SELECTION AND EVALUATION OF FOUR NOVEL NITROGEN REDUCTION PROBIOTICS ON GUT MICROFLORA, NITROGEN UTILIZATION, AND BROILER PERFORMANCE

A Dissertation

by

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ABSTRACT

The ruminal microorganisms are an excellent example of how the body adapts to different feeding sources on its own initiative. The ruminants, in contrast to monogastric creatures such as chickens, create an ideal environment for a group of microorganisms in the rumen compartment, which then uses their powers of cellulose digestion and nitrogen conversion to allow them to use the forge more efficiently than their monogastric counterparts, such as cattle (Davis, 1973; Wu, 1993). While the rumen microorganisms' nitrogen utilization is estimated to be greater than 67% of non-protein nitrogen (Firkins et al., 2007), the poultry nitrogen emission is estimated to be greater than 27 % of total emission (Battye et al., 1994). Based on that, the idea of presenting some nitrogen assimilation bacteria to broiler chickens was shaped to reduce the nitrogen emissions associated with poultry production. This study aims to investigate the effect of the bacteria strains *Selenomonas ruminantium, Butyrivibrio fibrisolvens A38, Ruminococcus albus strain 7,* and *Bacteroides ruminocola subsp. brevis B14* (new name *Prevotella bryantii B14*), on the broiler performance, dietary amino acids from bacterial protein availability, and the broiler gut microflora in three separate experiments. The first experiment was in vitro, the second experiment was in vivo, and the third was a bioinformatics experiment. To evaluate the effect of those novel probiotics on the broiler performance, nitrogen utilization, and the broiler cecal microflora's microbial diversity, richness, evenness, and composition. The results showed that the *R. albus 7* had the highest nitrogen utilization ($P > 0.05$) in the first experiment, the highest BWG ($P > 0.05$), slightly the lowest FCR, slightly the lower nitrogen emission, slightly the higher body N/ feed N ratio, and slightly

lower FC N/ feed N ratio in the second experiment. *R. albus 7* treatment showed no significant effect on the broiler cecal microflora's microbial diversity, richness, evenness, and composition in the bioinformatic study in the third experiment. Based on these results, it was concluded that *R. albus 7* is a potential novel nitrogen reduction probiotic.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professor John B. Carey, advisor and Professor Christopher A. Bailey of the Home Department and Professor Robin Anderson and Dr. J. Allen Byrd of Outside The Department

The student contributors helped to complete all other work conducted for the dissertation: Nasser Al Haj Ali, Talaat Khudhair Al-Alwani, and Koyle Knape

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DEDICATION

This dissertation is dedicated to my charming wife, Salwa; thank you from the depth of my heart for your tolerance, love, encouragement, and unconditional support throughout the good and bad times of my Ph.D. journey. To my uncle Ali, who supported me during this long journey to achieve my degree. To the memory of my father and mother, who were dreaming of witnessing this moment. To my family for their support and prayers. Without you, I would not have been able to achieve this degree. You are my inspiration and motivation. God bless you all

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NOMENCLATURE

NOx	Nitrogen Oxides
USEPA	The United States Environmental Protection Agency
$PM_{2.5}$	Fine Particles
CP	Crude Protein
In-vitro	Is Latin for in Glass or Lab
In-vivo	Is Latin for within the living things
ATP	Adenosine Triphosphate
NRC	National Research Council (Broiler Nutrient Requirements)
N.A.	Ammonia Andil
NFA	Ammonia-Free Andil
AA	Amino Acids
IACUC	Institutional Animal Care and Use Committee
PC	Positive Control
NC	Negative Control
BS	Bacterial strain
WRM	Whole Ruminant Microflora
BWG	Bodyweight Gain
FCR	Feed conversion Ratio
NRP	Nitrogen Reduction Probiotic
PCA	Principal Coordinates

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The global population has increased from 600 million people in 1700 to approximately 6.3 billion in 2003 (Cohen, 2003). Furthermore, with UN forecasting that the population by 2100 will reach as high as 11.2 billion people (Figure 1) (Roser, 2013; United Nations, 2019), food production must grow by 70-100 percent to meet the demand for food (Tian et al., 2021). As the number one source of animal protein, poultry products consumption has increased from 34.2 pounds per person a year in 1960 to approximately 113.3 pounds per person by 2020 (National Chicken Council, 2021). Poultry meat has gradually replaced beef consumption over the years, starting in the 1960s (figure 2) (National Chicken Council, 2021).

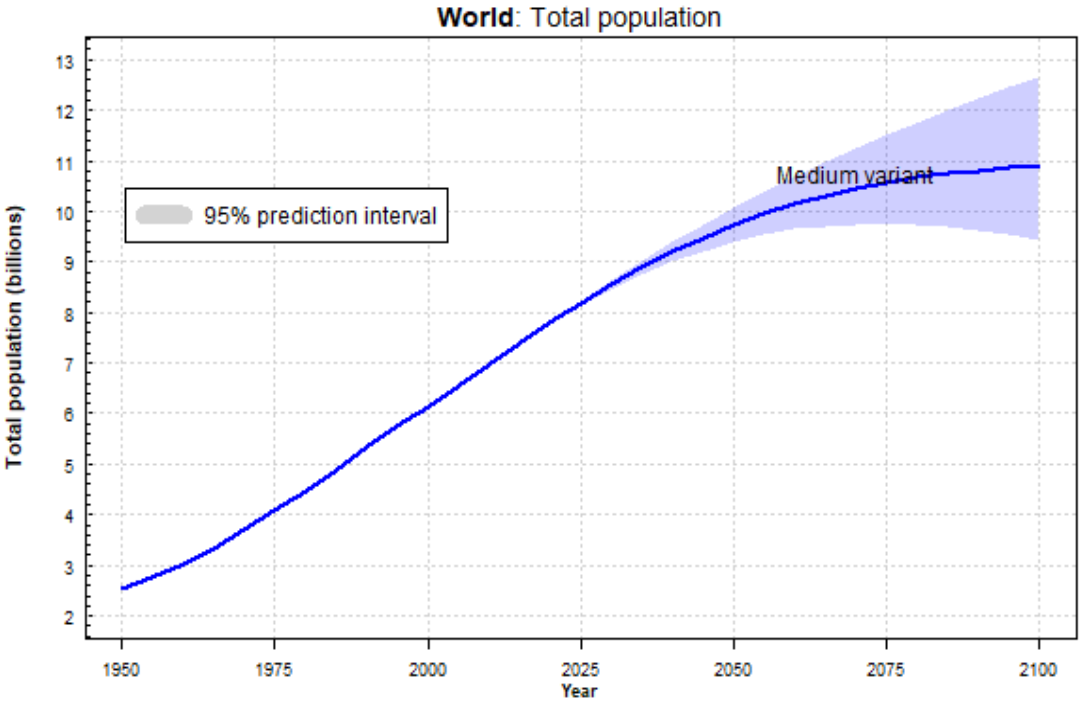

Figure 1: United Nations, DESA, Population. Word Population Prospects 2019 in the period between 1950 to forecast of 2100 (United Nations, 2019).

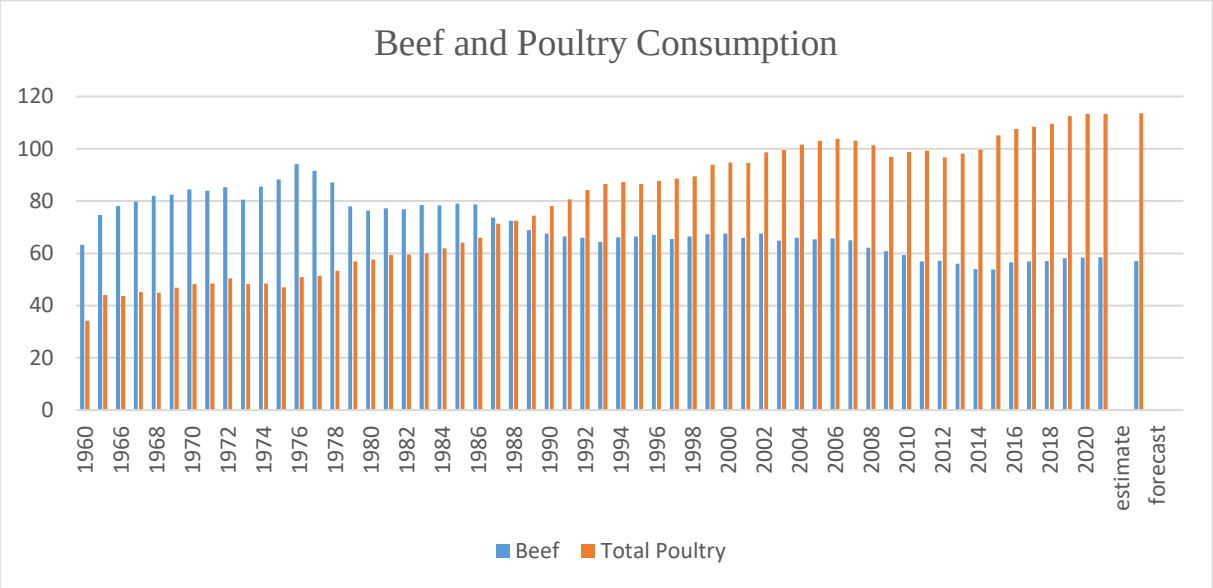

Figure 2 The Consumption of Poultry Meat compared to Beef (lb/person/year), 1960 to forecast 2022. (National Chicken Council, 2021)

That gave poultry meat and products the lead in the market. Throughout history, the high demand for poultry products has compelled those in the poultry industry to adopt intensive rearing methods that require high-tech and expensive housing to increase production rates as much as possible within the same amount of available space. The poultry industry started from small-scale individual backyard chickens growers in the 1800s and early 1900s into a small-scale commercial egg industry. Later, the early development of the broiler in the early 1920s-1930s until the mid of the 1970s, where the poultry industry was shaped in its modern style (National Chicken Council, 1999). As a revolutionary industry that changed food security rules, the poultry industry has an environmental impact that is correlated with the industry's size in different aspects. In general, animal-based food has a much more significant impact on the environment than plant-based food (Heller et al., 2013). The poultry industry's environmental consequences substantially impact climate

change, water and air pollution, land degradation, and biodiversity loss (Steinfeld et al., 2006). Examples include poultry farms introducing toxic levels of zinc, potassium, and other nutrients into nearby water bodies such as rivers (DeLaune & Moore Jr, 2016; Sharpley et al., 2018), and poultry production also contributes to climate change through the emissions of nitrous oxide, ammonia, fine particulate matter, and methane (Malomo et al., 2018). Because intensive poultry farming places large numbers of birds in considerably small spaces, producing vast amounts of manure, dead animals, microbiological pathogens, and feed additives are all released into the environment, posing a serious threat to public health. This type of poultry farming pollutes the soil, air, and water, endangering the health of both humans and wild animals. Because of the enormous amount of waste, composting, storing waste, and overfertilizing agricultural land all result in runoff of waste components into freshwater bodies such as lakes, rivers, and ponds, causing algae blooms, which is known as eutrophication, blocking sunlight from reaching the underwater and reducing the oxygen concentration in the water, killing all aquatic life, mainly fishes. (Blue, 2017). Land-independent industrial farming establishments have appeared, alongside an increase in the concentration of poultry operations, which also plays a major role in this growth. As the rising pressure to cut production costs and increase supplies led to increased use of animal genetics, optimized nutrition, efficient feed formulas, and new production technologies. Morovre, the key forces driving change in poultry production are no different from other livestock commodities. Innovation and economies of scale are fueled by consumer demand. Basically, all of those problems, can be summarized in three words (industrialization,

geographic concentration, and intensification) has resulted in a volume of waste that is far too large to be managed solely through land disposal, with some poor manure-management, the problem can be even worse. (Gerber et al., 2007).

