken meat is a function of its appearance, organoleptic attributes, and nutritional value, which can be affected by several factors, including diet, gut microbiota, and probiotics.

2.10.2 Diet and gut microbiota: effects on meat quality

The nutrient composition and fatty acid profiles of feed directly affect meat texture, flavour, and nutritional value (de Oliveira *et al*., 2023). Some diets contain high concentrations of unsaturated fatty acids whose metabolism, once absorbed, results in excessive generation of reactive oxygen species and oxidative stress (Lund *et al*., 2011), compromising meat quality. It is therefore important that the diet formulation targets mitigating potential diet-induced oxidative stress in the birds. Using feeding technologies, for example, fortifying broiler chicken diets with essential oils with antioxidant activity has been shown to modulate gut microbiota composition, with concomitant reductions in diet-induced oxidative stress and improved meat quality (Chen *et al*., 2022). The relationship between gut microbiota and diet significantly influences meat quality parameters, as the diet is digested in the stomach with the help of gut microbiota, ultimately affecting the meat quality of the product. Therefore, feeding broiler chickens a nutritionally balanced diet that promotes the proliferation of desirable gut microbiota is central to producing high-quality meat. It has been shown that fortifying broiler

chickens' diet with magnolol promoted the development of a balanced gut microbiota, characterised by increased caecal Faecalibacterium and reduced Coprobacillus, resulting in meat of high quality (Xie *et al*., 2022). Increased caecal *Faecalibacterium* population positively impacts gut health by increasing SCFA production, which provides energy to intestinal cells and helps maintain gut barrier function (Salahi *et al*., 2024).

2.10.3 Probiotics: effects on meat quality

Evidence shows that supplementing broiler chickens with probiotics enhances the pH, colour, water retention, fatty acid profile, and resistance to oxidation of their meat (Dong *et al*., 2024). The breast meat of broiler chickens supplemented with *E. faecium* probiotic had lower drip loss and cooking loss at 45 minutes and 24 hours post-slaughter (Zheng *et al*., 2014). Low post-slaughter drip loss contributes to maintaining meat tenderness and juiciness and prolongs shelf life. Furthermore, it has been shown that fortifying broiler chicken diets with probiotics containing *B. licheniformis* enhanced fresh meat colour, flavour, and juiciness (Popova, 2017) and the inclusion of probiotics (*Lactobacillus fermentum*) in the drinking water of broiler chickens significantly improved chicken breast meat redness but with no effect on both breast and thigh meat yellowness and lightness (Sobezak *et al*., 2018). Interestingly, supplemental probiotics impact meat chemical composition, as demonstrated by increased protein, essential amino acid, and flavour content, and a reduction in fat content in broiler chicken meat of birds fed a diet supplemented with *B. licheniformis* (Liu *et al*., 2012). Other studies have also found similar results, with low-fat and high-protein content in broiler chicken breast meat (Hossain *et al*., 2012; Kim *et al*., 2016). Even when added to drinking water for broiler chickens, lactic acid bacteria-based probiotics have been shown to lower the cholesterol content of broiler chicken meat (Popova, 2017). Two mechanisms explain the lower broiler chicken meat cholesterol content: absorption into the growing bacterial and attachment of the cholesterol to

the surface of the probiotic microorganism; processes that restrict cholesterol uptake and its accretion in muscle tissue (Ooi & Liong, 2010; Kumar *et al*., 2012; Khare & Gaur, 2020). Broiler chicken meat with a low cholesterol content is considered a healthy product, as it reduces consumers' risk of developing cardiovascular disease (Abdurrahman *et al*., 2016; Popova, 2017). Furthermore, the use of probiotics as a feeding technology has been shown to increase the desirable polyunsaturated fatty acid content at the expense of saturated fatty acids in broiler chicken meat (Kwiecień *et al*., 2021), thereby enhancing its nutritional value. In-feed or drinking water probiotics have been shown to increase the shelf life of broiler chicken meat by reducing lipid oxidation (Bai *et al*., 2016). In the aforesaid discourse, there is ample evidence that the use of probiotics as a feeding technology in broiler chicken production positively impacts the birds' productive performance, gut and overall health, and meat quality.

2.11 Summary, research gaps, and future directions

Poultry meat is in high demand due to its relative affordability compared to other meats. This prompts producers to strive to increase production to meet the growing demand and earn profits from increased sales volumes. To mitigate high broiler chicken production costs, producers frequently increase stocking density to produce more broiler chicken meat per unit area. Although higher stocking density can increase profits per unit area, it often reduces productivity and performance in chickens due to stress-induced by high stocking density. Following the ban on the use of AGP to mitigate stress in densely stocked broiler production, probiotics have gained attention as eco-friendly alternatives. *Bacillus* probiotics have attracted researchers' interest due to their spore-forming ability, which enables them to survive during feed processing and the harsh conditions of the GIT. However, studies have reported contradictory results regarding the effects of Bacillus probiotics, used as single strains or cocktails, in broiler chicken production. The contradictory outcomes observed with probiotic

use could be due to differences in probiotic doses, chicken rearing environment, and potential incompatibility of *Bacillus* probiotic strains.

As discussed in this literature review, while probiotics such as *B. subtilis* individually suppressed the growth of *C. perfringens*, no inhibition occurred when multiple *Bacillus* strains were combined. Therefore, thorough research is required to determine the compatibility of probiotic strains to avoid the risk of them suppressing each other's effects. The dose of *Bacillus* probiotics, whether used individually or in combination, varies among reported studies, leading to confusion among producers regarding the optimal amount needed for optimal efficacy. Therefore, further investigations are needed to determine the efficacious doses of *Bacillus* probiotics required for specific situations, for example, in high-density broiler chicken rearing. Furthermore, limited research has investigated the effects of incrementally dosed four-strain *Bacillus* probiotic mixtures in densely stocked broiler chickens. Therefore, this study sought to evaluate the effect of varying doses of a four-strain *Bacillus* probiotic on gut function and associated parameters in densely stocked Ross 308 broiler chickens.

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3 CHAPTER 3: EEFECTS OF INCREMENTAL DOSES OF A NOVEL *BACILLUS SUBTILIS* PROBIOTIC MIXTURE ON NUTRIENT DIGESTIBILITY, GROWTH PERFORMANCE, HAEMATO-BIOCHEMISTRY, AND CARCASS AND MEAT QUALITY

3.1 Abstract

Compared to other meats, broiler chicken is relatively cheaper, and as such, its demand is increasing. This increase in demand has prompted the search for alternative nutritional strategies to replace routine antibiotic use, fortifying broiler chicken feed to mitigate the public health challenge of antibiotic resistance and the negative effects of AGP on consumer and environmental health. *Bacillus subtilis* probiotic strains are emerging as promising natural feed additives in poultry nutrition. However, optimal strain combinations and doses require further investigation; thus, this study investigated the optimal dose of a four-strain *B*. *subtilis* probiotic in broiler chickens. Nine hundred one-day-old Ross 308 chicks were randomly assigned to six dietary treatments, with each treatment replicated six times in a completely randomized design. The dietary treatments, provided from the starter through to the finisher phase, comprised: **1.** a basal broiler diet without AGP or *B*. *subtilis* probiotic mixture (PRB0); **2.** the basal diet supplemented with 0.5% (w/w) AGP (POSC); and four versions of the basal diet supplemented with *B*. *subtilis* probiotic mixture at 0.3 (PRB3), 0.4 (PRB4), 0.5 (PRB5), or 0.6% (PRB6). Growth performance, nutrient digestibility, haemato-clinical biochemistry, viscera morphometry, and carcass and meat quality were determined. The *B*. *subtilis* probiotic blend at 0.5% (w/w) improved ($p < 0.05$) body weight gain and feed efficiency, with a positive quadratic effect on feed intake. Compared with controls, diets supplemented with a *B. subtilis* probiotic mixture significantly improved ($p < 0.05$) organic matter, neutral detergent fibre, and gross energy digestibility, but exhibited a negative quadratic effect on crude protein

digestibility. Significant differences ($p < 0.05$) in haematological and biochemical parameters were observed: platelet counts decreased, neutrophil percentage increased, and white blood cell counts indicated reduced stress and inflammation in broiler chickens fed the dietary treatments PRB3, PRB4, and PRB5. Increased WHC, decreased shear force, DL, and CL observed in the meat from birds fed PRB4, PRB5, and POSC indicate improved meat quality. These study findings suggest that fortifying Ross 308 broiler chicken diets with a *Bacillus* probiotic cocktail enhances nutrient digestibility, thereby improving growth performance. These probiotic-mediated effects are likely due to enhanced gut health, increased digestive enzyme production and nutrient absorption, and attenuated oxidative stress. In conclusion, the *B. subtilis* probiotic mixture at a 0.5% dose produced the best outcomes in broiler chicken performance, health, and meat yield and quality compared with unfortified control diets.

Keywords: Antibiotic growth promoters, *Bacillus subtilis*, growth performance, haemato-clinical biochemistry, meat quality

3.2 Introduction

Broiler chicken farming plays a vital role in the global food supply by providing a relatively affordable animal-source protein for human consumption, thereby significantly contributing to food and nutrition security (AgriSETA, 2023; Council, 2023). Due to their rapid growth and lower per-unit production cost, broiler chickens have gained popularity as an alternative to beef and pork (Nkukwana, 2018). As the global human population is projected to reach 9.6 billion by 2050, demand for broiler chicken meat is expected to increase by 70% (Wickramasuriya *et al*., 2022), making the future of broiler farming a crucial topic for research and innovation (Acheampong, 2024). The increase in global demand for broiler chicken meat prompts farmers to adopt intensive broiler production methods to maximize output and profit per unit area and meet growing meat demand (Wickramasuriya *et al*., 2022). However, intensive broiler

production systems are associated with high disease risks that must be mitigated to achieve optimal production. Traditionally, AGP are used to reduce disease risks, enhance immunity, and improve growth performance, particularly in densely stocked systems (Gadde *et al*., 2017) but their prolonged and routine use spawns antimicrobial resistance and deposition of antibiotic residues in broiler chicken meat and chicken waste that compromise human health and pollute the environment, respectively (Salim & Khalleduzzaman, 2021; Ahmad *et al*., 2022). These concerns have led to restrictions or bans on AGP in many parts of the world (Huyghebaert *et al*., 2011). Nonetheless, without AGP, the high incidence of diseases in densely stocked broiler chicken production systems compromises productive performance, health, and welfare of the birds (Cervantes, 2015; Abd El-Hack *et al*., 2022). Additionally, consumer awareness and demand for antibiotic-free, naturally produced poultry products that support better health outcomes are increasing (Mohammadi *et al*., 2023). As a result, more natural alternatives to synthetic antibiotics, such as probiotics, need to be explored as feeding technologies in broiler chicken production.

Probiotics are direct-fed microorganisms that provide health benefits to the host when administered in adequate amounts (Onyenweaku *et al*., 2016). In poultry production, use of probiotics as a feeding technology has been shown to improve feed intake and digestive efficiency (Ye *et al*., 2021) by increasing the synthesis and release of the endogenous digestive enzymes (amylase, protease, and lipase) in that gut resulting in increased hydrolysis of macromolecular feed nutrients to absorbable monomeric nutrients (Amenyogbe *et al*., 2024). Furthermore, in-feed probiotics suppress the growth of pathogenic gut bacteria with concomitant mitigation of gut inflammation, and they promote proliferation of desirable microbiota, including *Lactobacillus* and *Bifidobacterium* (Chandrasekekaran *et al*., 2024); processes that result in stronger intestinal mucosa whose enhances integrity and guards against transfer of gut luminal agents from crossing into the bloodstream (Latif *et al*., 2023). Due to

their modulation of the immune system, probiotics enhance the birds' immune response to infectious and physical stressors, such as high stocking density (Ayana & Kamutambuko, 2024). The probiotics induced increased endogenous enzyme production and secretion, enhanced intestinal integrity, promoted the growth of desirable gut microbiota, and increased immune system response, thereby improving bird health and welfare, increasing growth performance, and enhancing end-product quality. It has been reported that probiotics improve broiler chicken meat quality by mediating reduced accumulation of toxic pathogenic metabolites, promoting the deposition of desirable, health-beneficial fatty acids, and prolonging the meat's shelf life (Abdulla *et al*., 2018; Dong *et al*., 2024). *Bacillus* probiotic strains, whether used singly or in combination, have been tested for their effects on broiler chickens' nutrient digestion and absorption, growth performance, haemato-clinical biochemistry, and meat quality in birds reared under recommended stocking densities and have provided important insights on their bird productive performance, health, and welfare and meat quality (Tijani *et al*., 2015). However, there is a dearth of information regarding the effects of in-feed *Bacillus* probiotics as dietary supplements on the performance, health and meat quality from broiler chickens reared under stress-inducing high stocking densities hence the current study evaluated, in densely stocked Ross 308 broiler chickens, the effects of a four-strain *Bacillus* probiotic mixture on growth performance, nutrient digestibility, haemato-clinical biochemistry and meat quality. The study hypothesized that replacing zinc bacitracin as a broiler chicken feed dietary supplement with a *B. subtilis* probiotic mixture would maintain nutrient digestibility and absorption, growth performance, haematological and biochemical parameters, and meat quality in Ross 308 broiler chickens reared under high stocking density.

3.3 Materials and methods

3.3.1 Study site and research inputs

The six-week feeding trial was conducted at Rooigrond Farm, at GPS coordinates (25º55′19″ S, 25º48′07" E) situated in the North-West province of South Africa. The trial was conducted during February and April, during which local ambient temperature ranged from 10 to 36 ºC. Rainfall at the study site ranges from 300 to 600 mm per annum, with most falling during the summer months (September - March).

One-day-old Ross 308 broiler chicken chicks were purchased from Chicken Ranch of Centurion, Gauteng Province, South Africa. The four-strain novel *Bacillus* probiotic mixture (CPB 003, CPB 029, HP 1.6, and D014), in equal proportions, was supplied by the Council for Scientific and Industrial Research and OptimusBio, South Africa. Feed ingredients were purchased from Nutroteq Pty (Ltd) and SimpleGrow Agric Service Pty (Ltd), both in South Africa.

3.3.2 Experimental design and feed formulation

A total of 900, one-day-old male Ross 308 broiler chicken chicks were evenly and randomly distributed into 36 replicate pens. Subsequently, six dietary treatments, each replicated six times, were randomly assigned to the pens. While the recommended stocking density for broiler chicken is 15 birds/m² (SAPA, 2012), this study employed a higher density of 13 birds per 0.75 m² (~17.3 birds/m²) to evaluate the probiotic mixture's potential to mitigate stocking density-induced stress. Each experimental diet was prepared for starter (0 – 14 days), grower (15 – 35 days), and finisher (36 – 42 days) phases. The experimental diets comprised: **1**. a basal broiler diet without zinc bacitracin an AGP or *B*. *subtilis* probiotic mixture (PRB0); **2.** the basal diet supplemented with 0.5% (w/w) zinc bacitracin (POSC); and four versions of the basal diet

supplemented with *B. subtilis* probiotic mixture at 0.3 (PRB3), 0.4 (PRB4), 0.5 (PRB5), or 0.6% (PRB6).

Table 3.1: Gross ingredient and nutrient composition (g/kg) of dietary treatments.

Ingredients	Starter (0-14 d)	Grower (15-28 d)	Finisher (29-42 d)
Yellow Corn	44.03	48.12	54.37
Soybean meal (46% CP)	41.71	37.94	32.67
L-Lysine HCl (99% CP)	0.21	0.14	0.17
DL-Methionine (99% CP)	0.42	0.45	0.35
L-Threonine (98% CP)	0.12	0.09	0.08
Limestone fine	1.4	1.16	0.87
Monocalcium phosphate	2.17	1.77	1.47
Salt fine	0.24	0.26	0.25
Sodium bicarbonate	0.28	0.25	0.27
Poultry vitamin and minerals premix	0.5	0.5	0.5
Soy oil	8.42	8.82	8.5
Calculated nutritional composition (%)			
Crude Protein	23	21.5	19.5
Crude fat	10.78	11.23	11.01
Calcium	0.95	0.8	0.65
Available Phosphorus	0.50	0.42	0.36
AME Poultry	2 975	3050	3100
SID Methionine	0.71	0.72	0.76
SID Lysine	1.32	1.18	1.08
SID Tryptophan	0.26	0.24	0.21
SID Threonine	0.88	0.79	0.72
SID Isoleucine	0.87	0.81	0.73
SID Histidine	0.54	0.5	0.46
SID Valine	0.93	0.87	0.79
SID Leucine	1.62	1.54	1.43
SID Arginine	1.42	1.32	1.17
Linoleic acid	5.58	5.81	5.68
Sodium	0.18	0.18	0.18
Potassium	1.02	0.95	0.86
Chloride	0.23	0.23	0.23
SID Met + Cys	1	0.92	0.86

AME, apparent metabolized energy; CP, crude protein SID; HCl, hydrochloric acid; standard ileal digestibility; ** Vitamin and mineral premix: copper sulphate 8.0 mg; zinc sulphate 79 mg; ferrous sulphate 80 mg; niacin 30 mg; magnesium sulphate 100 mg; vitamin A 11,0 0 0 IU; potassium iodide 0.34 mg; pantothenic acid 10 mg; folic acid 0.7 mg; biotin 0.12 g; vitamin B6 5.1 mg; vitamin B1 2.5 mg; vitamin B2 4.5 mg; vitamin D3 2,500 IU; vitamin E 25 IU; vitamin K3 2.0 mg; and sodium selenite, 0.25 mg.

