Impact of phased nucleotide supplementation on gut function, performance, and meat quality in densely stocked broilers

Nicole Mmatsie Moreane

orcid.org/0009-0002-0747-069X

201861755

A dissertation submitted for the Master of Science degree in Agriculture

Supervisor: Prof. V. Mlambo

Co-supervisors: Prof. C.M. Mnisi [North-West University]

Prof. O.E. Fayemi [North-West University]

Dr G. Moonsamy [Council for Scientific and Industrial Research]

School of Agricultural Sciences

Faculty of Agriculture and Natural Sciences

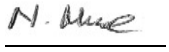
May 2026

UNIVERSITY OF MPUMALANGA

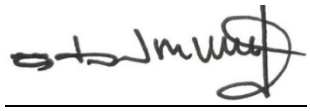
DECLARATION

I, Nicole Mmatsie Moreane, confirm that this MSc thesis has only been submitted to the University of Mpumalanga for examination. This work was done under the supervision of Prof. V. Mlambo, Prof. C.M. Mnisi, Prof. O.E. Fayemi, and Dr. G. Moonsamy. All information sources and materials have been fully acknowledged.

Student: Nicole Moreane (201861755)

Signature:  Date: 16/10/2025

Main Supervisor: Prof. V. Mlambo

Signature:  Date: 16/10/2025

Co-Supervisor: Prof. C.M. Mnisi

Signature:  Date: 20/10/2025

Co-Supervisor: Prof. O.E. Fayemi

Signature:  Date: 20/10/2025

Co-Supervisor: Dr. G. Moonsamy

Signature  Date: 20.10.2025

GENERAL ABSTRACT

Poultry production plays a significant role in ensuring food and nutrition security in South Africa and beyond. However, this enterprise relies heavily on antibiotic growth promoters (AGP) to control disease outbreaks and boost growth performance. The use of AGP is increasingly frowned upon due to their role in antimicrobial resistance and unsafe antibiotic-residue-rich poultry products. As such, there is a need for AGP alternatives that would ensure that sustainable poultry production is economically efficient, environmentally friendly, and promotes consumer health. Exogenous dietary nucleotides are increasingly investigated as potential alternatives to AGP; however, their continuous supplementation across the broiler production cycle may impose higher costs. Given that nucleotides are conditionally essential nutrients, required primarily during periods of rapid growth, stress, immunological challenge, or tissue repair; strategic, phase-specific inclusion rather than continuous provision may be both biologically justified and economically advantageous. Therefore, this study investigated the comparative effects of phased vs continuous yeast-based nucleotide supplementation on growth performance, physiological function, meat quality parameters, and gut microbiome in densely stocked Ross 308 broilers. This study is split into two experimental chapters (3 and 4). Chapter 3 presents the effects of phased yeast-based nucleotide supplementation on growth performance, haemato-biochemical parameters, and meat quality traits. Chapter 4 investigates how phased supplementation with yeast-based nucleotides affected visceral morphometry and gut microbiome of the broilers. The feeding trial was carried out at Rooigrond poultry farm, near Mahikeng (North West, South Africa). A total of 900, one-week-old, unsexed Ross 308 broilers were evenly allotted to 36 experimental units (25 birds/1.2 m^2), to which six feeding strategies were randomly allocated in a completely randomized design. The feeding strategies were: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of

AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF). Body weight and feed intake measurements were recorded weekly. Mortalities were recorded daily and replaced with birds of similar weights to maintain stocking density and correct the feed conversion ratio. The collection of blood was performed on day 40 of the feeding trial. Feeding strategies influenced ($p < 0.05$) feed intake, weight gain, and feed conversion ratio. The positive control outperformed ($p < 0.05$) all the feeding strategies in relation to growth performance, while phased and continuous supplementation with yeast-based nucleotides yielded similar results. In addition, phased yeast-based nucleotide supplementation significantly ($p < 0.05$) increased heterophils, platelet and monocyte count, and blood urea nitrogen, albumin, alanine aminotransferase, and total bilirubin. This indicates that phased supplementation with yeast-based nucleotides can enhance immunomodulatory responses and increase metabolic activity as a stress response mechanism, thereby rendering phased supplementation efficient. Birds were slaughtered at 6 weeks of age for post-mortem measurements and analysis. Meat tenderness and drip loss were the only parameters influenced by feeding strategies. No effects were observed on carcass yield, meat pH, colour, water holding capacity, and cooking loss. In Chapter 4, the sizes of proventriculus, ventriculus, spleen, liver, duodena, jejuna, and caeca were not affected by feeding strategy ($p > 0.05$). However, the positive control notably increased the ileal size. The alpha and beta diversity analysis revealed similarity across all feeding strategies. Statistical analysis revealed that the relative abundance of *Alistipes inops* species was higher in the negative control and yeast-based nucleotide-supplemented broiler caeca. Although they did not differ ($p > 0.05$) across the feeding strategies, the five most abundant phyla and genera indicated that birds had healthy gut functions. It was concluded that phased and continuous yeast-based nucleotide supplementation resulted in comparable

outcomes in broiler chickens. Therefore, phased supplementation of yeast-based nucleotides in broiler diets is recommended as a cost-cutting strategy.

Keywords: antibiotic growth promoters, economic efficiency, growth performance, gut microbiome, nucleotides

ACKNOWLEDGEMENTS

First and foremost, I thank the Lord Almighty for His grace and strength that enabled me to see this work through completion. Secondly, I would like to express my heartfelt gratitude to my supervisor, Prof. V. Mlambo, co-supervisors, Prof. C.M. Mnisi, Prof. O.E. Fayemi, Dr G. Moonsamy, and Dr V.O. Sumanu for their guidance, support, and patience throughout this journey, and most importantly, their availability and eagerness to always assist where needed.

I thank the National Research Foundation (NRF) for funding my tuition and the research itself, the North-West University for granting me access and usage of their facilities, and the University of Mpumalanga for the opportunity to further my studies. I also appreciate my colleagues and staff members at the North-West University for their selfless support, which greatly contributed to this success. The research activities reported herein were financially supported by the National Research Foundation of South Africa (Grant Number: CPRR23032387169).

Finally, I am grateful to my mother, Nicoline, for her unwavering support and encouragement. To my sister, Tasha, thank you for mimicking everything I do, which has driven me to strive to be the best, and thank you for cheering me on. Lastly, I want to thank my friends for their steady encouragement, support, and for always believing in me.

DEDICATION

This work is dedicated to myself; I persevered and overcame.

LIST OF ABBREVIATIONS

AAT	Alpla-1 antitypsin
ADP	Adenosine diphosphate
AGP	Antibiotic growth promoter
ALB	Albumin
ALT	Alanine transaminase
AMP	Adenosine monophosphate
AMR	Antimicrobial resistance
ANCOM	Analysis of compositions of microbiomes
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BUN	Blood urea nitrogen
BWG	Body weight gain
CoA	Coenzyme A
DL	Drip loss
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
FABP	Fatty acid binding proteins
FAD	Flavin adenine dinucleotide
FCR	Feed conversion ratio
FI	Feed intake
GIT	Gastrointestinal tract
GMP	Guanosine monophosphate
HCW	Hot carcass weight
HSD	High stocking densities
I-BABP	Ileal bile acid-binding protein
I-FABP	Intestinal fatty acid binding protein
IAP	Intestinal alkaline phosphatase
IgA	Immunoglobulin A

L-FABP	Liver fatty acid binding protein
LSD	Low stocking densities
MDR	Multidrug-resistant
mRNA	Messenger ribonucleic acid
MUC2	Mucin 2
MUFA	Monounsaturated fatty acids
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NSP	Non-starch polysaccharides
OECD-FAO	Organization for Economic Co-operation and Development – Food and Agriculture Organization
OTAP-92	Ovotransferrin antimicrobial peptide
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCFA	Short-chain fatty acids
SF	Shear force
TBIL	Total bilirubin
WHC	Water holding capacity
WHO	World Health Organization

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CONFERENCE PRESENTATIONS

1. N. Moreane, C.M. Mnisi, O.E. Fayemi, G. Moonsamy & V. Mlambo. Impact of phased yeast-based nucleotide supplementation on gut function, performance, and meat quality in densely stocked broilers. World Poultry Science Association Symposium, Council for Scientific and Industrial Research, Pretoria. 4-5 March 2025, Poster.
2. N. Moreane, C.M. Mnisi, O.E. Fayemi, G. Moonsamy & V. Mlambo. Impact of phased yeast-based nucleotide supplementation on gut function, performance, and meat quality in densely stocked broilers. South African Society for Animal Science Congress, Protea Hotel, The Ranch, Polokwane. 8-10 July 2025, Presentation and Poster.
3. N. Moreane, C.M. Mnisi, O.E. Fayemi, G. Moonsamy, R. Jha, V. Mlambo. Impact of phased yeast-based nucleotide supplementation on gut function, performance, and meat quality in densely stocked broilers. Poultry Science Association Pacific Rim Scientific Conference, Macau, China. 13-16 October 2025, Poster.

1. CHAPTER ONE: GENERAL INTRODUCTION

1.1 Background

Poultry products play a vital role in food and nutrition security, as they are among the most popular animal-based foods worldwide, especially in low-income countries (Mottet & Tempio, 2017). In South Africa, the poultry industry accounts for over 16% of the gross domestic product (South African Poultry Association, 2023). However, the industry faces numerous challenges that arise from the highly stressful, intensive commercial production systems.

Stressful rearing conditions, such as high stocking densities, are known to compromise gut health and functionality in birds (Salois *et al.*, 2016). The gut serves as a highly dynamic and multifunctional organ that is essential for maintaining the overall health and performance of birds. It is intricately involved in a wide range of physiological functions, including metabolism, immune defense, digestion, nutrient absorption, and hormonal regulation (Sumanu *et al*., 2023). These interconnected roles not only ensure efficient utilization of feed and energy but also support the bird's ability to resist disease, adapt to environmental stressors, and achieve optimal growth and productivity. As such, the gut functions as a central regulatory hub that influences nearly every aspect of avian health and performance (Svihus, 2014). Consequently, poor gut function is the direct cause of suboptimal bird welfare, disease outbreaks, poor growth performance, and compromised product (meat and eggs) quality and safety faced by the poultry industry (Thema *et al.*, 2024).

1.2 Problem statement

Traditionally, stress-induced challenges have been mitigated using antibiotic growth promoters (AGP) to effectively control dysbacteriosis and enteropathogens in the gastrointestinal tract (GIT) and enhance feed conversion efficiency and the growth performance of birds (Dibner &

Richards, 2005). However, the use of AGP has been discontinued or strictly regulated in several countries due to growing concerns over antibiotic resistance and its potential impacts on human health and the environment (Wallace *et al.*, 2010). Food products from chickens exposed to AGP have also been shown to contain higher levels of antibiotic residues that have effects ranging from hepatotoxicity, reproductive disorders, mutagenicity, allergies, nephropathy, bone marrow toxicity, carcinogenic effects, and genotoxic potential, among other detrimental effects in humans (Guetiya Wadoum *et al.*, 2016). The use of AGP contributes to the spread of pathogenic antimicrobial resistance genes in the environment, including soil, water, and air (Greko, 2001). Moreover, the routine administration of broad-spectrum antibiotics exerts selective pressure on the microbial community, promoting the emergence and proliferation of multidrug-resistant (MDR) bacteria. This not only complicates disease management in animals but also facilitates the development of new antibiotic-resistant strains, leading to more difficult and often less effective treatment options (Schjørring & Krogfelt, 2011). This has also caused an increase in morbidity and mortality in birds during disease outbreaks (Untari *et al.*, 2021). This growing concern has driven the demand for effective alternatives to AGP, such as nucleotides, fundamental structural units of DNA and RNA that play critical roles in cellular regeneration, energy metabolism, protein synthesis, and the regulation of hormonal signaling pathways (Chiofalo *et al.*, 2011).

1.3 Justification

Nucleotides support the immune system of broiler chickens by enhancing the production of key immune cells, such as leukocytes and macrophages (Dil & Qureshi, 2002). Consequently, dietary supplementation with nucleotides has demonstrated potential to promote growth performance, strengthen immune responses, and increase resistance to disease in poultry (Rutz *et al.*, 2008). Indeed, the use of dietary yeast nucleotides in chickens was shown to trigger rapid

production of antibodies following vaccination (Wu *et al.*, 2018). Nucleotide supplements were also shown to promote healthier gut flora, subsequently improving broiler chickens' performance metrics and product quality (Chiofalo *et al.*, 2011; Wang *et al.*, 2019). Most studies investigating the effectiveness of nucleotides have used the continuous supplementation strategy, where chickens receive the feed additive throughout the production cycle. This is particularly noteworthy given that nucleotides are considered semi-essential nutrients, as animals can typically synthesize them endogenously. However, under stressful conditions such as high stocking densities, rapid growth, or immune challenges, the body's capacity to produce sufficient nucleotides may be compromised, increasing the need for dietary supplementation (Rutz *et al.*, 2008).

Theoretically, animals should require higher dietary nucleotide levels during periods of rapid growth, stress, or a compromised immune system (Stein & Kil, 2006), when endogenous synthesis is inadequate (Maldonado *et al.*, 2001). Moreover, commercial broiler diets, which mostly consist of maize and soybean meal, are typically deficient in nucleotides (Mateo & Stein, 2004), making this additive essential in broiler nutrition, especially under high stocking densities. Accordingly, the supplementation of nucleotides as AGP alternatives is a recent beneficial practice in broiler production (Rutz *et al*., 2008). However, in comparison with conventional feed additives, nucleotides have higher cost implications that are dependent on the dosage and supplementation period. Additionally, the production process of nucleotides can be expensive and complex, thus adding to the cost implications (Huyghebaert *et al*., 2011). For an industry already dealing with increases in costs of production and price competition from imports, the South African broiler industry requires AGP alternatives that are cost-effective to remain profitable (Nkukwana, 2019). Although the cost of nucleotide supplementation could potentially be minimized by implementing a strategic, periodic

approach targeting the most stressful phases of the broiler's lifecycle, this method remains underexplored and lacks sufficient scientific validation. Therefore, this study is a comparative exploration of the impact of phased *versus* continuous nucleotide supplementation on growth performance, haematological and serum biochemical parameters, and gut health parameters in densely stocked broilers.

1.4 Overall aim

To evaluate the effectiveness of phased *versus* continuous supplementation of broiler chickens reared under high stocking densities with dietary nucleotides as alternatives to antibiotic growth promoters.

1.5 Specific objectives

i. To assess the impact of phased supplementation with dietary nucleotides as alternatives to zinc bacitracin on growth performance metrics, blood parameters, and meat quality traits in densely stocked Ross 308 broilers.

ii. To determine the influence of phased supplementation with dietary nucleotides in place of zinc bacitracin on gut microbial diversity and abundance in densely stocked Ross 308 broilers.

1.6 Hypotheses

i. Dietary nucleotide supplementation in lieu of zinc bacitracin improves growth performance, physiological responses, gut microbial diversity and abundance, and meat quality in densely stocked Ross 308 broilers.

ii. Phased dietary nucleotide supplementation has similar effects on growth performance, physiological responses, gut microbial diversity and abundance, and meat quality in densely stocked Ross 308 broilers as continuous supplementation.

1.7 References

Aziz, A.A.A., Aziz, E.S.A.A., Khairy, M.H., Fadel, C., Giorgi, M. & Abdelaziz, A.S., 2024. The effect of butyric acid and nucleotides supplementation on broiler (*Gallus gallus domesticus*) growth performance, immune status, intestinal histology, and serum parameters. *Open Veterinary Journal*, *14*(1), 324.

Chiofalo, B., Lo Presti, V., Savoini, G., D'Alessandro, E., Chiofalo, V., & Liotta L., 2011. Nucleotides in broiler chicken diet: effect on breast muscles quality. *Czech Journal of Food Science,* 29, 308-317.

Dibner, J.J. & Richards, J.D., 2005. Antibiotic growth promoters in agriculture: history and mode of action. *Poultry Science,* 84, 634–643.

Dil, N., & Qureshi, M.A., 2002. Different expression of inducible nitric oxide synthethase is associated with differential toll-like receptor-4 expression in chicken macrophages from difference genetic backgrounds. *Veterinary Immunology and Immunopathology,* 84, 191 – 207.

Greko, C., 2001. Safety aspects on non-use of antimicrobials as growth promoters. In: Piva, A., Bach Knudsen, K.E. and Lindberg, J.E. (eds), *Gut Environment of Pigs*, pp. 219–230. Nottingham University Press, Nottingham, UK.

Guetiya Wadoum, R.E., Zambou, N.F., Anyangwe, F.F., Njimou, J.R., Coman, M.M., Verdenelli, M.C., Cecchini, C., Silvi, S., Orpianesi, C., Cresci, A. & Colizzi, V., 2016. Abusive use of antibiotics in poultry farming in Cameroon and the public health implications. *British poultry science*, 57(4), 483-493.

Huyghebaert, G., Ducatelle, R. & Immerseel, F.V., 2011. An update on alternatives to antimicrobial growth promoters for broilers. *The Veterinary Journal*, 187(2), 182–188.

Kelly, L. & Alworth, L., 2013. Techniques for collecting blood from the domestic chicken. *Lab Animal,* 42, 359–361.

Marshall, B.M. & Levy, S.B., 2011. Food animals and antimicrobials: impacts on human health. *Clinical Microbiology Reviews*, 24, 718–733.

Mateo, C.D. & Stein, H.H., 2004. Nucleotides and young animal health: can we enhance intestinal tract development and immune function? In: Lyons, T.P., Jacques, K.A. (Eds.), Nutritional biotechnology in the feed and food industries. Proceedings of Alltech's 20th annual symposium. *Nottingham University Press*, Nottingham, UK, 159–168.

Mottet, A. & Tempio, G., 2017. Global poultry production: current state and future outlook and challenges. *World's Poultry Science Journal,* 73(2), 245 – 256.

Nkukwana, T.T., 2019. Global poultry production: Current impact and future outlook on the South African poultry industry. *South African Journal of Animal Science*, 48(5), 869–884.

Rutz, F., Xiaver, E., Anciuti, M. Roll., V., & Rossi, P., 2008. The role of nucleotides in improving broiler pre-starter diets. In: J. A. Taylor-Pickard & Spring. P., ed., Gut Efficiency: The Key Ingredient in Pig and Poultry Production: Elevating Animal Performance and Health, by Publisher: *Wageningen Academic Publishers*, The Netherlands, 155-163.

Salois, M.J., Cady, R.A. & Heskett, E.A., 2016. The environmental and economic impact of withdrawing antibiotics from US broiler production. *Journal of Food Distribution Research*, (1), 1–2.

SAPA., 2016. South African Poultry Association 2016, Industry Profile. http://www.sapoultry.co.za/

Sauer, N., Mosenthin, R. and Bauer, E., 2011. The role of dietary nucleotides in single-stomached animals. *Nutrition Research Reviews,* 24, 46 – 59.

Schjørring, S. & Krogfelt, K.A., 2011. Assessment of bacterial antibiotic resistance transfer in the gut. *International journal of microbiology*, (1), 312956.

Sumanu, V.O., Naidoo, V., Oosthuizen, M. & Chamunorwa, J.P., 2023. A technical report on the potential effects of heat stress on antioxidant enzymes activities, performance and small intestinal morphology in broiler chickens administered probiotic and ascorbic acid during the hot summer season. *Animals*, 13(21), 3407.

Svihus, B., 2014. Function of the digestive system. *Journal of Applied Poultry Research*, 23, 306–314.

Thema, K.K., Mlambo, V. & Mnisi, C.M., 2024. Stress-alleviating properties of dietary red grape pomace in Ross 308 broilers reared at a high stocking density. *South African Journal of Animal Science*, *53*(6), 883-893.

Untari, T., Herawati, O., Anggita, M., Asmara, W., Wahyuni, A.E.T.H. & Wibowo, M.H., 2021. The effect of antibiotic growth promoters (AGP) on antibiotic resistance and the digestive system of broiler chicken in Sleman, Yogyakarta. In *BIO Web of Conferences*, 33, 04005).

Wallace, R.J., Oleszek, W., Franz, C., Hahn, I., Baser, K.H.C., Mathe, A. & Teichmann, K., 2010. Dietary plant bioactives for poultry health and productivity. *British Poultry Science*, 51(4), 461–487.

Wang, Z., Cerrate, S., Coto, C., Sacakli, P., Yan, F., Costa, F. & Waldroup., 2019. Evaluation of Nupro yeast product in diets for broilers. *International Journal of Poultry Science*, 8, 515 – 520.

Wu, C., Yang, Z., Song, C., Liang, C., Li, H., Chen, W., Lin, W. & Xie, Q., 2018. Effects of dietary yeast nucleotides supplementation on intestinal function, intestinal microbiota, and humoral immunity in specific pathogen-free chickens. *Poultry Science*, 97 (11), 3837-3846.

2. CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

With the global population projected to reach approximately 9.6 billion by 2050, concerns over food security are becoming increasingly urgent, given that poultry products (meat and eggs) are one of the most globally consumed animal products. There is a need to increase their production to meet the expected demand (Mottet & Tempio, 2017; Maharjan *et al.*, 2021). Accordingly, commercial poultry production systems are geared towards highly intensive and thus stressful rearing conditions that compromise the bird welfare and efficiency of production. These stressful rearing conditions predominantly weaken the health and functionality of the gut (Salois *et al.*, 2016). The gut plays a key role in the overall performance of birds through its contribution to digestive, absorptive, immunological, metabolic, and endocrine functions (Svihus, 2014). Therefore, gut dysfunction leads to substandard bird welfare, disease outbreaks, poor growth performance, and product safety challenges (Thema *et al*., 2024). Traditionally, AGP have been used to optimize gut function and overall bird performance through their ability to effectively control dysbacteriosis, enteropathogens, and enhance feed conversion efficiency (Dibner & Richards, 2005). However, the overuse of AGP has led to antibiotic residues in poultry products, the rise of antimicrobial resistance, and environmental contamination. These issues have raised significant public health concerns, prompting the discontinuation or restriction of AGP use in several countries (Wallace *et al.*, 2010). Hence, there is a growing need to identify effective alternatives for enhancing gut health, with nucleotides emerging as promising candidates. As fundamental structural units of DNA, nucleotides play vital roles in cell regeneration, energy transfer, hormone signal mediation, and protein synthesis.

2.1 Factors influencing gut health in poultry

The chicken gut harbors a diverse and complex microbiome that plays a vital role in digestion, health, physiological functions, and the development and modulation of the immune system (Bindari & Gerber, 2022). Chicken gut health, functionality, and integrity are affected by several factors, including feed, the environment, and gut microbiota. The composition and function of the gut microbiota are determined by a variety of factors, which are discussed in the following sections.

2.1.1 Host factors (age, sex, genetic differences)

Research has shown that age is among the most significant determinants that influence the GIT's microbial composition (Lu *et al*., 2003). Post-hatch broiler chickens already have a microbial community as early as 1 day of age (Ballou et al., 2016). Additionally, changes occur in the microbial community makeup because of succession and establishment of more stable microbial taxa as the chicks age (Crhanova *et al.*, 2011). Lee *et al.* (2017) revealed that variation in microbial composition between sexes exists, with females comprising a high number of anaerobic *Firmicutes* and males showing many *Bacteroidetes* in the ceca. Age-related changes in the microbial community significantly influence gut functionality and the interaction between its structural and immune components. Stability in the microbial population enables the exclusion of pathogenic bacterial populations such as *Salmonella* (Feye *et al.*, 2020), thus minimizing the occurrence of dysbiosis. There is a lack of agreement when it comes to the age at which birds' microbial community reaches stability, with reports suggesting 15 to 22 days old. (Ranjitkar *et al.*, 2016) and 28 to 49 days (Lu *et al.*, 2003). Although this is restricted to specific taxa (Wen *et al.*, 2021), the genetic makeup of chickens also affects the proportion of gut microbiota (Tůmová *et al.*, 2021). Accordingly, Richards *et al.* (2019) observed significant differences between the Cobb and Hubbard breeds, with the

Cobb cecal microbiota dominated by *Clostridiaceae* and *Enterococcaceae*, while the Hubbard cecal microbiota was dominated by *Enterobacteriaceae* at day 0.

2.1.2 Diet

The nutritional composition and texture of feed play a critical role in determining nutrient digestibility and absorption within the gut (Apajalahti *et al.*, 2004) and consequently shape the constitution and function of the gut microbiome. A multitude of anti-nutritional constituents exist in different feed ingredients, with the non-starch polysaccharides (NSP) being the largest group. Typical NSP traits include the tendency to increase viscosity in the intestinal lumen, which causes the excretion of sticky droppings due to their ability to withstand the bird's digestive enzymes (Yegani & Korver, 2008). Through this, they also decrease the rate of digesta passage through the GIT and nutrient availability. These ultimately result in both digestive and health issues (Yegani & Korver, 2008). An approximate 10% difference in mortality rate was observed between birds fed finely ground wheat and those fed coarsely ground wheat, with the finely ground group showing higher mortality. These deaths were primarily associated with coccidiosis and necrotic enteritis (Yegani & Korver, 2008). Meanwhile, some studies reported no notable impacts of diets containing whole wheat on digesta passage rate (Svihus et al., 2002), feed conversion efficiency, and body weight gain (Svihus *et al.*, 2004). Plavnik *et al.* (2002) recorded a notable positive effect of a whole wheat-based diet on the efficiency of feed. From the findings above, one can assume that the incorporation of whole wheat in a healthy gut can promote or downgrade its function depending on feeding conditions. Feed availability, pellet size, and manufacturing methods such as processing temperature can significantly influence nutrient availability and utilization in poultry (Apajalahti *et al.*, 2004). Chicks that fed on pelleted starter feed in comparison to chicks that fed on mash were found by Engberg *et al.* (2002) to have had a distinctly different

microbial population, particularly Coliforms, *Clostridium perfringens*, and *Enterococci*. Microbial populations were significantly higher in pellet-fed chicks. While this increase may present certain challenges, it can also offer potential benefits to gut health and nutrient utilization. In a study by Engberg *et al.* (2002), total volatile fatty acids, acetate, and butyrate were found to be higher in the ceca of birds feeding on pelleted feed.

2.1.3 Rearing environment

The rearing environment in broiler production is inclusive of litter, stocking density, and micro-climatic conditions. The type of litter and its microbial composition have a direct effect on the development and colonization of the chicken gut microbiome (Torok *et al.*, 2009), especially in the first two weeks, due to 4% of the chicken diet comprising litter (Bindari & Gerber, 2022). The type of bedding material used in the broiler house also determines the composition of microbial communities. This was observed by Torok *et al.* (2009), where cecum microbiota in 4-week-old broilers raised in sawdust and chopped straw were significantly different. The condition of litter, wet or dry, also defines the biodiversity of microbiota, with wet litter comprising a higher diversity (Oakely *et al.*, 2013). However, wet litter is mostly linked to dysfunctional gut health (Collet, 2012). Other factors, including the management practices, biosecurity, and stocking densities, influence gut health and overall performance. Kamel *et al.* (2021) observed that chickens reared in high stocking densities had a decrease in mucosal thickness, reduced amounts of goblet cells, and shortened villi height in comparison to those under low stocking densities. This could consequently lead to low feed intake and nutrient deficiency (Bun *et al.*, 2011). On the other hand, biosecurity and effective management practices prevent the transfer of pathogens between humans and birds.

2.1.4 Pathogens

The inclusion of varying bacteria, viruses, infectious and non-infectious agents leads to a complex etiology of enteric infections (Reynolds, 2003). Bacterial and viral infections damage the integrity and structure of the gut and its contents, and as a result decrease weight gain, feed efficiency, and decrease the uniformity in flocks, and are likely to end in disease and high mortality rates (Yegani & Korver, 2008). However, the end results of these infections are determined by other infectious agents, nutrition, and virulence of the pathogen, management practices, and environmental factors.

2.2 Challenges associated with antibiotic growth promoters

The utilization of AGP in the poultry industry has significantly improved the efficiency of production and aided in fulfilling the increasing demand for broiler products (Miyakawa *et al.*, 2024) through improved growth performance and bird welfare. However, their overuse and misuse have led to challenges in the poultry industry, including antimicrobial resistance, antibiotic residues in food products, and environmental contamination.

2.2.1 Antimicrobial resistance

Antimicrobial growth promoters are natural, semisynthetic, or synthetic substances that are meant to interrupt the growth and development of undesirable bacterial microorganisms (Mohr, 2016) and enhance the presence of beneficial bacterial microorganisms in the chicken gut. Unfortunately, over the years, bacterial populations have undergone evolution and found mechanisms to counteract interruption from AGP. These microorganisms have developed the capacity to withstand the agents that inhibit growth, a phenomenon termed antimicrobial resistance (Mathur & Singh, 2005). Antimicrobial resistance (AMR) has been declared a global public health threat by the World Health Organization (WHO, 2015), due to the complex and

multifactorial mechanisms that drive the evolution and communication of resistance genes across bacterial populations, animals, humans, and the environment (Lugsomya *et al.*, 2017). Antimicrobial resistance has also caused an increase in morbidity and mortality during disease outbreaks (Untari *et al.*, 2021). It facilitates the uncontrollable spread of infectious diseases (Kalia *et al.*, 2022). Accordingly, even low concentrations of antibiotics in the environment are responsible for spontaneous random mutagenesis (Larsson & Flach, 2022). Bacteria can spread resistance genes between similar and varying species through conjugation, transduction, and transformation (Chang *et al.*, 2015), also termed lateral gene transfer. Furthermore, the application of broad-spectrum antibiotics places bacterial flora under selective pressure, resulting in high multidrug-resistant (MDR) bacteria counts and subsequently new antibiotic-resistant bacteria with unpleasant treatment cycles (Schjørring & Krogfelt, 2011).