Nitrogen Emission

In addition to methane, carbon dioxide, phosphorus, and microorganisms, poultry production generates significant amounts of ammonia and other oxides of nitrogen (NOx) emissions, which are harmful to the environment (Malomo et al., 2018). Among the well-known sources of ammonia are waste disposal, fertilizer, soils, forest fires, industry (including vehicles), the oceans, humans (including pets), livestock (including wild animals), and recycling activities. An estimated 44 million tons of ammonia (NH3) gas are emitted into the atmosphere each year from non-oceanic sources. It is generally agreed that agricultural activities are the most significant sources of these emissions, accounting for approximately 75% of total emissions. Although non-agricultural sources are usually not considered in most inventories, and only agriculture is held responsible, this is not always the case. Human food derived from sources other than agriculture, consumption and waste management, natural vegetation and wildlife, biomass and fuel combustion, mobile sources, industry, and other technical activities are all non-agricultural sources of ammonia emission (Table 1) (Sapek, 2013). Approximately $3x10^9$ kg of ammonia was produced by livestock waste in the United States The United States Environmental Protection Agency (USEPA) stated that livestock and poultry production accounted for approximately 18-40 percent of total ammonia emissions in 2005 (Patterson, 2005). According to Battye et al. (1994), the poultry industry alone is responsible for

approximately 27 percent of total nitrogen emissions across all source categories, including agriculture. While cattle are responsible for 44 percent of ammonia emission, and hogs and pigs, fertilizer application, accounting for about 10% and 9%, respectively, placing the poultry industry in the second-largest ammonia emitting source position. (figure 3). However, the work of Battye et al. (1994) accounted for all industries for zero nitrogen emission, and humans activities, and fuel combustion for 1% each of nitrogen emission, while, a few years later, Sapek (2013) claimed that humans' waste and fossil fuel combustion accounted for approximately 5.9 % and 0.2 % of total nitrogen emission, respectively

Table 1 Global sources of atmospheric ammonia gas emissions from terrestrial sources (Sapek, 2013)

Sources	Emissions MLn N-NH_3 .T.Y^{-1}	Percent of Total Emissions
Animal Manure	21.6	48.6
Mineral Fertilizer	9	20.3
Crops and Decomposition of Crops	2.6	5.9
Human Waste	2.6	5.9
Soils Under Natural Vegetation	2.4	5.4
Biomass Burning, Including biofuel	5.9	13.3
Fossil Fuel combustion	0.1	0.2
Industrial processes	0.2	0.5
Total	44.4	100

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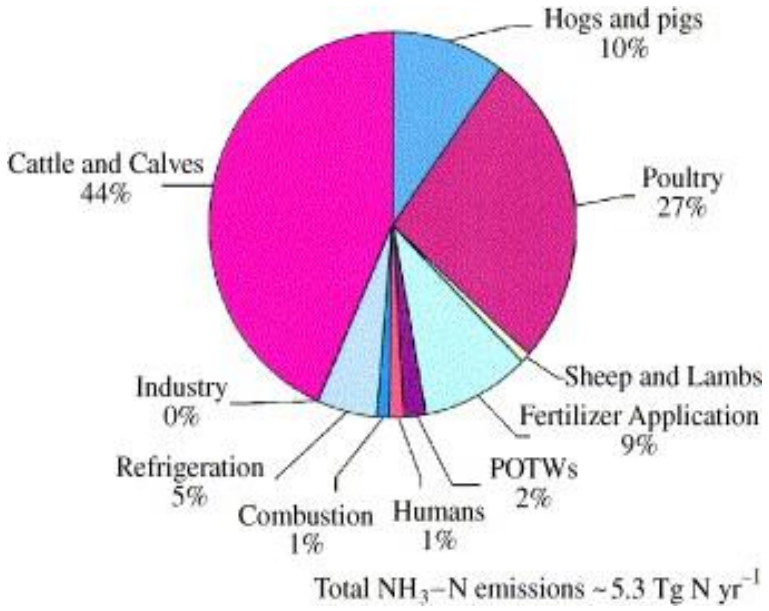

Figure 3 Total nitrogen source across all source categories in United State (Battye et al., 1994).

Based on Sobota et al. (2015) work in the United States, the potential damage costs associated with all human's activities N leakage ranged from $1.94 to $2255.00 per hectare a year in 2000, and about 75% of the damage costs were associated with leakage of agricultural nitrogen based on the location, which was exacerbated by adverse effects on aquatic habitat (eutrophication). Another 14–24% of the potential damage costs ($50 billion) were associated with fossil fuel combustion. Generally speaking, regions that experienced the greatest damage costs experienced the most significant N inputs and leakages, such as the upper Midwest and Central California. The costs of damages to human health/society, ecosystems, agriculture, and the climate differed based on the region. Intensive agricultural areas such as the upper Midwest received higher nitrogen per year than the mid-Atlantic, Pacific Northwest, and Southern California. On the other

hand, the high cost of air pollution to human health, potential damages to air and climate were more evenly distributed across the US than in the past. Nationally, the best estimates of possible damages ranged from $19 billion for drinking water impacts to $78 billion for freshwater ecosystem impacts (figure 4)(Sobota et al., 2015).

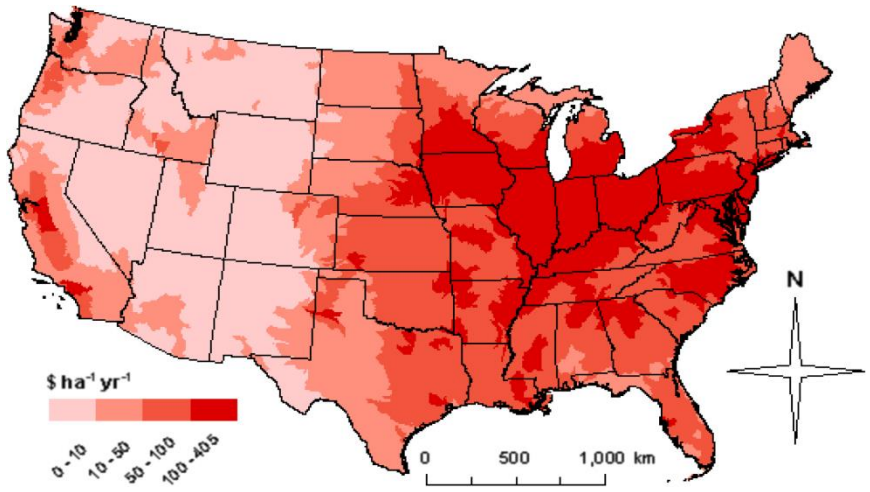

Figure 4 Distribution of potential damage costs represnted in a dollar per hactar per year in 2008 caused by humans activities' nitrogen leaked to the environment. (Sobota et al., 2015)

Because of the poultry industry's contribution to nitrous oxide pollution, many environmental effects have been documented. These include the neutralization of precipitation, cloud water, aerosols, water, and soil acidification, resulting in forest damage (Bouwman & Van Der Hoek, 1997; Lee & Dollard, 1994). Nitrogen oxide can be converted to nitric acid, which results in acid rain, which causes damage to buildings and infrastructure (Patterson, 2005). During the last two decades, nitrate violations (NO_3^-) have been reported on public drinking water supplies serving 1.5 million people throughout the United States (Garcia et al., 2017; Pennino et al., 2017), and high levels of

NOx contribute in part to the frequent exposure of vulnerable populations to fine particles ($PM_{2.5}$) and ozone at ground level. Furthermore, these fine particles are typically deposited deep within the lungs, where they can cause increased morbidity and/or mortality (Anderson et al., 2003; Di et al., 2016).

Factors affecting ammonia emission from the poultry industry

Different factors impacting NH_3 emissions from poultry operations have been identified, such as the amount of nitrogen in the feed and the proportion of various amino acids in the meal, and the conversion factor between nitrogen in poultry feed and nitrogen in meat and eggs (Battye et al., 1994). In other words, poultry consumes large amounts of nitrogen in their feed and excrete it in their excrement. The excess protein and amino acids fed to poultry are a significant contributor to the excretion of nitrogen. This excess occurs because the amino acid ratios in the feed fed to poultry are not balanced perfectly in the grains and ingredients used to formulate poultry diets. In order to meet the minimum requirements for one essential amino acid, some amino acids that are in abundance in excess in the feedstuffs are consumed by the poultry in overdosage. In addition, poultry does not digest all of the amino acids in their diet. A third of the nitrogen in practical poultry diets is processed into the tissues and eggs of the bird, with the remaining two-thirds excreted (Ritz et al., 2004). Also, the kind of poultry, how old it is, how much it weighs, and other important considerations include the housing system and how the manure is stored (pile, open/closed tanks) (Battye et al., 1994).