3.3.3 Measurement of growth performance

At day 0, chicks were weighed using an electronic weighing scale (Richter scale model 330 weighing, Pretoria, South Africa), and thereafter the birds were weighed once weekly to calculate body weight gain (BWG). The feed provided was weighed before feeding, and refusals were collected prior to the next feeding and weighed to determine average feed intake (AFI) by difference. Mortalities were recorded as they occurred, and the carcasses were weighed to adjust the feed conversion ratio (FCR). Computations for AFI, BWG, and FCR were done according to the respective equations by Omoor *et al*. (2024) as follows:

$$AFI = \frac{Feed\ offered - Feed\ refusals}{No.of\ animals}$$

$$BWG = \frac{Final\ weight - Initial\ weight}{No.of\ animals}$$

$$FCR = \frac{Feed\ intake}{Body\ weight\ gain}$$

3.3.4 Determination of nutrient digestibility

To assess nutrient digestibility, titanium dioxide (TiO_2), an indigestible marker, was added to the dietary treatment at 5 g/kg inclusion (w/w) as described by Sort *et al*. (1996) with minor modifications. In summary, nutrient digestibility was computed by comparing the TiO_2 concentration in the diet and in ileal digesta. After slaughter, ileal digesta was collected by gently flushing out the intestinal contents from the ileum into clean, labelled graduated Falcon tubes for each replicate pen. The samples from the same pen were pooled and immediately frozen at -20°C until they were oven-dried at 55°C overnight. Subsequently, the feed and ileal

samples were oven-dried at 135℃ to determine dry matter, and then combusted in a furnace at 550℃ for 16 hours to determine ash. A stock solution was made by dissolving 0.05 g of TiO_2 and 15 g of anhydrous sodium sulphate (Na_2SO_4) in 50 ml of 97% concentrated H_2SO_4. The solution was heated to 100 ºC for 15 mins, then cooled. After cooling, it was mixed with 200 mL of distilled water and filled up to the mark in a 500 mL volumetric flask. Calibration standards were prepared by pipetting increasing volumes of this solution into 5 ml Falcon graduated tubes, followed by the addition of concentrated H_2SO_4 and 400 µL of 30% hydrogen peroxide (H_2O_2). Feed ash (0.3 g) and ileal digesta ash (0.09 g) were weighed and digested in an acid containing 15 ml H_2SO_4 (5%) and 2.4 g anhydrous Na_2SO_4 in test tubes and placed on a heating block for 20 hours. After cooling, the digested samples were transferred into a 100 ml volumetric flask, and deionized water was added up to the mark. After settling overnight, the colour response of standards and samples was measured at 408 nm using a spectrophotometer (V-730, Japan Spectroscopic Company (JASCO), Tokyo, Japan). The concentration of TiO_2 in feed and digesta was calculated using the calibration standard curve. Nutrient digestibility (ND) percentage was calculated based on the formula by Faryad *et al*. (2021) as follows:

$$ND\ (\%) = 1 - [(TiO_2 \text{ in diet} / TiO_2 \text{ in digesta}) \times (\text{Nutrient in digesta} / \text{Nutrient in diet})] \times 100$$

3.3.5 Determination of haemato-clinical biochemistry

Two days before the feeding trial termination, blood was collected from two birds randomly selected per experimental unit. The blood samples were used to determine white blood cells (neutrophils, lymphocytes, monocytes, and eosinophils), red blood cells, haemoglobin, platelets, and platelet distribution width. Blood samples were collected via branchial vein puncture using a sterilised vein needle. The collected blood was then transferred into EDTA-coated blood collection tubes. An automated IDEXX LaserCyte Haematology Analyser

(IDEXX Laboratories (Pty) Ltd., Gauteng, South Africa) was then used to determine haematological indices following the manufacturer's instructions. Serum cholesterol, albumin, globulin and total protein, glucose, calcium, phosphate and total bilirubin concentrations, serum alanine aminotransferase, alkaline phosphatase and gamma-glutamyl transferase activities (markers of liver function) as well as serum amylase and lipase activities (markers of pancreatic damage) were determined using an automated IDEXX Catalyst One Chemistry Analyser (IDEXX Laboratories (Pty) Ltd., Gauteng, South Africa) following the manufacturer's instructions.

3.3.6 Carcass traits and viscera morphometry

3.3.7 Carcass traits and viscera morphometry

Twelve hours prior to day 42 of age, the chickens were fasted, placed in crates, and transported to an on-site abattoir at Rooigrond Farm. At the abattoir, the chickens were euthanized, electrically stunned, followed by severing of the jugular veins to allow complete bleeding. After bleeding, the carcasses were scalded using a scalding machine and then plucked with a mechanical feather plucker. Hot carcass weight was determined, and breast and thigh muscle pH of the carcasses was then measured using a digital pH meter with a spear-piercing electrode (HI98163 Professional Portable pH-temperature meter, Hanna instruments (Pty) Ltd, JHB, South Africa). The carcasses were then packaged and refrigerated at 4ºC. Twenty-four hours post-slaughter, after refrigeration, drumstick, thigh, breast, and wing weights were measured using an electronic balance (CPWplus 75, Adam Equipment, Milton Keynes, United Kingdom). Viscera (proventriculus, gizzards, spleen, liver, pancreas, and intestines with their contents) that were extracted at carcass dressing were also weighed. Carcass cuts and viscera weights expressed in g/100 g cold carcass weight (CCW).

3.3.8 Determination of physical meat quality attributes

3.3.8.1 Meat temperature, pH, and colour

Measurements of meat pH and temperature were taken at 1 and 24 hours post-slaughter in the central portion of the breast and thigh muscles using a calibrated combination pH-temperature probe. The colour of the chicken meat was measured on the surface of the meat samples (breast and thigh) for redness (a^*), yellowness (b^*), and lightness (L^*) using a calibrated Minolta colorimeter .

3.3.8.2 Drip loss

For each meat sample collected, weights were taken before and after refrigeration to calculate the amount of water lost during storage. The meat samples (5 – 10 g) were secured with a thin string, allowing them to hang vertically along the muscle fibres in a 250 ml laboratory blue-top bottle. The hanging was such that each meat sample did not come into contact with the bottle's walls. The samples were then stored in a fridge at 4°C for 24 hours. After 24 hours, the samples were weighed again, and drip loss was calculated and expressed as a percentage, following the procedure reported by Xiong *et al*. (2022).

$$Drip\ loss\ (\%) = \frac{Weight\ before\ drip - Weight\ after\ drip}{Weight\ before\ drip} \times 100$$

3.3.8.3 Cooking loss

Cooking loss was determined following a procedure described by Sari *et al*. (2021) with minor modifications. Briefly, 30–40 g breast meat samples were placed in a plastic cooking bag and then steam-cooked in a water bath at 80 °C for 60 minutes. Thereafter, the cooked samples were allowed to cool to room temperature and reweighed. Percent cooking loss was calculated using the following formula:

$$Cooking\ loss\ (\%) = \frac{Weight\ before\ cooking - Weight\ after\ cooking}{Weight\ before\ cooking} \times 100$$

3.3.8.4 Water holding capacity

The meat's water-holding capacity (WHC) was determined using the filter paper press method as described by Grau & Hamm (1953), with minor modifications. After 24 hours post-slaughter

$$WHC = 100 - \left[\left(\frac{Weight\ before\ pressing - Weight\ after\ pressig}{Weight\ before\ pressing}\right) \times 100\right]$$

3.3.8.5 Tenderness

Chicken breast samples were tested for meat tenderness by measuring shear force after refrigeration; the samples were cut into cubes (5–10 g). Each meat cube was carefully placed between layers of filter paper and compressed with a 60 kg weight for 5 minutes, followed by weighing the samples after compression. The formula used to calculate WHC was as follows: using a Texture Analyzer (TA-XT plus, Stable Micro Systems, Surrey, UK). Each breast muscle sample was placed perpendicularly on a Meullenet-Owens Razor Shear Blade (A/MORS) attached to a texture analyser, and shear force was measured using a 5 kg load cell at a crosshead speed of 10 mm/s. The average shear force measurements for each sample were presented in Newtons.

3.4 Statistical analysis

Levene's test and the NORMAL option in the Univariate Procedure statement of SAS were used to assess homogeneity of variance and normality, respectively. The relationship between diets (excluding the POSC) and response factors for all measured parameters was analysed using response surface regression (PROC RSREG; SAS, 2013). For estimation of optimum

inclusion rate of dietary probiotic blend, the following non-linear equation was used: $y = ax^2 + bx + c$

y = dependent variable, a, b, and c = coefficients of the quadratic equation, and x = dietary doses of probiotic blend. The x value for optimal response will be determined as: $x = \frac{-b}{2a}$

Cross-sectional data were analyzed using a one-way ANOVA with the candidate probiotic blend dose as the fixed factor. The significance of all measured parameters was determined at $p < 0.05$, and differences were compared using least-squares means.

3.5 Results

3.5.1 Mortality

Dietary *B. subtilis* probiotic mixture showed no significant effect on the mortality of Ross 308 broiler chickens.

3.5.2 Growth performance

Table 3.2 shows that there were no significant ($p > 0.05$) dietary effects on the growth performance metrics in the starter phase; however, a positive quadratic response was observed for FI [y = = 439.0 (±3.452) - 58.15 (±24.77) x + 92.59 (±40.86) x^2; $R^2 = 0.158$; $p = 0.032$]. Significant BWG and FCR differences were observed during the grower phase, with diet PRB5 promoting the highest BWG ($p < 0.05$) and the lowest FCR. Birds reared on a diet PRB6 showed the lowest BWG and the highest FCR. Weight gains in birds fed diets PRB0, POSC, PRB4, and PRB3 were statistically similar ($p > 0.05$). FCR for PRB4 did not differ significantly from PRB5, while PRB0, POSC, and PRB3 showed intermediate FCR values. No dietary treatment effects ($p > 0.05$) were observed in the finisher phase and overall growth performance metrics. However, cumulative FCR was significantly affected by dietary treatments, with diet

PRB6 (1.485) recording the highest ($p > 0.05$) FCR and PRB5 (1.412) the lowest, while PRB0, POSC, PRB3, and PRB4 were statistically similar ($p > 0.05$).

Table 3.2: Effects of incremental levels of a novel *B*. *subtilis* probiotic mixture on growth performance of male Ross 308 broiler chickens (n = 900).

[2]Parameters	[1]Diets						[3]SEM	Significance		
	POSC	PRB0	PRB3	PRB4	PRB5	PRB6		GLM	Linear	Quadratic
					Starter (0 - 14 days)					
Feed intake (g/bird)	426.9	438.7	431.3	430.8	430.3	439.0	3.862	0.186	0.539	0.032
BWG (g/bird)	249.5	240.3	2450	238.5	249.0	246.2	7.03	0.836	0.751	0.482
Feed conversion ratio	1.782	1.765	1.767	1.812	1.736	1.790	0.047	0.911	0.870	0.918
					Grower (15 – 28 days)					
Feed intake (g/bird)	1162	1174	1205	1189	1155	1179	13.57	0.147	0.920	0.627
BWG (g/bird)	712.8^{ab}	750.5^{ab}	723.3^{ab}	760.0^{ab}	783.3^{a}	699.0^{b}	17.45	0.017	0.388	0.075
Feed conversion ratio	1.552^{bc}	1.650^{abc}	1.669^{ab}	1.523^{c}	1.518^{c}	1.696^{a}	0.033	0.001	0.499	0.109
					Finisher (29 – 42 days)					
Feed intake (g/bird)	1618	1618	1640	1636	1664	1648	23.44	0.713	0.213	0.881
BWG (g/bird)	1233	1256	1295	1220	1293	1256	22.15	0.123	0.422	0.495
Feed conversion ratio	1.288	1.315	1.269	1.343	1.287	1.314	0.024	0.330	0.984	0.592
					Cumulative (0 – 42 days)					
Feed intake (g/bird)	3207	3231	3276	3222	3283	3267	33.47	0.490	0.404	0.938
BWG (g/bird)	2195	2246	2263	2218	2325	2201	31.88	0.066	0.324	0.185
Feed conversion ratio	1.428^{ab}	1.474^{ab}	1.448^{ab}	1.453^{ab}	1.412^{b}	1.485^{a}	0.016	0.027	0.528	0.081

[a, b, c] Within row means with different superscripts are significantly different ($p < 0.05$). [1]Diets: POSC = Positive control diet with 0.5 g/kg zinc bacitracin but without a novel *B*. *subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin and a novel *B*. *subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B*. *subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B*. *subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B*. *subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B*. *subtilis* probiotic mixture. [2]Parameters: BWG, body weight gain; [3]SEM: standard error of means.

3.5.3 Nutrient digestibility

Varying supplementation levels of the *B. subtilis* probiotic blend significantly affected nutrient digestibility (Table 3.3). Organic matter digestibility (OMD) differed ($p < 0.05$) among dietary treatments: chickens fed POSC had the lowest OMD, while those fed other diets had similar OMD. A negative quadratic response [$y = 71.16$ (±0.626) + 14.55 (±4.492) x - 21.34 (±7.412) x^2; $R^2 = 0.361$; $p = 0.014$] was observed in OMD. Neutral detergent fibre digestibility (NDFD) differed significantly: ($p < 0.05$) chickens fed probiotic supplemented diets had comparable NDF digestibility but PRB0 and POSC fed counterparts had the lowest NDFD ($p < 0.05$) percentage with an observed negative quadratic effect [$y = 58.60$(±1.233) + 54.43 (±8.776) x - 63.41 (±14.48) x^2; $R^2 = 0.845$; $p = 0.001$]. There were no significant differences ($p > 0.05$) observed in ether extract digestibility (EED) and crude protein digestibility (CPD) among the dietary treatments. However, a negative quadratic response [$y = 87.56$ (±0.641) + 13.90 (±4.600) x - 20.28 (±7.589) x^2; $R^2 = 0.331$; $p = 0.020$] was observed in CPD. Gross energy digestibility (GED) differed significantly ($p < 0.05$) among dietary treatments. Broiler chickens fed the POSC diet had the lowest GE digestibility, whereas counterparts reared on other diets had GE digestibility similar to that of the POSC group, except for PRB0, which had an intermediate GE digestibility. Gross energy digestibility increased linearly [$y = 72.83$ (±0.755) + 9.516 (±5414) x, $R^2 = 0.307$; $p = 0.033$] with increasing dietary probiotic inclusion dose.

Table 3.3: Impact of dietary supplementation with a novel *B. subtilis* probiotic mixture on nutrient digestibility (%) in Ross 308 male broiler chickens (n = 900).

	[1]Diets							Significance		
[2]Parameters	POSC	PRB0	PRB3	PRB4	PRB5	PRB6	[3]SEM	GLM	Linear	Quadratic
OMD	68.03[b]	71.16[a]	73.47[a]	73.90[a]	72.81[a]	72.29[a]	0.641	0.003	0.129	0.014
NDFD	55.35[b]	58.47[b]	70.50[a]	68.37[a]	70.63[a]	68.47[a]	1.026	<.0001	<.0001	0.001
EED	61.31	72.13	66.65	68.97	69.09	62.66	2.936	0.153	0.113	0.747
CPD	88.27	87.49	90.29	89.71	89.01	88.87	0.620	0.084	0.145	0.020
GED	70.03[b]	72.88[ab]	74.39[a]	75.69[a]	75.04[a]	75.05[a]	0.799	0.003	0.033	0.304

[a, b] Within row means with different superscripts are significantly different ($p < 0.05$). [1]Diets: POSC = Positive control diet with 0.5 g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture. [2]Parameters: OMD, organic matter digestibility; NDFD, neutral detergent fibre digestibility; EED, ether extract digestibility; CPD, crude protein digestibility; GED, gross energy digestibility. [3]SEM: standard error of means.

3.5.4 Heamato-clinical biochemistry

Dietary supplementation with *B. subtilis* probiotic blend differed significantly ($p < 0.05$) in the broiler chickens' haematological profile, except monocytes (Table 3.4). Diet PRB3 promoted the highest ($p < 0.05$) red blood cell (RBC) counts, while diet PRB6 resulted in the lowest RBC count. A negative quadratic effect [$y = 0.623$ (±0.075) + 2.529 (±0.827) x - 4.108 (±1.415) x^2; $R^2 = 0.456$; $p = 0.018$] was also observed for RBC counts as the probiotic dose increased. Broiler chickens supplemented with PRB4 exhibited significantly higher ($p < 0.05$) platelet counts compared to those fed PRB3, PRB5, and PRB6, while control chickens had intermediate platelet counts ($p > 0.05$). Haemoglobin concentrations did not differ ($p > 0.05$) across experimental diets but decreased linearly [$y = 12.30$ (±0.619) - 3.855 (±6.868) x; $R^2 = 0.526$; $p = 0.012$] with increasing probiotic supplementation.

A significant dietary effect on white blood cell (WBC) counts was observed. The highest ($p<0.05$) WBC counts were observed in birds fed the PRB0, and the lowest in chickens fed PRB4. A positive quadratic response was observed for WBC counts [$y = 119.1$ (±9.320) - 513 (±103.4) x + 623.7 (±177.03) x^2; $R^2 = 0.867$; $p = 0.007$] as probiotic dose increased. Varying doses of *B. subtilis* significantly affected lymphocyte percentage, with broiler chickens fed the diet containing PRB6 having the highest lymphocyte percentage ($p < 0.05$), and those fed the diet containing PRB4 having the lowest. A positive quadratic response [$y = 1.004$ (±0.132) – 3.035 (±0.950) x + 5.249 (±1.567) x^2; $R^2 = 0.294$; $p = 0.002$] on lymphocyte percentage was observed with the increasing dietary probiotic dose. Dietary treatment significantly affected ($p < 0.05$) the percentage of neutrophils in broiler chickens. Birds reared on PRB4 had the highest ($p < 0.05$) neutrophil percentage, with counterparts reared on PRB6 having the lowest neutrophil percentage and a negative quadratic effect observed [$y = 0.311$ (±0.087) + 2.252 (±0.622) x - 3.961 (±1.026) x^2; $R^2 = 0.355$; $p = 0.001$] with an increase in dietary probiotic dose.

Dietary treatment effects showed no significant effects ($p > 0.05$) on broiler chickens' eosinophil counts. However, a negative quadratic effect [$y = 0.202$ (±0.041) + 0.607 (±0.292) x - 1.217 (±0.481) x^2; $R^2 = 0.185$; $p = 0.018$] was observed. Dietary treatment significantly affected platelet distribution width (PDW), with the POSC and PRB6 groups showing the highest percentage ($p < 0.05$), the PRB4 group the lowest, and the remaining treatments were similar ($p > 0.05$). Furthermore, a positive quadratic response [$y = 0.395$(±0.065) - 1.650 (±0.465) x + 2.737 (±0.768) x^2; $R^2 = 0.318$; $p = 0.001$] was observed in PDW with an increase in dietary probiotic dose.

Table 3.4: Effects of dietary supplementation with a novel *B. subtilis* probiotic mixture on haematological parameters in Ross 308 male broiler chickens (n = 900).

	[1]Diet						Significance		
[2]Parameters	POSC	PRB0	PRB3	PRB4	PRB5	PRB6	GLM	Linear	Quadratic
RBC (M/µL)	0.515±0.130^{b}	0.603±0.130^{b}	1.257±0.184^{a}	0.760±0.130^{b}	0.805±0.160^{b}	0.460±0.130^{b}	0.018	0.333	0.018
Platelet (K/µL)	1395±238.1^{ab}	1205±238.1^{ab}	248.0±336.7^{b}	4092±238.1^{a}	426.9±291.6^{b}	595.8±238.1^{b}	0.018	0.160	0.260
HGB (g/dL)	10.45±0.949	12.02±0.949	9.550±0.949	11.33±0.949	11.47±0.949	8.783±0.949	0.162	0.012	0.8779
WBC(K/µL)	57.71±11.60^{ab}	101.9±11.60^{a}	23.36±11.60^{b}	19.91±11.60^{b}	40.43±11.60^{b}	20.37±11.60^{b}	0.001	<.001	0.007
LYM (%)	0.886±0.126^{ab}	0.972±0.126^{ab}	0.766±0.126^{ab}	0.511±0.126^{b}	0.628±0.126^{b}	1.194±0.126^{a}	0.009	0.989	0.002
NEU (%)	0.387±0.081^{ab}	0.329±0.081^{ab}	0.493±0.081^{ab}	0.716±0.081^{a}	0.472±0.081^{ab}	0.191±0.081^{b}	0.003	0.853	0.001
MONO (%)	0.433±0.086	0.424±0.086	0.382±0.086	0.523±0.086	0.446±0.086	0.247±0.086	0.361	0.424	0.181
EOS (%)	0.239±0.042	0.202±0.042	0.288±0.042	0.218±0.042	0.230±0.042	0.120±0.042	0.149	0.291	0.018
PDW (%)	0.399±0.062 ^{a}	0.393±0.062^{ab}	0.164±0.062^{ab}	0.159±0.062^{b}	0.246±0.062 ^{ab}	0.399±0.062 ^{a}	0.011	0.628	0.001

$^{a, b}$ Within row means with different superscripts are significantly different ($p < 0.05$). [1]Diets: POSC = Positive control diet with 0.5 g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture. [2]Parameters: RBC, red blood cells; PLT, platelet; HGB, hemoglobin; WBC, white blood cells; LYM, lymphocytes; NEU, neutrophils; MONO, monocytes; EOS, eosinophils; PDW, platelet distribution width. [3]SEM: standard error of means.