2.2.2 Human health

Antimicrobial resistance in food animals is linked to resistant pathogens in humans (Ma *et al.*, 2019). According to Recht *et al.* (2020), most of the recently found human illnesses have been proven to be of zoonotic origin. Antimicrobial-resistant pathogens have led to over 2 million infections and 23 000 deaths in the USA yearly (Li & Webster, 2018). This is a result of treatment failure, given that numerous antimicrobials that are taken by humans are also administered to animals (Abreu *et al.*, 2023). Moreover, the escalation of AMR is partly driven by the narrowing of effective treatment options and the enhanced virulence of pathogens, which arises from the co-selection of resistance and virulence traits due to the genetic linkage between resistance and virulence genes (Salam *et al.*, 2023). Some of the antimicrobial-resistant bacteria that are spread through animal food products include *Escherichia coli*, *Salmonella*, different enterococci, and methicillin-resistant *Staphylococcus aureus* (MRSA) (Ma *et al.*, 2019). These bacteria are also human pathogens that cause illnesses in consumers of animal products.

Furthermore, daily exposure of veterinary professionals, farm and slaughterhouse workers to infected animals increases their susceptibility to microbial infections (Ma *et al.*, 2019). Human health issues may also arise from antibiotic residues found in animal food products such as meat and eggs (Guetiya Wadoum *et al.*, 2016). These may range from hepatotoxicity, reproductive disorders, mutagenicity, allergies, e.g., penicillin, nephropathy, e.g., gentamycin, bone marrow toxicity, e.g., chloramphenicol, carcinogenic effects, e.g., oxytetracyclin, and the disruption of beneficial gut microflora, especially in children (Guetiya Wadoum *et al.*, 2016).

2.2.3 Environmental health

Antibiotic pollution refers to the widespread misuse of antibiotics, resulting in elevated concentrations of these drugs in the environment. The use of AGP contributes significantly to this contamination, as antibiotics enter the environment primarily through animal waste streams, affecting soil, water, and manure (Manyi-Loh *et al.*, 2018). The waste streams hold both resistance genes and antibiotics as they are not completely metabolised by the hosts (Larsson & Flach, 2022). Manure represents a major source of environmental antibiotic pollution (Muhammad *et al*., 2020). When applied as fertilizer, antibiotic residues can persist in soil and are readily transported via irrigation, farm wastewater, or rainfall into downstream rivers and sediments (Pruden *et al*., 2012). This risk is particularly pronounced for antibiotics with high water solubility, which facilitates their mobility and enhances their ecotoxic potential (Du & Liu, 2002). Moreover, improper disposal practices, such as discarding used antibiotic containers in refuse dumps, on bare ground, or in drainage systems, further exacerbate environmental contamination (Manyi-Loh *et al*., 2018). Nonetheless, the nature and utilization of nucleotides in rearing broilers presents potential solutions to these challenges.

2.3 Sources and functions of nucleotides

Nucleotides are synthesized endogenously through *de novo* and salvage pathways, nonetheless biotechnology can be applied to manufacture nucleotides through the enzymatic hydrolysis of yeast RNA and microbial fermentation (Ledesma-Amaro *et al.*, 2013). In addition, nucleotides play a pivotal role in physiological and biochemical processes (Wang *et al.*, 2024).

2.3.1 Nature and sources of nucleotides

Nucleotides are monomers of nucleic acids (Jung & Batal, 2012) that are manufactured in cells through a *de novo* procedure from precursor amino acids such as glycine, formate, aspartic acid, and glutamine, and sugars. They are also recycled through a salvage pathway, which is simply recovering nucleosides and bases from RNA and DNA degradation (Chandel, 2021). Dietary nucleic acids are converted by nucleases into nucleotides before the monomers are absorbed in the small intestines (Jung & Batal, 2012). Biotechnology can be used to produce nucleotides in addition to endogenous nucleotide production for exogenous supplementation. The biotechnological means of producing nucleotides involve RNA breakdown and mutagenesis, also known as strain improvement. In the former, RNA is extracted from cell cultures by ethanol precipitation and further hydrolysed to release free nucleotides; cells can either be produced for this procedure or gathered from by-products of other fermentation processes. Most of the yeasts utilised are *Candida*, *Hansenula*, *Pichia,* and *Saccharomyces* as they contain reasonably high amounts of RNA (Ledesma-Amaro *et al.*, 2013). Whereas in the latter, nucleotides are directly manufactured from cell cultures through the microbial fermentation of the improved strain acquired from mutagenesis (Ledesma-Amaro *et al.*, 2013). Mutagenesis is a procedure wherein cell DNA is altered, thus leading to a genetic mutation (Durland & Ahmadian-Moghadam, 2022).

2.3.2 Functions of nucleotides in cellular processes

Nucleotides have an essential role in several metabolic reactions, especially those that take place within cells. They are monomeric units that make up DNA and RNA. Nucleotides also have an impact on the cell cycle; in their absence, T-lymphocytes are detained in the growth phase, thus hindering their response to varying immunological signals that take place through transitioning into the synthesis of DNA (S phase) (Sauer *et al.*, 2011). In theory, animals may require increased levels of dietary nucleotides during periods of rapid growth, physiological stress, or immune challenges, when endogenous synthesis may be insufficient to meet metabolic demands (Maldonado *et al.*, 2001; Stein & Kil, 2006). Supplementing such animals with nucleotides can improve growth, immune function, and disease resistance. On the other hand, the maize-soybean broiler diets are typically nucleotide-deficient (Mateo & Stein, 2004), making nucleotides a conditionally essential nutrient, especially under stressful conditions. The adenine nucleotides, namely adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP), are crucial molecules in chemical energy transfer from energy-producing reactions to reactions that require energy (Sauer *et al.*, 2011). Cyclic AMP is an agent in biological control; it acts as a second messenger for transduction of intracellular signals, a process that entails the transfer of hormonal action, including adrenaline and glucagon, which are incapable of crossing the cell membrane (Zivkovic, 2007). Additional cyclic AMP traits involve the regulation of adrenaline and glucagon effects, activating protein kinase (Sauer et al., 2011) and regulation of calcium ion passage via ion channels (Zivkovic, 2007). Other differentiated nucleotide forms activate as co-enzymes and are crucial in protein, carbohydrate, and lipid synthesis, i.e., nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), and coenzyme A (CoA) (Sauer *et al.*, 2011).

2.3.3 Nucleotides as alternatives to antibiotic growth promoters

Nucleotides occupy a key role in major biological processes, including their role in cellular metabolism, namely cell signaling and transferring energy (ATP). Dietary nucleotides have been shown to modulate immunity by enabling expeditious and stronger responses against pathogens, through the increased manufacture of leukocytes and macrophages (Dil & Qureshi, 2002). Based on an observation by Rutz *et al.* (2008), the mortality rate of birds exposed to stressful conditions decreased by about 30% after nucleotide feeding. Dietary nucleotides foster an improved formation of antibodies in cooperation with vaccination (Wu *et al.*, 2018).

Gut integrity and improved growth and development are potential benefits of supplementary dietary nucleotides. Indeed, Wu *et al.* (2018) demonstrated that birds supplemented with nucleotides exhibited a higher abundance of lactic acid bacteria, along with a more diverse intestinal microbiota. Additionally, greater villus height and villus height to crypt depth ratio were demonstrated in broiler chickens (Jung & Batal, 2012). The availability of nucleotides can be a limiting factor in the rate at which cell division occurs, especially in juveniles. Accordingly, their supplementation resolves this problem and minimizes the amount of energy required for *de novo* production of nucleotides, thus boosting bird performance (Wang *et al.*, 2009). Jung & Batal (2012) recorded an increase in body weight, feed conversion rate (FCR), and daily body weight gains in broiler chickens, while Bonato *et al.* (2014) observed improved egg fertility, egg production, and hatchability in broiler breeders. These benefits altogether subsequently enhance meat quality through improved tenderness, a more consumer appealing redder colour and high nutritional value (Chiofalo *et al.*, 2011; Zhang *et al.*, 2008). Nucleotides are conditionally essential, especially under stressful conditions, during periods of rapid growth, and when the immune system is compromised (Stein & Kil, 2006). Broiler diets are mostly made of soybean meals and maize (Mateo & Stein, 2004), which are nucleotide-

deficient, hence the need to supplement chicken diets with dietary nucleotides. Therefore, there is potential for nucleotides to replace antibiotic growth promoters in broiler diets.

2.4 Dietary nucleotide supplementation in poultry

Nucleotides are conditionally essential nutrients, primarily required under stressful conditions and periods of rapid growth, thus necessitating supplemental exogenous supply through broiler diets (Gil *et al.*, 2002; Li *et al.*, 2007).

2.4.1 Effect on gut health and function

Gut histomorphology refers to the study of the structure and morphology of intestinal cells using histological techniques. The gut is a vital organ, playing a central role in both digestion and immune defense, thereby significantly influencing overall immune status and growth performance. It is important to ensure a balance in the ever-changing gut ecosystem to ensure optimum gut function (Kamel *et al.*, 2021). The availability of nucleotides controls the development of epithelial cells and ultimately villi through the production of DNA. Histological findings by Daneshmand *et al.* (2017a) demonstrated an increase in villus height and villus weight similar to those of Jung & Batal (2012). Improved villi development means increased absorption of nutrients (Daneshmand *et al.*, 2017a). Additionally, a study by Alizadeh *et al.* (2016) demonstrated that the supplementation of dietary nucleotides resulted in increased villus height and crypt depth. An increase in the production of goblet cells and a thicker mucous layer was also observed by Kamel *et al.* (2021). Maintenance of the balance of intestinal microbes, blockage of pathogens, enhancement of nutrient flow, and the modulation of microbial-host immune response are the vital roles played by the mucous layer (Birchenough *et al.*, 2015).

The pancreas is an accessory organ of the digestive tract; it is found in a loop within the duodenum. The pancreas produces digestive enzymes and bicarbonate, an intestinal pH buffer (Klasing, 1999). The production rate of digestive enzymes is a crucial indicator of gut health (Kamel *et al.*, 2021). Dietary nucleotide-supplemented diets have a positive effect on intestinal and pancreatic digestive enzymes. Lee *et al.* (2007) reported increased intestinal enzyme activity in birds supplemented with dietary nucleotides. Increased digestive enzyme activity, villi height, intestinal tissue length, and weight are also influenced by the age of birds (Iji *et al.*, 2001). Kamel *et al.* (2001) also reported improved digestive enzyme activity in birds under high stocking densities (HSD) with dietary nucleotide-supplemented diets.

Intestinal permeability is the direct flow of medium-sized molecules with hydrophilic traits through the intestinal epithelium via a concentration gradient (France & Turner, 2017). Damage to the intestinal mucosa, caused by pathogenic microbes, among other things, increases intestinal permeability (Binieda *et al.*, 2020). However, dietary nucleotides have been proven to optimize the integrity of intestinal mucosa (Kamel *et al.*, 2021), thus minimizing the chances of pathogenic invasion of the gut. In addition to supporting gut integrity, dietary nucleotides have been shown to exhibit anti-inflammatory properties that can positively impact poultry health. While inflammation is a natural immune response to injury or infection, excessive or prolonged inflammation may impair gut function, compromise immune balance, and negatively affect overall growth and performance. Nucleotides have been shown to modulate inflammatory responses by reducing the manufacture of pro-inflammatory cytokines, inhibiting the activation of inflammatory pathways, and enhancing the production of anti-inflammatory molecules (Wang et al., 2022). These anti-inflammatory effects promote better health and growth performance in poultry.

2.4.2 Influence of dietary nucleotides on blood parameters

Maintaining optimal blood parameters is essential for protecting birds against pathogens, as immune defense is primarily mediated by leukocytes (Daneshmand *et al.*, 2017b). Lymphocytes, macrophages, and plasma cells, just like epithelial cells, need large amounts of DNA and RNA for proliferation and maintenance (Zhou *et al.*, 2015). Beneficial effects of dietary nucleotides include the improved production of lymphocytes, which drives the conversion from intestinal B cells into plasma cells and secretion of immunoglobulin A (IgA), an antibody on the intestinal mucosal membrane surface (Sauer *et al.*, 2012). The availability of IgA in intestinal mucosa is one of the most paramount factors, as it offers further protection against disease (Sauer *et al.*, 2012; Zhou *et al.*, 2015). A study conducted by Daneshmand *et al.* (2017b) on broiler chickens supplemented with pyrimidine nucleosides demonstrated an increase in the weight of the Fabricius bursa and improved IgA secretion in the jejunum. The Fabricius bursa plays a key role in the manufacture of antibodies (Yegani & Korver, 2008). Nucleosides are simply nitrogenous bases that are linked to ribose or deoxyribose sugars without a phosphate group, while nucleotides are nitrogenous bases linked to ribose or deoxyribose sugars joined to at least one phosphate group.

2.4.3 Impact of dietary nucleotides on feed utilization and growth performance

The *de novo* production of nucleotides is an energy-consuming activity that can stall the productivity and growth performance of birds. Jung & Batal (2012) stated that the supplementation of dietary nucleotides is necessary to maintain and optimize the growth performance of broiler chickens, especially under stressful rearing conditions. Nucleotides enhanced the length of villi, which means a larger surface area for absorption to take place, thus increasing the digestion of nutrients and absorption (Khedr *et al*., 2020), resulting in faster growth and maturity. Feed utilization efficiency and body weight gain increase with the

supplementation of dietary nucleotides, but not feed intake (Salah *et al.*, 2019; Zahran *et al.*, 2020). However, De Souza *et al.* (2021) recorded a similar growth rate in control birds and those offered dietary nucleotide-supplemented diets. The discordance with previous studies could be a result of differences in chicken strains; other studies used the Ross 308 strain, while others used the CP 707 and Hubbard Efficiency strains.

2.4.4 Dietary nucleotides and meat quality traits

Salah *et al.* (2019) observed an increase in the iron content in breast meat and an increased water holding capacity (WHC) in meat from broilers kept under a hot environment with a dietary nucleotide-supplemented diet. This is in consensus with the results observed by Chiofalo *et al.* (2011), where dietary nucleotide supplementation did not have any impact on lightness, cooking loss, and pH. Nucleotides are involved in the absorption of iron through the transformation of AMP and guanosine monophosphate (GMP) into hypoxanthine, uric acid, and inosine; this may be linked to the increased iron concentrations in meat. This makes broiler meat and livers a good source of heme iron for consumers, most importantly, consumers struggling with iron deficiency (Rosenbloom, 2024). An increase in meat redness values was also observed in broiler meat from a dietary nucleotide-supplemented diet (Chiofalo *et al.*, 2011). Zhang *et al.* (2008) recorded an increase in meat redness values where broiler chickens were fed with inosinic acid, which improved the meat colour. This, too, could be related to increased iron absorption in the intestines. Additionally, myoglobin is a protein that consists of heme iron and gives meat its colour. The supplementation of dietary nucleotides improved crude fat content (Chiofalo *et al.*, 2011), which could be linked to the nucleotides' physiological effect to stimulate the synthesis of plasmatic α-lipoprotein (Mateo & Stein, 2007). Lower values for the sum of saturated fatty acids and higher values for the sum of monounsaturated fatty acids (MUFA) were recorded in dietary nucleotide-supplemented diets (Chiofalo *et al.*,

2011). The MUFA have beneficial health effects on humans as they minimize the risk of inflammatory and cardiovascular disease (Watkins & German, 2002). The supplementation of dietary nucleotides may have enhanced the production of elongation and desaturase enzymes in the intestines or liver because of an increase in protein synthesis required for metabolic digestion of acidic products.

2.5 Comparative studies on different nucleotide supplementation strategies

Zahran *et al.* (2020) reported findings from a study where nucleotides were supplemented for the first 10 days, first 25 days, as well as throughout the feeding trial (35 days) at 0.025% dietary nucleotide concentration; the weight gain and FCR increased significantly when nucleotides were supplemented. Birds supplemented in the first 10 days had the overall least weight gain, but feed intake remained the same for all treatments. Nucleotides seemingly do not have an impact on feed intake even when graded levels from 0 – 4% were investigated in pre-starter feeds (Zauk *et al.*, 2006). However, Esteve-Garcia *et al.* (2007) observed a difference in the feed intake between broilers receiving dietary nucleotide supplements at 0.5% in starter feed. Additionally, Shivkumar *et al.* (2009) observed that feeding broilers with dietary supplements at varying time intervals (7, 14, and 42 days) resulted in higher feed intake in the birds that received dietary nucleotide supplements on day 14, while lower feed intake was observed in the 7 and 42-day birds. Day 7 and 42 treatments also recorded a lower FCR but better weight gain in comparison to the control diet. The discordance in the results observed may be a result of environmental factors, including the broiler strains, stressors, and the source of nucleotides, as well as varying broiler management practices.

2.6 Gut health biomarkers

Gut health biomarkers are broadly defined as measurable changes in biological substances that reflect normal physiological conditions or pathological states. These indicators provide

invaluable insights into the functional status of the gastrointestinal tract and its response to various internal and external factors (De Meyer *et al.*, 2019). Biomarkers have the potential to streamline both research and field studies by offering objective, quantifiable data related to gut development, immune function, nutrient absorption, and disease progression. As such, they can play a crucial role in advancing strategies for disease prevention, early diagnosis, and the evaluation of interventions aimed at improving poultry gut health (De Meyer *et al.*, 2019). They may also be utilised to detect subclinical intestinal modifications. Early detection biomarkers have the potential to aid with prevention or relief from enteropathogens through diet changes (De Meyer *et al.*, 2019).

2.6.1 Intestinal biomarkers

Intestinal biomarkers are used as indicators of the intestine's functionality through measures including immunity status and barrier integrity (Rey *et al.*, 2025).

2.6.1.1 Intestinal walls

The intestinal wall structure is a representation of the immune status, integrity, and functionality of the intestines. High stocking densities (HSD) may increase intestinal permeability and damage the function of the intestinal barrier (Yu *et al.*, 2021). Rearing at HSD can induce heat stress, which may, as a result, reduce intestinal barrier function as it reduces intestinal blood flow (Lambert, 2009). Measurements of the villus length, crypt depth, and the ratio between villus length and crypt depth are the reference standard in intestinal health evaluation (Ducatelle *et al.*, 2018). Enteropathogens such as *Eimeria species* may cause epithelial cell death, thus a decrease in the villus length. The reduction in the villus length stimulates the proliferation of enterocytes, which consequently increases crypt depth. There are specific references that were recorded for broilers at 23 days old, i.e., villus length of 700, 900,

and 1400 mm, and a crypt depth of 160, 170, and 190 mm, and a villus length to crypt depth ratio of 5, 6, and 8 (Ducatelle *et al.*, 2018).

The gut barrier is composed of three layers, the first layer being the intestinal mucus, epithelial cells, and lastly the lamina propria. A healthy lamina propria consists of a high number of tolerogenic FoxP3 regulatory T-lymphocytes (Treg); however, these were proven to be deficient in inflammatory bowel disease (Sarrabayrouse *et al*., 2014). Quantifying Treg density can possibly represent a valuable standard for intestinal health in chickens. Additionally, the total T-lymph counts in the lamina propria of chickens were found to be linked with parallel villus shortening (Teirlynck *et al.*, 2009; Yacoubi *et al.*, 2018). According to Brudnicki *et al.* (2017), yeast nucleotide supplementation in broilers enhanced the structure of the small intestine. In addition, the supplementation of Japanese quail diets with yeast nucleotides was shown to improve nutrient absorption as a result of increased villus height (Abd El-Wahab *et al.*, 2019). Wu *et al.* (2018) also recorded an increase in villus height and villus height to crypt depth ratio in 0.5% yeast nucleotide-fed chickens. Phased dietary nucleotide supplementation could improve the structure and function of the intestinal walls.

2.6.1.2 Fibronectin

Fibronectin is a glycoprotein involved in normal tissue integrity preservation that is produced by various cells, including intestinal epithelial cells (Barekatain *et al.*, 2020). Its soluble form is found in the plasma, while the insoluble form is present in the extracellular matrix and the basement membrane of the intestinal wall. Damage in the intestines could possibly lead to the release of fibronectin into the intestinal lumen (De Meyer *et al.*, 2019; Barekatain *et al.*, 2020). According to Goo *et al.* (2019), HSDs are likely to disrupt intestinal barrier function, thus increasing intestinal permeability. The mechanism by which dietary nucleotides enhance

immune responses involves the improvement of mucosal thickness and the maintenance of intestinal structural integrity. This is achieved through increased goblet cell numbers, enhanced villus height, and reduced crypt depth (Kamel et al., 2021). These structural benefits may be linked to the regulation of fibronectin, a key extracellular matrix glycoprotein involved in cell adhesion, tissue repair, and epithelial barrier function.

2.6.1.3 Mucin 2

Mucin 2 (MUC2) is a glycoprotein that is involved in the formation of the intestinal barrier mucus layer (Chen *et al.*, 2015) and a key element of the intestinal mucus layer in chickens (Duangnumswang *et al.*, 2021). Additionally, MUC2 plays a notable role in the digestion and absorption of nutrients and functions as a substrate for the local microbiota in fostering the elimination of harmful bacteria through aggregation (Biasato *et al.*, 2019). Mucin 2 genes are transcribed to mRNA; the downregulation of the MUC2 gene expression is harmful to the intestinal structure and barrier function and could potentially be used as a gut barrier failure biomarker in chickens (Chen *et al.*, 2015). Zhang *et al.* (2017) observed that stress caused by heat can decrease the abundance of MUC2 mRNA in broiler jejunal mucosa. Feed composition, physiological status, and post-hatch development affect the dynamics of MUC2 in the intestines. The continuous supplementation of dietary nucleotides in broilers could facilitate the upregulation of MUC2 and its function as an immune response by increasing mucosal thickness. They may also increase feed efficiency through increased digestion and absorption and ultimately improve growth performance (Kamel *et al.*, 2021). Phased dietary nucleotide supplementation could potentially regulate MUC2 expression.

2.6.1.4 Occludins

Occludins are unique proteins that form a part of the intercellular junction, also known as tight junctions, which link intestinal epithelial cells and are, as a result, involved in paracellular

transfer of molecules (Mohamed *et al.*, 2020). Tight junctions play a pivotal role in maintaining paracellular permeability and barrier functions, thus making them an essential intestinal mucosal barrier component (Santos *et al.*, 2021). Similar to MUC2, heat stress in broilers can reduce the expression of occludin in mRNA jejunal mucosa (Mohamed *et al.*, 2020). Reduced occludin gene expression could badly impact the integrity of these intercellular junctions and consequently be correlated with impaired intestinal barrier function (Chen *et al.*, 2015). Disruption of intestinal tight junctions can lead to increased luminal antigen permeability and bacterial translocation, allowing harmful microbes and toxins to cross the gut barrier and enter systemic circulation (Ulluwishewa *et al.*, 2011). Continuous dietary nucleotide supplementation has been revealed to increase intestinal mucosal weight in broilers (Aziz et al., 2024). Therefore, it can be hypothesized that phased nucleotide supplementation may similarly enhance the expression and function of occludin, thereby strengthening the intestinal barrier.

2.6.1.5 Intestinal fatty acid binding proteins

Fatty acid binding proteins (FABP) have three isoforms that occupy the intestines (I-FABP/FABP2), liver (L-FABP/FABP1), and bile (I-BABP) (Sun *et al.*, 2015). Fatty acid binding proteins are intracellular proteins that take part in inflammatory and metabolic pathways (Barekatain *et al.*, 2020). Mucosal damage and subsequent intestinal permeability that occur as a result of pathogenic or non-pathogenic stress can increase I-FABP levels in the vascular system from the epithelium (Adriaanse *et al.*, 2013). Lesions in the intestines cause increased IFABP in the serum. Stable at room temperature, IFABP could indicate intestinal damage earlier, even at the subclinical stage (Cahyaningsih *et al.*, 2018). Literature on the relationship between intestinal permeability and I-FABP is limited. Given that continuous dietary nucleotide supplementation can enhance the function of the intestinal barrier (Mohamed

et al., 2020), an assumption that phased dietary nucleotide supplementation can potentially achieve the same results, thereby regulating I-FABP expressions, can be drawn.

2.6.1.6 Intestinal alkaline phosphatase

Intestinal alkaline phosphatase (IAP) is a brush border enzyme that is produced by enterocytes into the intestinal lumen. It is involved in intestinal mucosal defense and hydrolyses phosphate monoesters (Escobar *et al.*, 2022). It aids with the maintenance of intestinal homeostasis using various mechanisms, i.e., regulation of intestinal pH, absorption of lipids, counteracting intestinal inflammation, and bicarbonate secretion (Celi et al., 2019; Barekatain *et al.*, 2020), and minimizes translocation of bacteria and dephosphorylation of bacterial lipopolysaccharides (Escobar *et al.*, 2022). The production of digestive enzymes in broilers may be hindered by intestinal damage (Ducatelle *et al.*, 2018). Not much literature is available to make comparisons; however, IAP activity has been shown to lessen in broilers under stress (Al-Zgoul *et al.*, 2019). Intestinal alkaline phosphatase in excreta would potentially represent intestinal health status. Continuous dietary nucleotide supplementation in broilers has proved to enhance the function of the intestinal barrier (Aziz *et al.*, 2024); as such, the presumption that phased dietary nucleotide supplementation may maintain or enhance IAP activity may be made.

2.6.1.7 D-lactate

D-lactate is one of several metabolites produced by intestinal bacteria, namely *Lactobacilli* and *Bifidobacteria*; it is not metabolised by mammalian cells. In normal physiological conditions, an equilibrium exists between intestinal bacteria and their metabolites, and plasma D-lactate is not detectable (Santos *et al.*, 2021; Remund *et al.*, 2023). However, D-lactate concentrations tend to increase when broiler intestinal mucosa is injured (Cheng *et al.*, 2019). Environmental factors such as HSDs can exert stress in broilers, subsequently altering the microbiome composition, resulting in dysbiosis (Li *et al.*, 2022). Plasma D-lactate concentrations may serve

as great indicators for the evaluation of intestinal injury and dysfunction in the gut barrier that may occur as a result of dysbiosis (Lei *et al.*, 2013). Song *et al.* (2017) observed that heat stress in broilers exhibited damage in the intestinal mucosa and consequently increased intestinal permeability. Additionally, the vascular secretion of D-lactate in broilers resulting from heat stress involves the tip cells of the villus or mucosal surface, as they are the primary sources of D-lactate (Wu *et al.*, 2018). Accordingly, the influence of continuous dietary nucleotide supplementation in enhancing balance in the broiler gut microbiome and the structure of the villus or mucosal surface can impact the concentrations of plasma D-lactate. Dietary nucleotides supplemented continuously at 0.1% demonstrated an increase in broiler villus height (Aziz *et al.*, 2024), while dietary yeast nucleotides improved the composition of intestinal broiler microbiota through enhancing beneficial bacteria (Wu *et al.*, 2018). Phased dietary nucleotide supplementation could potentially achieve similar results.

2.6.2 Gut microbiome

The broiler gut is a host to a complex and diverse microbiota that holds a key role in the digestion and absorption of nutrients, development of the immune system, and pathogen resistance (Shang *et al.*, 2018). Immune stimulation and programming, competitive pathogen exclusion, and enhanced nutrition are benefits provided by commensal microbiota (Dibner & Richards, 2005). Commensal microbiota can facilitate the stimulation of the immune system development, including intestinal immune cells, epithelial monolayer, lamina propria, and mucus layer (Shang *et al.*, 2018). These tissues withstand harmful microorganisms and create barriers between the microbes and the host. Accordingly, the microbiota in the distal gut (colon and ceca) also synthesize energy and nutrients like short-chain fatty acids (SCFAs), vitamins, and amino acids that are eventually utilized by the host (Gaskins *et al.*, 2002; Dibner & Richards, 2005). Short-chain fatty acids can eliminate foodborne pathogens, such as

Salmonella, by hindering their growth (Ricke, 2003). In addition to being an energy source to the host, SCFAs can enhance gut epithelial cell development, thus broadening the surface area for absorption. Furthermore, gut microbiota facilitates the metabolism of nitrogenous substances; for instance, cecal bacteria can drive ammonia from uric acid, which can then be absorbed and used to generate amino acids (Shang *et al.*, 2018).

In contrast, the proximal gut (gizzard and small intestines) microbes and the host contend for protein and energy. The microbiota in both the proximal and distal guts catabolize bile acids and produce toxic metabolites such as amino acid catabolites, which may inhibit bird growth and reduce their ability to digest fat (Gaskins *et al.*, 2002). While the microbiota regulates the gut microbial composition and hinders enteropathogens from invading host intestinal epithelial cells, these processes are protein and energy-consuming and negatively affect bird growth and performance (Mitchell *et al.*, 2006; Suzuki & Nakajima, 2014). An imbalance in the gut microbiota is referred to as dysbiosis. This imbalance may cause a sequential reaction in the GIT, such as intestinal wall thinning and minimized intestinal barrier activity, leading to substandard digestibility of nutrients, an increased risk of bacterial translocation, and inflammatory responses in the GIT. Dysbacteriosis can occur because of pathogenic stressors such as *coccidiosis* or non-pathogenic stressors such as environmental stress, diet modification, mycotoxins, and nutritional imbalances (Teirlynck *et al.*, 2011). Increased intestinal permeability is linked with increased amounts of gut bacteria flooding the liver and the bloodstream; hence, bacterial count has been applied as an intestinal permeability biomarker in broilers (Tellez *et al.*, 2014). According to Chen *et al.* (2015), leakage of epithelial junction complexes allows bacterial macromolecules, such as lipopolysaccharides from gram-negative bacteria, to permeate the intestinal barrier. There is no lipopolysaccharide leakage via the paracellular pathway in healthy intestines because the alkaline phosphatase in the epithelial cells detoxifies and absorbs those (Guerville & Boudry, 2016). According to Mohamed *et al.*

(2020), the continuous supplementation of nucleotides improved intestinal histomorphology and the intestinal barrier function in chickens infected with *Clostridium perfringens*. This can be attributed to the mechanism of nucleotides to promote the dominance of probiotics such as *Bifidobacteria* and *Lactobacilli,* thus reducing the concentration of enterobacteria. Accordingly, the assumption that phased dietary nucleotide supplementation can achieve similar results may be made.