Furthermore, other factors such as temperature and relative humidity impact the ammonia emission from the litter after the secretion of the fecal matter into the bedding

materials. Emissions are generally higher as the temperature rises, but they will be lower with the humidity increase. Additionally, as the manure's characteristics (pH, viscosity, and dry matter content) improve, the amount of emission produced by the manure increases in general as the pH, viscosity, and dry matter content of the manure increase (Battye et al., 1994). Also, reusing the bedding materials (litter) in broiler production for a long time allows more ammonia to be released (Russ & Schaeffer, 2017)

Dietary methods to lower nitrogen emission

There was much research conducted aimed to reduce nitrogen emission from poultry operations that adapted many dietary techniques. There are many examples of these techniques, like lowering dietary crude protein (CP) by using N-balanced diets based on digestible amino acids, multi-phase feeding, supplementing with essential amino acids, and other suitable feed additives (Santonja et al., 2017). Research conducted using (CP) using N-balanced diets reported a 40% nitrogen emission reduction (Nahm, 2007). According to Kristensen and Wathes (2000), adding high-fiber ingredients to laying hen feeds can reduce NH3 emissions from manure. Increased bacterial fermentation of dietary fiber in the intestine produces acetate, butyrate, and propionate fatty acids, which lowers the pH of the manure by the development of more ammonium ion ($NH4^{+}$) occurs at lower pH shifts (Wathes, 1998). Liu et al. (2011) showed that diets containing three supplemental amino acids resulted in 12% lower nitrogen excretion by 12 % lower excretion and 23 % lower cumulative ammonia loss compared to diets containing only two supplemental amino acids. Other researchers used protease to lower nitrogen emission (Al-juboori, 2017; Leinonen & Williams, 2015; Oxenboll et al., 2011). Increased dietary

protein hydrolysis and, consequently, improved nitrogen utilization are the goals of adding proteases to animal feed. When animals can better utilize nitrogen, there is the possibility of lowering the protein content of their diets, which will, in turn, reduce the nitrogen content of their manure (Oxenboll et al., 2011).

The use of probiotics to reduce nitrogen emissions has never been attempted before, or at the very least, there has been very little research done on this subject before. From this point on, the concept for this research was conceived, to lower nitrogen emissions by selecting bacteria strains from the rumen compartment that have the highest ability to convert non-protein nitrogen, protein fragments, and some free amino acids into a bacterial protein that birds can digest and utilize in their gut. The bacteria strains selected from (Schaefer et al., 1980) will be tested in-vitro for their ability to convert ammonia as a nitrogen source into a protein content of their cells, which are theoretically going to be used as a protein source for the bird. When performing the second experiment, the nitrogen emission will be calculated using the nitrogen mass balance mothed, which will be accomplished by calculating the nitrogen inputs in the feed, the nitrogen outputs in the chicken's body, and the nitrogen in the feces. The nitrogen emission will be equal to the difference between the nitrogen input and the nitrogen output. Also, the impact of these bacteria on the microflora of the broiler gut will be investigated.

CHAPTER II

SELECTION AND EVALUATION OF FOUR NOVEL NITROGEN REDUCTION PROBIOTICS IN-VITRO

Introduction

Some ruminal microbes can utilize the available nitrogen resulting from the breakdown of non-protein and protein nitrogen sources that require ATP into microbial protein that provides approximately 50% to about all amino acids required for ruminants (Clark et al., 1992). According to Hume et al. (1970), the highest concentration of microbial protein in the rumen corresponded to an ammonia form of nitrogen concentration of 6.3 mM, and the most significant flow of microbial protein out of the rumen corresponded to an ammonia concentration of 9.5 mM. Schaefer et al. (1980) concluded that many bacterial species in the rumen require ammonia as their primary source of nitrogen for growth, and most of the species that have been studied have been able to utilize ammonia as their primary source of nitrogen in the laboratory. This experiment aims to test in vitro the ability of the four bacteria strains selected by Schaefer et al. (1980) to utilize the non-protein nitrogen, peptides, and free amino acids. In this study, four bacteria strains, including *Selenomonas ruminantium, Butyrivibrio fibrisolvens A38, Ruminococcus albus strain 7,* and *Bactetoides ruminocola subsp. brevis B14,* now known as *Prevotella bryantii B14,* were evaluated as novel nitrogen-fixing probiotics in two different media with and without ammonia and with and without additions of avian cecal microbes to determine the ammonia consumption and production rates throughout the experiments' timeline.

Additionally, the amino acid profile of these bacteria was analyzed to determine the essential dietary amino acids that may be available to birds from these bacteria.

Materials and Methods

Buffers

Three buffers used in this study, buffer A (ammonia-free ANDIL), buffer B (ammonia-free ANDIL supplemented with glucose, maltose, and cellobiose 12 mM), and buffer C (Ammonia-containing 12 mM ANDIL supplemented with glucose, maltose, and cellobiose 12 mM). Andil prepared anaerobically by bubbling with CO_2. The ammonia-free andil composition for one liter is as follows: mineral #1 75 mL (K_2HPO_4 (dibasic) 0.006 g/L), mineral #2 75 mL (K_2HPO_4 (monobasic) 0.006 g/L, NaCl 0.012 g/L, $MgSo_4{}^*7H_2O$ 0.0012 g/L, $CaCl_2{}^*6H_2O$ 0.0012 g /L, and $CaCl_2$ 0.006 g/L), resazurin 1.0 mL, H_2O 849 mL, Na_2CO_3 1.2 g, and Cysteine HCl 2.5 % SOL. The pH of the mixture was adjusted to 6.8 under CO_2 bubbling preheated to boiling.

Medium

Medium is modified Shaeffer's medium (Schaefer et al., 1980) containing per 90 mL, 3.75 mineral mix 1, 3.25 mL mineral mix 2, 1.0 mL Pfennings Trace mineral mix, 1.0 mL VFA mix 1-17, 1.0 mL Vitamin mix, 10 mL clarified rumen fluid, 0.1 mL resazurin mix, 69.4 mL water and prepared as described. The medium was adjusted to pH 6.8. Then boiled, cooled on ice while bubbling with O_2-free CO_2, and then distributed anaerobically (9 mL/tube) to 18 x 150 mm crimp top tubes and immediately sealed. Tubes were autoclaved and, when cooled, supplemented via syringe and needle with anaerobic solutions of 0.5 mL sterile 8% sodium carbonate, 0.5 mL 1% (wt/vol) dithiothreitol, and 0.7 mL of a

carbohydrate mixture containing (wt/vol) 3% D-glucose, 6% D-maltose and 6% cellobiose. Cultures inoculated with 2% volume from like-grown 48 hours old cultures were incubated upright at 39 ^{o}C without agitation.

Growth Curves

Growth curves were performed on pure cultures of rumen ammonia-assimilating bacteria. The cultures were inoculated, and the optical densities were read at 600nm at 3-hour intervals (table 2). Each bacterium was harvested after 24 hours of growth and was within mid to late log-phase growth

Table 2 Ammonia-assimilating bacteria optical densities means of reading representing the growth rate of all four bacterial strains throughout the timeline in hours (0, 3, 6, and12), N=4.

Bacterium	0 h	SD	3 h	SD	6 h	SD	12 h	SD
B. fibrisolvens	0.020	0.002	0.012	0.004	0.038	0.009	0.185	0.091
P. bryantii	0.072	0.040	0.098	0.025	0.107	0.067	0.085	0.031
R. albus 7	0.036	0.013	0.047	0.020	0.383	0.194	0.550	0.081
S. ruminantium	0.073	0.035	0.251	0.038	0.435	0.071	0.459	0.067

Preparation of washed cell suspensions of each of the pure cultures of ruminal ammonia-assimilating bacteria.

To harvest pure bacterial cells from 3 tubes of each pure bacterial cultures were harvested. Then the contents of three culture tubes were transferred into appropriately labeled 50 mL polycarbonate centrifuge tubes while maintaining a flow of CO_2 over transferred fluids. Then the tubes were centrifuged at 4 ^oC for 20 minutes at 10,000 rpm. After the first centrifuge run, supernatant fluids were carefully decanted into a waste beaker while maintaining a CO_2 flow over cells. Thirty mL of anaerobically prepared ammonia-free Andil was anaerobically added to each separate centrifuge tube containing packed cells of the respective bacteria agitated via vigorous vortexing to resuspend the bacterial cell pellet and re-centrifuged at 4 ^oC at 11,000 rpm for 20 minutes to complete a one-time washing of the cells of each pure bacterium. Then supernatant fluids resulting from the washing step were decanted into a waste beaker. The cells of each bacterium were again resuspended with 8 mL fresh anaerobically prepared ammonia-free Andil and placed on ice until ready to distribute to incubation vials. A 0.6 mL solution of CH_2O was added and mixed, then after distributing the first set a 3 -1 mL NH3-free Andil was added to vials, then 50 µL (0.05 mL) 1600 mM NH_4Cl was added to the remaining 5 mL suspension, then distributed 1 mL each to 3 vials for a test with 12 mM added ammonia.

Preparation of washed cell suspensions of mixed populations of the bovine rumen and avian cecal bacteria.

Rumen microbes

Rumen contents, approximately 500 mL, were collected from a cannulated Jersy steer early in the morning (8:00) and strained through a paint strainer to remove large particles. The strained fluid was collected in thermos containers and immediately caped to avoid air exposure**,** then returned to the lab and divided into four 50 mL centrifuge tubes (40 mL each), with maintaining a CO_2 flow over the tubes. Then tubes were centrifuged at 10,000 rpm for 20 minutes at 4 °C. Then the supernatant was decanted fluid into a waste beaker. After that, the cells of mixed rumen microbes were washed two times via resuspension in 30 mL anaerobic ammonia-free Andil and recentrifuged at 10,000 rpm for 20 min at 4 ^oC. The supernatant fluids were decanted into a waste beaker after each wash. After washing, the cell pellet in each of the four centrifuge tubes was resuspended in 10 mL anaerobic ammonia-free Andil under a CO_2 flow. Then caped and agitated to resuspend the cell pellet, then pooled to make a single-cell suspension containing 40 mL of a 4X concentrated suspension of rumen microbes which was caped and placed on ice until ready to distribute to incubation vials. Sixteen mL for vials (preserved remaining 24 mL) was pulled off to be used for ammonia assay by first adding 1.2 mL CH_2O mix to 16 mL, then distributing 1 mL to each of 6 vials for NH_3-free test. Then in each of the remaining six vials, a 100 µL of 1600-mM NH_4Cl was added to be tested with ammonia.

Avian microbes

Cecal contents were collected from ceca from another experiment (another student experiment, after they euthanized the birds for carcass study, the ceca were collected). All cecal contents were combined into one container while flushing with a CO_2 flow. Then equal volumes were distributed to 4 or more centrifuge tubes, then four mL ammonia-free Andil per each gram of cecal content were to each tube while maintaining a constant flow of CO_2, then caped. Mixed microbial population within the cecal contents within the tubes were balanced on a scale via appropriate additions of small volumes of ammonia-free Andil and centrifuged at 10,000 rpm at 4 ^oC for 20 minutes. When centrifuging was complete, the supernatant fluid was decanted into a waste beaker. After that, the cell pellets were washed two times, as were the cells of mixed rumen microbes. Upon completion of the washing steps, 23 mL of anaerobically prepared ammonia-free Andil was added to centrifuge tubes containing washed avian microbe cell pellets then capedand vortexed to resuspend the bacterial cell pellet. The volumes of the four tubes were combined to yield a 92 mL cell suspension. The 92 mL final tube contains a 1.5X concentrated avian microbe suspension. The final concentration factor was recorded. Then was caped and placed in ice until ready to distribute to incubation vials. 6.5 mL CH_2O mix will be added to 21 vials testing NH_4Cl-free Andil. 500 µL (0.5 ml) 1600 mM NH_4Cl mix was added, then 2 mL volumes were added to each remaining 21 vials to test NH_4-containing Andil.