There were no dietary effects ($p > 0.05$) on the broiler chickens' serum clinical biochemistry markers of general, kidney, liver and pancreatic health. However, incremental supplementation rates of *B*. *subtilis* probiotic blend induced positive quadratic response in phosphate [$y = 16.49$ (± 6.464)$x^2 - 8.300$ (± 3.927)$x + 8.562$ (± 0.509); $R^2 = 0.364$; $p = 0.029$] and calcium [$y = 7.272$ (± 2.393)$x^2 - 4.222$ (± 1.453)$x + 10.76$ (± 0.188); $R^2 = 0.480$; $p = 0.013$] concentration in serum.

Table 3.5: Impact of dietary supplementation with a novel *B. subtilis* probiotic mixture on serum biochemical parameters in Ross 308 male broiler chickens (n = 900).

	[1]Diet							Significance		
[2]Parameters	POSC	PRB0	PRB3	PRB4	PRB5	PRB6	[3]SEM	GLM	Linear	Quadratic
Glucose (mg/dL)	119.4	96.25	112.7	104.2	96.33	91.00	17.98	0.774	0.401	0.979
Phosphate (mg/dL)	7.720	8.525	7.450	7.600	8.583	8.567	0.548	0.287	0.268	0.029
Calcium (mg/dL)	10.48	10.70	10.45	10.46	10.67	10.55	0.227	0.916	0.975	0.013
TP (g/dL)	3.920	3.625	4.250	3.400	3.900	4.433	0.490	0.553	0.841	0.487
GGT (U/L)	7.620	4.125	8.850	10.40	3.167	11.50	3.849	0.420	0.533	0.904
TBL (mg/dL)	24.28	57.88	15.90	0.200	0.283	0.333	17.69	0.142	0.161	0.887
Cholesterol (mg/dL)	189.4	197.8	135.3	115.6	115.5	125.5	35.26	0.280	0.345	0.880
Globulins (g/dL)	2.420	2.733	2.733	1.860	2.233	2.767	0.482	0.459	0.624	0.687
Albumin (g/dL)	1.540	4.425	1.483	1.540	1.650	1.633	0.974	0.222	0.301	0.096
ALB/GLOB	0.900	0.567	0.533	3.280	0.950	0.600	1.157	0.255	0.440	0.994
ALT (U/L)	14.00	315.5	13.25	16.00	14.33	13.33	224.2	0.733	0.323	0.343
ALKP (U/L)	621.5	720.3	673.8	1233	693.8	1213	234.7	0.152	0.899	0.226
Amylase (U/L)	259.2	174.5	323.2	292.0	247.8	279.8	46.26	0.258	0.195	0.492
Lipase (U/L)	144.5	132.5	190.4	155.6	152.2	163.0	32.99	0.630	0.251	0.856

[1]Diets: POSC = Positive control diet with 0.5 g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture. [2]Parameters: TP, total protein; TBL, total bilirubin; GGT, gamma-glutamyl transferase; ALB/GLOB, albumin to globulin ratio; ALT, alanine aminotransferase; ALKP, alkaline phosphatase. [3]SEM: standard error of means.

3.5.5 Carcass traits and viscera morphometry

There were no significant dietary effects on broiler chickens' final body weight, cold carcass weight, and dressing percentage (Table 3.6). However, the relative duodenal weight showed a significant difference, with birds fed diets PRB0 and POSC having heavier duodena ($p < 0.05$), whereas counterparts fed diet PRB5 had the lightest duodena. Broiler chickens fed the PRB6 diet had longer jejunum ($p < 0.05$) than the rest. Furthermore, broiler chickens fed the PRB3-supplemented diet had the shortest jejunum ($p < 0.05$), whereas those fed PRB0, POSC, PRB4, and PRB5 had similar jejunum lengths ($p > 0.05$). Additionally, a positive quadratic response was observed in the jejunum length [$y = 92.04(\pm3.450) - 35.73(\pm23.26)\ x + 86.47(\pm36.58)\ x^2$; $R^2 = 0.340$; $p = 0.027$] with an increase in dietary probiotic dose.

Table 3.6: Impact of incorporating a novel *B. subtilis* probiotic mixture in a basal broiler diet on carcass traits and internal organs (g/100 g CCW) of Ross 308 male broiler chickens (n = 900).

	[1]Diets							Significance		
[2]Parameters	POSC	PRB0	PRB3	PRB4	PRB5	PRB6	[3]SEM	GLM	Linear	Quadratic
FBW (g)	2289	2238	2306	2260	2368	2243	31.83	0.066	0.939	0.671
CCW (g)	1373	1348	1315	1304	1428	1273	42.06	0.451	0.827	0.888
Dressing (%)	59.60	57.22	56.54	58.37	60.30	57.30	1.351	0.337	0.850	0.689
Breast	17.08	18.86	18.22	17.32	16.11	17.44	1.134	0.626	0.127	0.806
Wing	5.765	5.385	5.861	5.935	5.296	5.859	0.205	0.148	0.347	0.328
Thigh	8.223	7.562	8.064	8.120	7.557	7.965	0.309	0.513	0.506	0.340
Drumstick	7.066	6.991	7.254	7.143	6.727	7.121	0.257	0.777	0.810	0.625
Proventriculus	0.583	0.627	0.657	0.686	0.639	0.651	0.027	0.179	0.437	0.247
Gizzard	2.670	2.716	2.858	2.954	2.826	2.841	0.098	0.382	0.203	0.254
Spleen	0.179	0.163	0.158	0.167	0.189	0.187	0.018	0.773	0.060	0.477
Liver	3.010	3.122	2.910	3.094	2.964	3.053	0.126	0.841	0.891	0.416
Pancreas	0.373	0.358	0.372	0.338	0.324	0.385	0.025	0.500	0.869	0.611
Duodenum	1.648[a]	1.646[a]	1.472[ab]	1.420[ab]	1.385[b]	1.468[ab]	0.063	0.020	0.061	0.225
Jejunum	2.997	3.233	2.958	3.201	2.911	3.198	0.183	0.693	0.958	0.504
Ileum	2.149	2.391	2.257	2.276	2.178	2.289	0.109	0.678	0.423	0.473
SIW	6.794	7.269	6.687	6.897	6.474	6.956	0.303	0.569	0.537	0.385
Caeca	0.897	0.907	1.025	1.013	0.930	0.964	0.072	0.722	0.786	0.549
Duodenum (cm)	39.55	42.01	40.28	39.85	40.38	41.17	1.264	0.737	0.307	0.120
Jejunum (cm)	90.89[ab]	92.31[ab]	85.67[b]	91.04[ab]	98.22[ab]	99.86[a]	3.252	0.046	0.025	0.027
Ileum (cm)	98.2	100.6	97.3	97.6	101.4	101.8	2.899	0.748	0.951	0.133
Caeca (cm)	22.38	23.30	23.49	23.64	22.76	23.48	0.445	0.244	0.573	0.791

[a, b] Within row means with different superscripts are significantly different ($p < 0.05$). [1]Diets: POSC = Positive control diet with 0.5 g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture. [2]Parameters: FBW, final body weight; CCW, cold carcass weight; SIW, small intestines weight. [3]SEM: standard error of means.

3.5.6 Meat quality attributes

Supplementation of Ross 308 broiler chicken diets with *B. subtilis* probiotic blend resulted in a significant difference in some meat quality attributes (Table 3.7). Breast meat from broiler chickens fed PRB0 had the highest ($p < 0.05$) redness compared to the rest. The Ross 308 broiler chicken thigh meat colour was not affected ($p > 0.05$) by dietary treatment. While there was no significant difference ($p > 0.05$) in breast and thigh meat pH, the chickens' breast meat pH decreased linearly [$y = 5.932$ (±0.039) - 0.416 (±0.280) x; $R^2 = 0.160$; $p = 0.029$] after 24 hours. The breast meat of the broiler chickens fed PRB0 had the highest ($p < 0.05$) shear force value, whereas breast meat from chickens fed PRB4 had the lowest value. Furthermore, a positive quadratic effect was observed for shear force [$y = 0.486$ (±0.243) - 5.645 (±1.688) x + 7.528 (±2.785) x^2; $R^2 = 0.337$; $p = 0.012$] with an increase in dietary probiotic dose. The Ross 308 chickens' breast meat Water holding capacity (WHC) showed a positive quadratic response [$y = 89.54$ (±2.824) - 46.60 (±20.26) x + 64.53 (±33.43) x^2; $R^2 = 0.337$; $p = 0.012$] with an increase in dietary probiotic dose. Breast meat from the PRB0 and PRB5 groups exhibited the highest WHC ($p < 0.05$), while that from chickens fed PRB4 had the lowest WHC.

Dietary treatments significantly influenced drip loss (DL) in breast meat from chickens fed diet PRB4, which recorded the highest DL ($p < 0.05$), while counterparts fed POSC and PRB6 exhibited the lowest DL. A negative quadratic response [$y = 8.349$ (±1.280) + 46.11 (±9.188) x - 79.60 (±15.16) x^2; $R^2 = 0.505$; $p = <.0001$] was observed for DL with an increase in dietary probiotic dose Breast meat from the Ross 308 broiler chickens reared on POSC and PRB3 had the highest ($p < 0.05$) cooking loss (CL) while that of counterparts reared on PRB5 and PRB0 had the lowest CL. Furthermore, a negative quadratic effect was observed for breast meat cooking loss [$y = 27.75$ (±1.026) + 20.13 (±7.362) x - 33.26 (±12.15) x^2; $R^2 = 0.216$; $p = 0.018$] with an increase in dietary probiotic dose.

Table 3.7:Effects of a novel *B. subtilis* probiotic mixture supplemented in a basal broiler diet on meat quality attributes in Ross 308 male broiler chickens (n = 900).

	[1]Diets							Significance		
[2]Parameters	POSC	PRB0	PRB3	PRB4	PRB5	PRB6	[3]SEM	GLM	Linear	Quadratic
Thigh measurements										
*L**	47.49	47.66	47.54	48.48	48.46	47.30	0.440	0.265	0.894	0.093
*a**	4.862	4.787	4.937	4.640	4.696	4.576	0.166	0.640	0.183	0.746
*b**	4.378	4.141	4.371	4.292	4.599	4.394	0.263	0.896	0.328	0.585
Chroma	6.571	6.346	6.611	6.330	6.580	6.352	0.243	0.904	0.978	0.588
Hue	0.729	0.710	0.719	0.747	0.775	0.763	0.030	0.612	0.063	0.680
pH 1hr	6.156	6.072	6.097	6.054	6.052	6.038	0.061	0.774	0.625	0.665
pH 24hrs	6.232	6.042	6.132	6.037	5.939	6.119	0.074	0.131	0.971	0.998
Breast measurements										
*L**	48.06	46.83	47.44	46.72	47.02	45.72	1.121	0.752	0.257	0.306
*a**	3.116^{a}	3.196^{a}	2.564^{b}	2.907^{b}	3.039^{b}	2.717^{b}	0.150	0.043	0.390	0.538
*b**	4.647	4.691	4.937	4.986	5.017	5.134	0.236	0.655	0.152	0.727
Chroma	5.617	5.687	5.576	5.803	5.881	5.814	0.201	0.870	0.343	0.930
Hue	0.973	0.975	1.091	1.036	1.024	1.082	0.034	0.080	0.202	0.595
pH 1hr	5.839	5.937	5.954	5.815	5.834	5.864	0.069	0.628	0.254	0.982
pH 24hrs	5.850	5.929	5.854	5.843	5.774	5.843	0.042	0.270	0.029	0.416
SF (N)	3.773^{ab}	4.626^{a}	3.706^{ab}	3.370^{b}	3.857^{ab}	3.907^{ab}	0.237	0.024	0.018	0.012
WHC (%)	84.21^{ab}	89.57^{a}	82.92^{ab}	76.06^{b}	87.83^{a}	82.92^{ab}	2.478	0.011	0.141	0.064
DL (%)	7.650^{c}	8.497^{bc}	14.11^{ab}	14.57^{a}	12.33^{abc}	6.789^{c}	1.385	0.001	0.957	<.0001
CL (%)	32.80^{a}	27.43^{b}	32.58^{a}	30.02^{ab}	27.04^{b}	29.32^{ab}	0.867	<.0001	0.684	0.011

$^{a, b, c}$ Within row means with different superscripts are significantly different ($p < 0.05$). [1]Diets: POSC = Positive control diet with 0.5 g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture. [2]Parameters: *L**, lightness; *a**, redness; *b**, yellowness; SF, shear force; WHC, water holding capacity; DL, drip loss; CL, cooking loss. [3]SEM: standard error of means.

3.6 Discussion

3.6.1 Growth performance

Supplementing broiler chickens' diets with *Bacillus subtilis* probiotics aims to enhance gut health by promoting the growth of beneficial microbes under normal conditions. This improvement supports more efficient feed digestion and nutrient absorption, which, in turn, contributes to improved overall growth performance (Jiang *et al*., 2021). Given that high stocking density in broiler chickens compromises bird gut health, this study evaluated the effectiveness of *B. subtilis* probiotics in improving growth performance by enhancing gut health. FI, BWG, and FCR are key growth performance metrics in poultry production as they are used to assess productivity and profitability (Zhang *et al*., 2021). The current study found that dietary fortification of Ross 308 broiler chicken diets with a *B. subtilis* probiotic mixture did not affect FI compared with the control group. However, a positive quadratic response observed during the starter phase indicates that chicks receiving the high-intermediate dosage (0.4 and 0.5%) consumed less feed than those given either the low or the high dosage. The reduced feed intake in PRB5 is a positive indicator because it demonstrates that, despite consuming less feed, optimal productive performance was achieved. The observed increase in feed utilisation most likely resulted from the *B. subtilis* probiotic, which induced enhanced nutrient utilisation through improved digestion and absorption (Liu *et al*., 2022). These findings also indicate that the birds on PRB5 were able to convert their feed into body mass more efficiently, gaining weight while consuming less feed. Specifically, broiler chickens on a diet supplemented with the *B. subtilis* probiotic blend at 0.5% dietary inclusion exhibited improved BWG compared to counterparts reared on other diets. However, the lowest (0.3%) *B. subtilis* probiotic mixture dose resulted in high FI and low BWG, which were statistically similar to those recorded for Ross 308 broiler chickens fed the negative control. This negative finding

suggests that at a lower dietary inclusion dose, the *B. subtilis* probiotic mixture did not fully confer the beneficial effects typically provided by probiotics on BWG.

Interestingly, Ross 308 broiler chickens fed diets with the highest (0.6%) inclusion of the B. subtilis probiotic mixture had lower BWG than those fed lower probiotic doses. This outcome implies that high dietary supplementation with *B. subtilis* compromises broiler BWG. It has been shown that probiotics secrete antibacterial molecules that lower intestinal pH, which then suppresses the proliferation of pathogenic gut microbes (Kulkarni *et al*., 2022) but promotes increased growth of desirable gut microbiota. However, excessive amounts of these beneficial microbes may increase gut acidity, leading to a microbial imbalance that subsequently impairs nutrient digestion and absorption, thereby compromising nutrient supply to the birds and resulting in poor growth performance (Mohamed *et al*., 2022). The higher FI observed during the starter phase in broiler chickens reared on diet PRB6 may be attributed to the development of a microbial imbalance due to excessive populations of desirable gut microbiota. This deduction is key in light of the assertion that early-life probiotic supplementation affects later developmental stages in broiler chickens (Rubio, 2019). Moreover, the highest dietary probiotics inclusion might have caused diet nutrient dilution, resulting in increased FI and gut fill; a scenario that the increased intestinal passage rate implies that nutrients spend less time in contact with the absorptive surface, leading to reduced nutrient absorption (Ravindran & Abdollahi, 2021; Singh & Kim, 2021). Generally, these current study findings highlight the importance of determining an optimal dietary probiotic dose for optimal responses, as exceeding the optimal dose may compromise broiler chicken performance while also increasing production costs.

The significantly lower FCR observed in broiler chickens fed diets supplemented with the *B. subtilis* probiotic mixture at 0.4% and 0.5% dietary inclusion indicates that the birds were able to convert feed into body mass more efficiently, requiring less feed to gain a unit of weight.

However, the calculated optimal dosage of 0.3% resulted in a higher FCR than the positive control. This may be attributed to the mechanisms by which zinc bacitracin enhances nutrient retention by improving digestive health, whereas a lower dosage of B. subtilis may not have fully realized its probiotic benefits. These results suggest that the benefits of zinc bacitracin surpassed those of the lower *B. subtilis* probiotic mixture dose. The observed improvement in feed efficiency with the 0.5% *B. subtilis* probiotic mixture suggests enhanced digestive function and nutrient utilization, mediated by a healthier gut environment and the development of an optimal intestinal microbial ecosystem. As a result, the broiler chickens reared on this diet exhibited higher BWG with reduced FI, which can contribute to lower production costs and higher enterprise profitability. The current study's findings are in line with the reported observations in Cobb 500 broiler chickens fed diets fortified with *B. subtilis* at 500g/ton of feed (Yu *et al*., 2024). Similarly, Upadhaya *et al*. (2019) reported increased BWG and lower FCR in Ross 308 broiler chickens fed a diet containing 500 g/ton of *Bacillus*-based probiotics. However, Chen *et al*. (2024) found that *B. subtilis* at 1.8×10^9 cfu/g mediated lower FCR but had no effect on BWG. Similarly, Ramlucken *et al*. (2020) noted no significant differences in BWG and FI but an improved FCR of Ross 308 broiler chickens fed a diet supplemented with a novel *B. subtilis*-based probiotic at 50 and 100 g/ton of feed. The referenced studies revealed improved FCR with *B. subtilis* supplementation, but no apparent benefits on FI or BWG. Therefore, the current study findings show that supplemental *B. subtilis* probiotics increase both BWG and nutrient utilization efficiency, resulting in feed cost savings.