2.6.3 Faecal microbiota

Faecal microbiota is likely to be identical to caecal microbiota if contents are collected right after emptying the caecum. (Rychlik, 2020). In general, the crop, ileum, and caecum are the GIT sites that are predominated by microbiota. The caecal microbiota is more stable in comparison to the ileal microbiota and is the most diverse fragment of the gut (Borda-Molina *et al.*, 2018; Kers *et al.*, 2018). A healthy caecum is largely occupied by *Firmicutes* (45%), *Bacteroidetes* (45%)*, Actinobacteria* (2 – 3%)*,* and *Proteobacteria* (2 – 3%) (Rychlik, 2020). Additionally, fermentation of indigestible fibre also occurs in the caecum (Liu *et al.*, 2022). The fermentation of dietary fiber by gut microbiota produces short-chain fatty acids (SCFAs), which play a pivotal role in the maintenance of the intestinal epithelium integrity (Liu *et al.*, 2020). Zuo & Ng (2018) demonstrated the ability of *Lactobacillus* spp. and *Faecalibacterium* spp. to offer protection from intestinal inflammation in humans. Accordingly, heat stress may potentially destroy broiler intestinal integrity by increasing intestinal inflammation in broilers and subsequently alter the bacterial compositions and structure in ileal microbiota (Liu *et al.*, 2020; Sumanu *et al*., 2023).

Faecal microbiota is frequently used as a representative for intestinal microbiota, because they are qualitatively similar, with quantitative variations between groups (Stanley *et al.*, 2015).

Inflammation in the intestines enables the development of facultative anaerobic bacteria (Faber & Baumler, 2014) from the phylum Proteobacteria. There has been an undesirable correlation between performance parameters and the expansion of the Enterobacteriaceae family within the Proteobacteria phylum in birds (Eeckhaut *et al.*, 2016). High fecal amounts of enterobacteriaceae are associated with poor bird performance. Based on an observation by Zhang *et al.* (2017), cyclic heat stress changed the profile of intestinal microbiota by increasing *E. coli*, *Clostridium* spp., and *Salmonella* populations, and reducing *Bifidobacterium* spp. and *Lactobacillus* spp. populations. Continuous dietary nucleotide supplementation was shown to trigger an inflammatory immune response following an enteritis challenge. The intestinal mucosa and submucosa exhibited an infiltration of inflammatory cells in comparison to the challenge control group (Aziz *et al.*, 2024). Hence, an assumption that phased dietary nucleotides have the capacity to enhance the activity of *Lactobacillus, Faecalibacterium,* and related mechanisms can be drawn.

2.6.4 Liver

2.6.4.1 Ovotransferrin

Ovotransferrin is a glycoprotein manufactured in the oviduct and liver of broiler chickens. It plays a role in preventing microbial invasions through binding and isolating iron, as its unavailability hinders bacterial growth (Goosens *et al.*, 2018). Its cationic fragment, ovotransferrin antimicrobial peptide (OTAP-92), can damage the gram-negative bacteria's membrane (Ibrahim *et al.*, 2000). The manufacture and secretion of ovotransferrin into the bloodstream can be a result of damaging stimuli, i.e., inflammation, trauma, infection, or stress. Serum ovotransferrin can be used to approximate the degree of inflammation and infection in chickens, whilst fecal ovotransferrin may possibly be an intestinal barrier damage biomarker (Goosens *et al.*, 2018). Continuous dietary nucleotide supplementation can enhance the

intestinal barrier and its function (Mohamed *et al.*, 2020). The mechanism used by dietary nucleotides and its influence on the intestinal barrier and inflammation can be related to its impact on the regulation of ovotransferrin.

2.6.4.2 Alpla-1 antitypsin

Alpla-1 antitypsin (AAT) is a serum glycoprotein that is manufactured in the liver. AAT translocates into the intestinal lumen because of increased intestinal permeability that may occur because of pathogenic or non-pathogenic stress. Translocation takes place via extravasation from the serum (Barekatain *et al.*, 2020). High stocking densities can induce stress in poultry by increasing heat exposure and intensifying competition for feed. The makeup of AAT cannot be altered by proteolysis or intestinal microbiome, thus enabling the quantification of intact AAT in fecal matter (Schwiertz *et al.*, 2018). Alpla-1 antitypsin can be used to assess the loss of protein because of enteropathies and as an intestinal permeability biomarker (Wang *et al.*, 2015; Celi *et al.*, 2019). The capacity of continuous dietary nucleotide supplementation in alleviating the impact of stress by maintaining the intestinal barrier and subsequently permeability (Mohamed *et al.*, 2020) may be related to the AAT levels in the serum or intestinal lumen. As such, the assumption that phased dietary nucleotide supplementation can regulate the abundance of AAT may be made.

2.7 Summary

The poultry industry is one of the largest producers of meat worldwide. However, the drive to meet increasing global demand and enhance food security has led to intensified production systems. These intensive rearing conditions often compromise bird welfare and performance, primarily by negatively affecting gut health. Initially, AGP were applied to optimize gut health and increase production efficiency, but the extensive application resulted in public health concerns in terms of product safety challenges, antimicrobial resistance, and environmental

pollution, leading to their discontinuation or restriction. Alternatives such as dietary nucleotides give benefits that largely focus on a holistic growth performance approach. However, the literature shows mixed results regarding the effects of dietary nucleotide supplementation on broiler growth performance, and the cost of this alternative remains a potential barrier to its widespread adoption. This study was designed to fill this gap by carrying out a comparative analysis of phased dietary nucleotide supplementation targeted at transitional periods in the broiler life cycle on feed utilization, blood parameters, growth performance, gut histomorphology, intestinal permeability, intestinal and pancreatic digestive enzymes, gut microbes, and meat quality traits.

2.8 References

Abd El-Wahab, A.A., Mahmoud, R., Marghani, B. & Gadallah, H., 2019. Effects of yeast addition to the diet of Japanese quails on growth performance, selected serum parameters, and intestinal morphology as well as pathogens reduction. *Pakistan Veterinary Journal*, *40*(2), 219-223.

Abreu, R., Semedo-Lemsaddek, T., Cunha, E., Tavares, L. & Oliveira, M., 2023. Antimicrobial Drug Resistance in Poultry Production: Current Status and Innovative Strategies for Bacterial Control. *Microorganisms*, *11*(4), 953.

Adriaanse, M.P.M., Tack, G.J., Passos, V.L., Damoiseaux, J.G.M.C., Schreurs, M.W.J., Van Wijck, K., Riedl, R.G., Masclee, A.A.M., Buurman, W.A., Mulder, C.J.J. & Vreugdenhil, A.C.E., 2013. Serum I-FABP as marker for enterocyte damage in coeliac disease and its relation to villous atrophy and circulating autoantibodies. *Alimentary Pharmacology & Therapeutics*, *37*(4), 482-490.

Al-Zghoul, M.B., Saleh, K.M.M. & Jaradat, Z.W., 2019. Expression of digestive enzyme and intestinal transporter genes during chronic heat stress in the thermally manipulated broiler chicken. *Poultry Science*, *98*(9), 4113-4122.

Alizadeh, M., Rodriguez-Lecompte, J.C., Rogiewicz, A., Patterson, R. & Slominski, B.A., 2016. Effect of yeast-derived products and distillers dried grains with soluble (ddgs) on growth performance, gut morphology, and gene expression of pattern recognition receptors and cytokines in broiler chickens. *Poultry Science*, *95*, 507–517.

Apajalahti, J., Kettunen, A. and Graham, H., 2004. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *World's Poultry Science Journal*, *60*(2), 223-232.

Aziz, A.A.A., Aziz, E.S.A.A., Khairy, M.H., Fadel, C., Giorgi, M. & Abdelaziz, A.S., 2024. The effect of butyric acid and nucleotides supplementation on broiler (*Gallus gallus*

domesticus) growth performance, immune status, intestinal histology, and serum parameters. *Open Veterinary Journal*, *14*(1), 324.

Bailey, R., 2019. Gut Health in Poultry -The World Within: Update. Available at: https://eu.aviagen.com/assets/Tech_Center/Ross_Tech_Articles/RossNote-GutHealth-2019-EN.pdf. [Accessed: 20 March 2024].

Ballou, A.L., Ali, R.A., Mendoza, M.A., Ellis, J.C., Hassan, H.M., Croom, W.J. & Koci, M.D., 2016. Development of the chick microbiome: how early exposure influences future microbial diversity. *Frontiers in Veterinary Science*, *3*(2), 1-12.

Barekatain, R., Howarth, G.S., Willson, N.L., Cadogan, D. & Wilkinson, S., 2020. Excreta biomarkers in response to different gut barrier dysfunction models and probiotic supplementation in broiler chickens. *PLoS One*, 15(8), 1-17.

Biasato, I., Ferrocino, I., Grego, E., Dabbou, S., Gai, F., Gasco, L., Cocolin, L., Capucchio, M.T. & Schiavone, A., 2019. Gut microbiota and mucin composition in female broiler chickens fed diets including yellow mealworm (*Tenebrio molitor*, L.). *Animals*, 9(5), 213.

Bindari, Y.R. & Gerber, P.F., 2022. Centennial Review: Factors affecting the chicken gastrointestinal microbial composition and their association with gut health and productive performance. *Poultry Science*, *101*(1), 101612.

Binienda A., Twardowska A., Makaro A. & Salaga M., 2020. Dietary carbohydrates and lipids in the pathogenesis of leaky gut syndrome: An overview. *International Journal of Molecular Sciences*, *21*, 8368.

Birchenough, G.M., Johansson, M.E., Gustafsson, J.K., Bergström, J.H. & Hansson, G., 2015. New developments in goblet cell mucus secretion and function. *Mucosal immunology*, *8*(4), 712-719.

Bonato, M., Araujo, L., Santos, G., Lohrmann, T., Barbalho, R., 2014. Nucleotide supplementation in the diet of broiler breeders and their effect on hatchability and subsequent progeny performance. *International Poultry Scientific Forum*, Abstract T131.

Borda-Molina, D., Seifert, J. & Camarinha-Silva, A., 2018. Current perspectives of the chicken gastrointestinal tract and its microbiome. *Computational and Structural Biotechnology Journal*, *16*, 131-139.

Brudnicki, A., Brudnicki, W., Szymeczko, R., Bednarczyk, M., Pietruszynska, D. & Kirkillo-Stacewicz, K., 2017. Histo-morphometric adaptation in the small intestine of broiler chicken, after embryonic exposure to galactosides. *JAPS: Journal of Animal & Plant Sciences*, 27(4), 1075-1082.

Cahyaningsih, U., Satyaningtijas, A.S., Tarigan, R. & Nugraha, A.B., 2018. Chicken I-FABP as biomarker of chicken intestinal lesion caused by coccidiosis. IOP Conference Series: *Earth and Environmental Science*, 196, 12032.

Cameron, B.F., Wong, C.W., Hinch, G.N., Singh, D., Colditz, I. G., 2001. Effects of nucleotides on the immune function of early-weaned piglets. In Digestive Physiology of Pigs. Proceedings of the 8th Symposium. Wallingford: CABI Publishing. 66-68.

Cao, L., Yang, X., Sun, F., Liu, C. & Yao, J., 2012. Lactobacillus strain with high adhesion stimulates intestinal mucin expression in broiler. *The Journal of Poultry Science*, *49*(4), 273-281.

Celi, P., Verlhac, V., Pérez, C.E., Schmeisser, J. & Kluenter, A.M., 2019. Biomarkers of gastrointestinal functionality in animal nutrition and health. *Animal Feed Science and Technology*, *250*, 9-31.

Chandel, N.S., 2021. Nucleotide metabolism. *Cold Spring Harbor perspectives in biology*, *13*(7), 040592.

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M. & Hanage, W.P., 2015. Antibiotics in agriculture and the risk to human health: how worried should we be?. *Evolutionary Applications*, *8*(3), 240-247.

Chen, J., Tellez, G., Richards, J.D. & Escobar, J., 2015. Identification of potential biomarkers for gut barrier failure in broiler chickens. *Frontiers in Veterinary Science*, *2*(14), 1-10.

Cheng, Y.F., Chen, Y.P., Chen, R., Su, Y., Zhang, R.Q., He, Q.F., Wang, K., Wen, C. & Zhou, Y.M., 2019. Dietary mannan oligosaccharide ameliorates cyclic heat stress-induced damages on intestinal oxidative status and barrier integrity of broilers. *Poultry Science*, *98*(10), 4767-4776.

Chiofalo, B., Lo Presti, V., Savoini, G., D'Alessandro, E., Chiofalo, V. & Liotta L., 2011. Nucleotides in broiler chicken diet: effect on breast muscles quality. *Czech Journal of Food Science*, *29*, 308- 317.

Collett, S.R. 2012. Nutrition and wet litter problems in poultry. *Animal Feed Science and Technology*, 173(1-2), 65-75.

Cowieson, A.J., Perez-Maldonado, R., Kumar, A. & Toghyani, M., 2020. Possible role of available phosphorus in potentiating the use of low-protein diets for broiler chicken production. *Poultry Science*, *99*(12), 6954-6963.

Crhanova, M., Hradecka, H., Faldynova, M., Matulova, M., Havlickova, H. & Sisak, F., 2011. Immune response of chicken gut to natural colonization by gut microflora and to Salmonella enterica serovar enteritidis infection. *Infection and Immunity,* 79, 2755–63.

Daneshmand, A., Kermanshahi, H., Danesh Mesgaran, M., King, A.J. & Ibrahim, S.A., 2017b. Effect of purine nucleosides on growth performance, gut morphology, digestive enzymes, serum profile and immune response in broiler chickens. *British Poultry Science*, *58*(5), 536–543.

Daneshmand, A., Kermanshahi, H., Mesgaran, M.D., King, A.J., Ibrahim, S.A. & Klasing, K.C., 2017a. Combination of purine and pyrimidine nucleosides influences growth performance, gut morphology, digestive enzymes, serum biochemical indices and immune functions in broiler chickens. *Animal Feed Science and Technology*, *228*, 186-193.

De Meyer, F., Eeckhaut, V., Ducatelle, R., Dhaenens, M., Daled, S., Dedeurwaerder, A., De Gussem, M., Haesebrouck, F., Deforce, D., & Van Immerseel, F., 2019. Host intestinal biomarker identification in a gut leakage model in broilers. *Veterinary Research*, *50*(1), 46.

De Souza, C., Eyng, C., Viott, A.M., de Avila, A.S., Pacheco, W.J., Junior, N.R., Kohler, T.L., Tenorio, K.I., Cirilo, E.H. & Nunes, R.V., 2021. Effect of dietary guanidinoacetic acid or nucleotides supplementation on growth performances, carcass traits, meat quality and occurrence of myopathies in broilers. *Livestock Science*, *251*, 104659.

Denbow, D.M., 2015. Gastrointestinal anatomy and physiology. In *Sturkie's avian physiology*, 337-366. Academic Press.

Dhanani, K.C.H., Samson, W.J. & Edkins, A.L., 2017. Fibronectin is a stress responsive gene regulated by HSF1 in response to geldanamycin. *Scientific Reports*, *7*(1), 17617.

Dibner, J.J. & Richards, J.D., 2005. Antibiotic growth promoters in agriculture: history and mode of action. *Poultry Science, 84*, 634–643.

Dil, N. & Qureshi, M.A., 2002. Different expression of inducible nitric oxide synthethase is associated with differential toll-like receptor-4 expression in chicken macrophages from difference genetic backgrounds. *Veterinary Immunology and Immunopathology 84*, 191- 207.

Dolliver, H. & Gupta, S., 2008. Antibiotic losses in leaching and surface runoff from manure-amended agricultural land. *Journal of Environmental Quality*, *37*(3), 1227-1237.

Du, L. & Liu, W., 2012. Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. *Agronomy for Sustainable Development*, *32*, 309-327.

Duangnumsawang, Y., Zentek, J. & Boroojeni, F.G. 2021. Development and Functional Properties of Intestinal Mucus Layer in Poultry. *Frontiers in Immunology*, 12, 745849.

Ducatelle, R., Goossens, E., De Meyer, F., Eeckhaut, V., Antonissen, G., Haesebrouck, F. & Van Immerseel, F., 2018. Biomarkers for monitoring intestinal health in poultry: present status and future perspectives. *Veterinary Research*, *49*, 1-9.

Ducatelle, R., Goossens, E., Eeckhaut, V. & Van Immerseel, F., 2023. Poultry gut health and beyond. *Animal Nutrition*, *13*, 240-248.

Durland, J. & Ahmadian-Moghadam, H., 2022. *Genetics, Mutagenesis*. [online] PubMed. Available at: https://www.ncbi.nlm.nih.gov/books/NBK560519/. [Accessed: 5 Oct. 2024].

Eeckhaut, V., Wang, J., Van Parys, A., Haesebrouck, F., Joossens, M., Falony, G., Raes, J., Ducatelle, R. & Van Immerseel, F., 2016. The probiotic *Butyricicoccus pullicaecorum* reduces feed conversion and protects from potentially harmful intestinal microorganisms and necrotic enteritis in broilers. *Frontiers in Microbiology, 7*, 1416.

Engberg, R.M., Hedemann, M.S. & Jensen, B.B., 2002. The influence of grinding and pelleting of feed on the microbial composition and activity in the digestive tract of broiler chickens. *British Poultry Science*, *43*(4), 569-579.

Escobar, J., Dobbs, M., Ellenberger, C., Parker, A., Latorre, J.D. & Gabor, L., 2022. Oral supplementation of alkaline phosphatase in poultry and swine. *Translational Animal Science*, *6*(3), 079.

Esteve-Garcia, E., Martinez-Puig, D., Borda, E. & Chetrit, C., 2007. Efficacy of a nucleotide preparation in broiler chickens. *In Proceedings 16th European Symposium on Poultry Nutrition*. Strasbourg, France, 511-514.

Ewaschuk, J.B., Naylor, J.M. & Zello, G.A., 2005. D-lactate in human and ruminant metabolism. *The Journal of Nutrition*, *135*(7), 1619-1625.

Faber, F. & Baumler, A.J., 2014. The impact of intestinal inflammation on the nutritional environment of the gut microbiota. *Immunology Letters*, *162*, 48–53.

Feye, K.M., Baxter, M.F.A., Tellez-Isaias, G., Kogut, M.H. & Ricke, S.C., 2020. Influential factors on the composition of the conventionally raised broiler gastrointestinal microbiomes. *Poultry Science*, *99*(2), 653-659.

Fialho, D.M., Roche, T.P. & Hud, N.V., 2020. Prebiotic syntheses of noncanonical nucleosides and nucleotides. *Chemical Reviews*, *120*(11), 4806-4830.

France M.M. & Turner J.R., 2017. The mucosal barrier at a glance. *Journal of Cell Science*, *130*, 307–314.

Gao, J., Zhang, H. J., Yu, S. H., Wu, S. G., Yoon, I., Quigley, J., Gao, P.Y. & Qi, G. H., 2008. Effects of yeast culture in broiler diets on performance and immunomodulatory functions. *Poultry Science*, *87*(7), 1377- 1384.

Gaskins, H.R., Collier, C.T. & Anderson, D.B., 2002. Antibiotics as growth promotants: mode of action. *Animal Biotechnology*, *13*(1), 29-42.

Ghosh, S. & LaPara, T.M., 2007. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *The ISME Journal*, *1*(3), 191-203.

Greko, C., 2001. Safety aspects on non-use of antimicrobials as growth promoters. In: Piva, A., Bach Knudsen, K.E. and Lindberg, J.E. (eds), Gut environment of pigs, 219–230. *Nottingham University Press*, Nottingham, UK.

Goo, D., Kim, J.H., Choi, H.S., Park, G.H., Han, G.P. & Kil, D.Y., 2019. Effect of stocking density and sex on growth performance, meat quality, and intestinal barrier function in broiler chickens. *Poultry Science*, *98*(3), 1153-1160.

Goossens, E., Debyser, G., Callens, C., De Gussem, M., Dedeurwaerder, A., Devreese, B., Haesebrouck, F., Flugel, M., Pelzer, S. & Thiemann, F., 2018. Elevated faecal ovotransferrin concentrations are indicative for intestinal barrier failure in broiler chickens. *Veterinary Research*, *49*(1), 51.

Guerville, M. & Boudry, G., 2016. Gastrointestinal and hepatic mechanisms limiting entry and dissemination of lipopolysaccharide into the systemic circulation. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *311*(1), 1-15.

Guetiya Wadoum, R.E., Zambou, N.F., Anyangwe, F.F., Njimou, J.R., Coman, M.M., Verdenelli, M.C., Cecchini, C., Silvi, S., Orpianesi, C., Cresci, A. & Colizzi, V., 2016. Abusive use of antibiotics in poultry farming in Cameroon and the public health implications. *British Poultry Science*, *57*(4), 483-493.

Huang, Q., Wen, C., Yan, W., Sun, C., Gu, S., Zheng, J. & Yang, N., 2022. Comparative analysis of the characteristics of digestive organs in broiler chickens with different feed efficiencies. *Poultry Science*, *101*(12), 102184.

Huyghebaert, G., Ducatelle, R. & Immerseel, F.V., 2011. An update on alternatives to antimicrobial growth promoters for broilers. *The Veterinary Journal*, *187*(2), 182–188.

Ibrahim, H.R., Sugimoto, Y. & Aoki, T., 2000. Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. *Biochimica et Biophysica Acta (BBA)- General Subjects*, *1523*, 196-205.

Iji, P.A., Saki, A. & Tivey, D.R., 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 2. Development and characteristics of intestinal enzymes. *British Poultry Science*, *42*, 514–522.

Jung, B. & Batal, A.B., 2012. Effect of dietary nucleotide supplementation on performance and development of the gastrointestinal tract of broilers. *British Poultry Science*, *53*(1), 98-105.

Kalia, V.C., Shim, W.Y., Patel, S.K.S., Gong, C. & Lee, J.K., 2022. Recent developments in antimicrobial growth promoters in chicken health: Opportunities and challenges. *Science of the Total Environment*, *834*, 155300.

Kamel, N.F., Hady, M.M., Ragaa, N.M. & Mohamed, F.F., 2021. Effect of nucleotides on growth performance, gut health, and some immunological parameters of broiler chicken exposed to high stocking density. *Livestock Science*, *253*, 104703.

Kers, J.G., Velkers, F.C., Fischer, E.A., Hermes, G.D., Stegeman, J.A. & Smidt, H., 2018. Host and environmental factors affecting the intestinal microbiota in chickens. *Frontiers in Microbiology*, *9*, 235.

Klasing, K.C., 1999. Avian gastrointestinal anatomy and physiology. In *Seminars in Avian and Exotic Pet Medicine*, *8*, (2), 42-50. WB Saunders.

Lambert, G.P., 2009. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. *Journal of Animal Science*, *87*(14), 101-108.

Larsson, D.G. & Flach, C.F., 2022. Antibiotic resistance in the environment. *Nature Reviews Microbiology*, *20*(5), 257-269.

Ledesma-Amaro, R., Jiménez, A., Santos, M.A. & Revuelta, J.L., 2013. Biotechnological production of feed nucleotides by microbial strain improvement. *Process Biochemistry*, *48*(9), 1263-1270.

Lee, D.N., Liu, S.R., Chen, Y.T., Wang, R.C., Lin, S.Y. & Weng, C.F., 2007. Effects of diets supplemented with organic acids and nucleotides on growth, immune responses and digestive tract development in weaned pigs. *Journal of Animal Physiology and Animal Nutrition*, *91*, 508–518.

Lee, K.C., Kil, D.Y. & Sul, W.J., 2017. Cecal microbiome divergence of broiler chickens by sex and body weight. *Journal of Microbiology*, *55*, 939-945.

Lei, K., Li, Y.L., Yu, D.Y., Rajput, I.R. & Li, W.F., 2013. Influence of dietary inclusion of Bacillus licheniformis on laying performance, egg quality, antioxidant enzyme activities, and intestinal barrier function of laying hens. *Poultry Science*, *92*(9), 2389-2395.

Li, B. & Webster, T.J., 2018. Bacteria antibiotic resistance: New challenges and opportunities for implant-associated orthopedic infections. *Journal of Orthopaedic Research*, *36*(1), 22-32.

Li, Y., Xing, S., Wang, X., Li, X., Zhang, M. & Feng, J., 2021. Effects of Increasing Stocking Density on the Performance and Ileal Microbiota of Broilers. *The Journal of Poultry Science*, *59*(3), 291-296.

Liu, G., Zhu, H., Ma, T., Yan, Z., Zhang, Y., Geng, Y., Zhu, Y. & Shi, Y., 2020. Effect of chronic cyclic heat stress on the intestinal morphology, oxidative status and cecal bacterial communities in broilers. *Journal of Thermal Biology*, *91*, 102619.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J.J. & Lee, M.D., 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Applied and Environmental Microbiology*, *69*(11), 6816-6824.

Lugsomya, K., Chatsuwan, T., Niyomtham, W., Tummaruk, P., Hampson, D.J. & Prapasarakul, N., 2017. Routine prophylactic antimicrobial use is associated with increased phenotypic and genotypic resistance in commensal *Escherichia coli* isolates recovered from healthy fattening pigs on farms in Thailand. *Microbial Drug Resistance* (Larchmont, NY) *24*, 213–223.

Ma, F., Xu, S., Tang, Z., Li, Z. & Zhang, L. 2020. Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans. *Biosafety and Health*, *3*(1), 32-38.

Maharjan, P., Martinez, D.A., Weil, J., Suesuttajit, N., Umberson, Mullenix, G., Hilton, K.M., Beitia, A. & Coon, C.N., 2021. Review: Physiological growth trend of current meat broilers and dietary protein and energy management approaches for sustainable broiler production. *Animal*, *15*, Supplement 1, 100284.

Manyi-Loh, C., Mamphweli, S., Meyer, E. & Okoh, A., 2018. Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. *Molecules*, *23*(4), 795.

Marshall, B.M. & Levy, S.B., 2011. *Clinical Microbiology Reviews*, *24*, 718–733.

Martinez, J.L., 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environmental Pollution*, *157*(11), 2893-2902.

Mateo, C.D. & Stein, H.H., 2004. Nucleotides and young animal health: can we enhance intestinal tract development and immune function? In: Lyons, T.P., Jacques, K.A. (Eds.), Nutritional biotechnology in the feed and food industries. proceedings of Alltech's 20th annual symposium. Nottingham University Press, Nottingham, UK, 159–168.

Mathur, S. & Singh, R., 2005. Antibiotic resistance in food lactic acid bacteria—a review. *International Journal of Food Microbiology*, *105*(3), 281-295.

Mitchell, M.A., Moretó, M. & Perry, G.C., 2006. Absorptive function of the small intestine: adaptations meeting demand. *Oxford CABI Publishing*, 43-64.

Miyakawa, M.E.F., Casanova, N.A. & Kogut, M.H., 2024. How did antibiotic growth promoters increase growth and feed efficiency in poultry? *Poultry Science*, *103*(2), 103278.

Mohamed, F. F., Hady, M. M., Kamel, N. F., & Ragaa, N. M., 2020. The impact of exogenous dietary nucleotides in ameliorating *Clostridium perfringens* infection and improving

intestinal barriers gene expression in broiler chicken. *Veterinary and Animal Science*, *10*, 100130.

Mohr, K.I., 2016. History of antibiotics research. *How to Overcome the Antibiotic Crisis: Facts, Challenges, Technologies and Future Perspectives*, 237-272.

Mottet, A. & Tempio, G., 2017. Global poultry production: current state and future outlook and challenges. *World's poultry science journal*, *73*(2), 245-256.

Muhammad, J., Khan, S., Su, J.Q., Hesham, A.E.L., Ditta, A., Nawab, J. & Ali, A., 2020. Antibiotics in poultry manure and their associated health issues: a systematic review. *Journal of Soils and Sediments*, *20*, 486-497.

Oakley, B.B., Morales, C.A., Line, J., Berrang, M.E., Meinersmann, R.J., Tillman, G.E., Wise, M.G., Siragusa, G.R., Hiett, K.L. & Seal, B.S., 2013. The poultry-associated microbiome: network analysis and farm-to-fork characterizations. *PloS one*, *8*(2), 57190.

Omotoso, A.O., Reyer, H., Oster, M., Maak, S., Ponsuksili, S. & Wimmers, K., 2023. Broiler physiological response to low phosphorus diets at different stages of production. *Poultry Science*, *102*(2), 102351.

Pan, D., & Yu, Z., 2014. Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes*, *5*, 108–19.

Pelícia, V. C., Sartori, J. R., Zavarize, K. C., Pezzato, A. C., Stradiotti, A. C., Araujo, P. C., Mituo, M.A.O. & Madeira, L. A., 2010. Effect of nucleotides on broiler performance and carcass yield. *Brazilian Journal of Poultry Science*, *12*(1), 31-34.

Plavnik, I., Macovsky, B. & Sklan, D., 2002. Effect of feeding whole wheat on performance of broiler chickens. *Animal Feed Science and Technology*, *96*(3-4), 229-236.