The distribution of cell suspensions to incubation vials.

Vials numbers (1, 2, and 3) are the first control group (mixed avian microbes), vials numbers (4, 5, and 6) are the mixed avian+rumenial microbes control group, and vials number (7, 8, and 9) are the ruminal microbes control group. Then the vials were incubated for hours (0, 3, 6, 9, 12, and 24). After incubation time, an ammonia assay test was performed for ammonia content on samples collected from each vial. Tables (3 and 4) show ammonia-free incubation vials' distribution, and tables (5 and 6) show the distribution of ammonia added incubation vials.

Table 3 Distribution of cell suspensions to incubation vials with buffer B representing the three control groups (avian microbes alone, avian +ruminal microbes, and ruminal microbes alone). N=3.

CH2O added only			
Vial number	Suspension of avian microbes (µl)	Suspension of ruminal microbes (µl)	NH3-free Andil (buffer B) (µl)
1	2	0	1
2	2	0	1
3	2	0	1
4	2	1	0
5	2	1	0
6	2	1	0
7	0	1	2
8	0	1	2
9	0	1	2

Table 4 Distribution of nitrogen-assimilating bacteria to incubation vails with buffer B representing the four treatments groups (treatments 1, 2, 3, and 4)). N=3.

CH_2O added only

Vial number	Suspension of avian microbes (µl)	T1:Suspension of P. bryantii (µl)	T2: Suspension of B. fibrisolvens (µl)	T3:Suspension of R. albus (µl)	T4:Suspension of S. ruminantium(µl)
10	2	1	0	0	0
11	2	1	0	0	0
12	2	1	0	0	0
13	2	0	1	0	0
14	2	0	1	0	0
15	2	0	1	0	0
16	2	0	0	1	0
17	2	0	0	1	0
18	2	0	0	1	0
19	2	0	0	0	1
20	2	0	0	0	1
21	2	0	0	0	1

Table 5 Distribution of cell suspensions to incubation vials with buffer C representing the three control groups (avian microbes alone, avian +ruminal microbes, and ruminal microbes alone). N=3.

CH_2O added plus 12 mM NH_4Cl

Vial number	Suspension of avian microbes (µl)	Suspension of ruminal microbes (µl)	Buffer C (µl)
22	2	0	1
23	2	0	1
24	2	0	1
25	2	1	0
26	2	1	0
27	2	1	0
28	0	1	2
29	0	1	2
30	0	1	2

Table 6 Distribution of nitrogen-assimilating bacteria to incubation vails with buffer C representing the four treatments groups (treatments 1, 2, 3, and 4)). N=3.

CH_2O added plus 12 mM NH_4Cl

Vial number	Suspension of avian microbes (µl)	Suspension of P. bryantii (µl)	Suspension of B. fibrisolvens (µl)	Suspension of R. albus (µl)	Suspension of S. ruminantium (µl)
31	2	1	0	0	0
32	2	1	0	0	0
33	2	1	0	0	0
34	2	0	1	0	0
35	2	0	1	0	0
36	2	0	1	0	0
37	2	0	0	1	0
38	2	0	0	1	0
39	2	0	0	1	0
40	2	0	0	0	1
41	2	0	0	0	1
41	2	0	0	0	1

Ammonia Assay

Fluid samples were collected after 0, 3, 6, 9, 12, and 24 hours of incubation and assayed colorimetrically for the determination of ammonia content (Chaney & Marbach, 1962).

Amino acids profile evaluation.

Representative samples from all treatments collected at hour-0 and hour-9 were sent to Dr. Guoyao Wu's lab for analysis of amino acid profiles (Wu, 1993). Nine poultry essential dietary amino acids, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine, were selected to be evaluated in comparison between each of the treatment groups to the avian microbes control group. Each amino acid

percentage in the samples has equaled the division of that amino acid on the sum of all ten amino acids multiplied by 100.

$$AA\% = \frac{AA\ value}{\sum Ten\ AA\ values} \times 100$$

The conversions of ammonia to glutamate and glutamine occur primarily in the central ammonium assimilation network in ruminal bacteria. In addition, glutamate and glutamine act as nitrogen donors to facilitate cell nitrogen metabolism. The mutual transformation of glutamate and glutamine occurs due to the sequential actions of glutamate synthase (Dumonceaux et al., 2006) and glutamine oxidase (GOGAT) in a cycle known as the GS-GOGAT pathway. (figure 5) (Pengpeng & Tan, 2013). Thus, the glutamate value alone was used as an indicator of ammonia utilization in the bacterial cell.

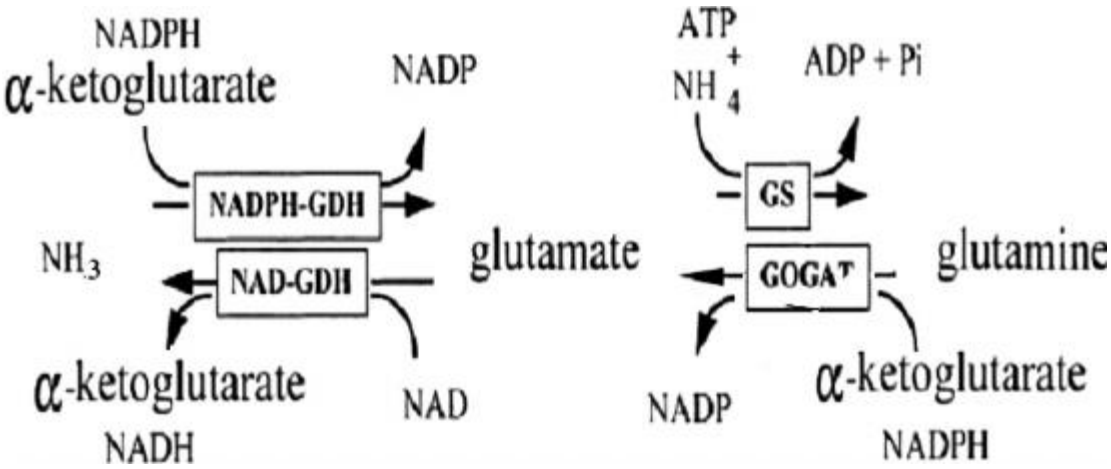

Figure 5 Interconversion of ammonia, glutamate, and glutamine in ammonia assimilation in the ammonia assimilation bacterial cell (Pengpeng & Tan, 2013).

Amino Acids analysis by HPLC

The amino acid profile analysis was conducted to understand the potential amino acids that our target bacteria can offer to the birds from their bacterial cells as a potential source of protein in the bird's gut due to nitrogen utilization by those bacteria. A total of nine essential dietary amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine) were selected from the entire amino acid profile of each bacterium to be studied. An HPLC mothed was used to analyze the amino acids from the bacterial culture, and according to the preceding description, the HPLC apparatus and the precolumn derivatization of amino acids with o-phthaldialdehyde were used (Wu, 1993). Supelco reversed-phase C18 columns (4.6 150 mm, internal diameter) were used to separate amino acids [with the exception of proline and cysteine]. A Supelco 40-mm reversed-phase C18 column was used to guard the 3-mm column (4.6 1 50 mm, i.d.; Supelco, Bellefonte, PA). 0.1 mol/L sodium acetate/0.5 percent tetrahydrofuran/9 percent methanol; pH 7.2) and solvent B (methanol) were used in the HPLC mobile phase, which flowed at a combined total flow rate of 1.1 milliliters per minute (mL/min). A gradient program with a total running time of 49 minutes (including the time required for column renewal) was created for the separation of amino acids with satisfactory results (0 min, 14 percent B; 15 min,14 percent B; 20 min, 30 percent B; 24 min, 35 percent B; 26 min, 47 percent B; 34 min, 50 percent B; 38 min, 70 percent B; 40 min, 100 percent B; 42 min, 100 percent B; 42.1 min, 14 percent B; 48.5 min, 14 percent B).

Hypothesis

The four selected rumen bacteria utilize the ammonia added to the media at the same rate and produce higher amino acids than the avian microbes alone.

Statistical analysis

All data were analyzed vails reading and amino acids percentage (AA%) for both ammonia assay reading and amino acids results as random variables, with SAS (SAS Inst. Inc., Cary, NC, USA; version 9.4) Satterthwaite approximation to determine the denominator df for the tests of fixed effects. Vails reading & AA % was assessed individually; therefore, Vaile reading & AA % were considered the experimental units. Quantitative data were analyzed with the MIXED procedure. Results are reported as least-square means and separated using Bonferroni-adjusted PDIFF to prevent type I errors. Significance was set at $P \leq 0.05$, and tendencies were determined if $P > 0.05$ and ≤ 0.10. Ammonia concentrations in the washed cell experiments were compared to controls at each sample time using a completely randomized analysis of variance with a Two-sided Dunnett's Multiple Comparisons test (Statistix9 Analytical Software, Tallahassee, FL).

Results

Ammonia assay results

The concentrations of ammonia in washed whole-cell incubations or mixed avian or rumen microbes incubated alone or together or, in the case of the avian microbes, with the addition of washed cells of 24 hours old cultures of the rumen bacteria *Prevotella bryantii* B14, *Butyrivibrio fibrisolvens* A38, *Ruminococcus albus* 7 or *Selenomonas ruminantium* 231-102-17 are shown in Figure 6. Chart A in (Figure 6) represents the results from cell suspensions incubated in ammonia-free anaerobic dilution solution, and chart B represents results from cell suspensions incubated in anaerobic dilution solution supplemented with ammonium chloride to achieve 12 mM of ammonia level. (Chart A) showed that the combined mixed avian+rumen and rumen microbes alone had significantly higher ammonia concentrations ($P > 0.05$) than the avian+*P.bryantii*, avian+*B.fibrisolvens*, avian+*R.albus*, and avian+*S.ruminantium* microbes in hours (3, 6, 9, and 12). However, the mixed avian+rumen is significantly higher ($P > 0.05$) than rumen microbes alone. Whereas the avian+*P.bryantii*, avian+*B.fibrisolvens*, avian+*R.ablus*, avian+*s.ruminantium*, and avian microbes alone are almost equal and all ≥ zero in hours (0, 3, 6, and 12), but avian microbes alone are significantly higher than all in hour 9. The higher concentrations of ammonia in all samples excluding (mixed avian+rumen and mixed rumen) were in hour 9, but mixed avian microbes were higher than others. A reduction in the ammonia concentration was noticed in hour 12 in all samples except avian+*S.ruminantium* microbes.