3.6.2 Nutrient digestibility

The digestibility of OM, NDF, and GE was greatly improved by the supplemental *B. subtilis* probiotic mixture. The improved digestibility in *B. subtilis*-supplemented diets may be attributed to documented evidence that probiotics mediate increased synthesis and release into

the gut of digestive enzymes, which then promotes improved bird growth performance due to increased availability of nutrients from the GIT (Jin *et al*., 1997; He *et al*., 2019; Idowu *et al*., 2025). These improvements in nutrient digestibility are directly related to the improved growth performance observed in broiler chickens fed the diet supplemented with 0.5% of the *B. subtilis* probiotic mixture. Current study findings are consistent with the report of Neijat *et al*. (2019), who observed similar outcomes, that is, increased DM, OM, CP, GE, and NDF digestibility, when they supplemented broiler chicken diets with incremental levels (0.05, 0.1, and 0.5%) of *B. subtilis* DSM29784. The increased nutrient digestibility was observed at the highest *B. subtilis* DSM29784 inclusion level. In contrast to the current study findings, Zhang & Kim (2014) reported that a multi-strain commercial probiotic containing *L. acidophilus*, *B. subtilis*, and *C. butyricum* at 1 to 2 × 10^2 cfu/g of feed had no effect on GE digestibility. The current study found that Ross 308 broiler chickens fed the control diet had the lowest NDF digestibility compared to those fed diets supplemented with *B. subtilis*, suggesting that the *B. subtilis* probiotic mixture can enhance the breakdown of high-fibre feed, unlike dietary zinc bacitracin fortification.

A negative quadratic response was observed on the CPD, indicating that digestibility was significantly higher at lower dosages and decreased at the highest dosage (0.6%). This finding aligns with the reported lower crude protein digestibility with higher inclusion of the probiotic mixture (*L. reuteri* DSM 16350, *E. faecium* DSM 16211, *Bifidobacterium animalis* DSM 16284, *Pediococcus acidilactici* DSM 16210, and *L. salivarius* DSM 16351) (Mountzouris *et al*., 2010). The lower CP digestibility may be linked to increased nutrient demands of the probiotic microbes, as they also require nutrients and energy for growth and proliferation, which can slightly decrease the nutrients available to the host (Mountzouris *et al*., 2010; Jha *et al*., 2020).

3.6.3 Haematological and serum clinical biochemistry indices

Haematological and serum clinical biochemistry assays assist in determining the chickens' nutritional and disease status, as well as overall physiological responses to interventions (Dlamini *et al*., 2023). In this study, the observed increase in RBC count in Ross 308 broiler chickens fed the PRB3 diet suggests that the *B. subtilis* probiotic mixture boosted RBC formation, thereby enhancing tissue perfusion and increasing metabolism. This finding is consistent with reported *B*. *subtilis* probiotics, which have been shown to mediate increased hematopoiesis and erythropoiesis in broiler chickens, thought to result from improved nutrient absorption and overall health status (Wang *et al*., 2017; Fairushin *et al*., 2022; El-Sayed *et al*., 2024). A significant decrease in RBC observed in the Ross 308 broiler chickens fed PRB6, most likely resulting in reduced oxygen-carrying capacity may be attributed to damaged intestinal mucosa resulting from excessive fermentation caused by the highest dosage of *B*. *subtilis* probiotic, which can lower intestinal pH levels, leading to impaired iron absorption, which is required for RBC production (Ogbuewu *et al*., 2022). Although haemoglobin concentrations were not significantly different, the decreasing linear effect suggests that higher probiotic dosages may suppress haemoglobin synthesis, possibly due to microbial competition for iron or altered mineral absorption (Abdel-Hafeez *et al*., 2016). These current study findings suggest that a lower probiotic dose is ideal for optimal haemoglobin concentration necessary for effective tissue perfusion and overall metabolic efficiency in broiler chickens reared under high stocking density.

High stocking density stress stimulates the nervous system of broiler chickens, increasing the release of noradrenaline and activating the bone marrow to increase hematopoietic stem cell production, especially WBCs (Nwaigwe *et al*., 2020). In broiler chickens, the normal WBC count ranges from 2.50 – 19.50 K/µL, and values above this are indicative of stressed chickens (Nwaigwe *et al*., 2020). In this study, WBC counts in the PRB0 group exceeded the normal

range, indicating that the Ross 308 broiler chickens fed supplement-free diets experienced elevated stress under HSD conditions. However, the normal WBC counts observed in chickens (PRB4) indicate a downregulation of systemic immune activation, likely due to improved gut barrier function, which mitigates the translocation of pathogens and their toxic metabolites into the bloodstream (Yosi & Metzler-Zebeli, 2023). Probiotics have been shown to reduce inflammation and support immune homeostasis by modulating cytokine production and enhancing intestinal integrity (Mountzouris *et al*., 2007; Li *et al*., 2018; Yousefi *et al*., 2019), thus it can be speculated that the increased percentage of neutrophils observed in broiler chickens fed diet PRB4 might have resulted from the *B. subtilis* probiotic-induced enhanced innate immune response, which would improve defence against infections. Furthermore, this observed improvement suggests reduced stress or pathogen exposure, which are key benefits in densely stocked rearing systems (Ibrahim *et al*., 2018).

Platelet distribution width is associated with platelet production and release during systemic inflammatory responses (Smith & Weyrich, 2011). A decrease in platelet counts suggests reduced inflammation and stress in broiler chickens (Nwaigwe *et al*., 2020), as observed in Ross 308 broiler chickens fed diets PRB3 and PRB5. However, the mechanisms of action by which *B*. *subtilis* probiotic decreases platelet counts remain unknown. The current study found that, for most haematology indices, the optimum *B. subtilis* probiotic mixture dose was 0.3% (the lowest dosage), whereas the optimum probiotic mixture dose for platelet and WBC counts was 0.4%. These findings suggest that a lower dose of *B. subtilis* probiotic mixture is ideal for attenuating stress and inflammation in broiler chickens raised under high stocking density.

Although no significant differences were found in serum clinical biochemical markers of health, a positive quadratic response in calcium and phosphate content suggests improved mineral metabolism at low (0.3%) to mid (0.5%) doses of the *B. subtilis* probiotic mixture. This could be attributed to enhanced nutrient absorption facilitated by a healthier gut microbiota,

enhanced intestinal integrity, and improved digestive enzyme activity (Awad *et al*., 2009; Sohail *et al*., 2010). Adequate calcium and phosphate concentrations are crucial for bone maintenance and bone metabolic health. Caution must be exercised when fortifying broiler chicken diets with a *B. subtilis* probiotic mixture, as higher-than-optimal doses can cause gut dysbiosis, disrupt nutrient absorption, and induce immune-related stress and overall poor health.

3.6.4 Carcass traits and viscera morphometry

The varying inclusion rates of the *B*. *subtilis* probiotic mixture had no significant effects on carcass traits of the Ross 308 broiler chickens. However, notable differences were observed in the chickens' mean duodenal weight and jejunal length, which varied with dietary treatment. Ross 308 broiler chickens fed a PRB5 diet had decreased duodenal weight, possibly due to nutrient channelling from improved digestion towards muscle growth and development, rather than maintaining a bulky gut (Haung *et al*., 2022). By implication, the observed increase in duodenal weight in birds fed PRB0 is most likely due to the associated increase in FCR, since this diet was not fortified with either zinc bacitracin or the *B. subtilis* probiotic mixture, which are known to improve nutrient digestion and absorption (Thema *et al*., 2019). The Ross 308 broiler chickens reared on the 0.6% *B. subtilis* probiotic blend fortified diet exhibited a significantly longer jejunum, while their counterparts reared with the diet fortified with the 03% probiotics blend had shorter jejuna compared to those of chickens fed a probiotic-free diet. This suggests that at higher dietary inclusion levels, the *B. subtilis* probiotic mixture stimulated the development of intestinal structures, potentially increasing the surface area available for nutrient absorption (Naeem & Bourassa, 2025). Evaluating the effects of different doses of the probiotic blend is critical to identifying the optimal dose that enhances intestinal surface area and improves nutrient absorption (Wijoyo & Nindria, 2024). Surprisingly, in the

current study, the broiler chickens fed the diet fortified with the highest *B. subtilis* probiotic dose had the longest jejunum, highest FCR, and lower BWG observations, which could have been caused by dietary nutrient dilution, which interferes with the availability of other essential nutrients, leading to impaired growth (Oikeh *et al*., 2019). When birds are fed a diluted diet, they often increase feed intake to meet their nutrient requirements, but gut capacity and metabolic limitations limit full compensation (Leeson *et al*., 1991; Taylor *et al*., 2021), resulting in higher FCR and reduced weight gain. Moreover, when diet dilution occurs, the birds use more energy for peristalsis, enzyme secretion, and the maintenance of larger digestive organs (Clauss *et al*., 2016) at the expense of muscle accretion. Therefore, higher maintenance energy required to support longer jejuna may have compromised energy supply for muscle growth (Yosi & Metzler-Zebeli, 2023). Similarly, Reis *et al*. (2017) found a decrease in the duodenum weight and elongated jejunum length in Ross 308 broiler chickens fed *B. subtilis* at 500 g/ton compared to those in the control. Overall, the current study's findings suggest that a lower dose of the *B. subtilis* probiotic mixture efficiently optimises muscle growth rather than visceral development.

3.6.5 Physical meat quality traits

While the dietary *B. subtilis* probiotic blend had no effect on the pH of breast and thigh meat in Ross 308 broiler chickens, a linear decrease suggests that pH declined as the probiotic dosage increased. A low pH in broiler chicken meat generally results in a lighter colour, while an increased pH is associated with a darker meat colour (Fletcher, 2002). The current study found that the pH of Ross 308 broiler chicken breast and thigh meat was within the recommended normal range of 5.2 - 6.2 (Ristic & Damme, 2013). In addition to pH, the different redox states of myoglobin, an oxygen store and transporter, also impact meat colour (Hoa *et al*., 2021). When oxygen concentration is low, iron in myoglobin exists in the Fe^{2+} state, resulting in a

purplish-red appearance of the meat (Zhang *et al*., 2024). Increased redness of chicken breast meat was observed in the breast meat from chicken diets that were not fortified with either zinc bacitracin or the *B. subtilis* probiotic mixture. Chicken breast meat, which appears redder, is perceived as not fresh. Dietary *B. subtilis* probiotic blend improved the broiler chickens' breast muscle colour as confirmed by the meat's redness, which fell within the normal range (1.0 to 3.0) of normal broiler chicken meat redness. *Bacillus subtilis* is known to enhance antioxidant defense by preserving myoglobin, particularly in broiler chicken subjected to heat stress (Al-Owaimer *et al*., 2014), the observed normal breast meat redness most likely was due to the probiotic's antioxidant activity.

The meat's WHC, DL, and CL are important since water loss may lead to the leaching of essential nutrients, potentially compromising juiciness, tenderness, and flavor (Yang *et al*., 2016; Ismail & Huda, 2024). Meat tenderness, measured as shear force, is one of the key sensory attributes that influence consumer preference (Bai *et al*., 2017). Typically, normal shear force values for broiler chicken breast muscle range from 2.24 to 4.04 N (Bowker & Zhuang, 2019). While the meat's shear force was within normal range, the supplemental *B. subtilis* probiotic mixture mediated a linear decline in the meat's shear force, an outcome ascribed to *B. subtilis* to enhancing proteolysis of the meat's myofibrillar proteins (Herich *et al*., 2025).

The diets supplemented with *B. subtilis* probiotic mixture (PRB5) increased broiler meat WHC. Similarly, other studies (Park & Kim, 2014; Mohammed *et al*., 2021) have reported results similar to those of the current study, indicating that supplementation with *B. subtilis* improves WHC. Furthermore, drip and cooking loss were reduced in breast meat from diet PRB5 and POSC. The reduced cooking loss observed in the POSC group could be due to zinc bacitracin's role as an antioxidant, which lowers oxidative damage to muscle proteins and lipids, thereby maintaining the integrity of muscle proteins crucial for water retention. Mohammed *et al*.

(2024) obtained similar results, where a *B. subtilis* probiotic at 0.5 g/kg exhibited higher WHC and lower CL in breast meat, indicating improved water retention. This was further corroborated by Wang *et al*. (2024). Cramer *et al*. (2018) suggested that this may be linked to the antioxidant capacity of *B*. *subtilis* probiotics, which is known to prevent protein and lipid oxidation by preserving the structural integrity of muscle fibres. Furthermore, the probiotic- and antibiotic-supplemented diets had similar effects on some breast meat quality attributes, which aligns with Attia *et al*. (2023), who reported that zinc bacitracin and *Lactobacillus acidophilus* decreased DL and CL in broiler breast meat.

While the current study's findings regarding the efficacy of the *B. subtilis* probiotic mixture are in line with previous research, Upadhaya *et al*. (2019) reported that the probiotic mixture had no significant effects on breast meat quality, a contrary finding. Such inconsistencies in research findings could be due to differences in probiotic strains, dose rate, and environmental conditions (Opazo *et al*., 2025). Nonetheless, most studies reported that *Bacillus* probiotics improve sensory attributes of broiler chicken meat (Zhou *et al*., 2015; Bai *et al*., 2017; Tang *et al*., 2021).

3.7 Conclusion

Supplemental *B. subtilis* probiotic mixture at 0.5% of the diet improved FI, BWG, and FCR during the grower phase. Furthermore, improved digestibility of OM, NDF, and GE, with a negative quadratic response in crude protein digestibility, indicates that probiotics enhanced digestive efficiency, resulting in more efficient nutrient absorption under high stocking density. Improved digestive efficiency and enhanced nutrient absorption contributed to the observed increased growth performance in broiler chickens reared on diets supplemented with *B. subtilis*. The reported improvement in haematology indices in broiler chickens reared on PRB3, PRB4, and PRB5 diets, all fortified by the *B. subtilis* probiotic mixture, is ascribed to its (probiotic

mixture) anti-inflammatory and anti-stress activities. Importantly, the lowest *B. subtilis* inclusion (0.3%) had the greatest positive impact on the chickens' haematology profile, indicating its efficacy in broiler chickens reared under high stocking density-induced stress. Interestingly, the low dose *of B. subtilis* inclusion resulted in the shortest jejunal length, while its highest dietary inclusion resulted in the longest jejunal length, but it (the highest probiotic dose) also resulted in the lowest BWG and poorest FCR. Further research is needed to elucidate how *B. subtilis* probiotics can achieve improved FCR with shorter jejunal lengths. The increased WHC, along with decreased shear force, DL, and CL observed in Ross 308 broiler chicken meat from birds fed PRB4, PRB5, and POSC, indicates improvements in meat quality. Nonetheless, further research is necessary to elucidate the mechanisms of how *B. subtills* enhances meat tenderness.

Gut function plays a crucial role in driving the improvements observed and reported in the current chapter. Thus, the next chapter evaluated the effects of varying supplementation rates of *B*. *subtilis* probiotic mixture inclusion on jejunal morphology and caecal microbiota in Ross 308 broiler chickens under high stocking density.

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4 CHAPTER 4: EFFECTS OF A NOVEL FOUR-STRAIN *BACILLUS* PROBIOTIC MIXTURE ON GUT FUNCTION OF DENSELY STOCKED ROSS 308 BROILER CHICKENS

4.1 Abstract

Bacillus probiotics as alternatives to AGP have been reported to enhance gut function in broiler chickens. This study sought to determine the optimal dose *Bacillus subtilis* probiotic mixture, a novel feed supplement, on various gut function indices. The day-old Ross 308 broiler chickens were allocated to the experimental diets as described in the previous chapter. On slaughter, about 5 cm sections of jejuna were collected for histology. Caeca digesta samples were obtained from three birds per dietary treatment for phylogenetic analysis of gut microbiota composition. Diet treatment significantly affected ($p < 0.05$) jejunal morphology, with jejuna from broiler chickens fed PRB5 exhibiting the largest villus absorptive surface area, and jejuna from counterparts fed PRB6 exhibiting the smallest. A significant dietary effect ($p < 0.05$) was noted on microbial diversity, but with no effect ($p > 0.05$) on alpha diversity. Caecal microbiota was dominated by the phyla *Verrucomicrobiota*, *Bacillota*, and Asgardarchaeota, while the genera Akkermansia, SOKP01, Exiguobacterium A, and *Rhizomicrobium* were most dominant across and in specific diets. A high abundance of *Bacillota* was associated with improved productive performance of the chickens. Broiler chickens fed PRB6 exhibited an unclassified phylum, SOKP01, which may have contributed to the reduced BWG observed in the previous chapter. Beneficial gut microbes, that is, *Bacillota* and *Verrucomicrobiota,* were most abundant in chickens fed PRB5, which mediated the development of a healthy gut. However, the role and impact of Rhizomicrobium in broiler chickens require further investigation. By promoting the development of a larger villi absorptive surface, supplement *B. subtilis* at 0.5% dietary inclusion (PRB5) improved nutrient uptake. In conclusion, the four-strain *B*. *subtilis* probiotic

blend is recommended for supplementation at 0.5% (w/w) in broiler chicken diets to improve gut health.

Keywords: Probiotics, phylogenetic composition, gut histomorphology, 16S report.

4.2 Introduction

The primary function of the gut is to digest feed into its basic components, allowing the bird to absorb and utilize them effectively to produce the energy required to maintain homeostasis and support productive performance (Bailey, 2019). Gut health relies on maintaining a delicate balance among the host, gut microbiota, the gut environment, and dietary components (Bailey, 2019; Shini & Bryden, 2021). Maintaining balance is crucial for optimal gut health, which is necessary for effective digestion and nutrient uptake (Bailey, 2019). Disruption of the host-gut microbiota-gut environment-diet balance compromises gut health and nutrient digestion and absorption, resulting in poor bird welfare and reduced productive performance. Globally, driven by the need to meet increasing demand for animal-source protein for human consumption, broiler chicken farmers adopt intensive production systems. Often, the intensive production systems are characterised by increasing stocking density to maximise profit. However, broiler chickens raised under high stocking density suffer from stress, which negatively affects their health and welfare, growth, and productivity. Stress, regardless of its cause, significantly shifts gut microbiota composition and proportion, compromises gut integrity, disrupts normal chicken physiology, and negatively affects the immune system (Rostagno, 2020). Rearing poultry under high stocking densities causes dysbiosis (Rezvani, 2023), resulting in inflammation, disruption of intestinal tight junctions, increased gut permeability, and concomitant leak of gut bacteria and their toxic products into the birds' systemic circulation (Barekatain *et al*., 2019) and increased risk of enteric diseases (Ducatelle *et al*., 2023). Furthermore, by compromising intestinal integrity, dysbiosis negatively affects

nutrient digestion and absorption, resulting in reduced nutrient uptake and lower bird productivity (Rassmidatta *et al*., 2024). Conventionally AGP have been and continue, in some instances, to be used to mitigate dysbiosis (Van der Klein *et al*., 2024) but routine use of AGP as feed supplements causes the public health challenge of antibiotic resistance, pollutes chicken and eggs as well as the environment with antibiotic residues (El-Fateh *et al*., 2024) There is therefore a dire need for natural and acceptable alternative to AGP, for examples, probiotics which have gained global interest (Kogut, 2019).