Pruden, A., Arabi, M. & Storteboom, H.N., 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. *Environmental Science and Technology*, *46*(21), 11541-11549.

Ranjitkar, S., Lawley, B., Tannock, G. & Engberg, R.M., 2016. Bacterial succession in the broiler gastrointestinal tract. *Applied and Environmental Microbiology*, *82*(8), 2399-2410.

Recht, J., Schuenemann, V.J. & Sanchez-Villagra, M.R., 2020. Host diversity and origin of zoonoses: The ancient and the new. *Animals*, *10*(9), 1672.

Remund, B., Yilmaz, B. & Sokollik, C., 2023. D-lactate: implications for gastrointestinal diseases. *Children*, *10*(6), 945.

Rey, A.I., Higueras, C., Olmeda, P., Sainz, A., Gálvez, B.G. & Larrosa, M., 2025. Markers of Gut Health in Small Animals: Focus on Fatty Acids and Amino Acids as Indicators of Intestinal Functionality and Microbiome Activity. *Animals*, *15*(13), 1927.

Richards, P., Fothergill, J., Bernardeau, M. & Wigley, P., 2019. Development of the caecal microbiota in three broiler breeds. *Frontiers in Veterinary Science*, *6*, 201.

Ricke, S.C., 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poultry Science*, *82*(4), 632-639.

Rodrigues, I. & Choct, M., 2018. The foregut and its manipulation via feeding practices in the chicken. *Poultry Science, 97*(9), 3188–3206.

Rosenbloom, C., 2023. 11 Healthy Foods That Are High in Iron. Available at: https://www.goodrx.com/well-being/diet-nutrition/foods-high-in-iron. [Accessed: 21 April 2024].

Rutz, F., Xiaver, E., Anciuti, M. Roll., V., & Rossi, P., 2008. The role of nucleotides in improving broiler pre-starter diets. In J. A. Taylor-Pickard and Spring. P., ed., Gut Efficiency: The Key Ingredient in Pig and Poultry Production: Elevating Animal

Performance and Health, by Publisher: Wageningen Academic Publishers, The Netherlands, 155-163.

Rychlik, I., 2020. Composition and function of chicken gut microbiota. *Animals*, *10*(1), 103.

Salah, M., Suprijatna, E., Djauhari, M.L. & Dwi, Y.V., 2019. The effects of nucleotide supplementation on the productivity, immune response and meat quality of broiler chicken reared under different environmental conditions. *Livestock Research for Rural Development*, *31*(11), 174.

Salam, M.A., Al-Amin, M.Y., Salam, M.T., Pawar, J.S., Akhter, N., Rabaan, A.A. & Alqumber, M.A., 2023. Antimicrobial resistance: a growing serious threat for global public health. *Healthcare*, *11*(13), 1946.

Santos, R.R., Oosterveer-van der Doelen, M.A., Tersteeg-Zijderveld, M.H., Molist, F. & Gehring, R., 2021. Induction of gut leakage in young broiler chickens fed a diet with low rye inclusion. *Heliyon*, *7*(12), 08547.

Salois, M.J., Cady, R.A. & Heskett, E.A., 2016. The environmental and economic impact of withdrawing antibiotics from US broiler production. *Journal of Food Distribution Research*, *47*(1), 1–2.

South African Poultry Association., 2016. Industry Profile. http://www.sapoultry.co.za/. [Accessed: 21 April 2024].

Sarrabayrouse, G., Bossard, C., Chauvin, J.M., Jarry, A., Meurette, G., Quevrain, E., Bridonneau, C., Preisser, L., Asehnoune, K., Labarriere, N., Altare, F., Sokol, H., & Jotereau, F., 2014. CD4CD8αα lymphocytes, a novel human regulatory T cell subset induced by colonic bacteria and deficient in patients with inflammatory bowel disease. *PLoS Biology 12*, 1001833

Sauer, N., Eklund, M., Bauer, E., Gänzle, M.G., Field, C.J., Zijlstra, R.T. & Mosenthin, R., 2012. The effects of pure nucleotides on performance, humoral immunity, gut structure,

and numbers of intestinal bacteria of newly weaned pigs. *Journal of Animal Science*, *90*, 3126–3134.

Sauer, N., R. Mosenthin & Bauer, E., 2011. The role of dietary nucleotides in single-stomached animals. *Nutrition Research Reviews*, *24*(1), 46–59.

Schjørring, S. & Krogfelt, K.A., 2011. Assessment of bacterial antibiotic resistance transfer in the gut. *International Journal of Microbiology*, *2001*(1), 312956.

Schwiertz, A., Spiegel, J., Dillmann, U., Grundmann, D., Bürmann, J., Faßbender, K., Schäfer, K.H. & Unger, M.M., 2018. Fecal markers of intestinal inflammation and intestinal permeability are elevated in Parkinson's disease. *Parkinsonism & Related Disorders*, *50*, 104-107.

Shang, Y., Kumar, S., Oakley, B. & Kim, W.K., 2018. Chicken gut microbiota: Importance and detection technology. *Frontiers in Veterinary Science*, *5*, 254.

Shivkumar, M.C., Narasimhamurthy, H.N. & Swamy, H.V.L.N., 2009. Effect of feeding NuPro at different time intervals on the performance of commercial broilers. Abstracts of papers, Nutrition–I, symposia and oral session. *International Poultry Scientific Forum*, 144.

Smith, J.A., 2011. Experiences with drug-free broiler production. *Poultry Science*, *90*, 2670–2678.

Song, Z., Cheng, K., Zhang, L. & Wang, T., 2017. Dietary supplementation of enzymatically treated Artemisia annua could alleviate the intestinal inflammatory response in heat-stressed broilers. *Journal of Thermal Biology*, *69*, 184-190.

Stanley, D., Geier, M.S., Chen, H., Hughes, R.J. & Moore, R. J., 2015. Comparison of fecal and cecal microbiotas reveals qualitative similarities but quantitative differences. *BMC Microbiology, 15*, 51.

Sumanu, V.O., Naidoo, V., Oosthuizen, M. & Chamunorwa J.P., 2023. A Technical Report on the Potential Effects of Heat Stress on Antioxidant Enzymes Activities, Performance and Small Intestinal Morphology in Broiler Chickens Administered Probiotic and Ascorbic Acid during the Hot Summer Season. *Animals (Basel)*, *13*(21), 3407.

Sun, X., Zhang, H., Sheikhahmadi, A., Wang, Y., Jiao, H., Lin, H. & Song, Z., 2015. Effects of heat stress on the gene expression of nutrient transporters in the jejunum of broiler chickens (*Gallus gallus domesticus*). *International Journal of Biometeorology*, *59*, 127-135.

Suzuki, K. & Nakajima, A., 2014. New aspects of IgA synthesis in the gut. *International Immunology*, *26*(9), 489-494.

Svihus, B., 2011. The gizzard: function, influence of diet structure and effects on nutrient availability. *World's Poultry Science Journal*, *67*(2), 207-224.

Svihus, B., 2014. Function of the digestive system. *Journal of Applied Poultry Research*, *23*, 306–314.

Svihus, B., Hetland, H., Choct, M. & Sundby, F., 2002. Passage rate through the anterior digestive tract of broiler chickens fed on diets with ground and whole wheat. *British Poultry Science*, *43*(5), 662-668.

Svihus, B., Juvik, E., Hetland, H. & Krogdahl, Å., 2004. Causes for improvement in nutritive value of broiler chicken diets with whole wheat instead of ground wheat. *British Poultry Science*, *45*(1), 5 5-60.

Teirlynck, E., Bjerrum, L., Eeckhaut, V., Huygebaert, G., Pasmans, F., Haesebrouck, F., Dewulf, J., Ducatelle, R. & Van Immerseel, F., 2009. The cereal type in feed influences gut wall morphology and intestinal immune cell infiltration in broiler chickens. *British Journal of Nutrition*, *102*, 1453–1461.

Teirlynck, E., Gussem, M.D.E., Dewulf, J., Haesebrouck, F., Ducatelle, R. & Van Immerseel, F., 2011. Morphometric evaluation of "dysbacteriosis" in broilers. *Avian Pathology*, *40*(2), 139-144.

Tellez, G., Latorre, J.D., Kuttappan, V.A., Kogut, M.H., Wolfenden, A., Hernandez-Velasco, X., Hargis, B.M., Bottje, W.G., Bielke, L.R. & Faulkner, O.B., 2014. Utilization of rye as energy source affects bacterial translocation, intestinal viscosity, microbiota composition, and bone mineralization in broiler chickens. *Frontiers in Genetics*, *5*, 339.

Torok, V.A., Hughes, R.J., Ophel-Keller, K., Ali, M. & MacAlpine, R., 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. *Poultry Science*, *88*(12), 2474-2481.

Tůmová, E., Chodová, D., Skřivanová, E., Laloučková, K., Šubrtová-Salmonová, H., Ketta, M., Machander, V. & Cotozzolo, E., 2021. Research note: The effects of genotype, sex, and feeding regime on performance, carcasses characteristic, and microbiota in chickens. *Poultry Science*, *100*(2), 760-764.

Untari, T., Herawati, O., Anggita, M., Asmara, W., Wahyuni, A.E.T.H. & Wibowo, M.H., 2021. The effect of antibiotic growth promoters (AGP) on antibiotic resistance and the digestive system of broiler chicken in Sleman, Yogyakarta. In *BIO Web of Conferences*, 33, 04005. EDP Sciences.

Verraes, C., Van Boxstael, S., Van Meervenne, E., Van Coillie, E., Butaye, P., Catry, B., De Schaetzen, M.A., Van Huffel, X., Imberechts, H., Dierick, K. & Daube, G., 2013. Antimicrobial resistance in the food chain: a review. *International Journal of Environmental Research and Public Health*, *10*(7), 2643-2669.

Wallace, R.J., Oleszek, W., Franz, C., Hahn, I., Baser, K.H.C., Mathe, A. & Teichmann, K., 2010. Dietary plant bioactives for poultry health and productivity. *British Poultry Science*, *51*(4), 461–487.

Wang, L., Llorente, C., Hartmann, P., Yang, A.M., Chen, P. & Schnabl, B. 2015. Methods to determine intestinal permeability and bacterial translocation during liver disease. *Journal of Immunological Methods*, *421*, 44-53.

Wang, Z., Cerrate, S., Coto, C., Sacakli, P., Yan, F., Costa, F. & Waldroup, P.W., 2009. Evaluation of Nupro yeast product in diets for broilers. *International Journal of Poultry Science*, *8*, 515-520.

Watkins, S.M. & German, J.B., 2002. Unsaturated fatty acids. In *Food lipids*, 578-607. CRC Press.

Wen, C., Yan, W., Mai, C., Duan, Z., Zheng, J., Sun, C. & Yang, N., 2021. Joint contributions of the gut microbiota and host genetics to feed efficiency in chickens. *Microbiome*, *9*, 1-23.

World Health Organization., 2015. *Global Action Plan on Antimicrobial Resistance*; World Health Organization: Geneva, Switzerland, 1–28.

Wu, G., 2013. Functional amino acids in nutrition and health. *Amino acids*, *45*, 407-411.

Wu, C., Yang, Z., Song, C., Liang, C., Li, H., Chen, W., Lin, W. & Xie, Q., 2018. Effects of dietary yeast nucleotides supplementation on intestinal function, intestinal microbiota, and humoral immunity in specific pathogen-free chickens. *Poultry Science*, *97*(11) 3837-3846.

Yacoubi, N., Saulnier, L., Bonnin, E., Devillard, E., Eeckhaut, V., Rhayat, L., Ducatelle, R., & Van Immerseel, F., 2018. Short-chain arabinoxylans prepared from enzymatically treated wheat grain exert prebiotic effects during the broiler starter period. *Poultry Science, 97*, 412–424.

Yadav, S., Caliboso, K.D., Nanquil, J.E., Zhang, J., Kae, H., Neupane, K., Mishra, B. & Jha, R., 2021. Cecal microbiome profile of Hawaiian feral chickens and pasture-raised

broiler (commercial) chickens determined using 16S rRNA amplicon sequencing. *Poultry Science*, *100*(7), 101181.

Yegani, M. & Korver, D.R., 2008. Factors Affecting Intestinal Health in Poultry. *Poultry Science*, *87*(10), 2052–2063.

Yu, D.G., Namgung, N., Kim, J.H., Won, S.Y., Choi, W.J. & Kil, D.Y., 2021. Effects of stocking density and dietary vitamin C on performance, meat quality, intestinal permeability, and stress indicators in broiler chickens. *Journal of Animal Science and Technology*, *63*(4), 815.

Zahran, R.H., Khader, N.E. & Ahmed, T.E., 2020. Effect of dietary nucleotide supplementation on broiler performance and economic efficiency. *Benha Veterinary Medical Journal*, *39*(1), 34-39.

Zauk, N.H.F., Lopes, D.C. M., Silva, L.M., Dallmann, P.R., Ribeiro, C.L.G., Pinto, J., Mielke, R.B., Anciuti, M.A. & Rutz, F., 2006. Performance and carcass traits of broilers fed pre-starter diets containing Nupro®. Nutritional Biotechnology in The Feed and Food Industries, Posters presented at Alltech's 22nd Annual Symposium (Suppl. 1). Lexington, Ky, USA, 10.

Zhang, C., Zhao, X.H., Yang, L., Chen, X.Y., Jiang, R.S., Jin, S.H. & Geng, Z.Y., 2017. Resveratrol alleviates heat stress-induced impairment of intestinal morphology, microflora, and barrier integrity in broilers. *Poultry Science*, *96*(12), 4325-4332.

Zhang, G., Ma, Q., & Ji, C., 2008. Effects of dietary inosinic acid on carcass characteristics, meat quality, and deposition of inosinic acid in broilers. *Poultry Science*, *87*, 1364-1369.

Zhang, H., Li, D., Liu, L., Xu, L., Zhu, M., He, X. & Liu, Y., 2019. Cellular Composition and Differentiation Signaling in Chicken Small Intestinal Epithelium. *Animals*, *9*, 870.

Zhou ,B., Liu, L., Liu, J., Yuan, F., Tian, E & Wang, H., 2015. Effect of Diclazuril on the Bursa of Fabricius Morphology and Siga Expression in Chickens Infected with *Eimeria tenella*. *Korean Journal of Parasitology*, *53*, 675–682.

Zivkovic, D., 2007. Investigations on 2,7-diamino-9-fluorenol photochemistry. PhD Thesis, University of Basel.

Zuo, T. & Ng, S.C., 2018. The gut microbiota in the pathogenesis and therapeutics of inflammatory bowel disease. *Frontiers in Microbiology*, *9*, 2247.

3. CHAPTER THREE: EFFECT OF PHASED NUCLEOTIDE SUPPLEMENTATION ON GROWTH, HAEMATOLOGY AND MEAT QUALITY IN DENSELY STOCKED ROSS 308 BROILERS

3.1 Abstract

The consistent demand for chicken meat has driven commercial farming toward intensive production systems. These production systems often alter optimal growth conditions for broilers, introducing stressors that can influence performance and meat quality, thereby warranting consideration of appropriate feed additives other than antibiotic growth promoters (AGP). Supplemental nucleotides have emerged as a promising alternative to AGP in rapidly growing or physiologically stressed broilers. In such birds, the demand for nucleotides often exceeds the capacity of both *de novo* synthesis and the salvage pathway, necessitating exogenous supplementation. However, continuous nucleotide supplementation can elevate production costs and may not always be essential. Hence, this study compared the effects of continuous vs phased yeast-based nucleotide supplementation on growth performance, haemato-biochemical parameters, and meat quality traits in densely stocked Ross 308 broiler chickens. A total of 900, one-week-old, unsexed Ross 308 broiler chicks were evenly allotted to 36 pens (25 birds/1.2 m^2) and reared on six isonitrogenous and isocaloric diets. The feeding strategies were: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF). Feed intake and growth performance were estimated for each feeding phase. Blood collection was done on day 40, and at six weeks of age, birds were slaughtered for meat quality measurements. Strategy NCS promoted the lowest ($p < 0.05$) feed intake (FI), similar to diets NCG, NCF, and NCC, while POSCON promoted the highest FI. In addition, POSCON promoted the highest ($p < 0.05$) body

weight gain (BWG), followed by NCG. Furthermore, NCC promoted the highest ($p < 0.05$) feed conversion ratio (FCR), whereas POSCON promoted the least FCR. Blood urea nitrogen (BUN), albumin (ALB), alanine transaminase (ALT), and total bilirubin (TBIL) were the only serum biochemistry parameters affected ($p < 0.05$) by feeding strategies, with NCG promoting the highest ALB, ALT, and TBIL, while POSCON promoted the lowest BUN, ALB, ALT, and TBIL. Drip loss and shear force were the only meat quality traits affected ($p < 0.05$) by feeding strategy. Supplementing yeast-based nucleotides on a phased basis had the same effects as continuous supplementation on growth performance and carcass traits. Therefore, phased supplementation of yeast-based nucleotides in broiler diets is recommended as a cost-cutting strategy.

Keywords: antibiotic growth promoters, *de novo*, growth performance, haemato-biochemical, nucleotides

3.2 Introduction

According to the OECD-FAO (2025), poultry meat is projected to account for 21% of total growth in meat protein production worldwide by 2034, underscoring its vital contribution to global food security. Its widespread consumption is fueled by the lack of cultural and religious barriers, affordability, and the nutritional advantages offered by broiler meat and related products (Jie *et al.*, 2024). To meet the ever-increasing demand for broiler meat, producers must rely on intensive production systems that produce more meat per unit area. However, intensive broiler production systems are often associated with multiple stressors that adversely impact the health, welfare, and growth performance of the birds. Consequently, the incidence of diseases tends to be higher in these systems, posing significant challenges to sustainable poultry production. In these production systems, antibiotic growth promoters have traditionally been used to control diseases, boost immunity, and enhance growth performance (Mehdi *et al.*, 2018). However, overreliance on antibiotics is associated with compromised food safety, environmental contamination, and the rise in antimicrobial resistance (Zhen *et al.*, 2023), leading to bans/restrictions in certain regions. Unfortunately, AGP-free production systems are characterized by substandard bird performance (Liu *et al.*, 2024), hence the need for alternatives such as nucleotides.

Nucleotides are key cell components and building blocks for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA); they also play a significant role in physiological and biochemical processes (Wang *et al.*, 2024). Although synthesized endogenously through *de novo* and salvage pathways (Hoffman, 2003), nucleotides may be inadequate under stressful conditions and periods of rapid growth, requiring supplemental exogenous supply through broiler diets (Gil *et al.*, 2002; Li *et al.*, 2007). Indeed, continuous supplementation of dietary nucleotides in broiler diets has been proven to enhance immune response (Boza & Martines-Augustin, 2002), through their ability to increase the manufacture of macrophages and leukocytes (Dil &

Qureshi, 2002). Similarly, improved growth performance (Wang *et al.*, 2009) and meat quality parameters (Chiofalo *et al.*, 2011) have been observed in broilers supplemented with exogenous nucleotides. Improved growth performance can be attributed to enhanced nutrient absorption and utilisation, as nucleotide supplementation has been proven to increase intestinal villi length (Pelícia *et al.*, 2010). Increased feed conversion rate and body weight gain result in better carcass yields (Rutz *et al.*, 2008) and better economic returns.

While continuous dietary nucleotide supplementation has been observed to effectively alleviate stress-related issues in poultry, its excessive cost limits its widespread adoption as an alternative to AGP. The cost of nucleotide supplementation could be reduced by adopting a periodic supplementation strategy, targeting the more stressful phases in the broiler's lifecycle, an approach that has not been sufficiently investigated. Therefore, this study is a comparative assessment of the effects of phased *versus* continuous supplementation of dietary nucleotides as an alternative to zinc bacitracin AGP. This study tested the hypothesis that phased dietary nucleotides supplementation will achieve similar results as continuous supplementation of dietary nucleotides as an alternative to zinc bacitracin AGP on growth performance metrics, blood parameters, and meat quality in densely stocked broiler chickens.

3.3 Materials and methods

3.3.1 Study site

A six-week feeding trial was conducted at Rooigrond Poultry Farm (25º92′76″S; 25º80′08″E) in the North-West province, South Africa. The temperatures in summer range between 22 and 34ºC, as well as 2 and 20ºC in winter.

3.3.2 Dietary treatments

The raw ingredients used to formulate experimental diets were acquired from Simplegrow Agric Services (pty) Ltd and Nutroteq (Centurion, South Africa). Basal broiler starter (day 7-14), grower (day 15-28), and finisher (day 29-42) diets were formulated to meet the daily nutritional requirements of Ross 308 birds (Aviagen, 2022). The following six experimental feeding strategies were designed: 1. A basal diet containing antibiotic growth promoters (AGP), fed from the starter phase through to the finisher phase [POSCON]; 2. A basal diet with neither AGP nor nucleotides, fed from the starter phase through to the finisher phase [NEGCON]; 3. A basal diet containing 0.05% nucleotides in place of AGP, fed continuously from the starter phase through to the finisher phase [NCC]; 4. A basal diet containing 0.05% nucleotides in place of AGP, fed in the starter phase only [NCS]; 5. A basal diet containing 0.05% nucleotides in place of AGP, fed in the grower phase only [NCG]; 6. A basal diet containing 0.05% nucleotides in place of AGP, fed in the finisher phase only [NCF]. Birds were fed the basal broiler diet outside the yeast-based nucleotide supplementation periods. The inclusion of nucleotides at 0.05% is commonly applied and considered to be economically efficient.

Table 3.1: Ingredients and nutritional composition (g/kg) of basal diets used in the feeding trial.

Ingredients	Starter (7-14 d)	Grower (15-28 d)	Finisher (29-42d)
Yellow maize	43.53	47.62	53.87
Soybean meal 46% CP	41.71	37.94	32.67
L-Lysine HCl	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	0.24	0.26	0.25
Sodium bicarbonate	0.28	0.25	0.27
Poultry VT Premix 15.8% Ca	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	0.5	0.42	0.36
AME Poultry	2975	3050	3100
SID Methionine	0.71	0.72	0.6
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 Methionine + SID Cysteine	1	0.92	0.86

VT = Vitamin; Av = Available; SID = Standard ileal digestibility; AME = Apparent metabolizable energy.

3.3.3 Experimental design and management

Nine hundred (and an additional replacement stock of 64 birds), one-week-old, unsexed Ross 308 chicks were acquired from Chicken Ranch in Pretoria (Gauteng, South Africa). The chicks were randomly and evenly distributed to 36 replicate pens [1.5 m L × 0.8 m W × 1.55 m H], to

which the experimental feeding strategies were randomly allocated in a completely randomized design. According to SAPA (2015), the recommended stocking density is 15 birds/m^2; thus, this study used a high stocking density of 1 bird/0.048 m^2 against 1 bird/0.067 m^2. Accordingly, each pen (experimental unit) in this study housed 25 birds. Each feeding strategy was replicated 6 times. A cleaning schedule was followed for the broiler house daily, and a daily washing and filling program was implemented to keep the drinkers clean. Environmental factors, including temperature, lighting, humidity, ventilation, and bedding, were managed through the Aviagen (2018) specifications. Additionally, the bedding was changed every 3-4 days from day 25 until the end of the trial.

3.3.4 Growth performance

One-week-old chicks were weighed to determine initial body weights and then weighed to determine body weight gain (BWG) for each feeding phase (starter from day 7 to 14, grower from day 15 to 28, and finisher from day 29 to 42). Feed offered and feed refused were measured at the end of each phase and used to calculate feed intake (FI). Mortalities were recorded on occurrence, and the carcasses were weighed to correct the feed conversion ratio, which was determined as a ratio of FI and BWG.

3.3.5 Blood collection and analysis

At day 40 of age, blood samples were collected from two birds randomly selected from each experimental unit to determine haematological, serum biochemical parameters, and stress biomarkers. The birds were starved for 6 hours before blood (4 mL) was collected by puncturing the wing vein using a 5 mL scalp vein needle set (Morishita, 2019). Approximately 2 mL of blood was transferred into two sets of sterilized tubes, with the haematological tubes containing ethylenediaminetetraacetic acid (EDTA) as the anti-coagulant and the serum biochemistry tubes containing silicon as the coagulant. Haematological parameters, namely

white blood cells, lymphocytes, haematocrits, monocytes, heterophils, and platelets, were determined using an automated IDEXX LaserCyte Haematology Analyser (IDEXX Laboratories, Johannesburg, South Africa). The clotted blood was centrifuged into a macro centrifuge (1 500 cfg for 15 minutes) to generate serum for the analysis of serum glucose, cholesterol, blood urea nitrogen, phosphorus, calcium, alanine transferase, γ-glutamyl transferase, alkaline phosphatase, amylase, lipase, total proteins, total bilirubin, albumin, globulin, and creatinine using an automatic biochemical analyzer using an automated IDEXX Vet Test Chemistry Analyser (IDEXX Laboratories, Johannesburg, South Africa).

3.3.6 Carcass and meat quality traits

At 42 days of age, feed was withdrawn from the broilers for 12 hours to empty the crop. The chickens were taken to Rooigrond abattoir, where they were stunned and then slaughtered by cutting the jugular vein using a sharp-ended knife. After bleeding ceased, they were de-feathered and packaged according to their experimental units. Immediately after slaughter, the carcasses were dissected to measure breast, thigh, drumstick, and wing weights, which were expressed as g/100 g hot carcass weight (HCW).

Breast meat pH and temperature were taken 24 hours post-slaughter using a Corning model 4 pH-temperature meter (Corning Glass Works, Medfield, MA), equipped with a spear-type electrode (Ingold Messtechnik AG, Udorf, Switzerland). The colour values: *L** (lightness), *a** (redness), and *b** (yellowness) were measured using a Minolta colorimeter (Spectrophotometer CM 2500c, Konika Minolta, Osaka, Japan) that has a 20 mm diameter measurement area with innovative 45º a:0 geometry optics. The colour measures were subsequently used to calculate hue angle and chroma values. The colorimeter was calibrated prior to taking measurements.

Water holding capacity (WHC) of breast meat was determined based on the amount of water lost when pressure was applied to the freshly cut pectoralis major muscles following a method described by Marareni & Mnisi (2020). Briefly, about 10 g freshly cut slices of breast meat samples were placed between a pre-weighed 18 Whatman filter-paper and pressed under a pressure of 60 kg for 5 minutes using dumbbell weights. The WHC was then calculated according to the following equation:

$$\text{WHC} = 100 - [\frac{\text{Weight before pressing} - \text{Weight after pressing}}{\text{Weight before pressing}} \; x \; 100]$$

Drip loss was determined following the method by Yang *et al.* (2025), with modifications, wherein the weight loss of a breast meat sample was measured following refrigeration. Briefly, about 10 g breast meat samples were placed in a sealed container and stored at 4°C for 72 hours. The breast meat samples were measured again at 72 hours, and drip loss was expressed as a percentage of the initial weight according to the following equation:

$$\text{DL} = [\frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}}] \; x \; 100$$

Cooking loss was determined following a method by Pang *et al.* (2020), with slight modifications, wherein about 40 g breast meat samples were cooked in a Henny Penny MCS-6 combi oven at 75 °C for 45 minutes. After cooking, samples were cooled for 5 minutes before reweighing to estimate for cooking loss calculated using the following equation:

$$\text{CL} = [\frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}}] \; x \; 100$$

To measure shear force (Newtons), raw breast meat samples were sheared perpendicular to the direction of muscle fibres using a Meullenet-Owens Razor Shear Blade (A/MORS) mounted on a Texture Analyser (TA XT plus, Stable Micro Systems, Surrey, UK).

3.4 Statistical analysis

Data were analysed for homogeneity of variance and for normality using Levene's test and the NORMAL option in the Procedure Univariate statement, respectively. Cross-sectional data were analysed using a one-way ANCOVA (SAS, 2010) with feeding strategy as the fixed factor using the following general linear model:

$$Yij = u + Ti + \beta\ (Xij - \bar{X}) + Eij$$

Yij = dependent variable, u = overall mean, Ti = effects of the fixed factor, β = regression coefficient of the covariate, Xij = covariate value (initial body weight), $\bar{X}$ = covariate mean, Eij = residual error. For all measured parameters, significance was declared at $p < 0.05$, and least squares means were compared by the probability of difference option.

3.5 Results

3.5.1 Mortality

There were no significant differences ($p > 0.05$) observed in bird mortality across all feeding strategies recording 3.642% mean for the entire flock.

3.5.2 Growth performance

3.5.2.1 Feed intake

The effect of feeding strategies on feed intake in Ross 308 broilers was significantly different ($p < 0.05$). Figure 3.1 presents the overall effects of feeding strategies on feed intake in Ross 308 broilers. In the starter phase, Ross 308 broilers reared on the NCF feeding strategy recorded the lowest ($p < 0.05$) FI with statistical similarities to POSCON, NCS, NCG, and NCC feeding strategies. Contrastingly, the Ross 308 broilers reared on the NEGCON recorded the highest FI, which did not differ from values observed in birds reared on POSCON, NCS, NCG, and

NCC feeding strategies. Additionally, in the grower phase, Ross 308 broilers on the NCG feeding strategy recorded the lowest feed intake ($p < 0.05$), with statistical similarities to NEGCON, NCS, NCF, and NCC feeding strategies. While the positive control recorded the highest FI. Finally, in the finisher phase, Ross 308 broilers reared on the NCS feeding strategy promoted the lowest ($p < 0.05$) FI, which was statistically similar to broilers on the NEGCON, NCG, and NCF feeding strategies. On the other hand, Ross 308 broilers reared on POSCON recorded the highest FI with statistical similarities to broilers reared on the NCC feeding strategy. Overall, the broilers reared on the NCS feeding strategy recorded the lowest ($p <0.05$) FI with statistical similarities to the negative control, NCG, NCF, and NCC feeding strategies. Whereas the positive control recorded the highest ($p < 0.05$) FI.