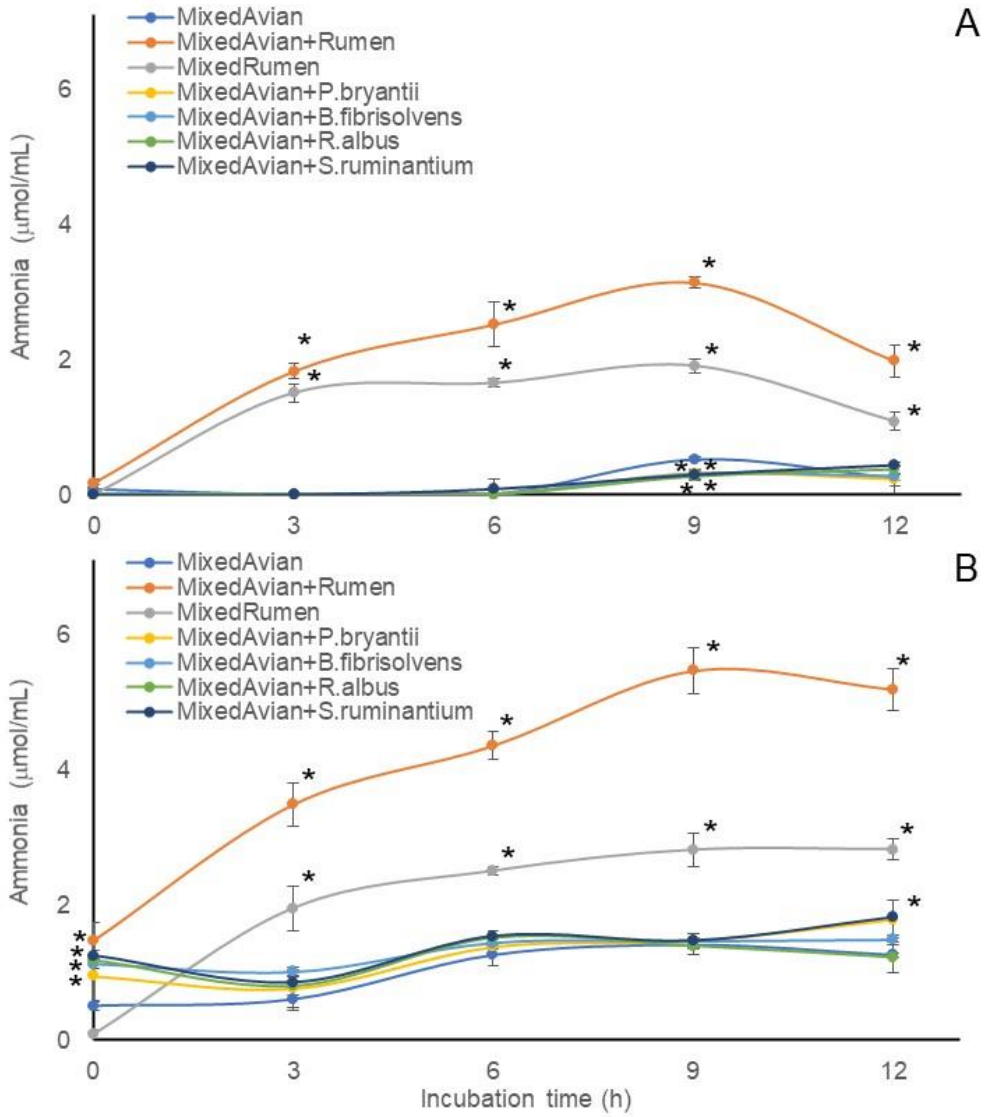

Figure 6 Ammonia concentrations within washed whole-cell incubations or mixed avian or rumen microbes incubated alone, together or in the case of the avian microbes, with the addition of washed cells of 24 h old cultures of the rumen bacteria *Prevotella bryantii* B14, *Butyrivibrio fibrisolvens* A38, *Ruminococcus albus* 7 or *Selenomonas ruminantium* 231-102-17. Chart A represents results from cell suspensions incubated in ammonia free anaerobic dilution solution lacking added ammonia, whereas Chart B represents results from cell suspensions incubated in anaerobic dilution solution supplemented with ammonium chloride to achieve 12 mM. Values are the mean + SD from $n = 3$ incubations. Asterisks reflect means differ ($P < 0.05$) from control values which are the suspension of mixed avian microbes alone when tested using a complete randomized analysis of variance.

(Figure 6, Chart B) showed that in hour zero, the ammonia concentration of mixed rumen microbes alone was ≥ zero, and the mixed avian microbes alone have significantly lower

ammonia concentration than other samples ($P > 0.05$). Whereas in hours (3, 6, 9, and12), the ammonia concentration of avian +rumen microbes was significantly higher than the mixed rumen microbes alone, and they are both significantly higher than other samples ($P > 0.05$). In addition, all other samples' ammonia concentrations were not close to each other in hours (3, 6, 9, and 12) except the avian+*S.ruminantium* and avian+*P.bryantii* were higher in hour 12.

Amino Acids

The nine essential dietary amino acids for poultry are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine. They were chosen from a pool of 22 amino acids in the amino acids profile analysis data to be evaluated. It was divided into two groups of seven treatments each, with the first seven treatments group receiving ammonia in the incubation solution NA (NH_3+Andil) and the second seven treatments group not receiving ammonia in the incubation solution NFA (NH_3 Free Andil). The avian microbes, rumen microbes, and avian+runen microbes are the control groups. (Figure 7) showed that methionine in all treatments in the NA group is significantly higher ($P \leq 0.05$) than the control treatment of avian microbes, and the NFA group is slightly higher ($P \leq 0.05$) or close to the control treatment of avian microbes. However, all treatments are not different from each other in both NA and NFA groups. (Figure 8) showed that lysine in the avian+*S.ruminantium* is slightly higher than the other three treatments in the NA group and significantly ($P \leq 0.05$) higher than the control treatment of avian microbes. Also, all four treatments are slightly higher than the control

treatment of avian microbes in the NA group. The lysine is either slightly higher or close to the avian microbes control group in the NFA treatments group.

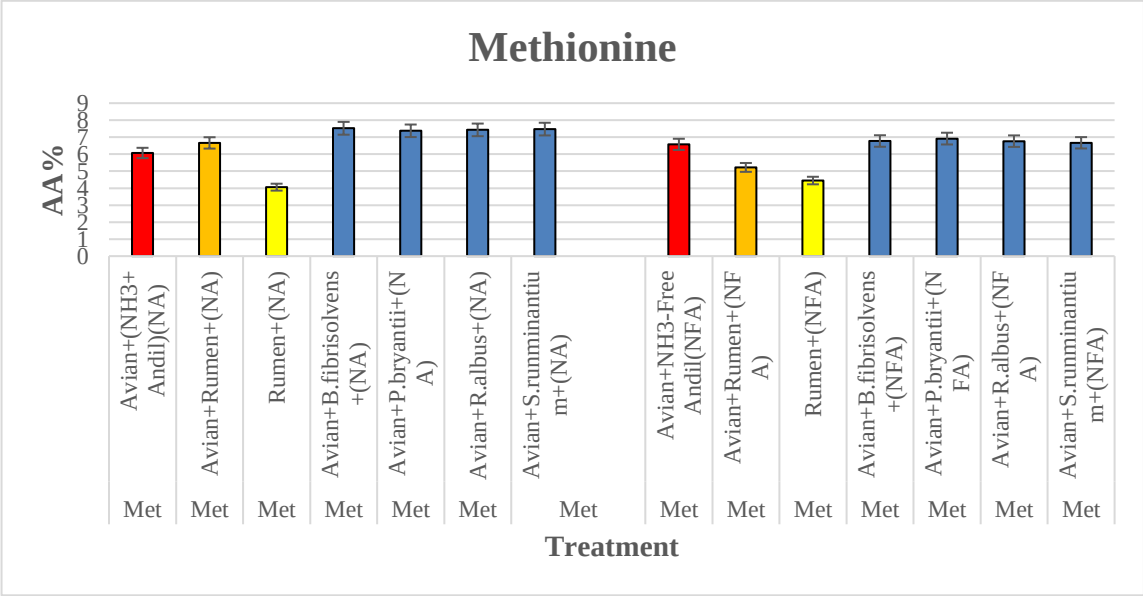

Figure 7 Methionine % in the novel four nitrogen reduction probiotics incubations' samples in both incubated with or without ammonia groups compared to control groups avian microbes alone, avian microbes+ruminal microbes, and ruminal microbes alone in red, orange, and yellow columns, respectively within washed whole-cell incubations. The first seven columns in the chart represent results from cell suspensions incubated in ammonia added anaerobic dilution solution NA, whereas the second seven columns in the chart represent results from cell suspensions incubated in ammonia-free anaerobic dilution solution NFA. Values are the means+SE from N=2 incubations. Asterisks reflect means differ ($P \leq 0.05$) from control values.