Probiotics benefit the host by functioning with the gastrointestinal tract (Abd El-Hack *et al*., 2020). They interact with the gut microbiota to regulate intestinal immune pathways, thereby improving poultry productivity, especially in flocks exposed to stress (Shini & Bryden, 2021). Fortification of probiotics modifies gut microbiota through by diminishing the multiplication of pathogens and promotes beneficial microbial populations (Ma *et al*., 2023). The microbial balance in the broiler chicken gut enhances nutrient digestion and absorption, supports immune system and curb diseases (Chandrasekaran *et al*., 2024). Through their functional properties, probiotics, have been reported as viable alternative to AGP in broiler diets, especially under stress-inducing conditions (Sugiharto, 2022). Most existing research has focused on probiotic effects in broiler necrotic enteritis and Salmonella challenged broiler chickens (Khalique *et al*., 2020; Sandvang *et al*., 2021; Zhao *et al*., 2022). In contrast, there is limited information available of *Bacillus* probiotics on gut function in broiler chickens under densely rearing conditions. Hence this paper evaluated the effects of incremental dose of a four-strain Bacillus probiotic blend through examination of gut histomorphology and phylogenetic composition of gut microbiota. These measures represent digestive efficiency, nutrient absorption and immune system performance (Linden, 2013; Celi *et al*., 2017). It was hypothesized that substituting dietary zinc bacitracin with a *B. subtilis* probiotic mixture would preserve the jejunal

morphology and caecal microbial composition of in broiler chickens reared under high stocking density.

4.3 Materials and methods

4.3.1 Study site

The study site, research consumables and material procurement, experimental design, feed formulation, and slaughter procedure are presented in the relevant sections of Chapter 3.

4.3.2 Jejunal histomorphology assessments

Post-slaughter, jejunum samples were randomly selected within each experimental unit. Following the protocol described by Viveros *et al*. (2011), the jejunum was cut into approximately 5 cm lengths from each selected bird. The samples were rinsed with physiological saline solution (1% sodium chloride) to remove intestinal contents and immediately placed in sterile bottles containing 10% formalin to preserve the tissue. The formalin-preserved jejuna processed and histologically assayed for villus height, crypt depth, the villus height-to-crypt depth ratio, villus base width, and absorptive surface area at the Department of Veterinary Science, University of Pretoria, South Africa. The villus height was measured from the villus apex to the junction of the villus and the crypt. The villus width was measured as the distance from the junction of the basement membrane of the epithelial cell at the bottom of the crypt to the bottom third of the length of the villus. The ratio of villus to crypt was estimated by dividing the average villus height by the average crypt depth. The absorptive surface area of the villus was estimated by considering the villus as a cylindrical structure, using the formula: villus absorptive area = $2\pi \times$ (average villus width/2) $\times$ villus height, as guided by Prakatur *et al*. (2019).

4.3.3 Determination of phylogenetic composition of gut microbiota

Following the method by Montso *et al*. (2022) with minor modifications. Caecal digesta samples were collected from three broiler chickens randomly selected per experimental unit followed by pooling of samples from the same dietary treatment into sterile Falcon tubes (50 ml) and stored in a freezer at -25°C pending analysis. Prior to DNA extraction, the frozen stored ceaca samples thawed for 30 minutes at room temperature. Caecal sample DNA extraction was done using the Zymo Research DNA Extraction Kit in compliance with the manufacturer's instructions. The ultra-pure DNA samples were checked for quality using a spectrophotometer (NanoPhotometer® NP80, Implen, Germany) and sent to Inqaba Biotec for a 16S metagenomic report.

In brief, following the manufacturer's instructions, Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, United States) and IDT Unique Dual Indexes were used to prepare DNA libraries (1 ng input). Genomic DNA was fragmented with the Nextera XT fragmentation enzyme, and unique dual indexes were added to each sample. The libraries were subsequently amplified by PCR for 12 cycles. Following amplification, the libraries were purified with AMPure magnetic beads (Beckman Coulter, Brea, CA, United States) and eluted in Qiagen EB buffer. Finally, library quantification was performed using the Qubit 4 fluorometer and the Qubit™ dsDNA HS Assay Kit. Library sequencing was performed on an Illumina HiSeq 4,000 platform (2 × 150 bp), generating paired reads.

4.4 Statistical analysis

For jejunal histomorphology, Levene's test and NORMAL option in the Univariate Procedure statement of SAS were used to analyse data for homogeneity of variance and normality. The relationship between diets and response factors for measured parameters was analysed using response surface regression analysis (PROC RSREG; SAS, 2013). For the estimation of the

optimum inclusion rate of the dietary probiotic blend, the following non-linear equation was used:

$$y = ax^2 + bx + c$$

y = dependent variable, a, b, and c = coefficients of the quadratic equation, and x = dietary dosages of probiotic blend. The x value for optimal response will be determined as: $x = \frac{-b}{2a}$

Cross-sectional data were analysed using a one-way ANOVA with probiotic blend dose as the fixed factor. The differences were compared using least-squares means, and a significant difference was determined at $p < 0.05$. Furthermore, for phylogenetic composition of gut microbiota, R Studio (4.5.1) software was used for the Kruskal-Wallis test to compare the alpha diversity indices (Observed, Simpson, Shannon, and Pielou evenness) of each group, while Bray-Curtis was used to analyze for beta-diversity, and Principal Coordinates Analysis was done to visualize the dissimilarity in microbial community among dietary treatments. PERMANOVA was used to compute beta-diversity and compare differences between groups. The significant difference was determined at $p < 0.05$. Relative abundance percentages were used to compare dominating microbial communities among experimental diets.

4.5 Results

4.5.1 Jejunal histomorphology

Except for villi absorptive surface area (VASA), the inclusion of *B. subtilis* probiotic mixture in Ross 308 broiler chicken diet showed no significant difference ($p > 0.05$) in jejunum morphometry (Table 4.1 and Figure 4.1). Broiler chicken reared on PRB5 exhibited the greatest ($p < 0.05$) VASA in the jejunum compared to counterparts broiler chickens reared on PRB4, but VASA of broiler chickens fed dietary treatments were similar ($p > 0.05$).

Table 4.1: Effects of dietary *B*. *subtilis* probiotic mixture supplement on male Ross 308 broiler chicken jejunal morphology (n = 900).

	[1]Diets							Significance		
[2]Parameters	POSC	PRB0	PRB3	PRB4	PRB5	PRB6	[3]SEM	GLM	Linear	Quadratic
Villus height	1244	1263	1145	1061	1280	1135	181.0	0.935	0.708	0.662
VBW	128.9	133.6	143.0	140.5	137.1	143.4	5.588	0.469	0.356	0.664
Crypt depth	289.8	312.6	310.3	259.3	292.2	311.3	23.56	0.609	0.675	0.397
V: C ratio	4.32	4.04	3.72	4.09	4.31	3.64	0.492	0.873	0.847	0.840
VASA	5039^{ab}	5301 ^{ab}	5148^{ab}	5117^{ab}	5513^{a}	4685^{b}	0.000	<.0001	0.768	0.325

$^{a, b}$ Within row means with different superscripts are significantly different ($p < 0.05$). [1]Diets: POSC = Positive control diet with 0.5 g/kg zinc bacitracin but without a novel *B*. *subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin and a novel *B*. *subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B*. *subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B*. *subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B*. *subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B*. *subtilis* probiotic mixture. [2]Parameters: VBW, villus base width; V: C, villus: crypt ratio; VASA, villi absorptive surface area. [3]SEM: standard error of means.

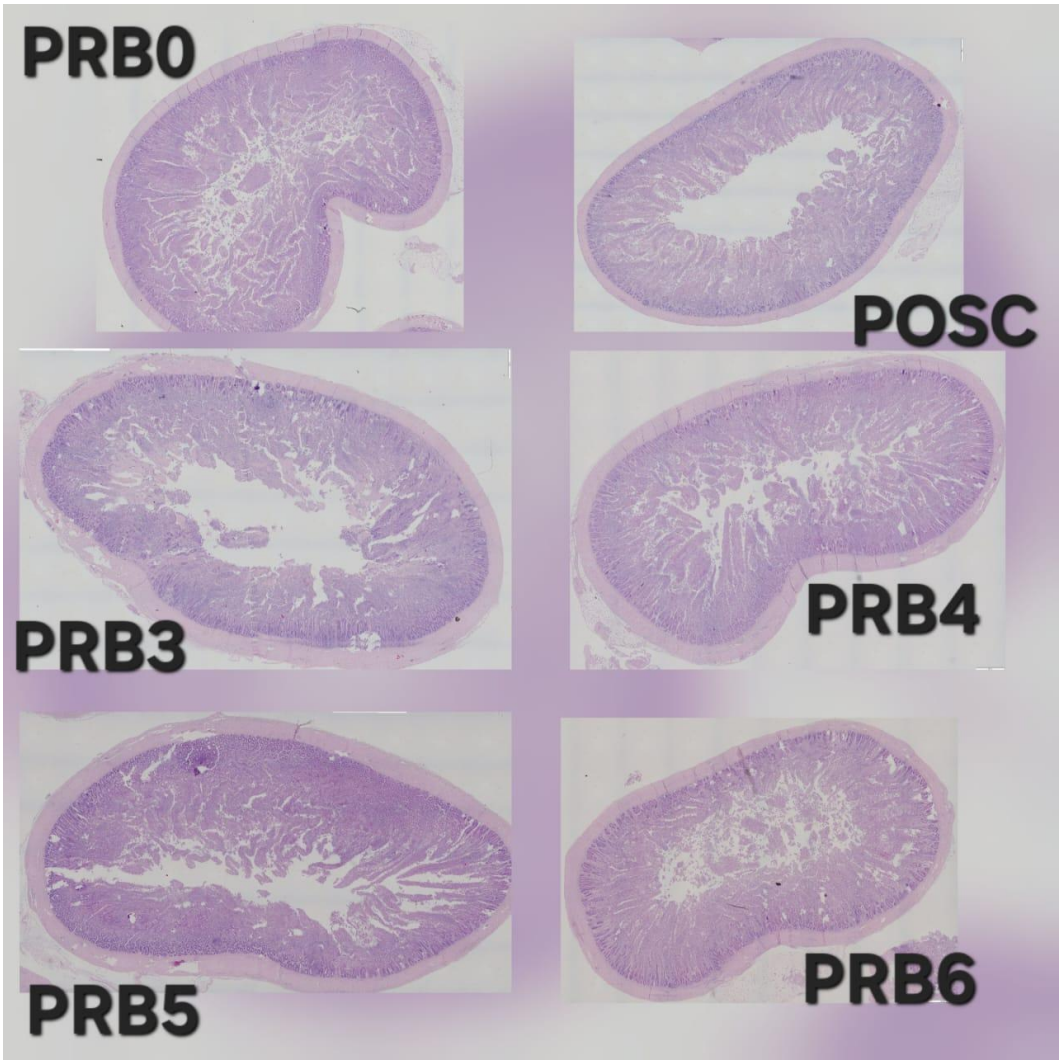

Figure 4.1: Effects of *B. subtilis* probiotic mixture on jejunal morphology of 308 broiler chickens.

Diets: POSC = Positive control diet with 0.5 g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture.

4.5.2 Phylogenetic composition of gut microbiota

4.5.2.1 Alpha diversity summary

There was no significant difference ($p > 0.05$) observed in the alpha diversity metrics (Shannon, Simpson, Observed, and Pielou_Evenness) in Ross 308 broiler chickens fed *B. subtilis*-supplemented diets compared to control groups (Table 4.2).

Table 4.2: Impacts of *B*. *subtilis* probiotic mixture supplementation on alpha diversity in caecal digesta of Ross 308 male broiler chickens (n = 900).

	[1]Diets						Significance
Metrics	**PRB0**	**POSC**	**PRB3**	**PRB4**	**PRB5**	**PRB6**	**P value**
Observed	6.667±0.882	6.5±0.5	6.667±0.667	5.667±0.333	7±2.517	6.333±0.882	0.851
Shannon	0.836±0.394	0.763±0.228	0.953±0.156	0.901±0.135	0.681±0.290	1.149±0.218	0.731
Simpson	0.386±0.198	0.378±0.139	0.469±0.080	0.456±0.093	0.361±0.161	0.571±0.113	0.766
Pielou	0.431±0.198	0.414±0.139	0.502±0.070	0.525±0.087	0.340±0.091	0.619±0.083	0.530

[1]Diets: POSC = Positive control diet with 0.5 g/kg zinc bacitracin but without a novel *B*. *subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin and a novel *B*. *subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B*. *subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B*. *subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B*. *subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B*. *subtilis* probiotic mixture.

4.5.2.2 Principal Coordinate Analysis

The inclusion rates of the *B. subtilis* probiotic mixture in broiler chicken diets resulted in a non-significant difference [$R^2 = 1$; $p = 0.04$] in Principal Coordinate Analysis (PCoA), as shown in Figure 4.2, indicating variation in microbial communities in caecal digesta amongst dietary treatments. The PCoA results show that the 1st principal coordinates accounted for 78.5%, while the 2nd coordinate accounted for 11.9%. The analysis revealed that the caecal microbiome from broiler chickens fed diet PRB5 distinctly separated from other dietary groups along PC1. The caecal microbiome of broiler chickens fed the PRB6 diet was closely related to those of the 0.3% and PRB4 groups along PC1 but was separated from them along PC2. Broiler chickens fed the PRB0, POSC, and PRB4 diets exhibited similar microbial profiles, clustering together along PC2. The PRB3 group showed a composition closely related to that of PRB0, POSC, and PRB4, clustering near them.

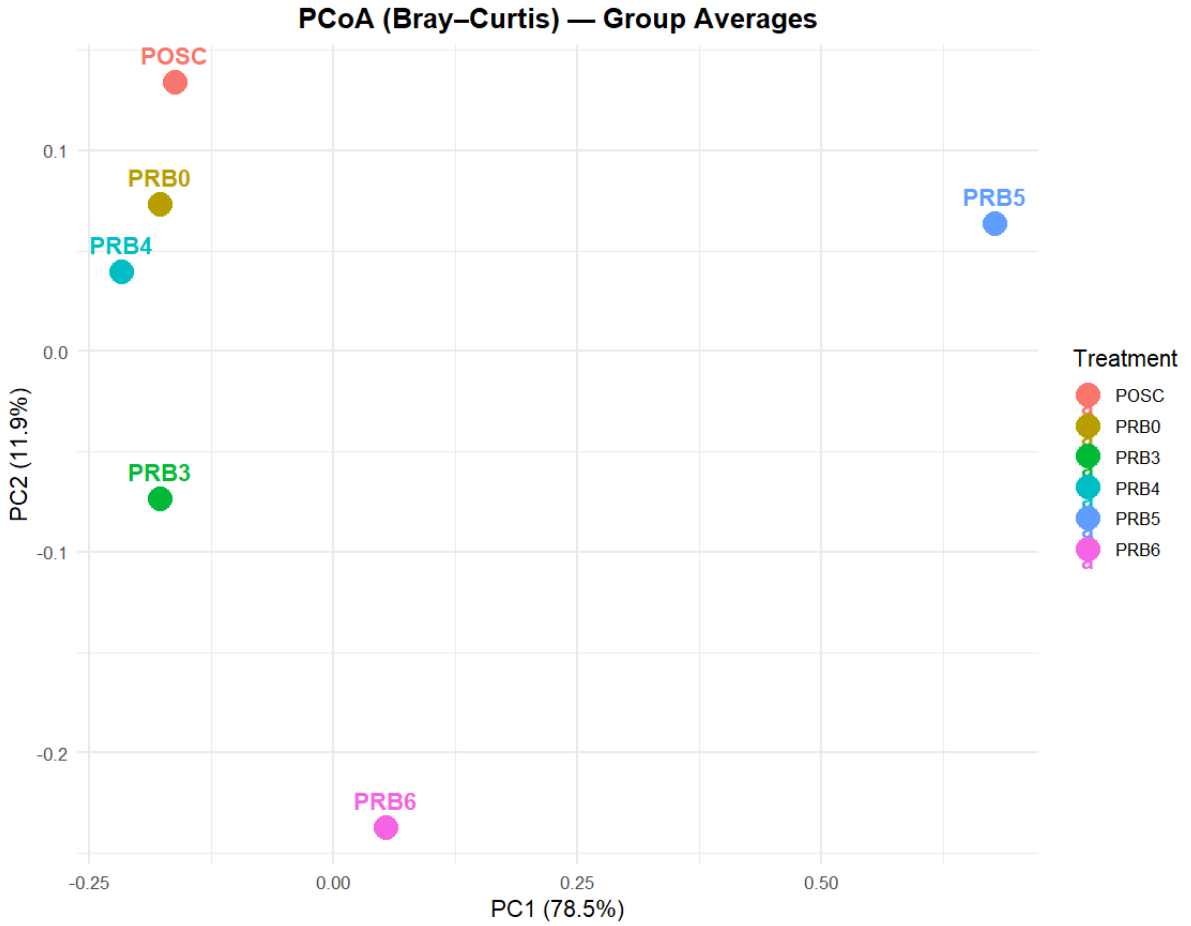

Figure 4.2: R-plot showing Principal Coordinate Analysis with Bray-Curtis dissimilarity based on dietary treatments supplemented with *Bacillus subtilis* probiotic blend on caecal digesta of broiler chickens.

[Treatment type: POSC = Positive control diet with 0.5 g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture.]

The dissimilarity index ($R^2 = 1$; $p = 0.04$) revealed significant variation among the treatments, as determined by PERMANOVA.

4.5.2.3 Kingdom

The 16S metagenomic report revealed an average of 91% bacteria and 8.5% unassigned microbes at the Kingdom level across all diets, with Archaea present at 28% in diet PRB6.

Although the differences were not statistically significant, diet PRB5 exhibited the highest percentage of unassigned microbes (Figure 4.3).