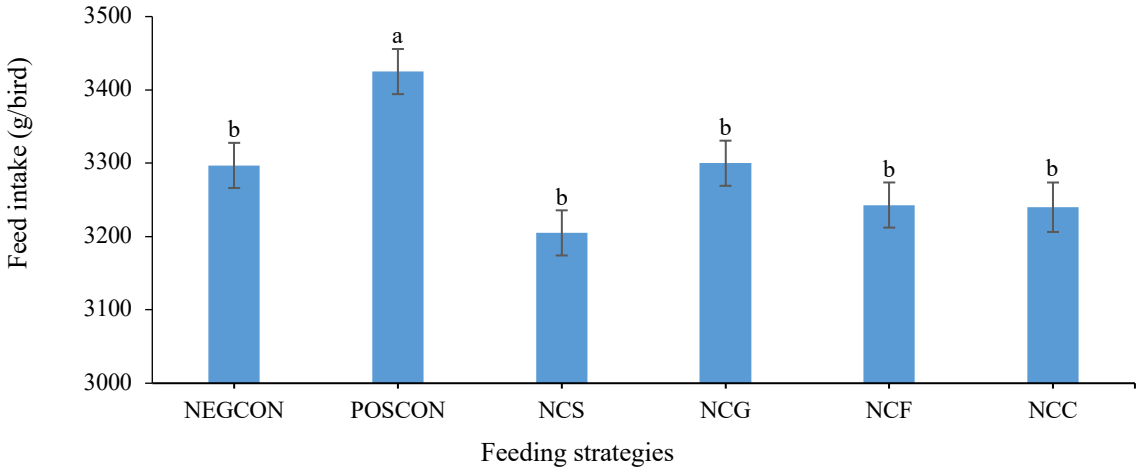


Figure 3.1: Effect of nucleotide feeding strategies on overall feed intake in Ross 308 broiler chickens.

abc Means with different superscripts are different ($p < 0.05$). Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

3.5.2.2 Body weight gain

The feeding strategies had significant effects on Ross 308 broiler weight gains ($p < 0.05$). Figure 3.2 presents the effects of feeding strategies on the overall weight gains of Ross 308 broiler chickens. In the starter phase, Ross 308 broilers on the NCG feeding strategy recorded the lowest ($p < 0.05$) body weight gain (BWG) with statistical similarities to the NEGCON, NCF, and NCC. POSCON promoted the highest ($p < 0.05$) BWG with statistical similarities to the NCF feeding strategy. While in the grower phase, Ross 308 broilers reared on the NCC feeding strategy recorded the lowest ($p < 0.05$) BWG, followed by NEGCON with statistical similarities to NCS, NCG, and NCF feeding strategies. Additionally, Ross 308 broilers on the POSCON strategy recorded the highest BWG with statistical similarities to phased supplementation. Furthermore, in the finisher phase, Ross 308 broilers on NCF recorded the lowest ($p < 0.05$) BWG and were statistically similar to the NEGCON, NCS, NCG, and NCC feeding strategies. In contrast, birds on POSCON recorded the highest ($p < 0.05$) BWG and were statistically similar to Ross 308 boilers on the NCS, NCG, and NCC feeding strategies. The final body weight ($p < 0.05$) was the lowest in Ross 308 broilers on the NEGCON, which were statistically similar to the NCS, NCG, NCF, and NCC feeding strategies.

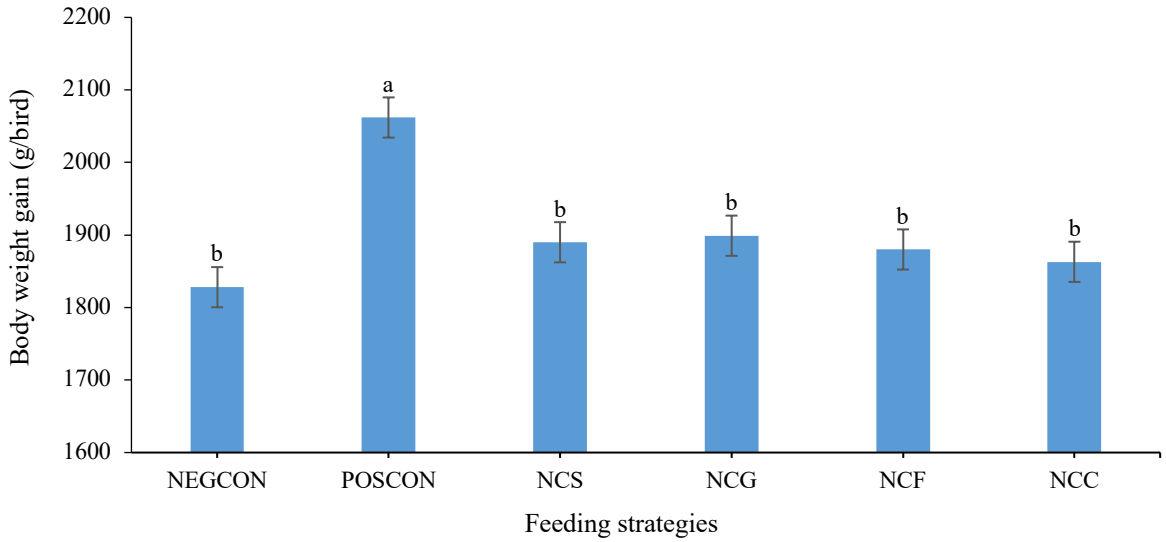


Figure 3.2: Impact of nucleotide feeding strategies on overall body weight gain in Ross 308 broiler chickens

abc Means with different superscripts are different ($p < 0.05$). Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

3.5.2.3 Feed conversion ratio

The effects of feeding strategies on feed conversion ratio (FCR) in Ross 308 broilers were statistically significant ($p < 0.05$). Figure 3.3 presents the effects of feeding strategies on overall FCR in Ross 308 broilers. In the starter phase, Ross 308 broilers on the NCF feeding strategy recorded the lowest FCR with statistical similarities to POSCON, NCG, and NCC diets. Broilers on the NEGCON recorded the highest FCR with statistical similarities to the NCS, NCG, and NCC feeding strategies. No differences were observed in FCR in the grower phase. Nonetheless, in the finisher phase, the Ross 308 broilers reared on NEGCON recorded the

highest ($p < 0.05$) FCR value, which was statistically similar to those reared on the NCF feeding strategy. In contrast, the Ross 308 broilers on the NCS feeding strategy recorded the lowest ($p < 0.05$) FCR value and were statistically similar to the positive control, NCG, and NCC feeding strategies.

Overall, the broilers reared on POSCON recorded the lowest ($p < 0.05$) FCR, while those on the NEGCON recorded the highest FCR with statistical similarities to the phased and continuous dietary yeast-based nucleotide supplementation feeding strategies.

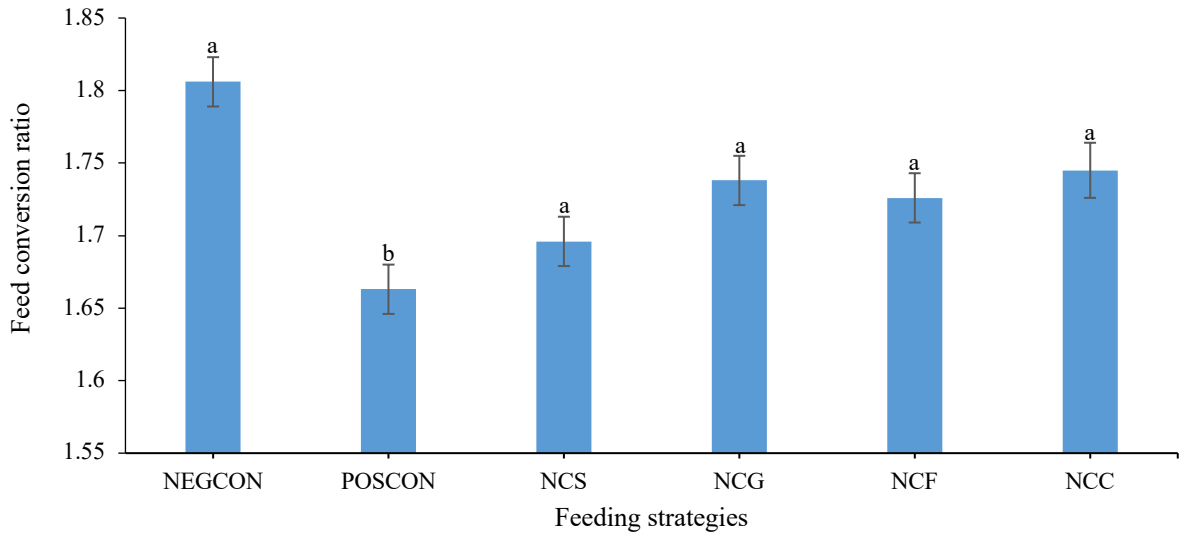


Figure 3.3: Effects of nucleotide feeding strategies on the overall feed conversion ratio of Ross 308 broilers.

^{abc} Means with different superscripts are different ($p < 0.05$). Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

3.5.3 Blood parameters

3.5.3.1 Serum biochemistry

Only serum albumin (ALB), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and total bilirubin (TBIL) were significantly ($p < 0.05$) affected by feeding strategies. The NCS feeding strategy promoted the highest ($p < 0.05$) BUN value, while the NCG feeding strategy promoted the highest ($p < 0.05$) ALB, ALT, and TBIL values. Additionally, POSCON promoted the lowest ($p < 0.05$) ALB, ALT, BUN, and TBIL values.

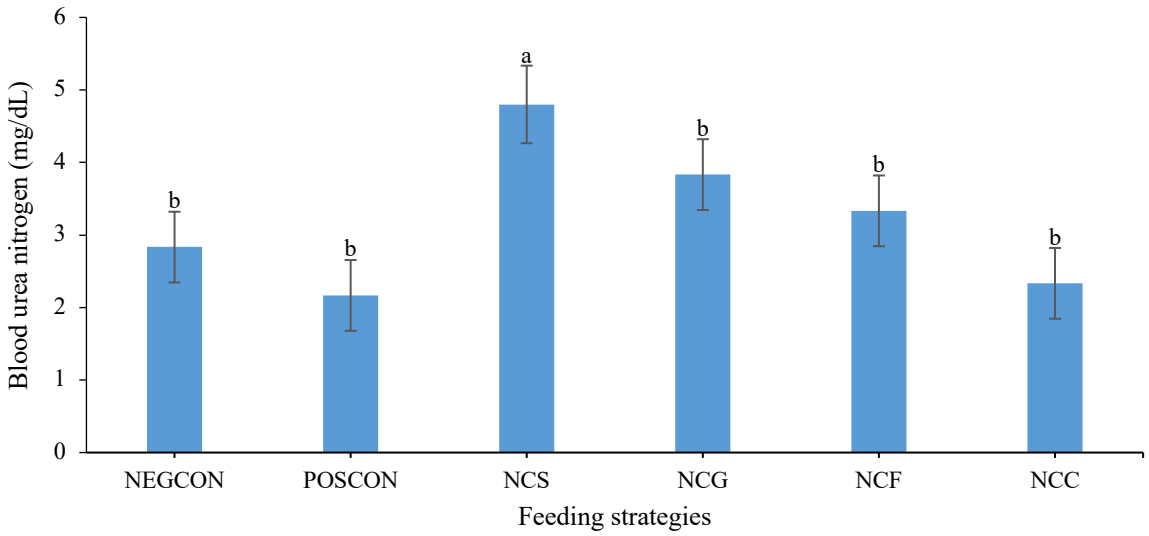


Figure 3.4: Serum blood urea nitrogen in Ross 308 broiler chickens supplemented with dietary nucleotides.

^{abc} Means with different superscripts are different ($p < 0.05$). Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

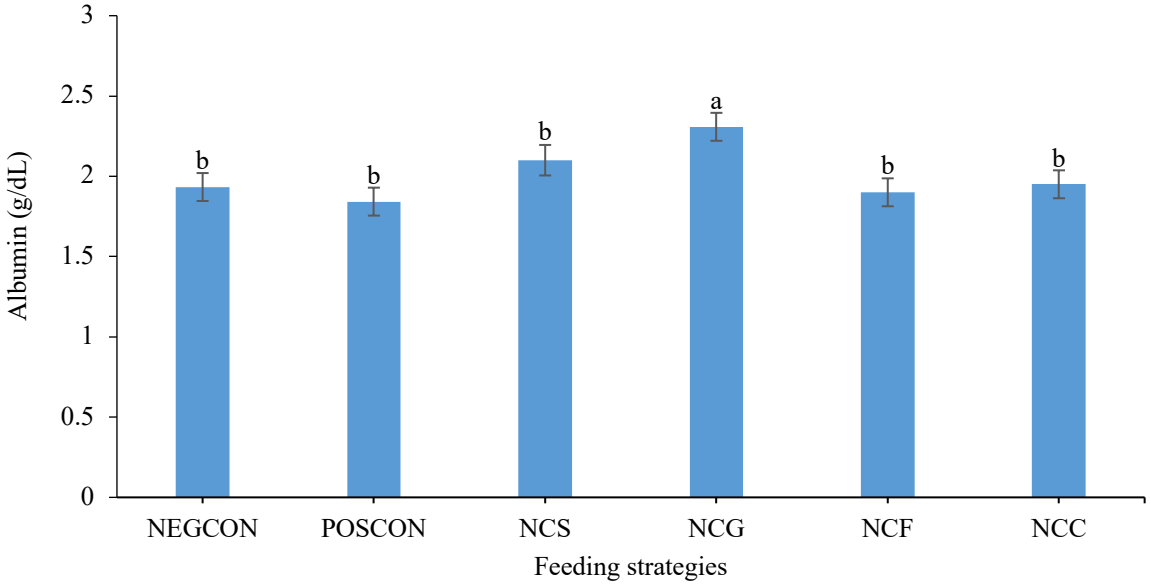


Figure 3.5: Serum albumin in Ross 308 broiler chickens supplemented with dietary nucleotides.

abc Means with different superscripts are different ($p < 0.05$). Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

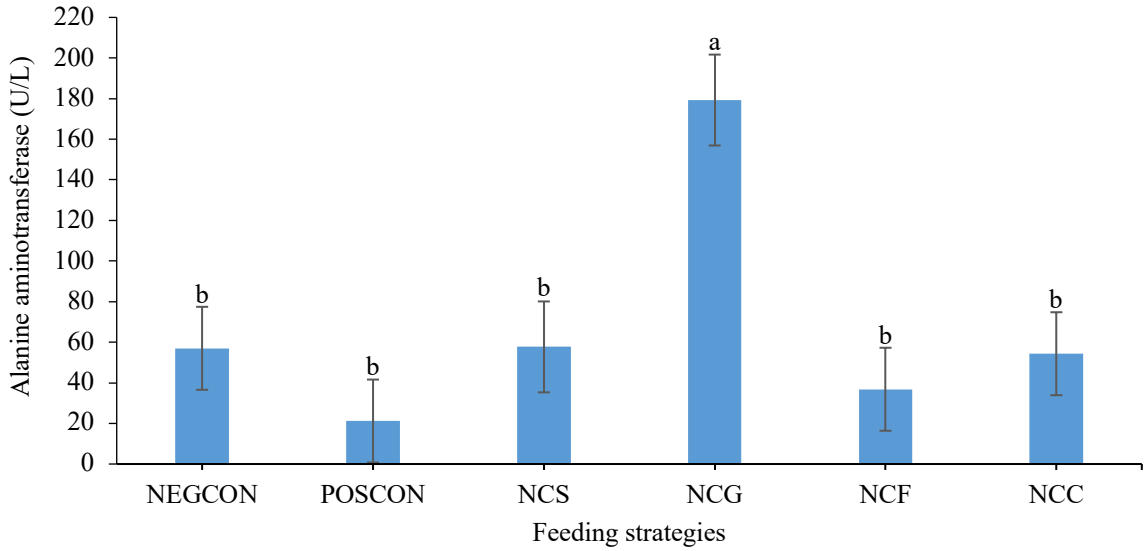


Figure 3.6: Serum alanine aminotransferase in Ross 308 broiler chickens supplemented with dietary nucleotides.

abc Means with different superscripts are different ($p < 0.05$). Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

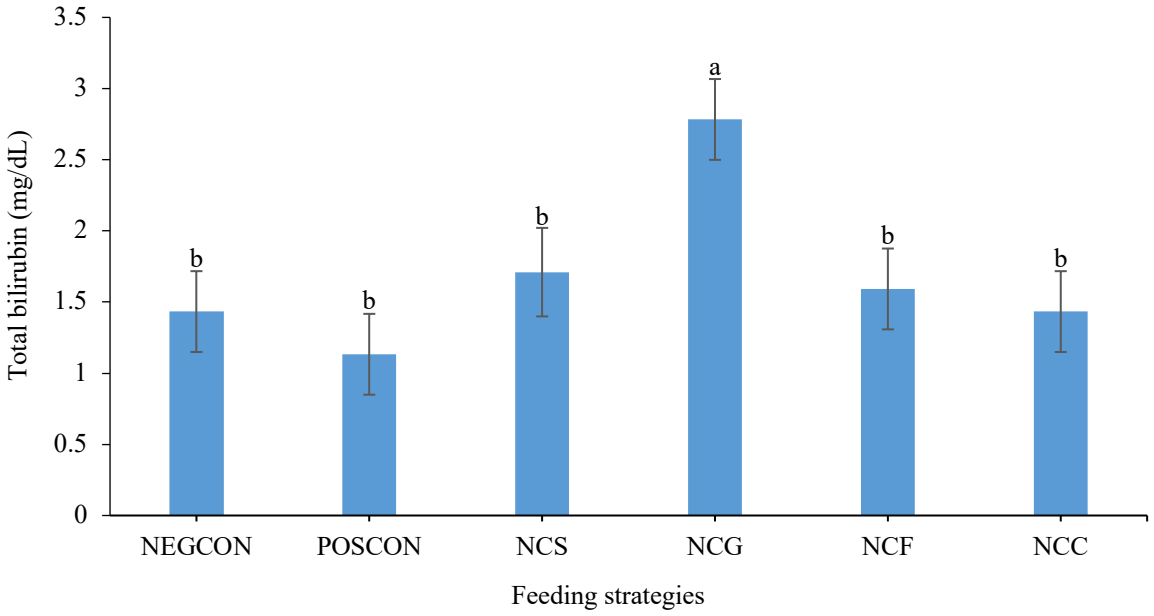


Figure 3.7: Serum total bilirubin in Ross 308 broiler chickens supplemented with dietary nucleotides.

abc Means with different superscripts are different ($p < 0.05$). Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

3.5.3.2 Haematological parameters

Platelet counts, heterophil percentage, and monocyte counts were the only parameters affected ($p < 0.05$) by nucleotide supplementation strategies. Table 3.2 presents the effects of feeding strategies on haematology parameters in Ross 308 broilers. The platelet count was lowest ($p < 0.05$) in birds reared on the POSCON with statistical similarities to NEGCON, NCS, NCG, and NCC feeding strategies, and was the highest ($p < 0.05$) in feeding strategy NCF. Again, the percentage of heterophils was the lowest ($p < 0.05$) in birds fed on the POSCON and highest ($p < 0.05$) in the birds fed on the NEGCON similar to phased and continuous yeast-based

nucleotide supplementation feeding strategies. Lastly, the absolute count of monocytes was the lowest ($p < 0.05$) in birds reared on the POSCON, with statistical similarities to the NEGCON, NCS, NCG, and NCC, and the highest ($p < 0.05$) in the feeding strategy that included yeast-based nucleotides in the finisher phase only.

Table 3.2: Impact of supplementation strategy for yeast-based nucleotides on haematological parameters in Ross 308 broilers.

Parameter	Feeding strategies						P-value
	NEGCON	POSCON	NCS	NCG	NCF	NCC	
White cell count (1×10^9/L)	21.35±2.625	15.57±2.397	17.09±2.397	22.80±2.397	24.24±2.397	21.47±2.397	0.113
Platelet count (1×10^9/L)	27.40±4.129^{b}	19.93±3.769^{b}	27.78±3.769^{b}	29.53±3.769^{b}	43.58±3.769^{a}	29.58±3.769^{b}	0.006
Haematocrit (%)	37.40±0.012	36.53±0.011	37.11±0.011	35.95±0.011	35.19±0.011	35.00±0.011	0.541
Heterophil (%)	80.47±0.036^{a}	64.45±0.033^{b}	72.07±0.033^{a}	78.61±0.033^{a}	75.93±0.033^{a}	74.98±0.033^{a}	0.011
Lymphocyte (%)	9.836±0.031	17.76±0.028	13.76±0.028	10.44±0.028	10.32±0.028	12.21±0.028	0.063
Monocyte (%)	9.248±0.022	10.80±0.020	12.34±0.020	9.776±0.020	12.67±0.020	11.63±0.020	0.375
Eosinophil (%)	3.493±0.038	5.242±0.027	2.538±0.033	3.031±0.033	1.785±0.038	4.469±0.046	0.363
Heterophil (1×10^9/L)	17.50±2.582	9.514±2.357	12.34±2.357	18.31±2.357	18.59±2.357	16.40±2.357	0.060
Lymphocyte (1×10^9/L)	1.999±0.350	2.689±0.319	2.426±0.319	2.173±0.319	2.441±0.319	2.457±0.319	0.750
Monocyte (1×10^9/L)	1.811±0.309^{b}	1.738±0.282^{b}	2.124±0.282^{b}	2.198±0.282^{b}	3.017±0.282^{a}	2.478±0.282^{b}	0.040
Eosinophil (1×10^9/L)	0.777±0.275	0.868±0.194	0.443±0.238	0.443±0.238	0.382±0.275	0.605±0.336	0.589
H/L Ratio	8.287±0.031	3.793±0.028	5.422±0.028	8.232±0.028	8.122±0.028	7.321±0.028	0.124

^{abc} Means with different superscripts are different ($p < 0.05$). Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

3.5.4 Carcass and meat quality traits

3.5.4.1 Carcass traits

Table 3.3 presents the effects of feeding strategies on carcass traits in Ross 308 broilers. There were no significant effects ($p > 0.05$) across all feeding strategies in terms of dressing percentage and carcass cuts of Ross 308 broilers.

Table 3.3: Effects of nucleotide feeding strategies on carcass traits (g/100g HCW, unless stated otherwise) of Ross 308 broiler chickens

Parameter	Feeding strategies						P-value
	NEGCON	POSCON	NCS	NCG	NCF	NCC	
HCW (g)	1644	1669	1585	1685	1620	1593	0.353
Dressing (%)	71.77	70.91	74.04	76.78	72.19	70.72	0.097
Drumstick	7.177	7.037	6.959	6.957	7.493	7.095	0.246
Thigh	8.320	8.782	8.966	8.813	9.732	8.544	0.115
Wing	5.420	5.471	5.435	5.234	5.728	5.472	0.439
Breast	21.84	21.32	21.91	21.74	23.53	22.12	0.376

Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF). Parameters: HCW = hot carcass weight.

3.5.4.2 Meat pH

Table 3.4 presents the pH values for breast and thigh meat in Ross 308 broilers. Feeding strategies had no notable effects on the pH of meat from both thighs and breasts ($p > 0.05$) in Ross 308 broilers.

Table 3.4: Influence of yeast-based nucleotides on breast and thigh meat pH (24 hours post-slaughter) in Ross 308 broiler chickens.

Parameter	Feeding strategies						P-value
	NEGCON	POSCON	NCS	NCG	NCF	NCC	
Breast	5.986	5.739	6.027	5.916	5.888	5.925	0.616
Thigh	6.221	5.857	5.931	5.829	5.966	5.873	0.287

Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

3.5.4.3 Meat colour

Table 3.5 presents the breast meat colour values in Ross 308 broilers. No significant differences ($p > 0.05$) were observed across all feeding strategies on Ross 308 broiler breast colour parameters.

Table 3.5: Effects of feeding strategies on Ross 308 broiler chicken breast meat colour.

Parameter	Feeding strategies						P-value
	NEGCON	POSCON	NCS	NCG	NCF	NCC	
*L**	46.62	49.66	47.37	48.99	47.69	50.02	0.527
*a**	3.030	3.496	4.011	3.327	3.548	3.431	0.575
*b**	7.338	7.438	7.634	7.257	7.697	7.808	0.965
Chroma	7.940	8.241	8.671	8.004	8.483	8.578	0.899
Hue	1.178	1.134	1.093	1.138	1.141	1.154	0.694

Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF). Parameters: *L** = lightness, *a** = redness, *b** = yellowness.

Table 3.6 presents the thigh meat colour values in Ross 308 broilers. Similarly, no significant differences ($p > 0.05$) were observed across all feeding strategies on Ross 308 broiler thigh colour parameters.

Table 3.6: Impact of nucleotide feeding strategies on Ross 308 broiler chicken thigh meat colour.

Parameter	Feeding strategies						P-value
	NEGCON	POSCON	NCS	NCG	NCF	NCC	
*L**	49.59	50.93	47.60	51.88	51.77	52.52	0.406
*a**	4.830	4.773	5.546	5.638	5.368	5.592	0.279
*b**	6.433	6.513	5.804	5.878	7.183	6.518	0.386
Chroma	8.093	8.090	8.101	8.164	9.002	8.638	0.558
Hue	0.927	0.938	0.799	0.809	0.925	0.853	0.147

Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF). Parameters: *L** = lightness, *a** = redness, *b** = yellowness.

3.5.4.5 Water holding capacity, cooking loss, and drip loss

No notable variations ($p > 0.05$) were observed across all feeding strategies on water holding capacity (WHC) and cooking loss of the meat. However, the effects of feeding strategies on drip loss significantly differed ($p < 0.05$). Breast meat from birds reared on the NEGCON recorded the lowest ($p < 0.05$) water retention, while breast meat from birds reared on nucleotide-containing diets recorded the highest ($p < 0.05$) water retention, regardless of the timing of supplementation.

3.5.4.6 Shear force

Table 3.7 presents the effects of feeding strategies on Ross 308 broiler chicken breast meat drip loss and shear force. Feeding strategies had a distinct effect on breast meat shear force ($p < 0.05$) in Ross 308 broilers. Breast meat from birds reared on the NCG feeding strategy recorded the lowest ($p < 0.05$) shear force with statistical similarities to the NEGCON, NCS, and NCF feeding strategies. While the Ross 308 broiler breast meat reared on the POSCON recorded the highest ($p < 0.05$) shear force, it was statistically similar to the NCC feeding strategy.

Table 3.7: Effects of nucleotide feeding strategies on drip loss (DL) and shear force (SF) in Ross 308 broiler chicken breast meat.

Parameter	Feeding strategies						P-Value
	NEGCON	POSCON	NCS	NCG	NCF	NCC	
DL (%)	3.198±0.353^{a}	2.782±0.432^{b}	2.246±0.432^{b}	4.253±0.387^{a}	3.612±0.387^{b}	2.258±0.353^{b}	0.007
SF (N)	4.226±0.321^{b}	6.281±0.393^{a}	4.712±0.393^{b}	3.803±0.351^{b}	4.762±0.321^{b}	5.314±0.351^{a}	0.001

^{abc} Means in the same row with different superscripts are different (P <0.05); Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

3.6 Discussion

Weight gains directly reflect the bird's growth performance and are both economically important and crucial in managing bird health. Accordingly, regularly recording body weight gains is essential for optimizing the feed conversion ratio (FCR) and making informed decisions about feed selection. By closely monitoring growth performance, producers can assess the efficiency of different feed formulations, identify potential nutritional deficiencies or imbalances, and pinpoint areas that require improvement. This data-driven approach allows for precise adjustments to feed composition, ensuring that birds receive the optimal nutrients necessary for healthy growth and maximizing overall production efficiency (Li *et al.*, 2023). This study provides valuable insights into how intermittent supplementation of yeast-based nucleotides influences growth performance in broilers raised under high stocking densities. Among the different feeding strategies tested, supplementation with yeast-based nucleotides during the grower phase yielded the highest overall body weight gains, with an average of 1899 g. Interestingly, birds that received continuous nucleotide supplementation throughout their lifecycle showed the lowest numerical weight gain, averaging 1863 g. These findings imply that targeted, phase-specific nucleotide supplementation may be more effective in promoting growth than continuous feeding. A preliminary study that aimed at exploring the role of nucleotides in enhancing pre-starter broiler diets discovered that supplementing nucleotides in the first three weeks increased weight gains (Rutz *et al.*, 2008). *De novo* and salvage pathway synthesis of nucleotides is compromised during rapid growth, such that the demand for nucleotides is higher. This suggests that supplementation of nucleotides under rapid growth periods is most efficient as they have the capacity to enhance growth and performance through their ability to promote cellular functions and support energy metabolism (Chandel, 2021).

Nonetheless, the results indicate that both phased and continuous supplementation with yeast-based nucleotides produced comparable effects on body weight gains, feed intake, and feed conversion ratio, suggesting flexibility in supplementation strategies without compromising overall performance. This aligns with Leung *et al.* (2019) and Salah *et al.* (2019) findings that proved that feed intake, body weight gains, and feed conversion ratio were not significantly influenced by yeast-based nucleotide supplementation. These implications, therefore, support this study's hypothesis that continuous and phased supplementation of nucleotides promotes similar results on performance if phased supplementation is timed to coincide with highly stressful periods. Furthermore, the costs of nucleotide supplementation can be reduced through phased supplementation, as the quantity of nucleotides required is reduced while their efficiency is increased. Overall, the positive control outperformed all the feeding strategies. It recorded the highest body weight gains (2062 g) and the most efficient feed conversion ratio (1.663). This can be attributed to the ability of zinc bacitracin to thicken the gut, thereby improving the absorption of nutrients and ultimately promoting better feed conversion ratio, growth, and performance (Rafiq *et al.*, 2022). Although there have been positive reports on the usage of nucleotides, these findings suggest that they are still inferior to AGP in ameliorating stress and promoting efficient growth performance.