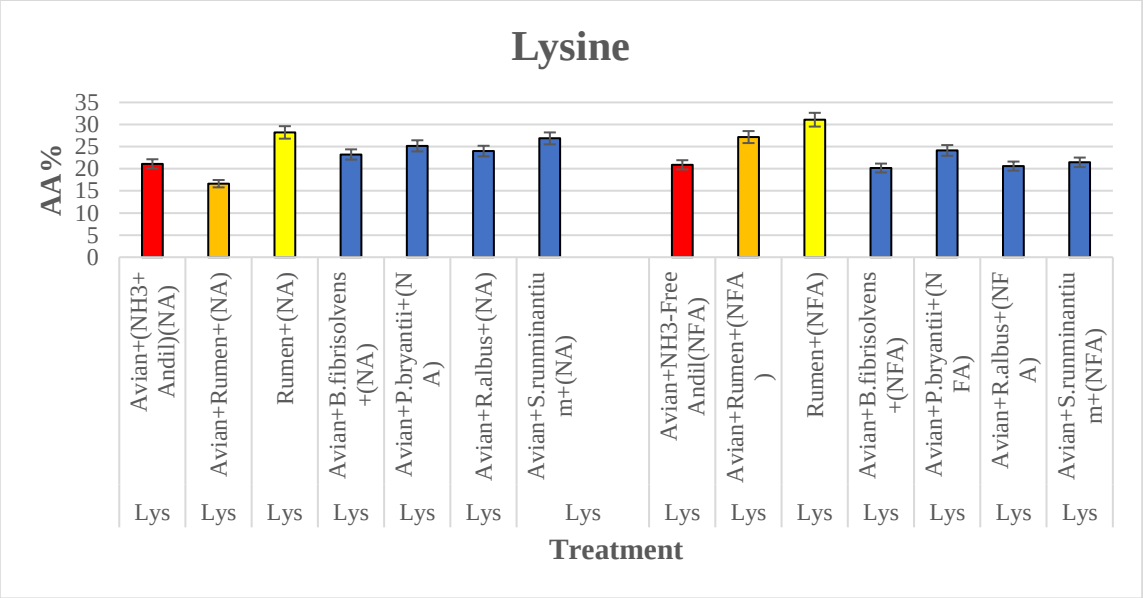

Figure 8 Lysine % in the novel four nitrogen reduction probiotics incubations' samples in both incubated with or without ammonia groups compared to control groups avian microbes alone, avian microbes+ruminal microbes, and ruminal microbes alone in red, orange, and yellow columns, respectively within washed whole-cell incubations. The first seven columns in the chart represent results from cell suspensions incubated in ammonia added anaerobic dilution solution NA, whereas the second seven columns in the chart represent results from cell suspensions incubated in ammonia-free anaerobic dilution solution NFA. Values are the means+SE from N=2 incubations. Asterisks reflect means differ (P≤0.05) from control values.

According to (Figure 9), the threonine concentration in all treatments is significantly lower ($P \leq 0.05$) than the control treatment of avian microbes in the NA group. In the NFA treatments group, however, the avian+*pbyarntii* treatment is significantly lower ($P \leq 0.05$) than the other three treatments and the control treatment of avian microbes. (Figure 10) showed that isoleucine is not significantly different from the control treatment of avian microbes in all NA treatment groups. In the NFA group, isoleucine is equal to the control treatment of avian microbes.

According to Alvarez et al. (2018), only a few bacterial strains can produce D-amino acids, including arginine, histidine. Also, arginine can be a processor to another amino acid. However, in this test, arginine was significantly higher ($P \leq 0.05$) than the control treatment of avian microbes in both NA, and NFA groups (Appendix A).

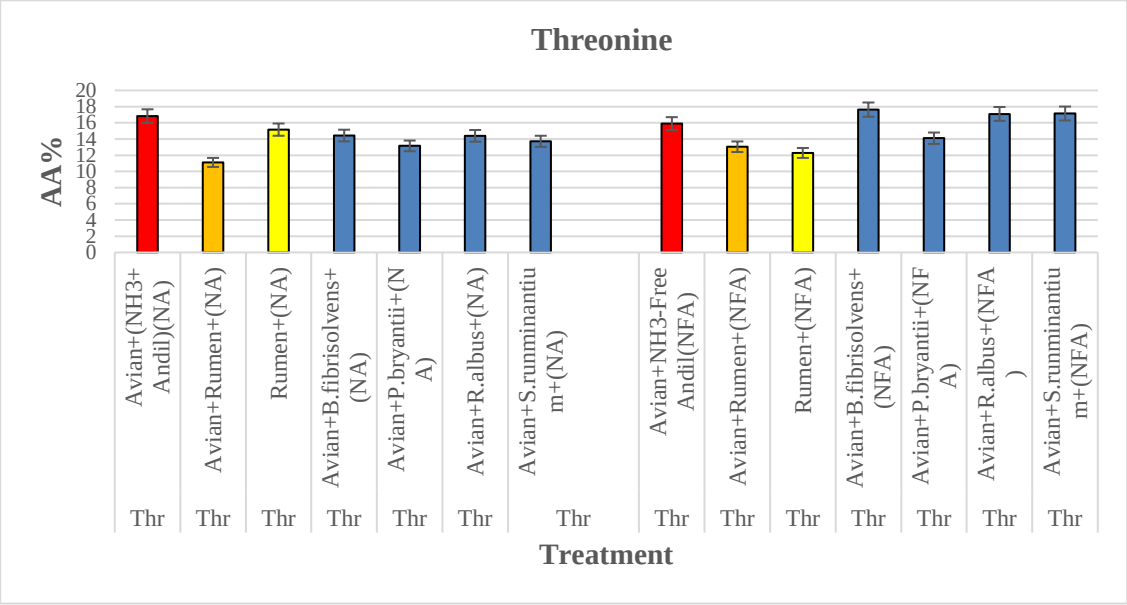

Figure 9 Threonine % in the novel four nitrogen reduction probiotics incubations' samples in both incubated with or without ammonia groups compared to control groups avian microbes alone, avian microbes+ruminal microbes, and ruminal microbes alone in red, orange, and yellow columns, respectively within washed whole-cell incubations. The first seven columns in the chart represent results from cell suspensions incubated in ammonia added anaerobic dilution solution NA, whereas the second seven columns in the chart represent results from cell suspensions incubated in ammonia-free anaerobic dilution solution NFA. Values are the means+SE from N=2 incubations. Asterisks reflect means differ ($P \leq 0.05$) from control values.

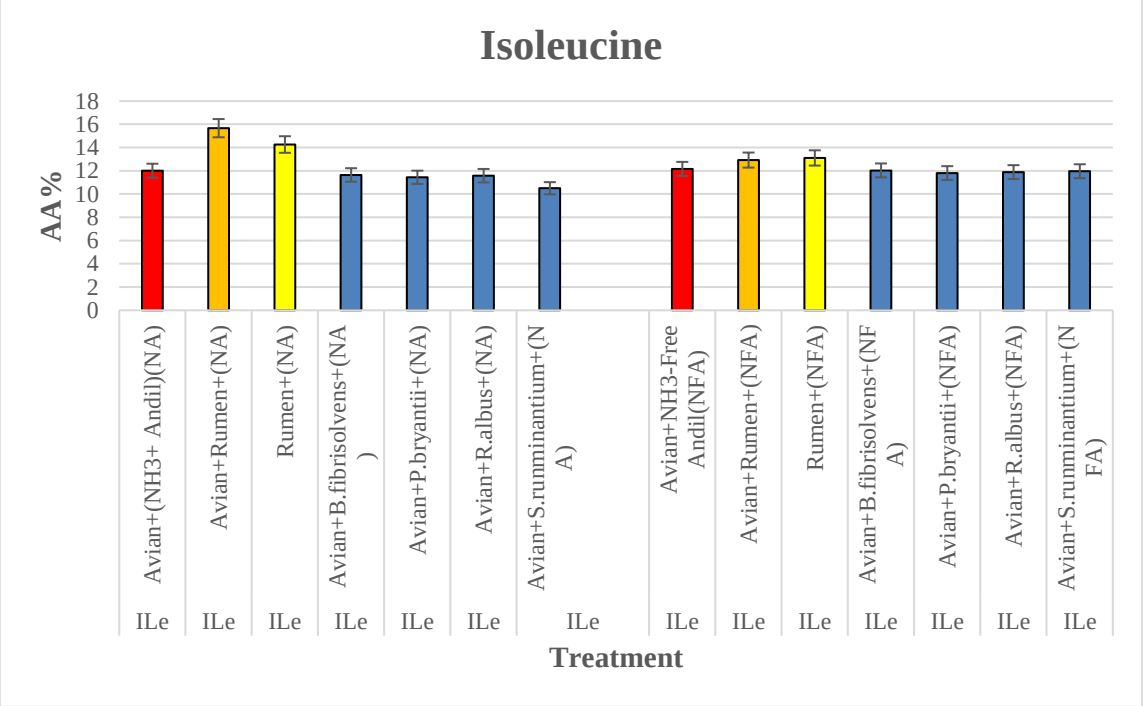

Figure 10 Isoleucine % in the novel four nitrogen reduction probiotics incubations' samples in both incubated with or without ammonia groups compared to control groups avian microbes alone, avian microbes+ruminal microbes, and ruminal microbes alone in red, orange, and yellow columns, respectively within washed whole-cell incubations. The first seven columns in the chart represent results from cell suspensions incubated in ammonia added anaerobic dilution solution NA, whereas the second seven columns in the chart represent results from cell suspensions incubated in ammonia-free anaerobic dilution solution NFA. Values are the means+SE from N=2 incubations. Asterisks reflect means differ (P≤0.05) from control values.

Leucine among all treatments is equal or close to the control treatment of avian microbes in both NA and NFA groups (Appendix A). Furthermore, phenylalanine among all treatments is equal or close to the control treatment of avian microbes in both NA, and NFA groups (Appendix A). On the other hand, valine in all treatments is significantly lower ($P \leq 0.05$) than the control treatment of avian microbes in the NA group and slightly lower than the control treatment of avian microbes in the NFA gorup (Appendix A).

Glutamate & Glutamine

The samples in treatments (1-7) incubated with ammonia added to the incubation solution showed in (Figure 11) that the avian+*B.fibrisolvens* has a significantly higher glutamate level than the other three treatments. Based on (Figure 11) the avian+*S.ruminantium* is significantly higher than avian+*P.bryantii* and avian+*R.albus*, and the last two are not different from each other. However, the treatments (8-14) showed in (Figure 11) that avian+*B.fibrisolvens* has a significantly higher glutamate level than all treatments, and the other three treatments are not different from each other.