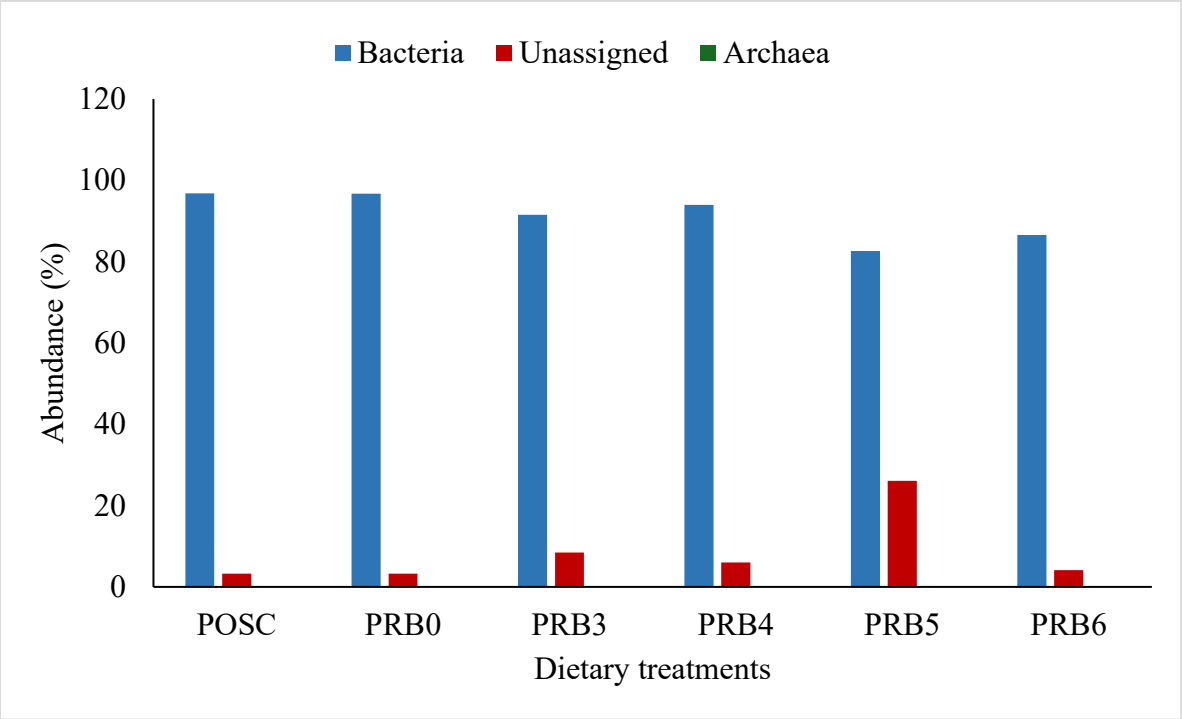

Figure 4.3: Bar graph showing microbial communities found in caecal digesta of broiler chickens fed incremental dosages of *Bacillus subtilis* probiotic mixture at kingdom rank. [Dietary treatments: POSC = Positive control diet with 0.5g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin antibiotic and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture.]

4.5.2.4 Phylum

Among the identified bacteria at the kingdom rank, 14 phyla were detected. Figure 4.4 shows that some microbial phyla were consistently present across diets, whereas others were observed only in specific diets. The phylum *Verrucomicrobiota* was the most abundant phylum overall and was detected in all but dietary treatment PRB5. The relative abundance of these species within the diets ranged from 48% to 79%. P*seudomonadota*, was ranked as the second most abundant phylum found across the diets, excluding PRB3 and PRB4 (58%), with the highest percentage in the PRB5 *B. subtilis*-supplemented diet. *Bacillota*, *Actinomycota*, *Bacteroidota*,

and *Bacillota A* were detected across all diets, with average abundances of 17%, 11%, 6%, and 2%, respectively. In contrast, the phylum *Asgardarchaeota* was detected only in the caecal digesta from broiler chickens receiving PRB6 supplementation at 29%.

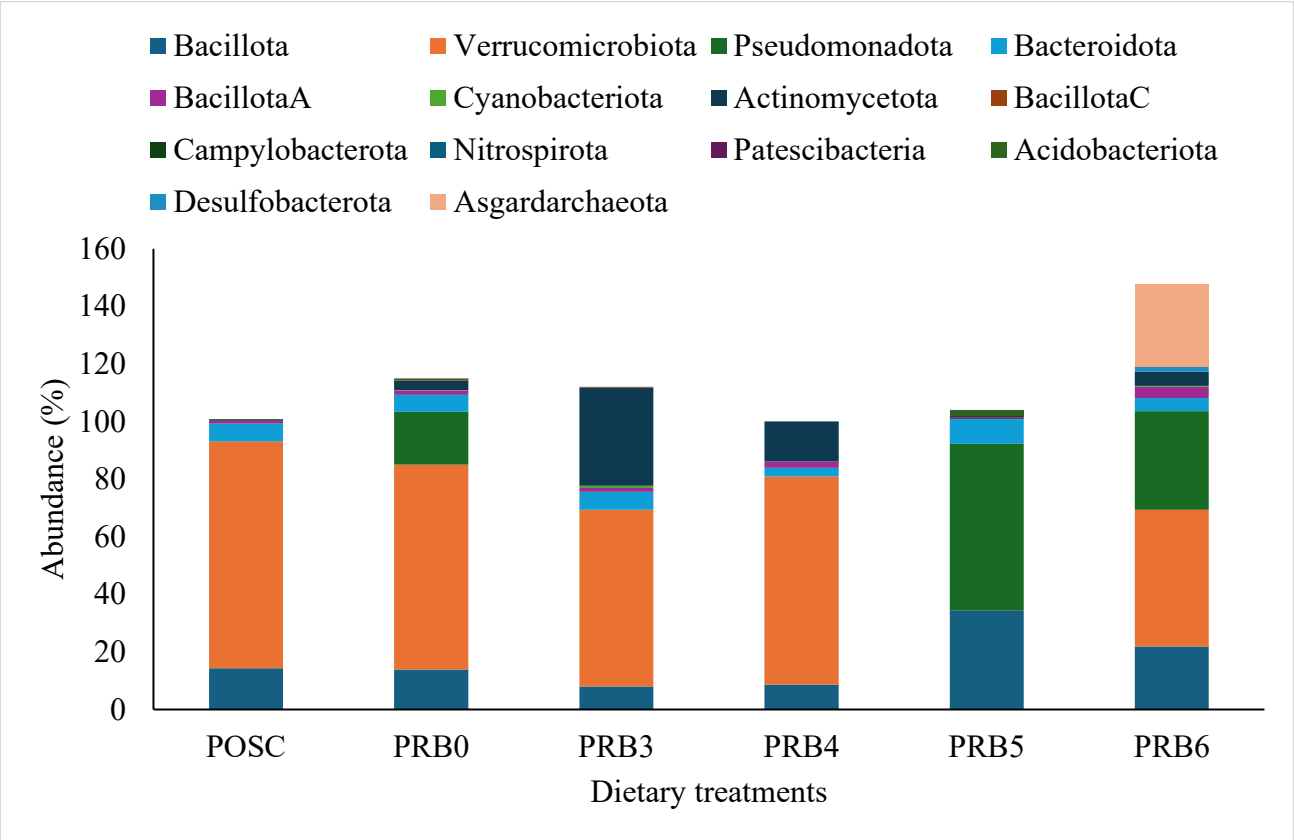

Figure 4.4: Bar graph showing the most abundant microbial communities found in caecal digesta of broiler chickens fed incremental dosages of *Bacillus subtilis* probiotic mixture at the phylum level.
[Dietary treatments: POSC = Positive control diet with 0.5 g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin antibiotic and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture.]

4.5.2.5 Class

Nineteen (19) microbial communities were detected at the class taxonomic rank (Figure 4.5).

The most dominant class in caecal digesta was *Verrucomicrobiae*, which was found in all diets

except diet PRB5, with a 65% abundance. *Lokiarchaeia*, ranked second, was observed only in PRB6 at 29%, while *Gammaproteobacteria* (26%) was observed solely in PRB5 and PRB6. *Alphaproteobacteria* (18%) were present in other diets but absent in caecal digesta from broiler chickens receiving PRB3 and PRB4 diets. Excluding diet PRB5, *Coriobacteria* (13%) were present in all diets, whereas *Bacilli*, *Bacteroidia*, and *Clostridia* were present in all dietary treatments with abundances of 17%, 5%, and 2%, respectively. *Clostridia* were the least dominant across the identified classes in the top ten.

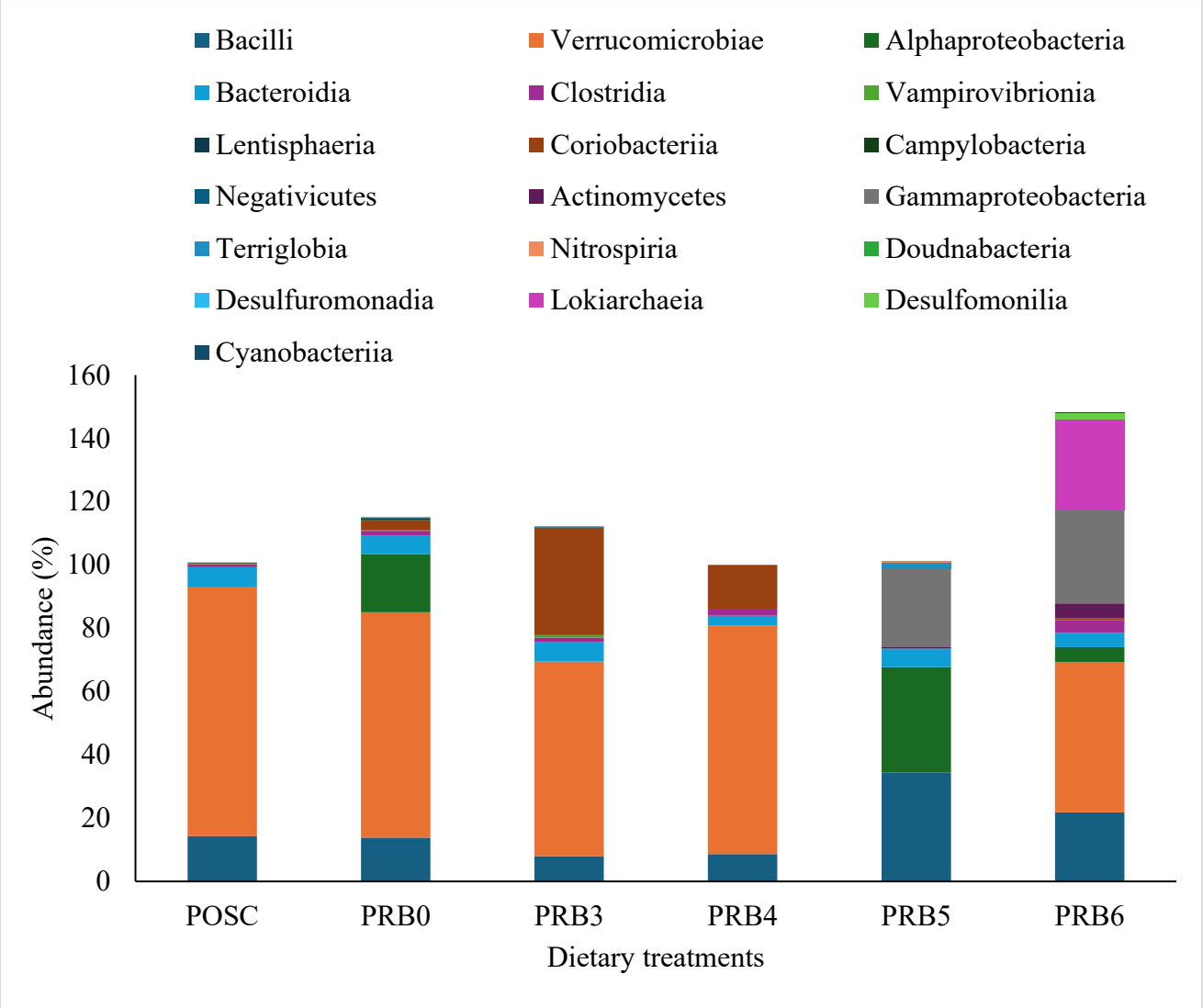

Figure 4.5: Bar graph showing the most abundant microbial communities found in caecal digesta of broiler chickens fed incremental dosages of *Bacillus subtilis* probiotic mixture at the class level.
[Dietary treatments: POSC = Positive control diet with 0.5 g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin antibiotic and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture.]

4.5.2.6 Order

The 16S report identified 48 orders (Figure 4.6), with the most abundant at the order taxonomic rank being *Verrucomicrobiales* (71%), observed in all dietary treatments except diet PRB5. In diet PRB6, the subsequent relative abundance was observed for *Sigynarchaeales* (29%) and *Exiguobacterales* (26%), but in chickens fed PRB5, *Burkholderiales* (29%) and *Micropepsales* (16%) were the predominant orders. Caecal digesta from the PRB0 group uniquely contained *RF32* at 18%. Furthermore, *Lactobacillales* (19%) were present in all dietary treatments, whereas *Coriobacteriales* were absent in diet PRB5, with a relative abundance of 16% in the other diets. *Rhizobiales* was detected in PRB5- and PRB6-fed chickens with a relative abundance of 10%. Moreover, *Chitinophagales* was the least abundant among the top 10, appearing only in diet PRB5 at 8%.

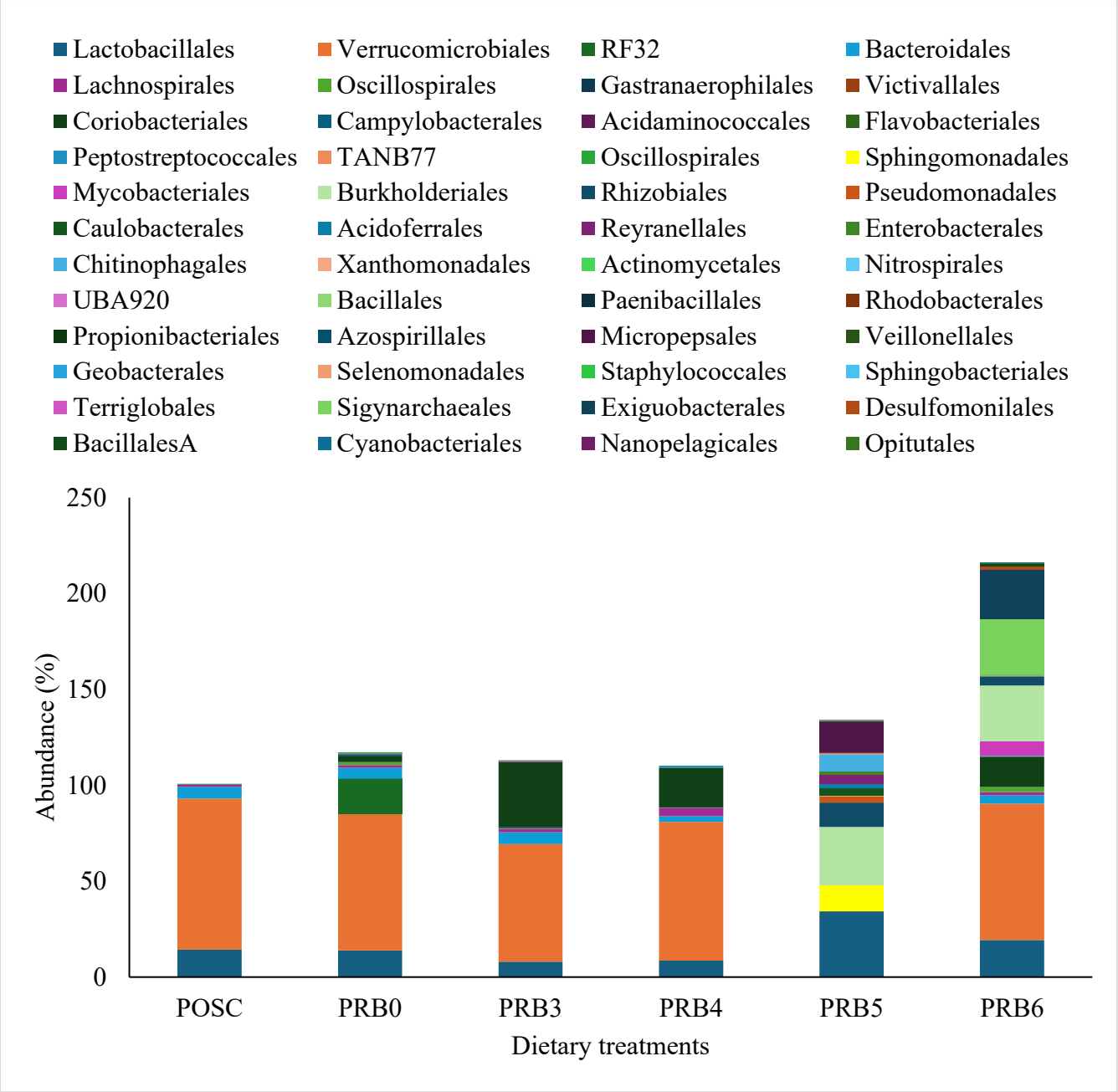

Figure 4.6: Bar graph showing the most abundant microbial communities found in caecal digesta of broiler chickens fed incremental dosages of *Bacillus subtilis* probiotic mixture at the order level.

[Dietary treatments: POSC = Positive control diet with 0.5g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin antibiotic and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture.]

4.5.2.7 Family

Analysis of caecal digesta at the family taxonomic rank identified 75 microbial families, as shown in Figure 4.7. The *Akkermansiaceae* family was the most dominant, with a relative abundance of 70% across all diets, except for the PRB5 diet. The second and subsequent abundant families were SOKP01, *Exiguobacteraceae*, and *Micropepsaceae*, at 29, 26, and 16%, respectively, which were observed in caecal digesta from broiler chickens fed PRB6. *Burkholderiaceae* had a relative abundance of 27% in diets PRB5 and PRB6 only, while UBA3637 was exclusively observed in diet PRB0 at 18%. Family *Atopobiaceae* was absent from the caecal digesta of chickens fed diets POSC, PRB5, and PRB6, but present in that of caecal digesta of chickens fed PRB0, PRB3, and PRB4 at 15%. The families detected solely in diet PRB5 consisted of *Xanthobacteraceae* and *Sphingomonadaceae*, with relative abundances of 13% and 8%, respectively. Across all the dietary treatments, *Lactobacillaceae* (10%) was the least abundant of the top ten.