Earlier studies by Daneshmand *et al.* (2017) and Hassanein *et al.* (2023) explored the effects of nucleotide supplementation in broiler chickens and found that they improved bird mortality. However, the current study shows that yeast-based nucleotide supplementation had similar bird mortalities as the POSCON. Instead, the results were statistically similar across all feeding strategies and numerically lower in the Ross 308 broiler chickens reared on the POSCON. This is despite the role of nucleotides in immunological, endocrinological, and gastrointestinal functions (Jung & Batal, 2012).

Haematology analysis is a good indicator of a bird's physiological status. It allows the tracking of responses of birds to different physiological conditions, including nutrition, stress, and disease (Ifelayo *et al.*, 2020). This study found that supplementing yeast-based nucleotides exclusively during the finisher phase resulted in an increase in platelet count. In birds, platelets function similarly to those in mammals by playing a critical role in hemostasis. When vascular injury occurs, avian platelets accumulate at the site of damage and release hypercoagulable factors, initiating the blood clotting process to prevent excessive bleeding. The observed elevation in platelet count may indicate enhanced readiness for vascular repair and immune defense during the finisher phase, potentially contributing to improved health and resilience as the birds approach market weight (St. Paul *et al.*, 2012). It has also been proven that platelets play a role in bird immunity through phagocytosis; however, the mechanisms have not yet been fully studied (Nagasawa *et al.*, 2014). Abo-Sriea *et al.* (2024) also revealed that continuous supplementation of dietary nucleotides in broiler diets improved phagocytic activity and immunity. Additionally, the negative control, phased and continuous supplementation with yeast-based nucleotides, increased the heterophil count. Heterophils are specialized leukocytes in birds that serve as a critical first line of defense against infections. They are functionally similar to mammalian neutrophils, rapidly responding to invading pathogens by engulfing and destroying them through phagocytosis and releasing antimicrobial substances (Kogut, 2022). An elevated heterophil percentage suggests an enhanced innate immune response, potentially improving the bird’s ability to combat infectious agents during this crucial growth stage. They partake in all roles played by innate immune response, including tissue remodelling, inflammation, and defence against pathogens (Kogut, 2022). Conversely, the birds reared on the positive control recorded the lowest platelet and heterophil percentage. The individual mechanisms of heterophils have not been explored.

However, heterophils and lymphocytes are often analyzed together as the heterophil-to-lymphocyte (H/L) ratio, which serves as a widely recognized indicator of physiological stress in poultry. An elevated H/L ratio typically reflects increased stress levels, as heterophil counts tend to rise while lymphocyte counts decrease under stress conditions. This ratio provides a valuable tool for assessing the welfare and immune status of birds in response to environmental, nutritional, or management-related stressors (Sumanu *et al*., 2022). The absence of significant differences in the H/L ratio observed in this study could be attributed to several factors. First, the birds across all treatment groups may have experienced similar levels of physiological stress, resulting in a stable H/L ratio. Additionally, optimal management and environmental conditions during the study could have minimized stress, thereby suppressing any potential effects of yeast-based nucleotide supplementation on this stress indicator. It is also possible that the supplementation modulated immune responses in a way that balanced heterophil and lymphocyte counts, maintaining the ratio within a consistent range. Moreover, the duration or dosage of supplementation might not have been sufficient to induce detectable changes in the H/L ratio. Finally, natural biological variability among individual birds can sometimes obscure subtle shifts in immune parameters, making it challenging to observe statistically significant differences.

Monocytes are classified as white blood cells and are crucial components of the immune system. They mature into macrophages and dendritic cells, which help regulate homeostasis by eliminating pathogens and regulating inflammation (Yánez *et al.*, 2017). Monocytes move from the bone marrow into the bloodstream in response to infection and inflammation and further into the tissues (Espinoza & Emmady, 2023). This current study's findings revealed that the positive control promoted lower monocyte count, similar to the negative control, NCS, NCG, and NCC feeding strategies. While this may be attributed to the ability of zinc bacitracin

as an AGP to balance microbial by suppressing the severity of enteropathogens and ultimately hindering inflammation and the need for inflammatory responses (de Morais Castro *et al.*, 2025), and the role of nucleotides in cell regeneration and repair in response to inflammation (Chiofalo *et al.*, 2011), it may also be that the physiological stress levels across these diets were similar. Contrastingly, exclusive supplementation with yeast-based nucleotides in the finisher phase only was the highest. This outcome may be associated with the activation of an immunomodulatory response triggered by enteropathogens, rendering late-stage yeast-based nucleotide supplementation less efficient.

Serum biochemistry parameters aid in the monitoring of nutritional status, stress levels, and diagnosis and identification of diseases, but are rarely used in avian species. Nonetheless, the difficulty in clinically diagnosing metabolic disorders makes serum biochemical analysis an important tool (Rezende *et al.*, 2017). The present study observed distinct effects of yeast-based nucleotide supplementation at different growth phases on key blood biochemical parameters. Specifically, supplementation during the starter phase led to an increase in blood urea nitrogen (BUN) levels, which may indicate enhanced protein metabolism or altered nitrogen excretion during early development. In contrast, supplementation during the grower phase resulted in elevated levels of albumin (ALB), alanine aminotransferase (ALT), and total bilirubin (TBIL). Increased albumin suggests improved liver synthetic function and nutritional status, while elevated ALT, a liver enzyme, could reflect heightened liver activity or mild hepatic stress. The rise in total bilirubin, a breakdown product of hemoglobin, may indicate changes in liver processing or red blood cell turnover. Together, these biochemical shifts highlight how the timing of nucleotide supplementation can differentially influence metabolic and liver function markers in broiler chickens. Interestingly, birds in the positive control recorded the lowest values for these parameters. Similarly, Prakash *et al.* (2016) recorded an

increase in ALB as a result of continuous nucleotide supplementation in Japanese quail. This aligns with this study's findings; similarly, the feeding strategy that included yeast-based nucleotides in the finisher phase only recorded the lowest cholesterol, although not statistically different. Aziz *et al.* (2024) also recorded an increase in ALB caused by nucleotide supplementation in broilers. This can be attributed to the capacity of nucleotides to increase metabolic activity in the liver because they partake in energy transfer, are nucleic acid precursors, and function as co-enzymes (Aziz *et al.*, 2024).

Meat quality parameters, such as tenderness and texture, are a direct reflection of poultry production management. In particular, bird nutrition plays a key role in poultry meat quality and safety (Mir *et al.*, 2017). This study gives insights into the effects of supplementing yeast-based nucleotide feeding strategies on the carcass and meat quality parameters of densely stocked broilers. The lack of significant differences in carcass yield, meat pH, color, water-holding capacity, and cooking loss across the different feeding strategies suggests that yeast-based nucleotide supplementation, regardless of timing or duration, did not negatively impact the physical and chemical properties of the meat. This could be because these quality traits are influenced by multiple factors, including genetics, overall nutrition, and management practices, which were likely consistent across all groups. Additionally, the supplementation levels and feeding durations may not have been sufficient to cause measurable changes in muscle metabolism or post-mortem muscle chemistry that directly affect these parameters. Therefore, while nucleotide supplementation may influence growth and immune function, its effects on meat quality appear neutral under the conditions of this study. These outcomes are in alignment with Pelícia *et al.* (2010) findings on the effects of incremental levels of continuous nucleotide supplementation at levels varying from 0.04 to 0.07% in broilers, wherein carcass yield and meat cuts did not differ. Salah *et al.* (2019) also observed no significant effects of continuous

nucleotide supplementation at 0.05% and 0.1% on broiler meat pH, cooking loss, lightness, and redness under various environmental conditions, while yellowness and water holding capacity increased. Furthermore, Rutz *et al.* (2006) revealed that yeast-based nucleotides had a numerical improvement on carcass yield, drumstick, breast, thigh, and wing yield, like the feeding strategy that included yeast-based nucleotides in the grower phase only. Yeast-based nucleotides supplementation strategies significantly affected shear force and drip loss. Shear force indicates the tenderness in meat, an important element of consumer perception of meat quality. Phased supplementation of yeast-based nucleotides improved the shear force values, while the positive control and continuous yeast-based nucleotide supplementation recorded the highest values. The finding that phased yeast-based nucleotide supplementation promoted better meat tenderness compared to continuous supplementation and antibiotic growth promoters suggests that targeted timing of nucleotide inclusion may positively influence muscle development and meat quality. This could be because intermittent supplementation aligns more closely with critical growth phases, allowing for improved protein synthesis and muscle fiber formation without overstimulation that might occur with continuous feeding. Additionally, nucleotides play a role in cellular regeneration and repair, which may enhance muscle texture when provided at specific stages. In contrast, continuous supplementation with yeast-based nucleotides or antibiotic growth promoters might not provide the same benefit, potentially due to metabolic adaptation in the case of nucleotides, whilst AGP primarily focus on gut microflora and do not have a direct effect on meat quality. Generally, phased supplementation appears to offer a strategic advantage in optimizing meat tenderness. These outcomes can be attributed to the influence of nucleotides on calpain enzymatic activity during rigor mortis. Calpain-induced proteolysis during rigor mortis stimulates histochemical changes in the muscle that directly affect meat tenderness (Chiofalo *et al.*, 2011). Nucleotides as energy

carriers influence the activity and/or stability of calpain activity, thus determining the rate and degree to which protein degradation occurs, and thus determining meat tenderness.

Drip loss is the exudation of myofibres and loss of iron, protein, and water during rigor mortis (Guo & Dalrymple, 2017). Drip loss is directly related to meat weight and lowers its tenderness (Ali *et al.*, 2016). Conversely, the observation that drip loss was significantly higher when yeast-based nucleotides were supplemented only during the grower phase, despite this group also recording the lowest shear force value, suggesting a complex interaction between muscle water retention and tenderness. Elevated drip loss indicates greater fluid release from the meat, which can be associated with changes in muscle structure or cell membrane integrity during growth. However, the concurrently low shear force value points to increased tenderness, implying that muscle fibers may be more relaxed or less densely packed, facilitating easier chewing. This paradox may arise because nucleotide supplementation during the grower phase enhances protein turnover and muscle remodeling, which improves tenderness but may also affect the muscle's ability to retain water. These results highlight the nuanced effects of timing in nucleotide supplementation on different aspects of meat quality.

3.7 Conclusion

It can be concluded that phased and continuous supplementation of yeast-based nucleotides promoted notably similar results. Exclusive supplementation with yeast-based nucleotides in the grower phase only enhanced the most weight gains through their ability to promote cellular functions and support energy metabolism. Moreover, phased yeast-based nucleotide supplementation also had distinct positive effects on haemato-biochemical parameters that stimulated immunomodulatory responses and improved metabolic liver function. Yeast-based

nucleotide supplementation had no discernible effect on carcass yield, meat pH, colour, water holding capacity, and cooking loss. However, supplementation of yeast-based nucleotides in the grower phase only improved meat tenderness and conversely increased drip loss attributed to the complex interaction between nucleotides, protein turnover, and muscle remodeling. These findings confirm the hypothesis that yeast-based nucleotides have similar effects on the parameters of interest. It is, therefore, recommended that yeast-based nucleotides be supplemented on a phased basis. Moreover, yeast-based nucleotides were revealed to be an incomparable substitute for AGP.

3.8 References

Abo-Sriea, T.M., Ismael, E., Sobhi, B.M., Hassan, N.H., Elleithy, E.M., Omar, S.A., Soliman, A.M., Fahmy, K.N.E.D. & Ramadan, A., 2024. Impact of dietary-nucleotides and Saccharomyces cerevisiae-derivatives on growth-performance, antioxidant-capacity, immune-response, small-intestine histomorphometry, caecal-Clostridia, and litter-hygiene of broiler-chickens treated with florfenicol. *International Journal of Veterinary Science and Medicine*, *12*(1), 11-24.

Ali, S., Rajput, N., Li, C., Zhang, W. & Zhou, G., 2016. Effect of Freeze-Thaw Cycles on Lipid Oxidation and Myowater in Broiler Chickens. *Revista Brasileira de Ciência Avícola*, *18*(1), 35-40.

Aviagen, 2018. Ross broiler: The pocket guide.

Aviagen., 2022. *Ross Broiler: Nutrition Specifications*. USA.

Aziz, A.A.A., Aziz, E.S.A.A., Khairy, M.H., Fadel, C., Giorgi, M. & Abdelaziz, A.S., 2024. The effect of butyric acid and nucleotides supplementation on broiler (*Gallus gallus domesticus*) growth performance, immune status, intestinal histology, and serum parameters. *Open Veterinary Journal*, *14*(1), 324.

Boza, J.J. & Martinez-Augustin, O., 2002. Role and function of nucleotides in Infant nutrition. In: Infant Formula: closer to the reference (Räihä, N.C.R. and F.F. Rubaltelli, eds.), *47*, 165-182.

Chandel, N.S., 2021. Nucleotide metabolism. *Cold Spring Harbor Perspectives in Biology*, *13*(7), 040592.

Chiofalo, B., Lo Presti, V., Savoini, G., D'Alessandro, E., Chiofalo, V., & Liotta L., 2011. Nucleotides in broiler chicken diet: effect on breast muscles quality. *Czech Journal of Food Science*, *29*, 308- 317.

Daneshmand, A., Kermanshahi, H., Mesgaran, M.D., King, A.J., Ibrahim, S.A. & Klasing, K.C., 2017. Combination of purine and pyrimidine nucleosides influences growth performance, gut morphology, digestive enzymes, serum biochemical indices and immune functions in broiler chickens. *Animal Feed Science and Technology*, *228*, 186-193.

de Morais Castro, K.L., Rohloff Júnior, N., Santos, E.T., Valentim, J.K., Garcia, R.G., Rieger, G. & Sgavioli, S., 2025. Zootechnical Additives Associated with Antimicrobials: Effects on Immune Response and Intestinal Histomorphometry in Broiler Chickens. *Veterinary Sciences*, *12*(6), 581.

Espinoza, V.E. & Emmady, P.D., 2023. Histology, monocytes. In *StatPearls*. StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK557618/. [Accessed 21 Jul. 2025]

Frankic, T., Rezar, V. & Salobir, J., 2006. Nucleotide supplementation eliminated leukocyte DNA damage induced by T-2 toxin and deoxynivalenol in broiler chickens. *Krmiva, 48* (6), 323-326.

Gil, A., 2002. Modulation of the immune response mediated by dietary nucleotides. *European Journal of Clinical Nutrition*, *56*(3), 1-4.

Hassanein, I.I., Metwally, A.E. & Abd Elbaky, H.E.M., 2023. Effects of dietary nucleotides supplementation on the performance of broiler chicks. *Slovenian Veterinary Research/Slovenski Veterinarski Zbornik*, *60*(25), 159-171.

Hernandez, J.R., Gulizia, J.P., Vargas, J.I., Thuekeaw, S., Guzman, E.G., Simões, C.T. and Pacheco, W.J., 2024. Effect of metabolizable energy levels and conditioning temperatures on broiler performance, processing yield, footpad lesions, and nutrient digestibility from 1 to 42 d of age. *Journal of Applied Poultry Research*, *33*(2), 100414.

Hoffmann, K., 2007. The use of Nucleotides in animal feed. *Feed Mix*, *6*, 25–27.

Ifelayo, I.I., Onize, I.H., Faith, U. & Wodi, S.M., 2020. Haematology and serum biochemistry of broiler strains (Cobbs and Arbor-acre) fed ginger (*Zingiber officinale*). *GSC Biological and Pharmaceutical Sciences*, *11*(02), 320-326.

Jie, Y., Wen, C., Huang, Q., Gu, S., Sun, C., Li, G., Yan, Y., Wu, G. &Yang, N., 2024. Distinct Patterns of Feed Intake and Their Association with Growth Performance in Broilers. *Poultry Science*, 103974.

Jung, B. & Batal, A. B., 2012. Effect of dietary nucleotide supplementation on performance and development of the gastrointestinal tract of broilers. *British Poultry Science*, *53*(1), 98-105.

Kamel, N.F., Hady, M.M., Ragaa, N.M. & Mohamed, F.F., 2021. Effect of nucleotides on growth performance, gut health, and some immunological parameters of broiler chicken exposed to high stocking density. *Livestock Science*, *253*, 104703.

Kogut, M.H., 2022. Avian granulocytes. In *Avian immunology,* Academic Press, 197-203.

Leung, H., Yitbarek, A., Snyder, R., Patterson, R., Barta, J.R., Karrow, N. & Kiarie, E., 2019. Responses of broiler chickens to Eimeria challenge when fed a nucleotide-rich yeast extract. *Poultry Science*, *98*(4), 1622-1633.

Li, P., Gatlin III, D.M. & Neill, W.H., 2007. Dietary supplementation of a purified nucleotide mixture transiently enhanced growth and feed utilization of juvenile red drum, *Sciaenops ocellatus*. *Journal of the World Aquaculture Society*, *38*(2), 281-286.

Li, X., Wu, J., Zhao, Z., Zhuang, Y., Sun, S., Xie, H., Gao, Y. & Xiao, D., 2023. An Improved Method for Broiler Weight Estimation Integrating Multi-Feature with Gradient Boosting Decision Tree. *Animals*, *13*(23), 3721.

Liu, Y., Xiong, M., Hu, X., Li, Y., Zhang, W., He, W., Luo, S., Zang, J., Yang, W. & Chen, Y., 2024. Dietary *Bacillus velezensis* KNF-209 supplementation improves growth

performance, enhances immunity, and promotes gut health in broilers. *Poultry Science*, *103*(9),103946.

Marareni, M. & Mnisi, C.M., 2020. Growth performance, serum biochemistry and meat quality traits of Jumbo quails fed with mopane worm (*Imbrasia belina*) meal-containing diets. *Veterinary and Animal Science*, *10*, 100141.

Mehdi, Y., Létourneau-Montminy, M.P., Gaucher, M.L., Chorfi, Y., Suresh, G., Rouissi, T., Brar, S.K., Côté, C., Ramirez, A.A. & Godbout, S., 2018. Use of antibiotics in broiler production: Global impacts and alternatives. *Animal Nutrition*, *4*(2), 170-178.

Mir, N.A., Rafiq, A., Kumar, F., Singh, V. & Shukla, V., 2017. Determinants of broiler chicken meat quality and factors affecting them: a review. *Journal of Food Science and Technology*, *54*, 2997-3009.

Nkukwana, T.T., 2019. Global poultry production: Current impact and future outlook on the South African poultry industry. *South African Journal of Animal Science*, *48*(5), 869–884.

OECD/FAO, (2025). *OECD-FAO Agricultural Outlook 2025-2034*, OECD Publishing, Paris/FAO, Rome.

Pang, B., Bowker, B., Zhuang, H., Yang, Y. & Zhang, J., 2020. Research Note: Comparison of 3 methods used for estimating cook loss in broiler breast meat. *Poultry Science*, *99*(11), 6287-6290.

Pelícia, V., Sartori, J., Zavarize, K., Pezzato, A., Stradiotti, A., Araujo, P., Mituo, M. & Madeira, L., 2010. Effect of nucleotides on broiler performance and carcass yield. *Revista Brasileira de Ciência Avícola*, *12*(1), 31–34.

Rafiq, K., Tofazzal Hossain, M., Ahmed, R., Hasan, M.M., Islam, R., Hossen, M.I., Shaha, S.N. & Islam, M.R., 2022. Role of different growth enhancers as alternative to in-feed antibiotics in poultry industry. *Frontiers in Veterinary Science*, *8*, 794588.

Rutz, F., Xiaver, E., Anciuti, M. Roll., V., & Rossi, P., 2008. The role of nucleotides in improving broiler pre-starter diets. In J. A. Taylor-Pickard and Spring. P., ed., Gut Efficiency: The Key Ingredient in Pig and Poultry Production: Elevating Animal Performance and Health, by Publisher: Wageningen Academic Publishers, The Netherlands, 155-163.

St. Paul, M., Paolucci, S., Barjesteh, N., Wood, R.D., Schat, K.A. & Sharif, S., 2012. Characterization of chicken thrombocyte responses to Toll-like receptor ligands. *PLOS One, 7*(8), 43381.

Sumanu, V.O., Naidoo, V., Oosthuizen, M.C., & Chamunorwa, J.P., 2022. Adverse effects of heat stress during summer on broiler chickens production and antioxidant mitigating effects. *International Journal of Biometeorology, 66*(12), 2379-2393.

Salah, M., Suprijatna, E., Djauhari, M.L. & Dwi, Y.V., 2019. The effects of nucleotide supplementation on the productivity, immune response and meat quality of broiler chicken reared under different environmental conditions. *Livestock Research for Rural Development*, *31*(11), 174.

Thiam, M., Wang, Q., Barreto Sánchez, A.L., Zhang, J., Ding, J., Wang, H., Zhang, Q., Zhang, N., Wang, J., Li, Q. & Wen, J., 2022. Heterophil/lymphocyte ratio level modulates Salmonella resistance, cecal microbiota composition and functional capacity in infected chicken. *Frontiers in Immunology*, *13*, 816689.

Wang, Z., Cerrate, S., Coto, C., Sacakli, P., Yan, F., Costa, F. & Waldroup, P.W., 2009. Evaluation of Nupro yeast product in diets for broilers. *International Journal of Poultry Science*, *8*, 515-520.

Wang, Y., Wang, J., Liu, L., Xu, H., Liang, H., Wang, Z. & Gu, J., 2024. Effects of dietary yeast nucleotides on the growth performance and muscle quality of juvenile largemouth bass (*Micropterus salmoides*). *Aquaculture Reports*, *36*, 102159.

Yáñez, A., Coetzee, S.G., Olsson, A., Muench, D.E., Berman, B.P., Hazelett, D.J., Salomonis, N., Grimes, H.L. & Goodridge, H.S., 2017. Granulocyte-monocyte progenitors and monocyte-dendritic cell progenitors independently produce functionally distinct monocytes. *Immunity*, *47*(5), 890-902.

Yang, J., Shi, Y., Li, X., Li, J., Shi, X., Zhang, C., Wang, M., Qin, Y. & Zhang, J., 2025. Optimization of processing parameters of 50% aged paddy rice replace of corn in feed formula: effects of grinding sieve opening, conditioning temperature and time on the growth performance, meat quality and serum biochemical parameters in broilers. *Poultry Science*, *104*(5), 105019.

Zhen, W., Zhu, T., Wang, P., Guo, F., Zhang, K., Zhang, T., Jalukar, S., Zhang, Y., Bai, D., Zhang, C. & Guo, Y., 2023. Effect of dietary Saccharomyces-derived prebiotic refined functional carbohydrates as antibiotic alternative on growth performance and intestinal health of broiler chickens reared in a commercial farm. *Poultry Science*, *102*(6), 102671.

4. CHAPTER FOUR: VISCERAL MORPHOMETRY AND GUT MICROBIAL DIVERSITY IN DENSELY STOCKED ROSS 308 BROILERS SUPPLEMENTED WITH YEAST-BASED NUCLEOTIDES

4.1 Abstract

The broiler gut is a complex organ responsible for digestion, nutrient absorption, and immune function. Maintaining a balanced relationship between the intestinal tract and its microbiota is essential for optimal health and productivity. However, stressors such as high stocking densities can disrupt this balance, leading to dysbiosis and negatively impacting bird health. Dietary supplementation with nucleotides has been shown to support gut integrity, development, and growth in broilers, offering a promising alternative to antibiotic growth promoters (AGP). Nucleotides are conditionally essential nutrients, with increased demand during periods of rapid growth, stress, and transition. Exogenous supplementation may be necessary to meet nucleotide requirements when endogenous synthesis is insufficient, but the appropriate timing and effect on gut function are largely underexplored in densely stocked broilers. Hence, this study was designed to determine the influence of phased supplementation with yeast-based nucleotides on visceral morphometry and the gut microbiome in densely stocked Ross 308 broilers. A total of 900, one-week-old, unsexed, Ross 308 broiler chicks were randomly allotted to 36 pens (25 birds/1.2 m^2) and reared on six isonitrogenous and isocaloric diets. The feeding strategies included a basal broiler diet with: 1. neither AGP nor nucleotides, continuously (NEGCON); 2. 0.05% AGP, continuously (POSCON); 3. 0.05% nucleotides in place of AGP, continuously (NCC); 4. 0.05% nucleotides in place of AGP, fed in starter phase only for a week (NCS); 5. 0.05% nucleotides in place of AGP, fed in grower phase only (NCG); and 6. 0.05% nucleotides in place of AGP, fed in finisher phase only (NCF). At six weeks of age, birds were slaughtered for visceral morphometry and gut microbiome measurements and analysis. Post-slaughter, weights and lengths of internal organs were measured with their

contents, except the gizzard. Caecal digesta were stored prior to DNA extraction. DNA extraction and purification were done using a QIAamp DNeasy PowerFecal DNA Kit (Qiagen, Germantown, MD) and a Nanodrop One (Thermofisher Scientific). Extracted DNA samples were further analysed to generate reads. Feeding strategies only influenced ($p < 0.05$) ilea length, with the positive control promoting the longest ilea and NCS promoting the shortest ilea. Feeding strategies yielded similar results on gut microbiome alpha and beta diversity and at kingdom, phylum, order, family, and genus levels ($p > 0.05$). However, feeding strategies caused variation in the species composition. The negative control promoted the highest abundance of *Alistipes inops* species, similar to phased and continuous yeast-based nucleotide supplementation, while POSCON promoted the least abundance of *A. inops* species. Phased and continuous yeast-based nucleotide supplementation demonstrates similar outcomes on the visceral morphometry and gut microbiome. Based on these findings, it is recommended that yeast-based nucleotides be supplemented on a phased basis; however, yeast-based nucleotides are an ineffective alternative to AGP.

Keywords: alpha diversity, exogenous nucleotides, gut function, gut integrity, microbiome.

4.2 Introduction

The broiler gut is a complex organ with digestive, absorptive, immunological, metabolic, and endocrinological functions (Svihus, 2014). Gut equilibrium is the balanced relationship between the intestinal tract and its microbiota, essential for maintaining animal health, immune function, and productivity. Disruptions to this balance can lead to health issues and reduced performance, highlighting the importance of supporting a stable gut environment (Celi *et al.*, 2019). The gut microbiota, predominated by bacteria (Wei *et al.*, 2013), plays a significant role in maintaining homeostasis and protecting the host animal from pathogens (Carrasco *et al.*, 2019). These microbes and their metabolites also interact with the bird's immune system (Klindworth *et al.*, 2013) and contribute to the development of the lamina propria, epithelial monolayer, and mucous layer (Oakley *et al.*, 2014). However, gut equilibrium is easily disrupted by stressors, such as high stocking density, commonly encountered in intensive broiler production systems, resulting in dysbiosis. As a result, bacterial infections in densely stocked birds are common, compromising poultry health and productivity. Gut dysbiosis is characterized by suboptimal bird welfare, increased disease incidence, poor growth rates, and a decline in the microbiological safety of meat products (Thema *et al.*, 2024). High stocking densities have been shown to disrupt gut microbiota balance, thus impeding digestion and nutrient absorption while increasing the susceptibility of broilers to diseases and infections (Rassmidatta *et al.*, 2024). Indeed, increased prevalence of enteropathogens impairs gut integrity (Aruwa & Sabiu, 2024). Antibiotic growth promoters (AGP) have traditionally been used to prevent dysbiosis; however, growing concerns over AGP residues, antimicrobial resistance, and their impacts on human and environmental health have led to their restricted use or complete ban in some regions (Yu et al., 2024). As a result, non-drug dietary supplements such as nucleotides have gained popularity as AGP alternatives.

Nucleotides play a significant role in various biochemical processes in living organisms, including broilers. They are components of DNA and RNA, provide cellular energy, regulate hormonal action, and function as coenzymes (Ying *et al.*, 2017). Continuous dietary nucleotide supplementation has been previously shown to promote the integrity, development, and growth of the broiler gut (Rutz *et al.*, 2008; Wu *et al.*, 2018). Nucleotides are conditionally essential nutrients, with demand rising under stressful conditions and during periods of rapid growth and transition (Abo-Sriea *et al.*, 2024). Under such conditions, the *de novo* and salvage pathways may be insufficient to meet the animal's nucleotide requirements, necessitating exogenous supplementation. Continuous nucleotide supplementation can be economically limited because of the relatively high cost of nucleotides compared to other feed additives. As a result, implementing a phased supplementation strategy targeting specific growth stages or periods of increased physiological demand may provide a more cost-effective approach. This method has the potential to deliver similar benefits in enhancing gut function and overall bird performance, while reducing the financial burden associated with continuous feeding. However, few studies have directly compared the efficacy of continuous versus phased nucleotide supplementation for the improvement of gut function in broiler chickens. Hence, this study is a comparative assessment of the effects of phased versus continuous supplementation of dietary nucleotides, replacing zinc bacitracin AGP, on visceral morphometry and gut microbiome. These parameters are important determinants of digestive, absorptive, and immunological gut functions. This study tested the hypothesis that phased and continuous dietary nucleotide supplementation in place of zinc bacitracin AGP would result in similar effects on visceral morphometry and gut microbiome diversity and abundance in densely stocked Ross 308 broilers.