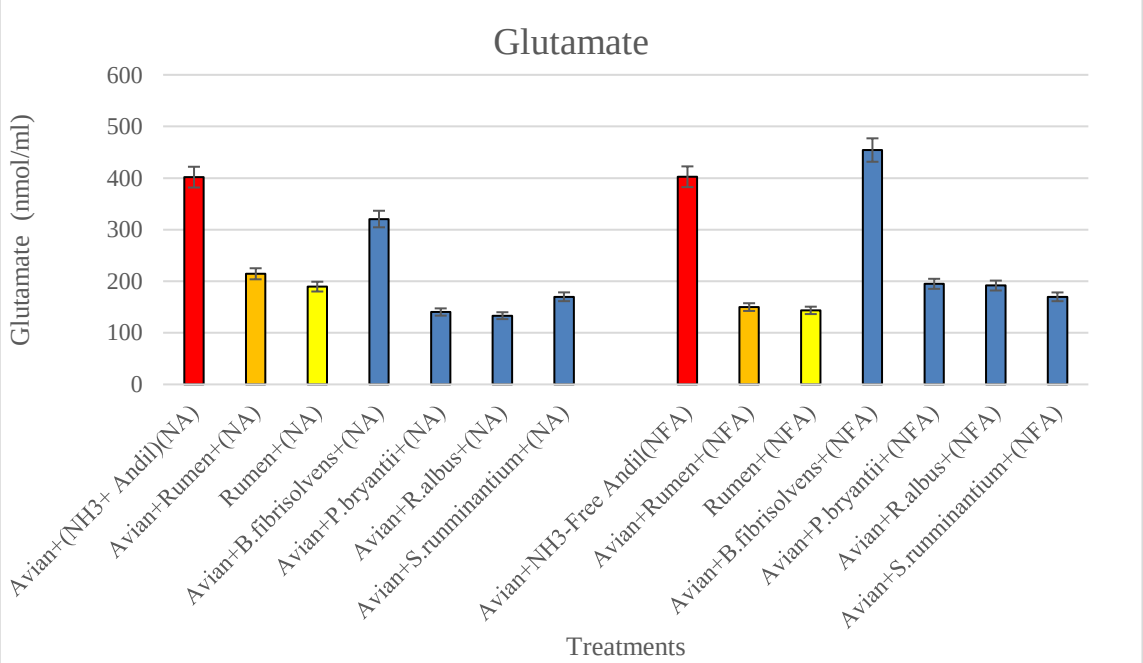

Figure 11 Glutamate concentration in the novel four nitrogen reduction probiotics incubations' samples in both incubated with or without ammonia groups compared to control groups avian microbes alone, avian microbes+ruminal microbes, and ruminal microbes alone in red, orange, and yellow columns, respectively within washed whole-cell incubations. The first seven columns in the chart represent results from cell suspensions incubated in ammonia added anaerobic dilution solution NA, whereas the second seven columns in the chart represent results from cell suspensions incubated in ammonia-free anaerobic dilution solution NFA. Values are the means+SE from N=2 incubations. Asterisks reflect means differ ($P \leq 0.05$) from control values.

Furthermore, the samples in treatments (1-7) incubated with ammonia added to the incubation solution showed in (Figure 12) that the avian+*B.fibrisolvens* has a significantly higher glutamine level than the other three treatments. Based on (Figure 12) the avian+*B.bryntii* is significantly lower than avian+*S.ruminantium* and avian+*R.albus*, and the last two are not different from each other. However, the treatments (8-14) showed in (Figure 12) that avian+*B.fibrisolvens* has a significantly higher glutamine level than all treatments, and the avian+*B.bryntii* is significantly lower than the other two treatments a last two are not different from each other, whereas the avian+*R.albus* is slightly higher than avian+*S.ruminantium*.

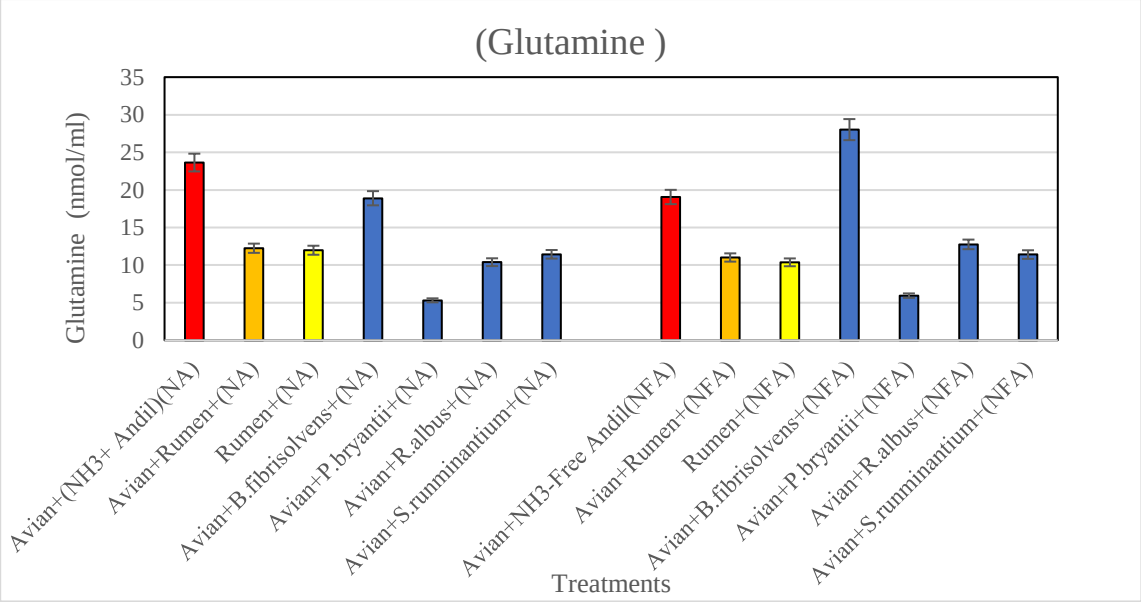

Figure 12 Glutamine concentration in the novel four nitrogen reduction probiotics incubations' samples in both incubated with or without ammonia groups compared to control groups avian microbes alone, avian microbes+ruminal microbes, and ruminal microbes alone in red, orange, and yellow columns, respectively within washed whole-cell incubations. The first seven columns in the chart represent results from cell suspensions incubated in ammonia added anaerobic dilution solution NA, whereas the second seven columns in the chart represent results from cell suspensions incubated in ammonia-free anaerobic dilution solution NFA. Values are the means+SE from N=2 incubations. Asterisks reflect means differ ($P \leq 0.05$) from control values.

Discussion

Ammonia assay

As a first step in evaluating the four selected ruminal bacteria, an ammonia assay test was conducted to investigate how those bacteria would behave in the incubation media with & without the presence of the ammonia. The results showed in (Figure 6- A) ammonia accumulation in the samples incubated in an ammonia-free incubation solution were significantly higher with the mixed avian+rumen microbes than mixed rumen microbes alone, and they both had significantly higher ammonia accumulation than all other treatments in hours (3, 6, 9, and 12;) However, the mixed avian and the mixed rumen microbial incubations both showed a decline in the ammonia accumulation between hours (9, and 12). All others, including our target four ruminal bacteria treatments, showed little ammonia accumulation in hours (0, 3, and 6). However, in hour 9, the mixed avian microbes alone had a significantly higher ammonia accumulation than our four target bacteria, giving initial evidence that those bacteria had some ammonia utilization activities. For sample incubated collected from incubation with the added ammonia (Figure 6-B), the mixed avian+rumen microbes have significantly higher ammonia accumulation than mixed rumen microbes alone, and they are both significantly higher in ammonia accumulation than all other treatments in hours (3, 6, 9, and 12;) However, only the incubation of mixed avian+ruman, microbes had a decline in ammonia accumulation between hours (9-12).

Furthermore, in hour zero, the mixed avian microbes alone show significantly lower ammonia content than all other treatments. However, in hours (3, 6, and 9), the four

selected bacteria plus the mixed avian alone were not different from each other but were lower than control groups, the avian-rumen and the rumen alone. It gives evidence of some nitrogen utilization compared to mixed avian+rumen & rumen alone microbes. At hour (12), the avian+*S.ruminantium* and avian+*P.bryantii* showed a significantly higher ammonia level compared to avian microbes alone, avian+*B.fibrisolvens*, and avian+*R.albus*. Thus, the avian+*B.fibrisolvens* and avian+*R.albus* are slightly lower than the avian microbes alone and significantly lower in ammonia production than all other treatments, and it is another evidence that those two treatments are potential nitrogen probiotics. However, further research is needed to prove this hypothesis.

Amino acids

With the amino acid study, we hope to determine whether any of those four novel probiotics will offer any of those nine essential dietary amino acids required by the bird compared to avian bacteria alone. The findings revealed that all treatments are considerably or somewhat higher in just methionine, lysine, and arginine than the control treatment of avian microbes alone, indicating that those bacteria can be a good prospective source of those amino acids in the future. The concentrations of histidine, isoleucine, leucine, phenylalanine, threonine, and valine are either equal to or lower than the concentrations observed with the control treatment of avian microbes only. However, at this point in the test, it is difficult to determine how much of the bacterial protein produced by these bacteria will be consumed by the bird, which means it is difficult to assume that this bacterial protein will meet the bird's requirement of those essential dietary amino acids. On the other hand, given the restricted supply of NH3 and energy available in the

test vails, the growth rate of these mcriobeal communities, and the metabolic circulation of those amino acids, it is hard to accept those results. Those results, on the other hand, provide a solid starting point for further experiments.

Glutamate & Glutamine

The results showed in the incubation without added ammonia the avian+*B.fibrisolvens* incubation had the highest glutamate level among the other microbial cell incubation. Also, the avian+*S.ruminantium* incubation was slightly higher than avian+*P.bryantii* and avian+*R.albus* incubations and these did not differ from each other, indicating the ammonia assimilation activities in incubation with avian+*B.fibrisolvens* being ranked number one and avian+*S.ruminantium* number two. However, in the incubation without added ammonia group, the avian+*B.fibrisolvens* is also the highest in glutamate level, and the avian+*P.bryantii* and avian+*R.albus* are the second-highest, followed by avian+*S.ruminantium* in the third position. For the glutamine, the results showed that avian+*B.fibrisolvens* incubation in both groups again incubation with ammonia added and incubation without ammonia added had the highest glutamine level and the avian+*P.bryantii* incubation had the lowest level of glutamine, whereas the avian+*R.albus* and avian+*S.ruminantium* did not differ, and they had the second-highest level of glutamine. All microbial cell incubations had some ammonia utilization activities. However, the avian+*B.fibrisolvens* exhibited the highest activity, followed by avian+*S.ruminantium* and avian+*R.albus*, indicating ammonia assimilation occurred in those microbial cell incubations. Based on their level of ammonia assimilation, the

avian+*B.fibrisolvens*, avian+*S.ruminantium*, and avian+*R.albus* incubations may be potential nitrogen reduction probiotics.

Based on the results of this experiment, the hypothesis of this experiment was rejected because the four nitrogen assimilation bacteria did not utilize ammonia at the same rate, and not all amino acids in their amino acid profile were higher than NRC.