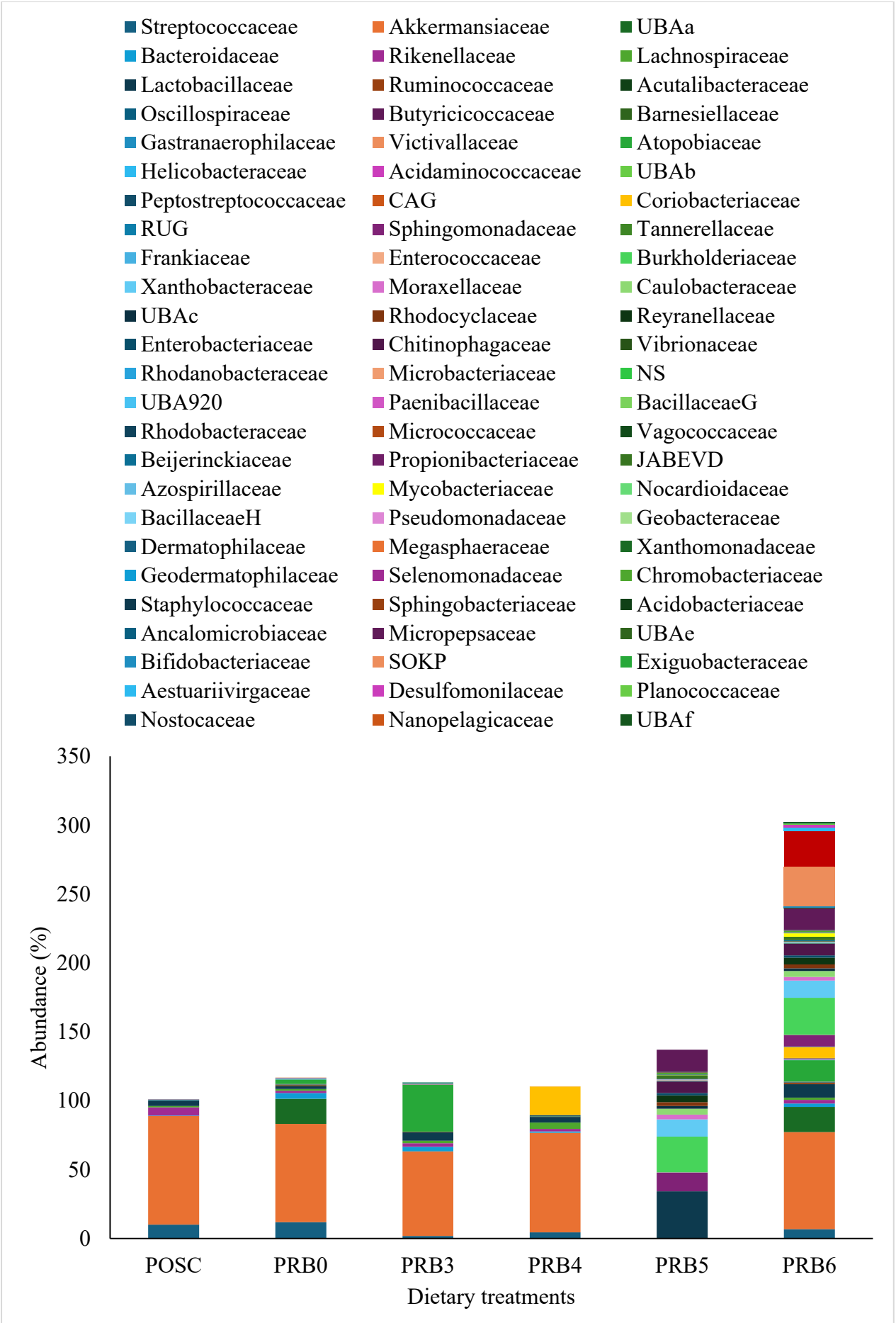
Streptococcaceae
Bacteroidaceae
Lactobacillaceae
Oscillospiraceae
Gastranaerophilaceae
Helicobacteraceae
Peptostreptococcaceae
RUG
Frankiaceae
Xanthobacteraceae
UBAc
Enterobacteriaceae
Rhodanobacteraceae
UBA920
Rhodobacteraceae
Beijerinckiaceae
Azospirillaceae
BacillaceaeH
Dermatophilaceae
Geodermatophilaceae
Staphylococcaceae
Ancalomicrobiaceae
Bifidobacteriaceae
Aestuariivirgaceae
Nostocaceae
Akkermansiaceae
Rikenellaceae
Ruminococcaceae
Butyricicoccaceae
Victivallaceae
Acidaminococcaceae
CAG
Sphingomonadaceae
Enterococcaceae
Moraxellaceae
Rhodocyclaceae
Chitinophagaceae
Microbacteriaceae
Paenibacillaceae
Micrococcaceae
Propionibacteriaceae
Mycobacteriaceae
Pseudomonadaceae
Megasphaeraceae
Selenomonadaceae
Sphingobacteriaceae
Micropepsaceae
SOKP
Desulfomonilaceae
Nanopelagicaceae
UBAa
Lachnospiraceae
Acutalibacteraceae
Barnesiellaceae
Atopobiaceae
UBAb
Coriobacteriaceae
Tannerellaceae
Burkholderiaceae
Caulobacteraceae
Reyranellaceae
Vibrionaceae
NS
BacillaceaeG
Vagococcaceae
JABEVD
Nocardioidaceae
Geobacteraceae
Xanthomonadaceae
Chromobacteriaceae
Acidobacteriaceae
UBAe
Exiguobacteraceae
Planococcaceae
UBAf
350
300
250
200
150
100
50
0
Abundance (%)
POSC
PRB0
PRB3
PRB4
PRB5
PRB6
Dietary treatments

Figure 4.7: Bar graph showing the most abundant microbial communities found in caecal digesta of broiler chickens fed incremental dosages of *Bacillus subtilis* probiotic mixture at the family level.

[Dietary treatments: POSC = Positive control diet with 0.5g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin antibiotic and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture.]

4.5.2.8 Genus

Analysis of caecal digesta of broiler chickens reared on *B. subtilis*-supplemented and control diets revealed 137 genera (Figure 4.8). *Akkermansia* was the most dominant genus, accounting for 70% of the relative abundance and detected in all diets except PRB5. The second most abundant genus, *Limosilactobacillus* (59%), was detected only in PRB5, along with *Rhizomicrobium* (16%) and *Bradyrhizobium* (12%). *Collinsella* (40%) was detected solely in PRB4, while RGIG was present only in PRB0 at 18%. Genera detected exclusively in PRB6 included SOKP01 (29%), *Exiguobacterium A* (26%), and *Limnohabitans* (12%). Lastly, *Thermophilibacter* (15%), was observed in PRB0, PRB3, and PRB4.

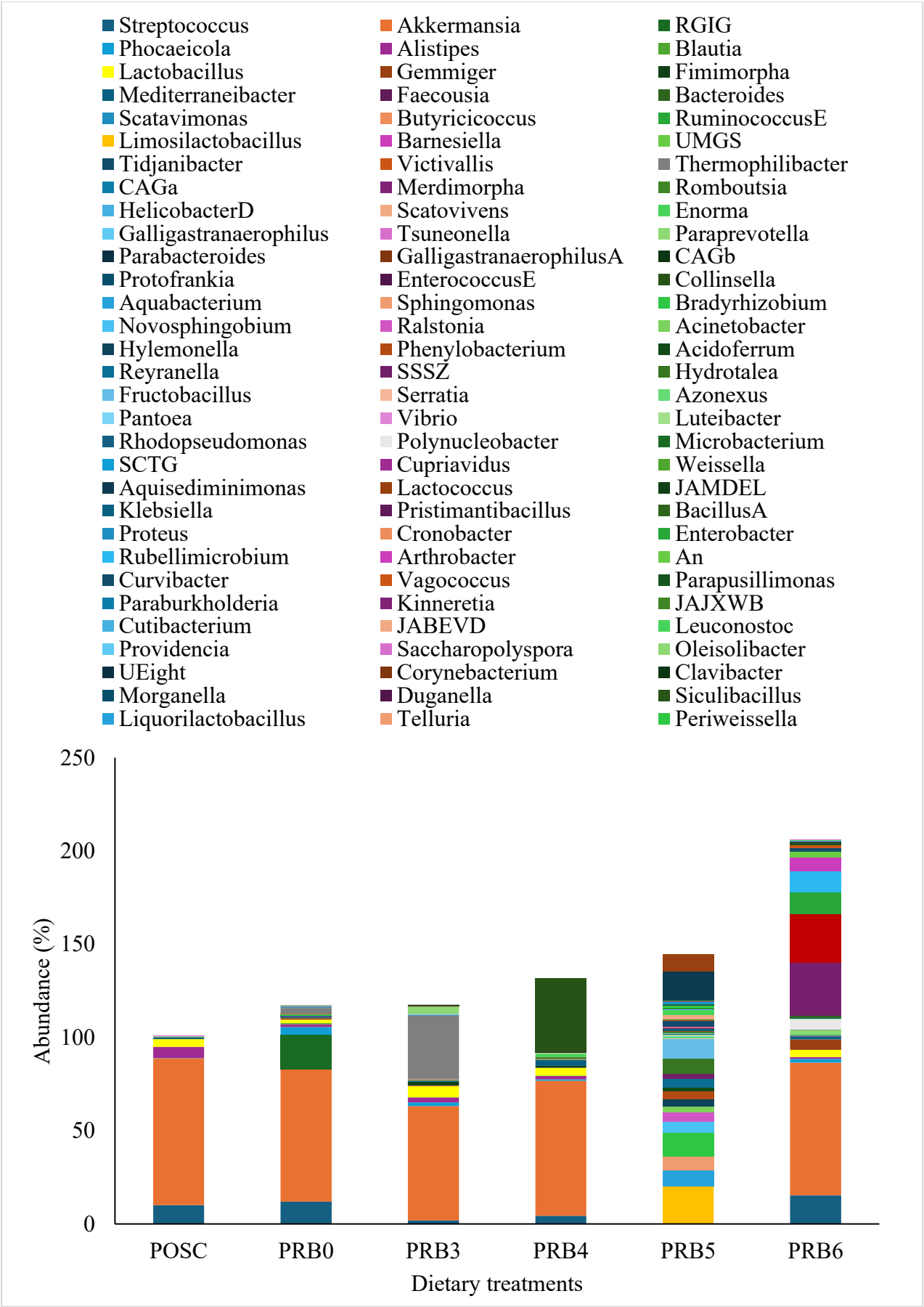
Streptococcus
Phocaeicola
Lactobacillus
Mediterraneibacter
Scatavimonas
Limosilactobacillus
Tidjanibacter
CAGa
HelicobacterD
Galligastranaerophilus
Parabacteroides
Protofrankia
Aquabacterium
Novosphingobium
Hylemonella
Reyranella
Fructobacillus
Pantoea
Rhodopseudomonas
SCTG
Aquisediminimonas
Klebsiella
Proteus
Rubellimicrobium
Curvibacter
Paraburkholderia
Cutibacterium
Providencia
UEight
Morganella
Liquorilactobacillus
Akkermansia
Alistipes
Gemmiger
Faecousia
Butyricicoccus
Barnesiella
Victivallis
Merdimorpha
Scatovivens
Tsuneonella
GalligastranaerophilusA
EnterococcusE
Sphingomonas
Ralstonia
Phenylobacterium
SSSZ
Serratia
Vibrio
Polynucleobacter
Cupriavidus
Lactococcus
Pristimantibacillus
Cronobacter
Arthrobacter
Vagococcus
Kinneretia
JABEVD
Saccharopolyspora
Corynebacterium
Duganella
Telluria
RGIG
Blautia
Fimimorpha
Bacteroides
RuminococcusE
UMGS
Thermophilibacter
Romboutsia
Enorma
Paraprevotella
CAGb
Collinsella
Bradyrhizobium
Acinetobacter
Acidoferrum
Hydrotalea
Azonexus
Luteibacter
Microbacterium
Weissella
JAMDEL
BacillusA
Enterobacter
An
Parapusillimonas
JAJXWB
Leuconostoc
Oleisolibacter
Clavibacter
Siculibacillus
Periweissella
250
200
150
100
50
0
Abundance (%)
POSC
PRB0
PRB3
PRB4
PRB5
PRB6
Dietary treatments

Figure 4.8: Bar graph showing the most abundant microbial communities found in caecal digesta of broiler chickens fed incremental dosages of *Bacillus subtilis* probiotic mixture at the genus level.
[Dietary treatments: POSC = Positive control diet with 0.5g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin antibiotic and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture.]

4.5.2.9 Species

The metagenomic report identified 208 species; the top 10 most abundant are shown in Figure 4.9. *Akkermansia intestinigallinarum* was the most dominant species, detected in the caecal digesta of chickens across all diets except the PRB5 diet, with a relative abundance of 70%. *Thermophilibacter avicola* was found solely in caecal digesta of chickens fed PRB3, with a relative abundance of 65%, whereas Collinsella was detected only in caecal digesta from broiler chickens receiving PRB4 at 40%. SOKP01, *Exiguobacterium A acetylicum*, and *Limnohabitans* were exclusively found in diet PRB6, with relative abundances of 34%, 17%, and 14%, respectively. Furthermore, *Limosilactobacillus fermentum* (20%), *Rhizomicrobium electricum* (16%) were identified only in the caecal digesta from the chickens reared on PRB5 only, while *RGIG* (18%) was present only in diet PRB0. Finally, *Phocaeicola Coprocola* (14%) was the least dominant of the top 10 and detected in PRB0, PRB3, and PRB6.

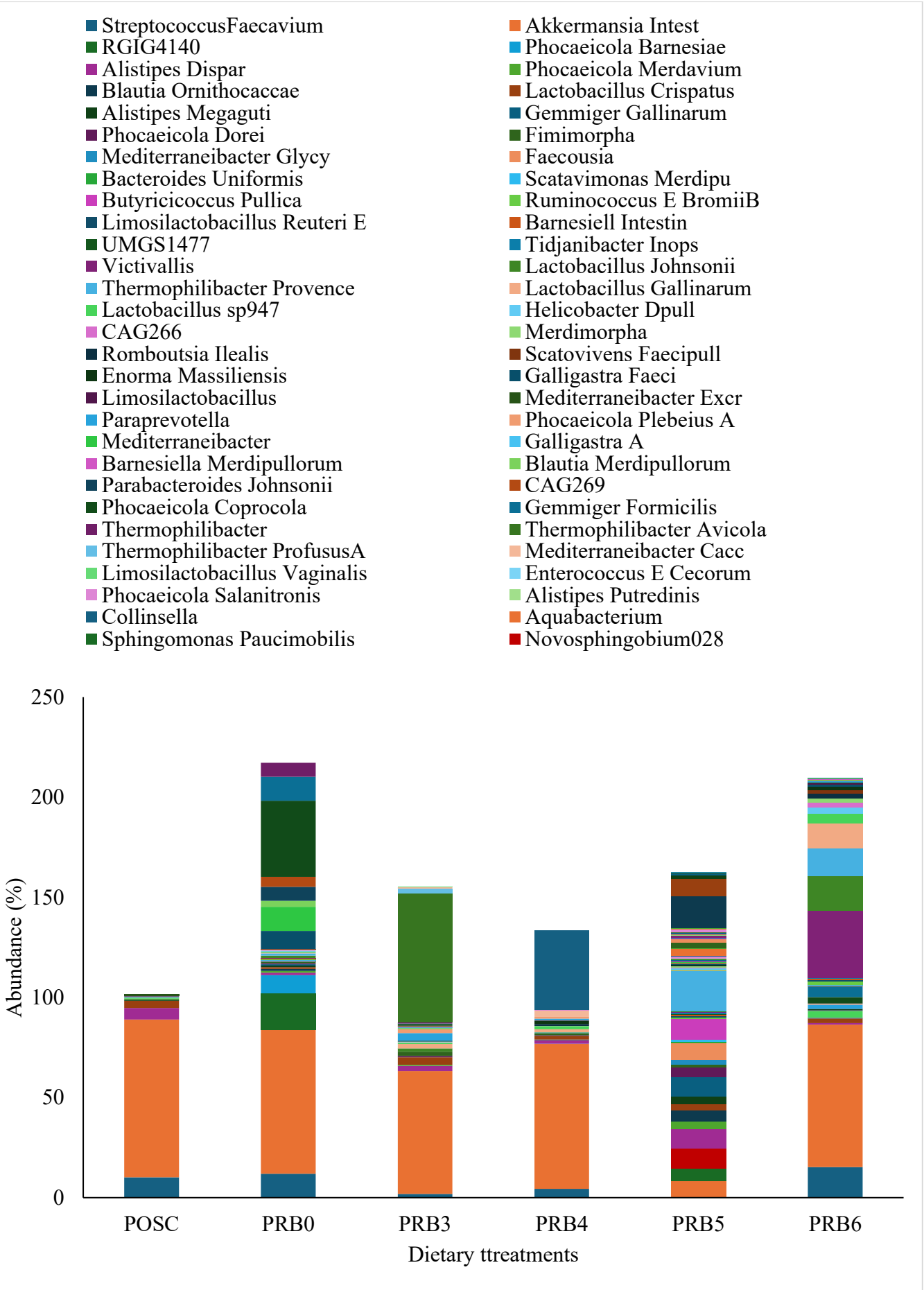
StreptococcusFaecavium
RGIG4140
Alistipes Dispar
Blautia Ornithocaccae
Alistipes Megaguti
Phocaeicola Dorei
Mediterraneibacter Glycy
Bacteroides Uniformis
Butyricicoccus Pullica
Limosilactobacillus Reuteri E
UMGS1477
Victivallis
Thermophilibacter Provence
Lactobacillus sp947
CAG266
Romboutsia Ilealis
Enorma Massiliensis
Limosilactobacillus
Paraprevotella
Mediterraneibacter
Barnesiella Merdipullorum
Parabacteroides Johnsonii
Phocaeicola Coprocola
Thermophilibacter
Thermophilibacter ProfususA
Limosilactobacillus Vaginalis
Phocaeicola Salanitronis
Collinsella
Sphingomonas Paucimobilis
Akkermansia Intest
Phocaeicola Barnesiae
Phocaeicola Merdavium
Lactobacillus Crispatus
Gemmiger Gallinarum
Fimimorpha
Faecousia
Scatavimonas Merdipu
Ruminococcus E BromiiB
Barnesiell Intestin
Tidjanibacter Inops
Lactobacillus Johnsonii
Lactobacillus Gallinarum
Helicobacter Dpull
Merdimorpha
Scatovivens Faecipull
Galligastra Faeci
Mediterraneibacter Excr
Phocaeicola Plebeius A
Galligastra A
Blautia Merdipullorum
CAG269
Gemmiger Formicilis
Thermophilibacter Avicola
Mediterraneibacter Cacc
Enterococcus E Cecorum
Alistipes Putredinis
Aquabacterium
Novosphingobium028
250
200
150
100
50
0
Abundance (%)
POSC
PRB0
PRB3
PRB4
PRB5
PRB6
Dietary ttreatments

Figure 4.9: Bar graph showing the most abundant microbial communities found in caecal digesta of broiler chickens fed incremental dosages of *Bacillus subtilis* probiotic mixture at the species level.
[Dietary treatments: POSC = Positive control diet with 0.5g/kg zinc bacitracin but without a novel *B. subtilis* probiotic mixture; PRB0 = a negative control diet without a zinc bacitracin antibiotic and a novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of a novel *B. subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of a novel *B. subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of a novel *B. subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of a novel *B. subtilis* probiotic mixture.]

4.6 Discussion

4.6.1 Jejunal histomorphology

Several factors, among them gut microbiota and morphology, are important indicators of gut health in broiler chickens. Crypt depth and villus height are essential determinants of intestinal cell maturity, nutrient digestion, and absorption, respectively (Zhang *et al*., 2021). In the current study, a supplemental *B. subtilis* probiotic was shown to affect jejunal VASA in broiler chickens, with chickens fed PRB5 exhibiting the largest jejunal VASA, and counterparts fed PRB6 having reduced jejunal VASA compared to the rest. These observed jejunal VASA suggest that the *B. subtilis* probiotic blend induced structural changes in the intestines, which are associated with enhanced nutrient absorption, a strengthened immune response, and overall gut health (Memon *et al*., 2021; Berto *et al*., 2024).