4.3 Materials and methods

4.3.1 Study site and treatment design

The study site, treatment design, experimental design, bird management, and bird slaughter were presented in Chapter Three.

4.3.2 Visceral morphometry

Post-slaughter, the weights of the liver, gizzard, proventriculus, spleen, and pancreas, and both the weights and lengths of small (duodenum, jejunum, and ileum) and large intestines (caeca) were measured along with their contents. The relative weights of the internal organs were expressed as g/100 g HCW.

4.3.3 Gut microbiome

Caecal samples were collected from 2 birds per experimental unit after slaughter. Samples were stored in a 50 ml sterile Falcon tube, and liquid nitrogen was used to snap freeze and keep them at -80˚C prior to analysis. DNA extraction was done using a QIAamp DNeasy PowerFecal DNA Kit (Qiagen, Germantown, MD) and further purified using a Nanodrop One (Thermofisher Scientific). The full-length 16S rRNA gene was amplified using universal primers and sequenced using the PacBio Sequel II platform to generate HiFi (high-fidelity) reads. Libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, United States) and IDT Unique Dual Indexes. Fragmentation of genomic DNA was done using Nextera XT Fragmentation Enzyme (Illumina). Libraries were then sequenced on the Illumina HiSeq 4,000 platform, 2×150 bp, and reads were generated.

4.4 Statistical analysis

Visceral morphometry data were analysed for homogeneity of variance and for normality using Levene's test and the NORMAL option in the Procedure Univariate statement, respectively. Data were then analysed using a one-way ANCOVA with feeding strategy as the fixed factor according to the following statistical model:

$$Yij = u + Ti + \beta\,(Xij - \bar{X}) + Eij$$

Yij = dependent variable, u = overall mean, Ti = effects of the fixed factor, β = regression coefficient of the covariate, Xij = covariate value (initial body weight), $\bar{X}$ = covariate mean, Eij = residual error. For all measured parameters, significance was declared at $p < 0.05$, and least squares means were compared by the probability of difference option.

Alpha diversity metrics (Shannon diversity, Pielou evenness, and Simpson index) were calculated to assess within-sample diversity, while beta diversity was analyzed using Bray–Curtis and weighted UniFrac distance matrices. Principal Coordinates Analysis was performed to visualize differences in community structure between treatment groups. Taxonomic composition was summarized at phylum and genus levels, and differentially abundant taxa were identified using the ANCOM method within QIIME 2. All visualizations and statistical analyses were conducted using QIIME 2 and R (vX.X) with the phyloseq, vegan, and ggplot2 packages as described by Montso *et al.* (2022).

4.5 Results

4.5.1 Visceral morphometry

The ilea lengths were significantly ($p < 0.05$) affected by feeding strategies. Ross 308 broilers reared on the POSCON had the longest ($p < 0.05$) ilea, while those supplemented with yeast-based nucleotides in the starter phase had the shortest ilea, similar to other phased and continuous yeast-based nucleotide supplementation feeding strategies ($p < 0.05$). Figure 4.1 presents the effects of feeding strategies on ilea lengths in Ross 308 broilers, and Table 4.1 presents the impact of feeding strategies on internal organ sizes.

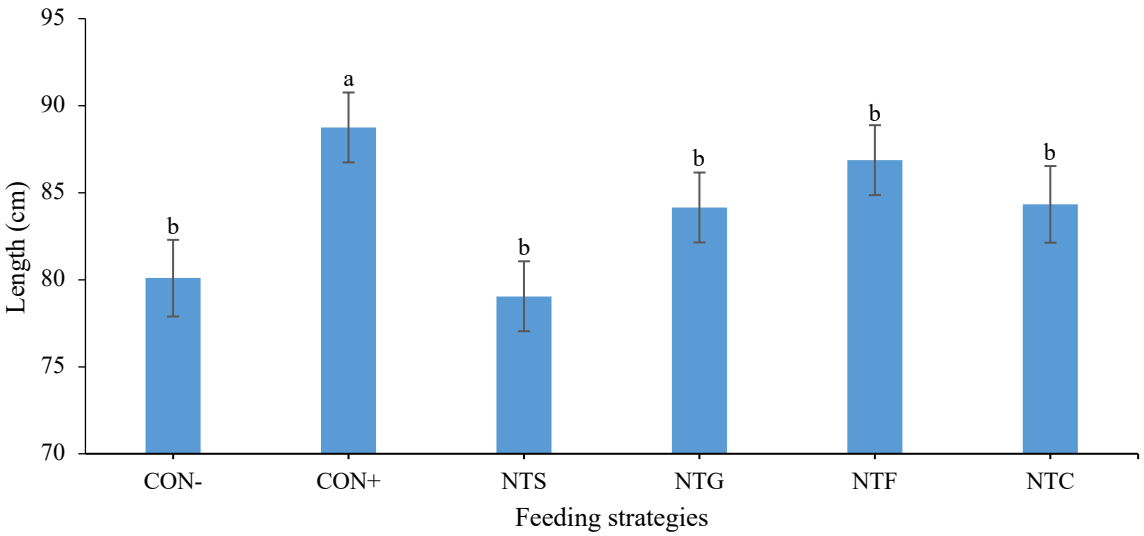


Figure 4.1: Effects of yeast-based nucleotide feeding strategies on the ilea lengths in Ross 308 broilers.

^{abc} Means with different superscripts are different ($p < 0.05$). Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

Table 4.1: Impact of yeast-based nucleotide supplementation on internal organ sizes (g/100 g HCW) in Ross 308 broilers.

Parameter	Feeding strategies						P-value
	NEGCON	POSCON	NCS	NCG	NCF	NCC	
Proventriculus	0.575±0.021	0.582±0.020	0.608±0.020	0.551±0.020	0.530±0.020	0.613±0.022	0.055
Gizzard	2.065±0.090	2.025±0.082	2.185±0.082	2.019±0.082	2.031±0.082	2.111±0.90	0.687
Spleen	0.169±0.008	0.173±0.007	0.176±0.007	0.161±0.007	0.180±0.007	0.160±0.008	0.306
Liver	2.363±0.115	2.289±0.105	2.407±0.105	2.338±0.105	2.380±0.105	2.319±0.115	0.974
Pancreas	0.214±0.033	0.240±0.027	0.226±0.027	0.228±0.027	0.232±0.027	0.223±0.030	0.994
Duodenum	1.123±0.056	1.004±0.051	0.952±0.051	0.973±0.051	0.949±0.051	1.024±0.056	0.234
Jejunum	1.626±0.117	1.602±0.107	1.725±0.107	1.773±0.107	1.660±0.107	1.779±0.117	0.795
Ileum	1.446±0.071	1.421±0.065	1.393±0.065	1.329±0.065	1.292±0.065	1.246±0.071	0.303
Caeca	0.686±0.035	0.727±0.032	0.653±0.032	0.648±0.032	0.634±0.032	0.738±0.035	0.154

Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

4.5.2 Gut microbial diversity

4.5.2.1 Alpha and beta diversity

Alpha and beta diversity were not affected by feeding strategies ($p > 0.05$). Figure 4.2 presents the effects of feeding strategies on Ross 308 broilers' microbiome index, and Table 4.1 presents the effects of feeding strategies on Ross 308 broilers' alpha and beta diversity.

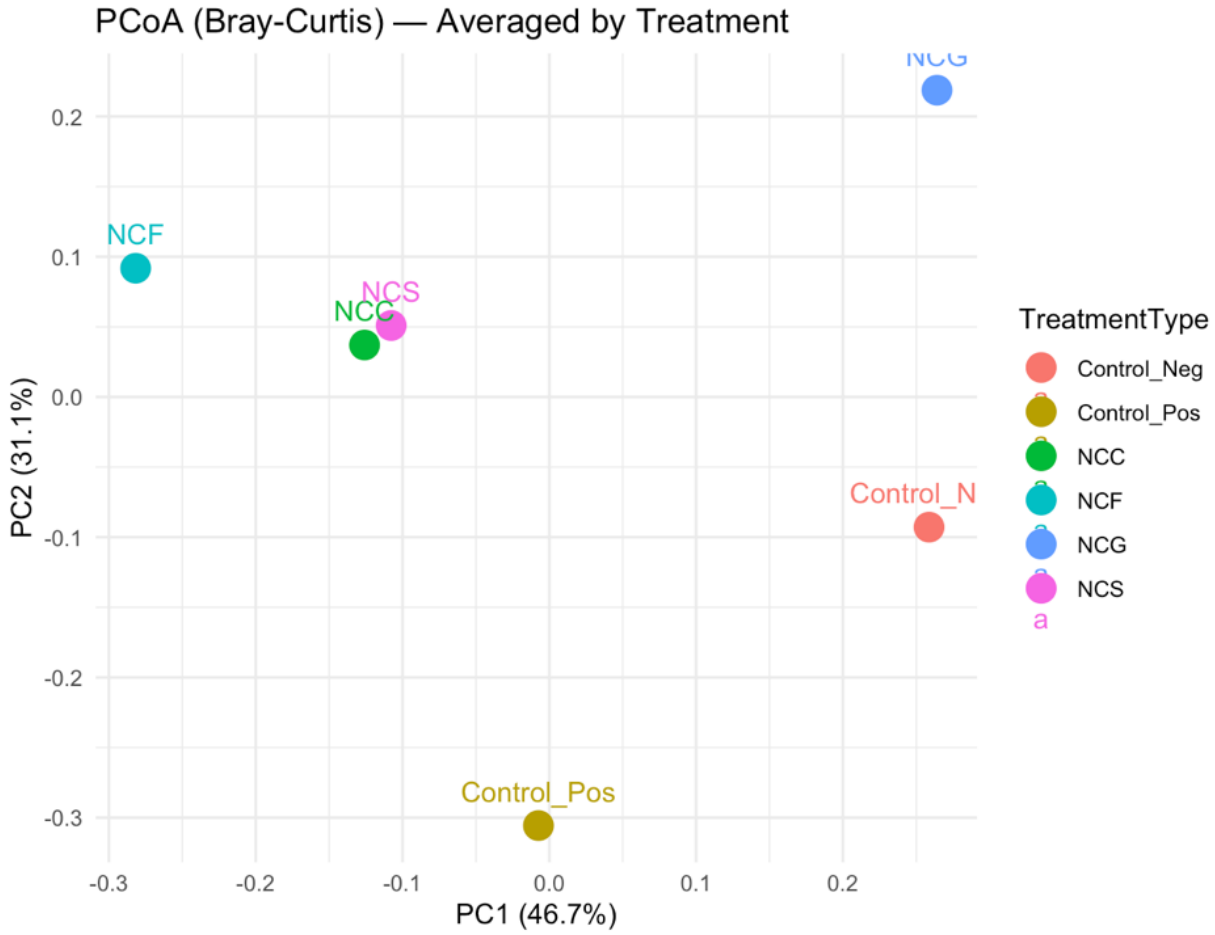


Figure 4.2: Principal Coordinates Analysis

[Feeding strategies: a basal diet without AGP or nucleotides (Control_Neg) fed continuously, a basal diet with AGP (Control_Pos) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).]

The Principal Coordinates Analysis shows that the effects of feeding strategies on the microbiome index did not differ. The similarity index (R^2 = 0.355; P = 0.152) did not differ across the feeding strategies when assessed with PERMANOVA, and the pairwise results were not significant.

Table 4.2: Summary of gut microbiome alpha diversity in Ross 308 broiler chickens supplemented with nucleotides.

	Mean Observed	Mean Shannon	Mean Simpson	Mean Pielou Evenness
NEGCON	42.00	2.038	0.746	0.545
POSCON	46.33	1.962	0.753	0.515
NCS	40.33	1.826	0.667	0.499
NCG	34.33	2.232	0.816	0.634
NCF	28.67	1.576	0.594	0.461
NCC	31.00	1.809	0.702	0.528
P-value	0.525	0.655	0.595	0.514

Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).

4.5.2.2 Kingdom level

The results show that bacteria were the only identified and predominant kingdom; however, the effects of feeding strategies on bacterial abundance were non-significant ($p > 0.05$). Figure 4.3 presents the effects of feeding strategies on the relative abundance of the assigned and unassigned kingdoms in caeca digesta samples of Ross 308 broilers.

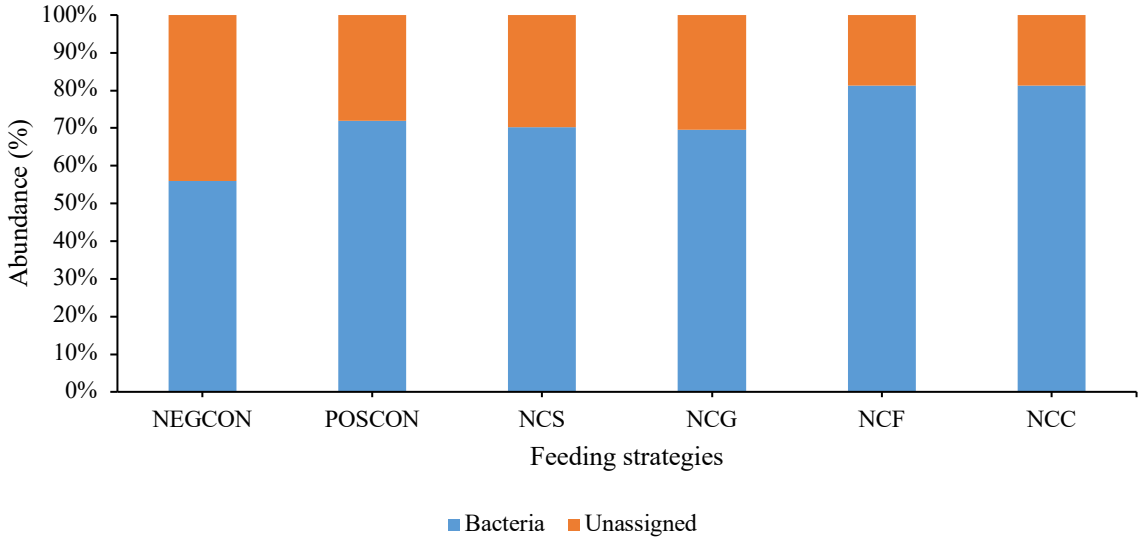


Figure 4.3: Effects of yeast-based nucleotides on the relative abundance (%) of microbial kingdoms in caeca digesta samples of Ross 308 broilers.

[Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).]

4.5.2.3 Phylum level

The effects of feeding strategies on the phyla were insignificant ($p > 0.05$). Figure 4.4 presents the relative abundance of phyla in caeca digesta samples of Ross 308 broilers. Out of 12 phyla, Bacillota A, Bacillota, Bacteroidota, Synergista, and Actinomycota were the top five (44.88, 38.69, 11.43, 11, and 2.671 %) abundant phyla. Bacillota A, Bacillota, Bacteroidota, and Actinomycota were present across all feeding strategies, while Synergista were only found in birds continuously supplemented with yeast-based nucleotides.

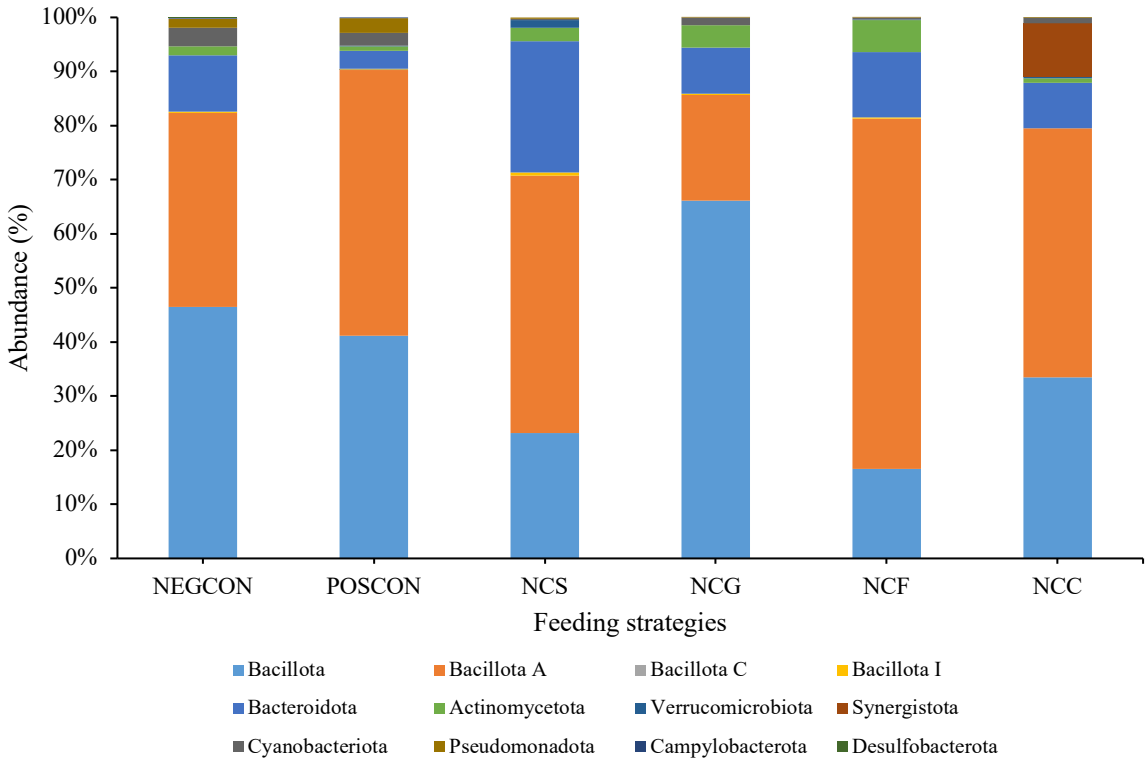


Figure 4.4: Effects of feeding strategies on the relative abundance (%) of phyla in caeca digesta samples of Ross 308 broilers.

[Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).]

4.5.2.4 Class level

Feeding strategies did not influence the class abundance ($p > 0.05$). Figure 4.5 presents the relative abundance of classes in caeca digesta samples of Ross 308 broilers. A total of 14 classes were identified; the top five abundant classes were Clostridia, Bacilli, Bacteroida, Actinomycetes, and Vampirovibrionia at 44.88, 38.69, 11.43, 2.962, and 1.753%, respectively. All the classes were evenly distributed across all feeding strategies; however, Actinomycetes

were only found in caecum digesta of broilers reared on phased yeast-based nucleotide strategies.

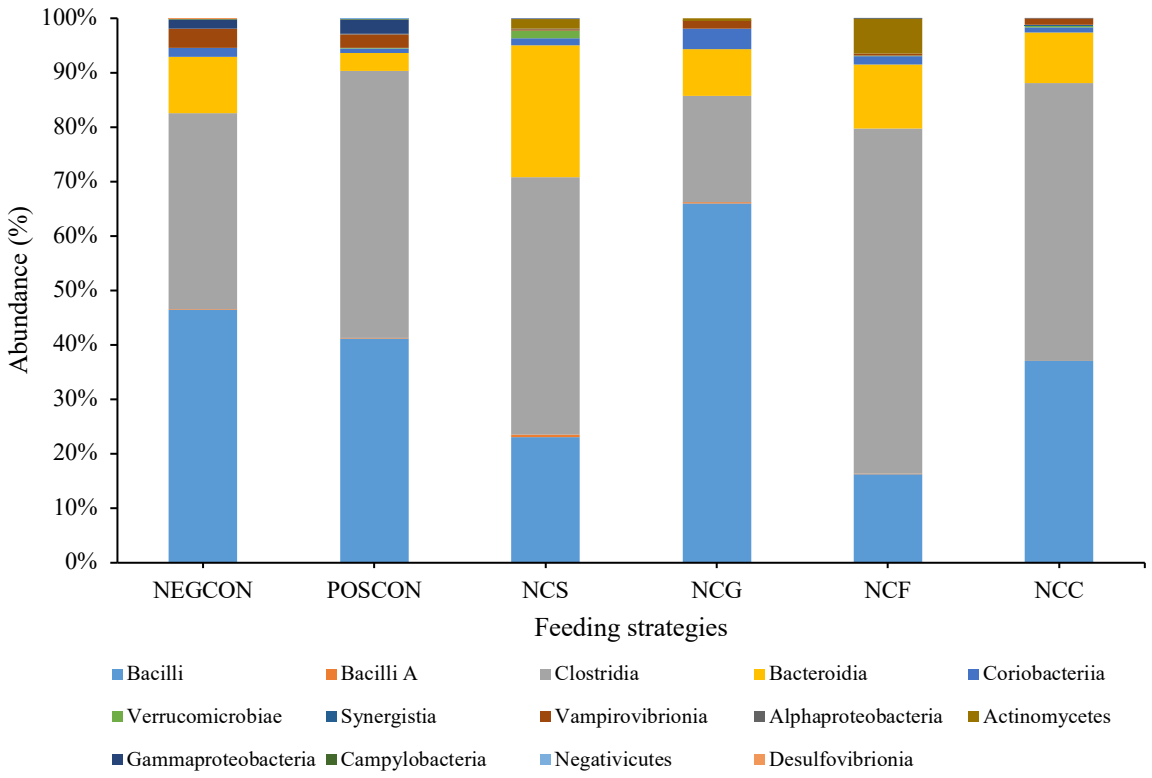


Figure 4.5: Effects of yeast-based nucleotides in shaping the relative abundance (%) of classes in caeca digesta samples of Ross 308 broilers.

[Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).]

4.5.2.5 Order level

Feeding strategies did not affect the gut microbiome at the order level ($p > 0.05$). Figure 4.6 presents the effects of feeding strategies on the relative abundance of orders in caeca digesta samples of Ross 308 broilers. Accordingly, a total of 26 orders were identified: Lactobacilales,

Peptostreptococcales, Bacteroidales, Oscillospirales, and Lachnospirales (38.75, 36.00, 11.26, 5.27, and 3.73%, respectively) were the most abundant across all feeding strategies. Xanthomonadales relative abundance was less than 0.1% and was only present in broilers' caeca reared on the NCS feeding strategy.

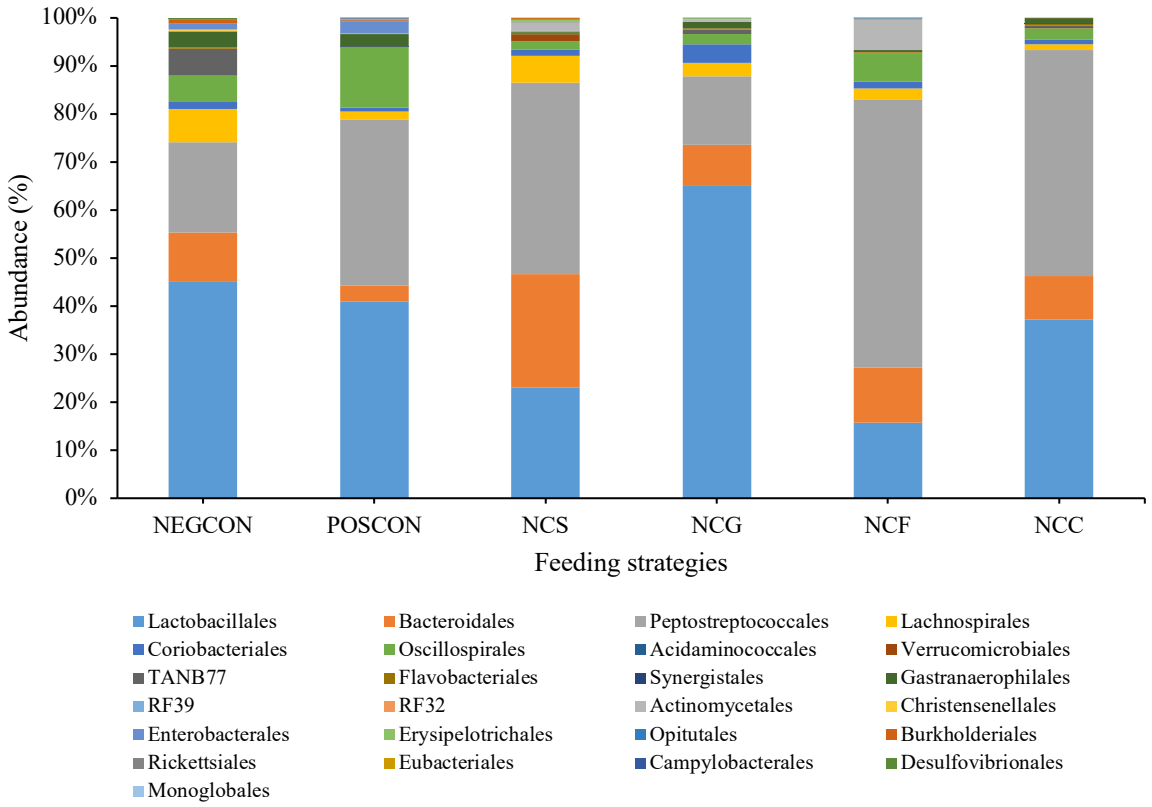


Figure 4.6: Influence of feeding strategies on the relative abundance (%) of orders in caeca digesta samples of Ross 308 broilers.

[Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).]

4.5.2.6 Family level

Feeding strategies did not significantly impact the gut microbiome at the family level ($p > 0.05$). Figure 4.7 shows the effects of feeding strategies on the relative abundance of families in caeca digesta samples of Ross 308 broilers. Overall, 46 families were identified. The

Peptostreptococcaceae, *Lactobacillaceae*, *Enterococcaceae*, *Streptococaceae*, and *Bacteroidaceae* were the most abundant at 35.98, 31.34, 9.06, 6.70, and 6.66% respectively. *Peptostreptococcaceae*, *Lactobacillaceae*, *Streptococaceae*, and *Bacteroidaceae* were present across all feeding strategies; however, *Enterococcaceae* were absent in the continuously phased yeast-based nucleotide-supplemented broiler caecum. *Xanthomonadales* relative abundance exclusively in the broiler caeca digesta reared on the NCS feeding strategy was less than 0.1%.

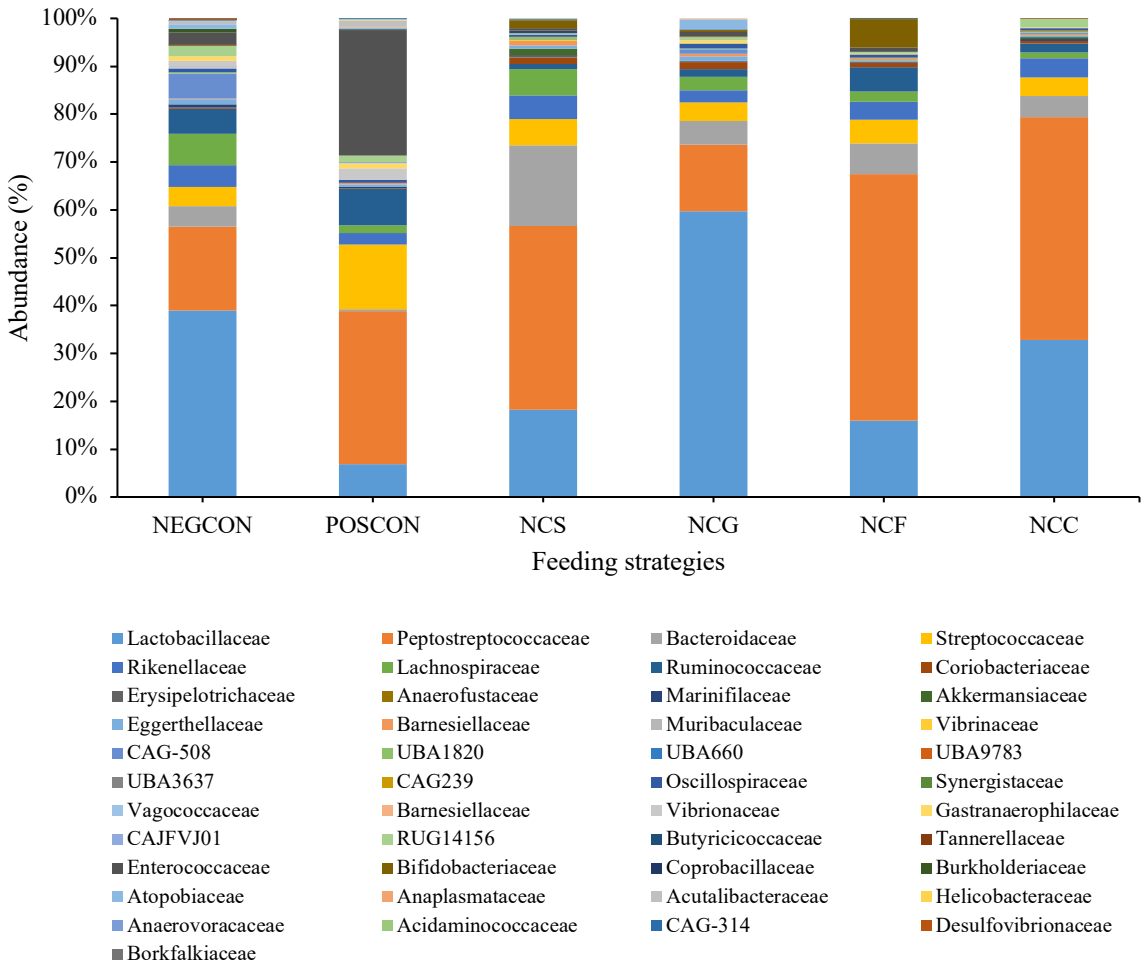


Figure 4.7: Effects of yeast-based nucleotides in shaping the relative abundance (%) of families in caeca digesta samples of Ross 308 broilers.
[Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).]