In a separate study, although probiotics did not affect VH:CD in broiler chickens, they increased VASA by stimulating epithelial cell proliferation (Olnood *et al*., 2015; Idowu *et al*., 2025). The observed increase in jejunal VASA in Ross 308 broiler chickens fed PRB5 is speculated to have increased the jejunal absorptive area, resulting in the observed improved growth performance. However, in counterparts reared on PRB6, the reduced VASA was most likely due to dilution of the diet by the increased probiotic content, which may have

compromised nutrient supply to the birds, resulting in reduced BWG and higher FCR. The current study's findings are in line with the observed improvement in the VH:CD ratio in Cobb 500 broiler chickens fed diets fortified with *B. subtilis* DSM 32315 and *Bacillus amyloliquefaciens* (Berto *et al*., 2025). Similarly, Oladokun & Adewole (2023) and Bromfield *et al*. (2024) reported significant improvements in jejunal morphology in broiler chickens. Current study findings provide some insight into the mechanisms by which probiotics mediate positive outcomes in broiler chickens (Berto *et al*., 2024; Rodrigues *et al*., 2024), which strengthens the argument for using them as a feeding technology in place of AGP in broiler chicken production.

4.6.2 Gut microbiota phylogenetic composition

Several factors, among them the surrounding environment, dietary supplementation, pathological conditions, breed, genetics, age, and others, affect intestinal microbial composition (Xiao *et al*., 2017). Phylum *Verrucomicrobiota*, *Pseudomonadota*, *Asgardarchaeota*, and *Bacillota* were the most dominant microflora. Some of these bacterial communities benefit the host by suppressing pathogenic proliferation, supporting the growth and integrity of intestinal mucosa, strengthening barrier function, and reducing intestinal toxins (Liu *et al*., 2025). For instance, *Verrucomicrobiota* has recently been documented to possess substantial glycoside hydrolase genes, enabling the breakdown of complex carbohydrates into monosaccharides and amino acids that support the proliferation of other beneficial microbes (Wang *et al*., 2024). Furthermore, among this phylum, *Akkermansia muciniphila* is the most prominent species, recognized for its role in mucin degradation, which helps preserve the mucosal layer, stimulates the production of prebiotics and SCFA, and thereby improves intestinal health and nutrient absorption (Wang *et al*., 2024).

Conversely, Feng *et al*. (2023) reported a decrease in *Verrucomicrobiota* and an increase in Firmicutes and Bacteroidota with *Bacillus* probiotics supplementation. Pseudomonadota, formerly known as Proteobacteria, was abundant in PRB5. Some members of this phylum can be pathogenic, while others aid in maintaining a healthy gut by lowering the redox potential, which favours the growth of microbes associated with SCFA (Zhan *et al*., 2025). The abundance of Pseudomonadota seems to correlate positively with improved BWG and reduced FCR in broiler chickens with a diet containing PRB5. In addition, *Bacillota* was more dominant in caecal digesta from broiler chickens fed a diet containing PRB5, which is associated with improved BWG (Deryabin *et al*., 2024). Asgardarchaeota is an understudied group in relation to its role in broiler chickens; therefore, further investigation is needed to explore the role of this phylum at the species taxonomic rank in the chicken gut microbiota. At the genus taxonomic rank, *Akkermansia*, SOKP01, *Collinsella*, *Exiguobacterium A*, and *Rhizomicrobium* were most abundant, with others being present in other groups while absent in others. *Exiguobacterium A* species are known to synthesize several enzymes, including alkali-tolerant esterase, among others, which contribute to the biodegradation of complex compounds (Pandey, 2020).

Furthermore, *Collinsella* belongs to the phylum Actinobacteria, which is known to be present at low abundance but is vital for producing vitamins, particularly vitamin B. Additionally, Collinsella contributes to gut health by hindering the growth of pathogenic microbes through the production of antimicrobial compounds, ultimately improving immune function (Obianwuna *et al*., 2024). This genus was detected only in broiler chickens provided a diet supplemented with PRB4, and its function may have contributed to reduced inflammation and stress, as reflected in haemato-clinical biochemistry. *Bacillus* probiotics are known to induce shifts in the gut microbiota of broiler chickens (Jacquier *et al*., 2019). In this study, the detection of SOKP01 exclusively in birds fed PRB6 provides novel evidence of its presence, as it has not

been previously reported. The highest dose of *B. subtilis* in broiler chickens fed PRB6 likely disrupted the balance of beneficial microbes by competing for nutrients, potentially allowing the introduction of new species. This microbial imbalance may have contributed to the observed poor performance in this group.

Furthermore, *Rhizomicrobium* is commonly found in soil and is typically absent from the animal gut, as it has not been widely studied in the broiler gut. In this study, this genus was detected exclusively in PRB5, which improved growth performance; however, the primary mechanism or correlation remains unknown. This necessitates further research on this genus, which may benefit broiler gut health. While previous studies reported Firmicutes and Bacteroidota as the dominant phyla in *Bacillus* probiotics fed to broiler chickens (Feng *et al*., 2023), with *Verrucomicrobiota* being less abundant but beneficial, this study found *Verrucomicrobiota* to be the most abundant across all diets, except for the PRB5 diet. However, the abundance of *Bacillota* and *Pseudomonadota* detected in the PRB5 group suggested that *Bacillota* and the introduced microbes likely contributed to achieving growth and reducing inflammation.

4.7 Conclusion

Supplementing broiler diets with *B. subtilis* probiotic mixture at 0.5% improved jejunal VASA, indicating better absorptive capacity for nutrient uptake and intestinal health. This improvement in jejunal morphology might have been supported by the shifts in gut microbial composition, with beneficial genera including *Akkermansia*, *Limosilactobacillus*, and *Collinsella* in broilers that received probiotic supplemented diets. The PRB5 dietary treatment demonstrated a combined enhancement of villus structure and beneficial microbes, supporting efficient nutrient absorption and immune function. Caecal digesta from PRB6 treatment group various scientifically unknown microbes, which might have disrupted microbial balance, hence

smallest VASA. *Rhizomicrobium*, *Asgardarchaeota* and SOKP01 require further investigation for their roles in broiler gut. The supplementation of *B. subtilis* probiotic mixture at 0.5% may serve as a sustainable alternative to antibiotics for improving intestinal health in broiler production under high stocking density.

4.8 References

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5 CHAPTER 5: GENERAL DISCUSSION, RECOMMENDATIONS, AND CONCLUSIONS

5.1 General discussion

Compared to other meats from other livestock, poultry meat is relatively more affordable by unit weight (Kleyn & Ciacciariello, 2021) and its demand is expected to increase in response to the growing human population (Gu *et al*., 2021). Optimizing broiler chicken production is critical to meeting the demand for animal-source foods and enhancing food security. Intensification of broiler chicken production should target to reduce and eventually eliminate itself from heavy dependence on AGP since their routine use is the major contributor to the public health challenge of antibiotic resistance (Nduku *et al*., 2025), tainting of poultry meat and eggs from antibiotic residues and pollution of the environment from poultry waste containing with toxic AGP metabolites (Izah *et al*., 2025). To mitigate consumer and bird health challenges, as well as the product and environmental pollution caused by AGP use, safe, natural, and acceptable alternatives to AGP need to be explored and evaluated. Among potential alternatives are probiotics, such as *Bacillus subtilis*, which can be used to fortify poultry feeds. *Bacillus subtilis* forms spores and can survive feed processing and gut environment, including the low gastric pH (Williams & Weir, 2024). ~~*Bacillus subtilis*~~These species have been shown to improve bird health and welfare (Sikandar *et al*., 2020), increase ileal nutrient digestive and absorptive function (Chen *et al*., 2025), enhance gut mucosal integrity (Zheng *et al*., 2025) and modulate immune system function (Guo *et al*., 2020) hence this study sought to determine, in densely stocked broiler chickens, the impact of *B. subtilis* probiotic blend efficacy as a feed broiler chicken feed supplements by evaluating its effects on Ross 308 broiler chickens growth performance, ~~gut function~~physiological response, meat quality and microbiota proportion and composition.

Dietary supplementation with *B. subtilis* at 0.5% improved growth performance in Ross 308 broiler chickens, as demonstrated by increased BWG and decreased FCR. The observed improvements are ascribed to the mediation of (i) stress reduction, (ii) gut health and integrity, and (iii) increased ileal digestive production and secretion. The *B. subtilis* probiotic mediated increased ileal digestive enzyme production and secretion (Ghimire *et al*., 2024). This is in tandem with improved gut health and integrity, which are likely drivers of the observed increases in OM, NDF, and GE digestibility, along with the negative quadratic response in CP digestibility, contributing to improved nutrient uptake and growth performance. A reduction in WBC observed in birds fed PRB4 compared to PRB0 indicates that probiotics may help reduce inflammation and support immune homeostasis. This effect is likely achieved through modifying cytokine production and strengthening the intestinal barrier (Naeem & Bourassa, 2025). A decrease in platelet counts suggests reduced inflammation and stress in broiler chickens, as observed in those fed diets PRB3 and PRB5. These improvements may also have contributed to greater growth in birds fed diets PRB4 and PRB5. Broiler chickens fed the PRB5 diet showed a significantly lower duodenum weight, indicating that *B. subtilis* improved nutrient digestion and absorption, enhanced feed conversion efficiency, and redirected more energy toward muscle growth rather than gut maintenance. Moreover, the shorter jejuna observed in broiler chickens fed a PRB3 diet resulted in a lower FCR than in those fed a PRB6 diet, which had longer jejuna. It is suggested that a lower *B. subtilis* probiotic mixture dose might improve FCR despite the shorter jejunum associated with decreased absorptive surface area.

The probiotic increased water-holding capacity while reducing shear force, drip loss, and cooking loss, likely through antioxidant activity associated with *B. subtilis*. However, breast muscles from broilers fed probiotic-supplemented diets showed meat quality values comparable to the POSC and PRB0 groups, indicating no clear improvement in overall meat

quality. The study hypothesized that replacing AGP with a mixture of novel *B. subtilis* probiotics would maintain growth performance, nutrient digestibility, haemato-clinical biochemistry, and meat quality. The findings supported this hypothesis: *B. subtilis* supplementation improved growth performance and haematological parameters, particularly at the PRB5 level, and enhanced nutrient digestibility at all incremental doses compared with control diets. Nevertheless, probiotic-supplemented diets produced meat quality outcomes similar to those observed with zinc bacitracin supplementation for most quality traits.

Chapter 4 evaluated how a *B. subtilis* probiotic mixture alters jejunal histology and the phylogenetic composition of the caecal microbiota in broiler chickens raised under high stocking density. Crypt depth and villus height directly determine epithelial cell maturity, nutrient digestion, and absorption (Wang *et al*., 2025). The observed changes in jejunal morphology demonstrate that supplemental *B. subtilis* probiotic mixture induces structural adaptations in the jejunum (Berto *et al*., 2024). Broiler chickens fed the PRB5 diet showed increased jejunal VASA, reflecting improved nutrient digestibility that directly enhanced growth performance. Reduced stress and inflammation likely further improved digestibility, nutrient uptake, and growth. The gut microbiome regulates immune function and suppresses pathogenic microbes; shifts in its composition modulate gut health (Cheng *et al*., 2025). PCoA showed that chickens fed PRB5 and PRB6 harboured distinct caecal microbial communities absent in other diets, confirming that probiotic supplementation actively shifted the gut microbiome by introducing new microbial populations in broilers reared under densely-stocked conditions.

Some of the detected genera have unclear functions, but others support gut health by strengthening the intestinal barrier, producing antimicrobial compounds, aiding the breakdown of complex substrates, and synthesizing vitamins essential for growth and overall bird welfare (Broom & Kogut, 2018). The caecal microbial communities directly supported improved

growth performance, enhanced nutrient digestibility, and better jejunal histomorphology, while also reducing stress and inflammation in broiler chickens reared under high stocking density. Broilers fed diets fortified with *B. subtilis* probiotic at 0.4% and 0.5% outperformed the control diets, whereas the 0.6% inclusion was less effective.

This chapter hypothesized that replacing AGP with a *B. subtilis* probiotic mixture would maintain jejunal histomorphology and the phylogenetic composition of the caecal microbiota in broilers raised under high density. The results supported this hypothesis: a 0.5% inclusion of *B. subtilis* increased jejunal VASA, indicating improved nutrient absorption, while PRB6 produced the lowest VASA relative to the control diets. Broilers fed *B. subtilis*–supplemented diets predominantly contained beneficial microbes, although the functions of some taxa, particularly in the PRB5 group, remain unclear. In contrast, the PRB6 diet increased the abundance of microbes with unknown scientific classification.

5.2 Conclusion

In conclusion, supplementation with a *B. subtilis* probiotic mixture improves growth performance by promoting efficient digestion and absorption and enhancing overall health by modulating the gut microbiota. However, new microbial communities detected in caecal digesta from broiler chickens fed a *B. subtilis* probiotic mixture require further investigation to better understand their role in the digestive tract and the overall physiology of broiler chickens. The optimum *B. subtilis* probiotic mixture dose in Ross 308 broiler chicken feeds for birds reared under high stocking density is 0.5%, as the higher (0.6%) inclusion level resulted in poor growth performance, likely driven by nutrient dilution and an imbalance in the gut microbiota.

5.3 Recommendations

It is recommended that the *B. subtilis* probiotic mixture be supplemented in broiler feed at 0.5% to improve birds' welfare and productive performance. Additionally, it is not recommended to supplement the broiler diet with excessive doses of a *B. subtilis* probiotic mixture, as this may be detrimental to broiler health and impair growth performance. Further research is needed to assess the efficacy of the genus *Rhizomicrobium* in broiler chicken production, as it is abundant in soil and readily available. The phyla *Asgardarchaeota* and SOKP01, which were observed exclusively in PRB6, require further investigation to determine their roles at the species level in the chicken gut microbiota. Furthermore, the mechanisms by which the *B. subtilis* probiotic mixture improves meat water retention in chicken breast require further investigation. In densely reared broiler chickens, it is recommended that poultry feed producers fortify broiler chicken diets with a *B. subtilis* probiotic mixture at 0.5%, as the current study findings have shown this to be the most efficacious dose/dietary inclusion level for promoting broiler chicken health and welfare and productive performance.

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APPENDICIES

Appendix 1: Ethics approval of the study

UNIVERSITY OF MPUMALANGA
Creating Opportunities

Prof. V. Mlambo
School of Agricultural Sciences
Mbombela Campus.

Dear Prof. Mlambo,

Protocol Reference Number: UMP/Mlambo/1/2024

Project Title: "Gut functionality biomarkers and related parameters in broilers fed nucleotides and novel probiotics".

Approval Notification in response to your application in March 2024. After considering the above-mentioned application and protocol, the Research Ethics Committee-Animal Sciences has granted **FULL APPROVAL**.

Any alteration/s to the approved research protocol i.e. Questionnaire/Interviews Schedule, Informed Consent form, Title of the project, Location of the study, Research Approach and methods must be reviewed and approved through the amendment/ modification prior to its implementation. In case you have further queries, please quote the above reference number.

PLEASE NOTE: Research data should be stored securely in the School/ division for a period of 5 years. The Ethical Clearance certificate is only valid for a period of 3 years from 2024 to 2026. Thereafter, Recertification must be applied for on an annual basis.

Wishing you the best with your study.

Yours faithfully,

25/04/24

....

T. Mwabvu (Chairperson) **Date**

Appendix 2: Publication in Veterinary and Animal Science

Appendix 2: Publication in Veterinary and Animal Science

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Dietary supplementation with a multi-strain *Bacillus subtilis* probiotic improves digestive efficiency, growth performance, and meat quality in densely stocked broilers

Zamile Mahlangu [a], Caven Mguvane Mnisi [b], Victory Osirimade Sumanu [a,b], Ghaneshree Moonsamy [c], Omolola Esther Fayemi [b], Rajesh Jha [b,d], Victor Mlambo [a,b,*]

[a] School of Agricultural Sciences, Faculty of Agriculture and Natural Sciences, University of Mpumalanga, Mbombela, South Africa
[b] Food Security and Safety Focus Area, Faculty of Natural and Agricultural Sciences, North-West University, Mafikeng, South Africa
[c] Council for Scientific and Industrial Research (CSIR), Future Production: Chemicals, Meiring Naude Drive, Pretoria, South Africa
[d] Department of Human Nutrition, Food and Animal Sciences, University of Hawaii at Manoa, USA

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ABSTRACT

Bacillus subtilis has attracted attention as a robust probiotic candidate to replace antibiotic growth promoters (AGPs) in poultry, owing to its spore-forming resilience and capacity to modulate gut function. However, inclusion levels and the efficacy of multi-strain *B. subtilis* mixtures remain underexplored, particularly at high stocking densities, when birds are more susceptible to physiological and environmental stressors. This study evaluated the effects of incremental levels of a multi-strain *B. subtilis* probiotic mixture on growth performance, nutrient digestibility, physiological responses, carcass traits, and meat quality of broiler chickens reared under intensive conditions. A total of 900 one-day-old Ross 308 broiler chicks were assigned to six dietary treatments in a completely randomized design, with 36 pens (13 birds/0.75 m^2) serving as experimental units. Diets comprised a basal diet without additives (PRB0), a positive control containing zinc bacitracin (POSC), and four diets supplemented with 0.3, 0.4, 0.5, or 0.6% of the *B. subtilis* probiotic mixture (PRB3 – PRB6). Diets were fed from the starter to the finisher phases. Probiotic supplementation influenced growth responses across the graded inclusion levels. Birds receiving the 0.5% inclusion level consistently exhibited the most favourable body weight gain and feed conversion efficiency compared with the other treatments. Probiotic supplementation enhanced the digestibility of organic matter, neutral detergent fibre, and gross energy, whereas crude protein digestibility exhibited a quadratic response. Haemato-biochemical profiles suggested improved physiological status in birds receiving mid-level probiotic inclusions (PRB3 – PRB5). Carcass yield and major meat quality traits were unaffected by dietary treatments, while minor effects on gut morphological indices were observed. Collectively, these findings suggest that a 0.5% inclusion level may be a promising probiotic supplementation strategy for broiler production under high stocking density.

Introduction

Broiler production is a key agricultural subsector globally, providing an affordable source of dietary protein to support the needs of an expanding human population. Due to high production costs and increased demand for affordable poultry products, poultry producers have increased broiler production by raising stocking density. However, growth promoters (AGPs) have been widely implemented to mitigate diseases and oxidative stress associated with high-density rearing conditions, while also enhancing feed efficiency, growth rates, and the overall health of broiler chickens (Nduku et al., 2025). However, growing concerns about antibiotic-resistant microorganisms and the accumulation of antibiotic residues in poultry products have led most countries to ban AGPs in food-producing animals, with exceptions