4.5.2.7 Genus level

Feeding strategies did not significantly impact the gut microbiome at the family level ($p > 0.05$). Figure 4.8 presents the effects of feeding strategies on the relative abundance of genera in caeca digesta samples of Ross 308 broilers. Accordingly, *Romboutsia* (33.63%), *Paraprevotella* (16.03%), *Ligilactobacillus* (13.46%), *Limosilactobacillus* (11.99%), and *Lactobacillus* (9.80%) were the most abundant. *Romboutsia*, *Ligilactobacillus*, *Limosilactobacillus*, and Lactobacillus were evenly distributed across all feeding strategies, while Paraprevotella were only abundant in NCS and NCC feeding strategies.

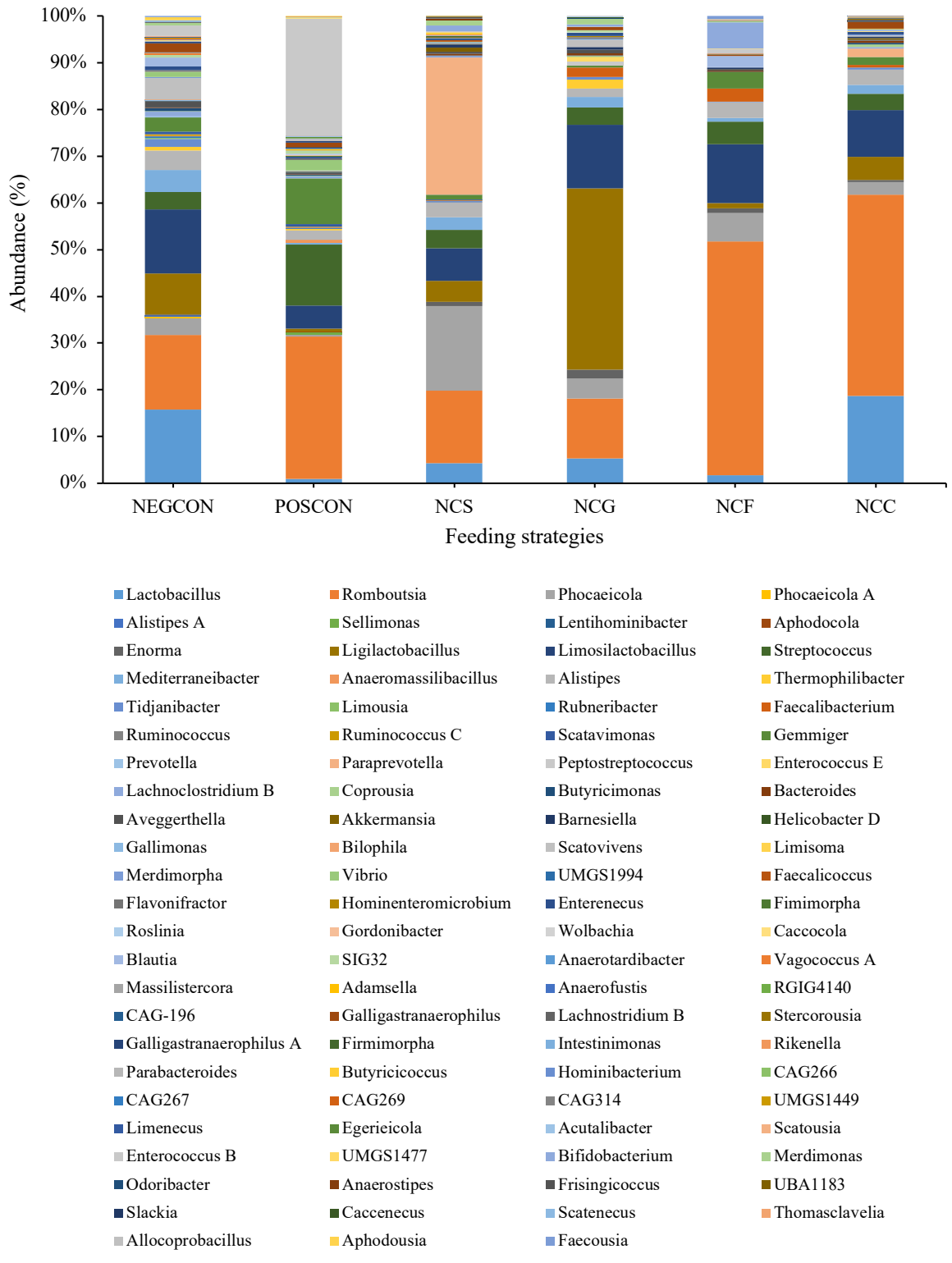


Figure 4.8: Influence of feeding strategies on the relative abundance (%) of genera in caeca digesta samples of Ross 308 broilers.

[Feeding strategies: a basal diet without AGP or nucleotides (NEGCON) fed continuously, a basal diet with AGP (POSCON) fed continuously, and a basal diet containing 0.05% nucleotides in place of AGP fed either continuously (NCC), during the starter phase only (NCS), during the grower phase only (NCG), or during the finisher phase only (NCF).]

4.5.2.8 Species level

A total of 155 species were identified. Feeding strategies had an impact ($p < 0.05$) on *Tidjanibacter inops* species, also known as *Alistipes inops*. The *A. inops* species was least abundant ($p < 0.05$) in POSCON birds, which was similar to NCS, NCF, NCG, and NCC birds, while the NEGCON birds recorded the highest ($p < 0.05$) population of this species. In addition, the five most abundant species across all diets were *Romboutsia ilealis* (35.96%), *Ligilactobacillus aviarius* (10.29%), *Ligilactobacillus aviarius B* (7.834%), *Limosilactobacillus vaginalis* (6.93%), and *Ligilactobacillus Aviarius C* (6.92%). Species classified under 'other' recorded less than 1% across all the feeding strategies. Figure 4.9 presents the effects of feeding strategies on the relative abundance of species in caeca digesta samples of Ross 308 broilers.

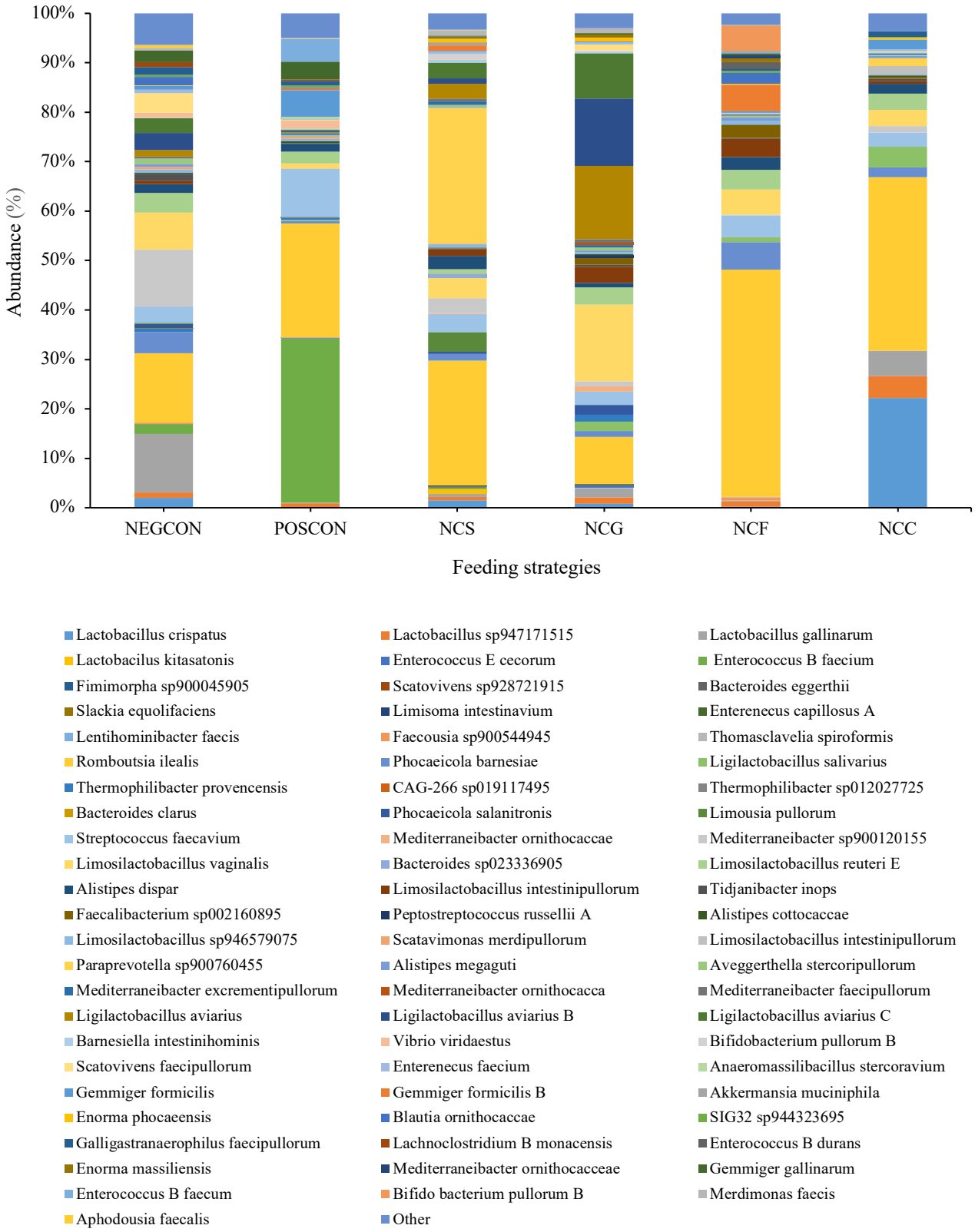


Figure 4.9: The impact of nucleotide feeding strategies on the relative abundance (%) of species in caeca digesta samples of Ross 308 broilers.

4.6 Discussion

The small intestines are made of three compartments: the duodenum, jejunum, and ileum. The ileum is the last fragment, usually with a similar length to that of the jejunum. It is primarily the site for water and mineral absorption and additionally digests and absorbs starch, fat, and protein (Svihus, 2014). This study found that broilers fed diets supplemented with zinc bacitracin exhibited longer ilea compared to those receiving either phased or continuous yeast-based nucleotide supplementation. While yeast-based nucleotides support gut health through cellular regeneration and immune modulation, their impact on ileum length may be less pronounced or require longer supplementation periods to manifest significant morphological changes during this study. Villus height and width determine the extent to which absorption occurs and eventually bird productivity (Ariyadi *et al.*, 2019), as reflected in the growth performance earlier.

Alistipes inops, although not as well-characterized, is part of the *Rikenellaceae* family and has been isolated from avian sources (Shkoporo *et al.*, 2015). The genus Alistipes is exclusively gram-negative anaerobic bacteria (Ogata *et al.*, 2021), and a primary source of lipopolysaccharides. Lipopolysaccharides are harmful endotoxins that cause inflammation and, as a result, affect gut functionality (Eeckhaut *et al.*, 2016). The suppression of *A. inops* in the positive control could indicate the ability of antibiotics to exert selective pressure that limits the proliferation of potentially opportunistic bacteria, while nucleotides similar to the negative control lacked the capacity.

Bacillota A, Bacillota, and Bacteroidota constituted over 90% of the microbiome in alignment with the literature (Pan & Yu, 2013). Bacillota and Bacteriodota are the most dominant phyla in a healthy gut. Both phyla are involved in physiological homeostasis, gut barrier function, transit, and immune system regulation through competitive interaction (Wilde *et al.*, 2024).

Bacillota are butyrate-producing bacteria; as a result, they enhance anti-inflammatory responses and regulate energy metabolism (Magne et al., 2022). These include most of the top 5 genera: Romboutsia, Ligilactobacillus, Limosilactobacillus, and Lactobacillus. While Bacteroidota play a significant role in dietary fibre and polysaccharides fermentation, thus serving as the primary source for acetate and propionate short-chain fatty acids (SCFAs) that determine gut mucous integrity and immunity (Gao *et al.*, 2024). These results generally illustrate that phased nucleotide supplementation can achieve similar results in shaping the gut microbiome as continuous supplementation, potentially enhancing beneficial bacterial populations when timed appropriately. This aligns with broader literature emphasizing the role of diet and feed additives in modulating gut microbial ecology to improve poultry health and productivity (Oakley *et al.*, 2014; Yang *et al.*, 2019).

4.7 Conclusions

These findings indicate that both phased and continuous supplementation with yeast-based nucleotides yielded indifferent results on digestive organs, including the spleen, liver, proventriculus, ventriculus, pancreas, duodenum, jejunum, and ceca sizes and lengths. Similarly, strategic-phased and continuous supplementation demonstrated similar outcomes on the gut microbiome, characterized by enhanced populations of fiber-fermenting microbes and increased metabolic activity related to carbohydrate and bile acid metabolism, energy harvesting, and nutrient provision for epithelial cells. As a result, this contributed to improved gut health. Based on these findings, it is recommended that yeast-based nucleotides be supplemented in a phased manner to promote optimal gut health and maintain microbiome balance.

4.8 References

Abo-Sriea, T.M., Ismael, E., Sobhi, B.M., Hassan, N.H., Elleithy, E.M., Omar, S.A., Soliman, A.M., Fahmy, K.N.E.D. & Ramadan, A., 2024. Impact of dietary-nucleotides and Saccharomyces cerevisiae-derivatives on growth-performance, antioxidant-capacity, immune-response, small-intestine histomorphometry, caecal-Clostridia, and litter-hygiene of broiler-chickens treated with florfenicol. *International Journal of Veterinary Science and Medicine*, *12*(1), 11-24.

Ariyadi, B., Sudaryati, S., Harimurti, S., Wihandoyo, Sasongko, H., Habibi, M.F. & Rahayu, D., 2019. Effects of feed form on small intestine histomorphology of broilers. In *IOP Conference Series: Earth and Environmental Science*, *387*(1), 012047.

Aruwa, C.E. & Sabiu, S., 2024. Interplay of poultry–microbiome interactions–influencing factors and microbes in poultry infections and metabolic disorders. *British Poultry Science*, *65*(5), 523-537.

Carrasco, J.M.D., Casanova, N.A. & Miyakawa, E.F., 2019. Microbiota, gut health and chicken productivity: What is the connection? *Microorganisms, 7*, 3-15.

Celi, P., Verlhac, V., Calvo, E.P., Schmeisser, J. & Kluenter, A.M., 2019. Biomarkers of gastrointestinal functionality in animal nutrition and health. *Animal Feed Science and Technology*, *250*, 9-31.

Kiarie, E.G., Mohammadigheisar, M. & Schulze, H., 2022. Effects of early feeding of enzymatically treated yeast on growth performance, organ weights, intestinal histomorphology, and ceca microbial metabolites in broiler chickens subjected to Eimeria challenge. *Poultry Science*, *101*(8), 101967.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. & Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1), 1.

Magne, F., Gotteland, M., Gauthier, L., Zazueta, A., Pesoa, S., Navarrete, P. & Balamurugan, R., 2020. The firmicutes/bacteroidetes ratio: a relevant marker of gut dysbiosis in obese patients?. *Nutrients*, *12*(5), 1474.

Maina, A.N., Thanabalan, A., Gasarabwe, J., Mohammadigheisar, M., Schulze, H. & Kiarie, E.G., 2022. Enzymatically treated yeast bolstered growth performance of broiler chicks from young broiler breeders linked to improved indices of intestinal function, integrity, and immunity. *Poultry Science*, *101*(12), 102175.

Montso, P.K., Mnisi, C.M. & Ayangbenro, A.S., 2022. Caecal microbial communities, functional diversity, and metabolic pathways in Ross 308 broiler chickens fed with diets containing different levels of Marama (*Tylosema esculentum*) bean meal. *Frontiers in Microbiology*, *13*, 1009945.

Oakley, B.B., Lillehoj, H.S., Kogut, M.H., Kim, W.K., Maurer, J.J., Pedroso, A., Lee, M.D., Collett, S.R., Johnson, T.J. & Cox, N.A., 2014. The chicken gastrointestinal microbiome. *FEMS Microbiology Letters*, *360*(2), 100-112.

Rassmidatta, K., Theapparat, Y., Chanaksorn, N., Carcano, P., Adeyemi, K.D. & Ruangpanit, Y., 2024. Dietary Kluyveromyces marxianus hydrolysate alters humoral immunity, jejunal morphology, cecal microbiota and metabolic pathways in broiler chickens raised under a high stocking density. *Poultry Science*, *103*(9), 103970.

Rubin, J.A., Runge, J.J., Mison, M., Mehler, S., Echols, M.S., Lam, N.K.Y, Speer, B.L., Bennet, R.A., Ponder, J.B. & Redig, P., 2016. CHAPTER 21 - Surgery, Current Therapy in Avian Medicine and Surgery, 631-668.

Rutz, F., Xiaver, E., Anciuti, M. Roll., V., & Rossi, P., 2008. The role of nucleotides in improving broiler pre-starter diets. In: J. A. Taylor-Pickard & Spring. P., ed., Gut Efficiency: The Key Ingredient in Pig and Poultry Production: Elevating Animal Performance and Health, by Publisher: *Wageningen Academic Publishers*, The Netherlands, 155-163.

Shkoporov, A.N., Chaplin, A.V., Khokhlova, E.V., Shcherbakova, V.A., Motuzova, O.V., Bozhenko, V.K., Kafarskaia, L.I. & Efimov, B.A., 2015. Alistipes inops sp. nov. and Coprobacter secundus sp. nov., isolated from human faeces. *International Journal of Systematic and Evolutionary Microbiology*, *65*(12), 4580-4588.

Svihus, B., 2014. Function of the digestive system. *Journal of Applied Poultry Research*, *23*, 306–314.

Stein, H. H., & Kil, D. Y., 2006. Reduced dietary nucleotide concentrations in growing pigs and their effects on intestinal development and health. *Animal*, *1*(6), 905-912.

Svihus, B., 2011. The gizzard: function, influence of diet structure and effects on nutrient availability. *World's Poultry Science Journal*, *67*(2), 207-224.

Thema, K.K., Mlambo, V. & Mnisi, C.M., 2024. Stress-alleviating properties of dietary red grape pomace in Ross 308 broilers reared at a high stocking density. *South African Journal of Animal Science*, *53*(6), 883-893.

Wilde, J., Slack, E. & Foster, K.R., 2024. Host control of the microbiome: mechanisms, evolution, and disease. *Science*, *385*(6706), 3338.

Wei, S., Morrison, M. & Yu, Z., 2013. Bacterial census of poultry intestinal microbiome. *Poultry Science*, *92*(3), 671-683.

Wu, C., Yang, Z., Song, C., Liang, C., Li, H., Chen, W., Lin, W. & Xie, Q., 2018. Effects of dietary yeast nucleotides supplementation on intestinal function, intestinal microbiota,

and humoral immunity in specific pathogen-free chickens. *Poultry Science*, *97*(11), 3837-3846.

Yang, Y., Iji, P. A., & Choct, M., 2019. Dietary modulation of gut microbiota in broiler chickens: a review of the role of probiotics, prebiotics, and synbiotics. *Animal Nutrition*, *5*(4), 353-362.

Ying, H., Cao, Z., Chen, Y., Chen, X., Wu, J. & Xie, J., 2017. Nucleotide production process. *Google Patents*. [online] Available at: https://patents.google.com/patent/US20170130254A1/en [Accessed 31 Jul. 2025].

Yu, C., Xu, H., Jiang, J., Tan, Z., Peng, X., Liu, S. & Yang, C., 2024. Effects of benzoic acid or benzocal-50 supplementation on growth performance and intestinal health in mixed-sex Ross 308 chickens under a high stocking density. *Journal of Applied Poultry Research*, *33*(3), 100454.

5. GENERAL DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

5.1 General discussion

The world population is projected to reach 9.6 million in the year 2050 (Maharjan *et al.*, 2021). Accordingly, the rapid increase in the human population means an increase in the global food demand, particularly animal protein (Zampiga *et al.*, 2021). Poultry meat remains the main driver for growth in global meat production. Its widespread consumption is driven by the lack of cultural and religious barriers, affordability, and the nutritional advantages it offers (Jie *et al.*, 2024). Producers rely on intensive production systems that produce more meat per unit area to meet this ever-rising demand. However, intensive production systems are associated with stressful rearing conditions that impair the health and functionality of the gut and ultimately the health, welfare, and growth performance of the birds (Salois *et al.*, 2016; Thema *et al.*, 2024). As a result, disease outbreaks are often higher in such systems, posing challenges to sustainable poultry production. Antibiotic growth promoters (AGP) were traditionally employed to optimize gut function and overall performance due to their ability to effectively control dysbacteriosis, enteropathogens, and enhance feed conversion efficiency (Dibner & Richards, 2005). Nonetheless, excessive use of antibiotics compromises food safety, contributes to increased antimicrobial resistance and environmental contamination (Zhen *et al.*, 2023), prompting their discontinuation or restriction in certain countries (Wallace *et al.*, 2010). Necessitating the identification of effective alternatives for optimizing gut health, functionality, and overall bird performance. Supplemental nucleotides are a promising candidate.

Nucleotides are the fundamental structural units of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) that play vital roles in physiological and biochemical processes in cell regeneration, hormone signaling, protein synthesis, and energy transfer (Wang *et al.*, 2024).

They are synthesized endogenously via *de novo* and salvage pathways but may be rendered inadequate under stress or during rapid growth, requiring exogenous supplementation through broiler diets. Moreover, commercial broiler diets, which mostly consist of maize and soybean meal, are typically deficient in nucleotides (Mateo & Stein, 2004), making this additive essential in broiler nutrition, especially under high stocking densities.

The influence of continuous supplementation with nucleotides in broiler diets has been proven to enhance immune response (Boza & Martines-Augustin, 2002), improve growth performance (Wang *et al.*, 2009), and meat quality parameters (Chiofalo *et al.*, 2011). However, high-cost limits of nucleotides in comparison to other feed additives make their widespread adoption as an alternative to AGP. The cost of nucleotide supplementation could be minimized by implementing a periodic supplementation strategy, targeting the more stressful phases in the broiler's lifecycle, an approach that has not been sufficiently investigated. Therefore, this study is a comparative exploration of the impact of phased *versus* continuous nucleotide supplementation on densely stocked Ross 308 broilers.

Chapter three investigated the comparative effects of yeast-based nucleotides on growth performance, blood parameters, and meat quality traits over a five-week period. The growth performance results obtained revealed that strategic-phased and continuous supplementation with yeast-based nucleotides yielded neutral effects. It is notable that birds reared on the positive control outperformed the yeast-based nucleotide-supplemented broilers. Birds reared on AGP achieved the highest body weight gains and the most efficient feed conversion. Moreover, this study's findings imply that both yeast-based nucleotide and AGP supplementation under high stocking densities counteract the primary rationale for intensive production systems, as broilers reared on both AGP and nucleotides could not reach the average

required market weight of Ross 308 broilers (2.3 - 3.0 kg) (Aviagen, 2022). Thus, questioning the efficiency of high stocking densities. Additionally, phased yeast-based nucleotide supplementation in the starter phase elevated blood urea nitrogen, while exclusive supplementation in the grower phase increased albumin, alanine aminotransferase, and total bilirubin as a result of increased liver function (Aziz *et al.*, 2024). Similarly, exclusive supplementation with yeast-based nucleotides in the finisher phase increased haematological immune responses by elevating platelet count and monocyte count. Furthermore, yeast-based nucleotides supplementation in the grower phase improved meat tenderness through their ability to positively influence muscle development. Conversely, supplementation with yeast-based nucleotides in the grower phase increased drip loss, suggesting a complex interaction between muscle water retention and tenderness. These findings support the hypothesis that phased and continuous supplementation of yeast-based nucleotides promote notably similar results in growth performance, while phased supplementation yielded better immune responses.

Chapter four revealed that feeding strategies had no discernible effects on the proventriculus, ventriculus, spleen, liver, pancreas, small and large intestine weights, and duodena, jejuna, and caeca lengths. Additionally, feeding strategies did not affect the gut microbiome alpha and beta diversity and the gut composition at kingdom, phylum, class, order, family, and genus levels. *Alistipes inops* species differed across feeding strategies. The greater presence of this species in the negative control, phased and continuous supplementation emphasizes the inability of yeast-based nucleotides to suppress specific opportunistic bacteria. Nonetheless, the predominant phyla and genera were constituted by phyla and genera that are involved in physiological homeostasis, gut barrier function, immune system, and energy metabolism regulation (Magne *et al.*, 2022; Wilde *et al.*, 2024). These findings underscore the ability of

phased yeast-based nucleotide to achieve similar results in shaping the gut microbiome as continuous supplementation, potentially enhancing beneficial bacterial population when timed appropriately. This is in alignment with the hypothesis that continuous and phased yeast-based nucleotide supplementation yields similar results in all parameters of interest.

5.2 Conclusions

In conclusion, this study shows that phased and continuous yeast-based nucleotide supplementation promoted comparable results in specific parameters, while phased supplementation improved certain immunomodulatory responses, explaining the moderate differences in the body weight gains. Most importantly, phased yeast-based nucleotide supplementation has the potential to shape the gut microbiome comparable to continuous yeast-based nucleotide supplementation and AGP. This effect was characterized by enhanced populations of fiber-fermenting microbes and increased metabolic activity. These findings support this study's hypothesis that phased and continuous yeast-based nucleotide supplementation yields comparable results in densely stocked birds; nonetheless, yeast-based nucleotides are an incomparable alternative to AGP.

5.3 Recommendations

Given that phased yeast-based nucleotide supplementation promoted immunomodulatory responses and increased metabolic activity, it is recommended that yeast-based nucleotides be supplemented on a phased basis to ensure economic efficiency while supporting optimal bird welfare and overall growth performance under less intense production systems.

5.4 References

Aviagen., 2022. *Ross Broiler: Nutrition Specifications*. USA.

Aziz, A.A.A., Aziz, E.S.A.A., Khairy, M.H., Fadel, C., Giorgi, M. & Abdelaziz, A.S., 2024. The effect of butyric acid and nucleotides supplementation on broiler (*Gallus gallus domesticus*) growth performance, immune status, intestinal histology, and serum parameters. *Open Veterinary Journal*, *14*(1), 324.

Boza, J.J. & Martinez-Augustin, O., 2002. Role and function of nucleotides in Infant nutrition. In: Infant Formula: closer to the reference (Räihä, N.C.R. and F.F. Rubaltelli, eds.), *47*, 165-182.

Chiofalo, B., Lo Presti, V., Savoini, G., D'Alessandro, E., Chiofalo, V., & Liotta L., 2011. Nucleotides in broiler chicken diet: effect on breast muscles quality. *Czech Journal of Food Science, 29*, 308-317.

Dibner, J.J. & Richards, J.D., 2005. Antibiotic growth promoters in agriculture: history and mode of action. *Poultry Science*, *84*, 634–643.

Jie, Y., Wen, C., Huang, Q., Gu, S., Sun, C., Li, G., Yan, Y., Wu, G. &Yang, N., 2024. Distinct Patterns of Feed Intake and Their Association with Growth Performance in Broilers. *Poultry Science*, *103*(9), 103974.

Maharjan, P., Martinez, D.A., Weil, J., Suesuttajit, N., Umberson, Mullenix, G., Hilton, K.M., Beitia, A. & Coon, C.N., 2021. Review: Physiological growth trend of current meat broilers and dietary protein and energy management approaches for sustainable broiler production. *Animal*, *15*, Supplement 1, 100284.

Mateo, C.D. & Stein, H.H., 2004. Nucleotides and young animal health: can we enhance intestinal tract development and immune function? In: Lyons, T.P., Jacques, K.A. (Eds.), Nutritional biotechnology in the feed and food industries. proceedings of

Alltech's 20th annual symposium. Nottingham University Press, Nottingham, UK, 159–168.

Mottet, A. & Tempio, G., 2017. Global poultry production: current state and future outlook and challenges. *World's poultry science journal*, *73*(2), 245-256.

Salois, M.J., Cady, R.A. & Heskett, E.A., 2016. The environmental and economic impact of withdrawing antibiotics from US broiler production. *Journal of Food Distribution Research*, *47*(1), 1–2.

Thema, K.K., Mlambo, V. & Mnisi, C.M., 2024. Stress-alleviating properties of dietary red grape pomace in Ross 308 broilers reared at a high stocking density. *South African Journal of Animal Science*, *53*(6), 883-893.

Wallace, R.J., Oleszek, W., Franz, C., Hahn, I., Baser, K.H.C., Mathe, A. & Teichmann, K., 2010. Dietary plant bioactives for poultry health and productivity. *British Poultry Science*, *51*(4), 461–487.

Wang, Y., Wang, J., Liu, L., Xu, H., Liang, H., Wang, Z. & Gu, J., 2024. Effects of dietary yeast nucleotides on the growth performance and muscle quality of juvenile largemouth bass (*Micropterus salmoides*). *Aquaculture Reports*, *36*, 102159.

Wang, Z., Cerrate, S., Coto, C., Sacakli, P., Yan, F., Costa, F. & Waldroup, P.W., 2009. Evaluation of Nupro yeast product in diets for broilers. *International Journal of Poultry Science*, *8*, 515-520.

Zampiga, M., Calini, F. & Sirri, F., 2021. Importance of feed efficiency for sustainable intensification of chicken meat production: implications and role for amino acids, feed enzymes and organic trace minerals. *World's Poultry Science Journal*, *77*(3), 639-659.

Zhen, W., Zhu, T., Wang, P., Guo, F., Zhang, K., Zhang, T., Jalukar, S., Zhang, Y., Bai, D., Zhang, C. & Guo, Y., 2023. Effect of dietary Saccharomyces-derived prebiotic refined functional carbohydrates as antibiotic alternative on growth performance and intestinal

health of broiler chickens reared in a commercial farm. *Poultry Science*, *102*(6), 102671.

APPENDICES

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**