CHAPTER III

THE EFFECT OF FOUR NOVEL NITROGEN REDUCTION PROBIOTICS ON NITROGEN UTILIZATION AND BROILER PERFORMANCE

Introduction

The ruminal microorganisms are an excellent example of how the body adapts to different feeding sources on its own initiative. The ruminants, in contrast to monogastric creatures such as chickens, create an ideal environment for a group of microorganisms in the rumen compartment, which then uses their power of cellulose digestion and nitrogen conversion to allow them to use the forge more efficiently than their monogastric counterparts, such as cattle (Davis, 1973). While the rumen microorganisms' nitrogen utilization is estimated to be greater than 67 % of non-protein nitrogen (Firkins et al., 2007), poultry nitrogen emission is estimated to be greater than 27 % of total emission (Battye et al., 1994). Based on that, the idea of the experiment was shaped by feeding some nitrogen assimilation bacteria to broiler chickens in order to reduce the nitrogen emissions associated with poultry production. This study aims to investigate the effect of the bacteria strains *Selenomonas ruminantium, Butyrivibrio fibrisolvens A38, Ruminococcus albus* strain *7,* and *Bacteroides ruminocola subsp. brevis B14* (new name *Prevotella bryantii B14*), and the whole microflora of the rumen compartment of a cow fed to a broiler cobb 500 on the nitrogen utilization and broiler performance parameters (FCR and BWG) during the stater feeding phase. Moreover, to understand the function of those bacteria Body N/Feed N ratio and the FM N/Feed N ratio will be investigated.

Materials and Methods

Experiment Design

The animal use for this experiment was approved by IACUC Experimental Animal protocol No. (2020-008) on Friday, August 14, 2020. A total of 168 birds were randomly assigned to seven treatment groups (Table: 7). Six replicates of each treatment were placed in battery cages, with four birds per cage in each treatment. The whole microflora of the rumen compartment of a cow (WMR), as well as four bacteria strains with the highest nitrogen utilization ability (Schaefer et al., 1980), were used in this experiment as five different treatment groups, as shown in the (Table: 8). The bacteria were gavaged to the birds on days 1, 2, 4, 6, and 14. The bacteria groups were resuspended into modified Shaeffer's liquid medium solution (pH 7.4), which contained the bacteria doses shown in (Table: 9). The birds in negative control and positive control groups received the same amount of a blank modified Shaeffer's medium on the same days. Four samples of feed and litter, as well as four birds from each pen, were collected. Birds were euthanized on day one, week1 one, week2, and week 3 for the nitrogen utilization calculation. The amount of feed consumed and body weight gain were calculated on day one, week one, week two, and week three. This experiment was repeated with only one difference that the remaining extra bird samples used for cecal collection for the third experiment in this project, which was used in the genome study in the fourth experiment. In this experiment, two diets were used, negative control NC (22.71% protein) and positive control PC (23.28% protein) (Pilgrim's, 2017) (Appendix B, Tables 1 and 2).

Table 7 Birds distribution on the seven treatments.

Treatment	Bacteria Strains	Quantity of the Birds
TX1	Negative control diet (NC) +Bacteria Strain BS1 (*S. ruminantium*)	24 (one day old) broiler Chicks
TX2	(NC)+BS2 (*B. fibrisolvens* A38)	24 (one day old) broiler Chicks
TX3	(NC.)+BS3 (*R. albus* strain 7)	24 (one day old) broiler Chicks
TX4	(NC.)+BS4 (*P. bryantii* B14)	24 (one day old) broiler Chicks
TX5	(NC.)+WMR	24 (one day old) broiler Chicks
TX6	(NC) Negative Control (low protein diet)	24 (one day old) broiler Chicks
TX7	(PC.) Positive control diet (Broiler Standard Diet)	24 (one day old) broiler

Table 8 The four novel nitrogen reduction probiotics bacterial Strains (Schaefer et al., 1980).

Treatment	Bacteria Strains
BS1	*Selenomonas ruminantium*
BS2	*Butyrivibrio fibrisolvens A38*
BS3	*Ruminococcus albus 7*
BS4	*Bactetoides ruminocola subsp. brevis B14* (now name *Prevotella bryantii B14*)
WMR	whole microflora of the rumen compartment

Table 9 Gavage days, doses, and concentrations.

Gavage Day	Dose	Bacteria Concentration
1	100µL	10^8 cfu/100 µL
2	200 µL	10^8 cfu/200 µL
4	400 µL	10^8 cfu/400 µL
6	600 µL	10^8 cfu/600 µL
14	1.00mL	10^8 cfu/1mL

Feed, Birds, and Fecal matter samples analysis

All samples were dried at 100ºC for 24 hours in a convection oven. All subsequent laboratory analyses were performed on a dry matter basis, and all litter samples were frozen to reduce the volatilization of nitrogen. Feed and litter samples were finely ground after drying using a flour mill. Bird's carcasses were homogenized before drying using a meat grinder, and then they were further homogenized in a food processor. After drying, all carcass samples were ground using a flour mill into a very fine powder. All feed, litter, and birds' carcass samples were analyzed for total N content using an Elementar-N/Protein Analyzer (Elementar, 2021). The nitrogen emission equals the difference between nitrogen input in the feed and output in the bird's carcass + in fecal matter. The remaining extra bird samples were euthanized using CO_2 and used for nitrogen analysis. The nitrogen input and output were calculated using the following equations: the calculation is per pen as an experimental unit.

$$\text{N input from feed} = \text{feed consumed} \times \left(\frac{\text{Feed N\%}}{100}\right)$$

$$\text{Meat wet weight N\%} = \left(\frac{\text{meat DM Ng}}{\text{meat samples wet wt.}}\right) \times 100$$

$$\text{N in body weight gain} = \text{body weight gain} \times \left(\frac{\text{meat wet weight N\%}}{100}\right)$$

$$\text{Fecal Samples wet N\%} = \left(\frac{\text{fecal samples DM Ng}}{\text{fecal samples wet wt}}\right) \times 100$$

$$\text{N in fecal material} = \text{fecal material total wet} \times \left(\frac{\text{fecal samples wet N\%}}{100}\right)$$

Hypothesis

The four selected rumen bacteria were oral gavaged to birds in four different treatments that will utilize ammonia at the same rate and improve the BWG, FCR, and nitrogen emission at the same rate, and all four treatments are higher in all tests than negative and positive controls.

Statistical analysis.

All data were analyzed using bird or the whole pen as a random variable, with SAS (SAS Inst. Inc., Cary, NC, USA; version 9.4) and Satterthwaite approximation to determine the denominator df for the tests of fixed effects. Birds were assessed individually or pen assessed individually. Therefore, the bird or pen was considered the experimental unit between the different parameters; for example, only in the BWG, the bird is the experimental unit and the FCR, and nitrogen emission tests the pen is the experimental unit. Quantitative data were analyzed with the MIXED procedure. The model statement used for bird performance analysis contained the effect of the four novel probiotics: BWG, FCR, and nitrogen emission as a separate test. Results are reported as least-square means and separated using Bonferroni-adjusted PDIFF to prevent type I errors. Significance was set at $P \leq 0.05$, and tendencies were determined if $P > 0.05$ and ≤ 0.10.

Results

Bodyweight gain (BWG)

The data analysis results in (Figure 13) showed that the BWG among all treatments in week one was not different ($P > 0.05$), and the same thing in week two, all treatments are close to each other with no significant differences. In week three, treatments (1, 2, 4, 5, and 6) are close with no significant difference. However, treatment three (R. albus 7) has the significantly highest BWG among all other treatments ($P \leq 0.05$), and treatment seven (PC) has the significantly lowest BWG compared to treatments (3, 4, and 5).

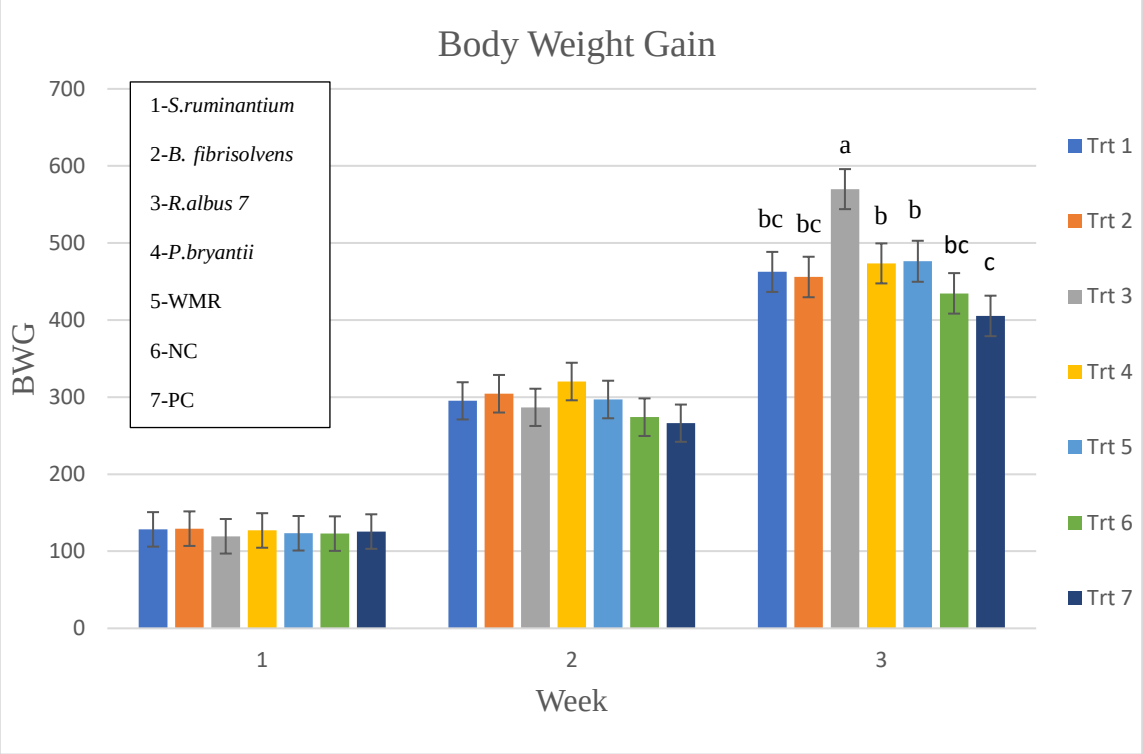

Figure 13 The body weight gain of the four nitrogen reduction bacterial strains and the PC and NC in weeks (1, 2, and 3) showed that treatment three in week three was significantly higher (a) than other treatment and controls(b, bc, and c). Values are the mean+SE, N=48, and means differ at ($P \leq 0.05$) from PC and NC.

Feed Conversion Ratio (FCR)

The data analysis results in (Figure 14) showed that the FCR among all treatments in weeks (1, 2, and 3) was not significantly different ($P > 0.05$). However, in week one treatment, four fellowed by treatment three are slightly lower than other treatments. In week two, all treatments are close to each other. Moreover, in week three, treatment three (*R. albus 7*) has the lowest FCR slightly. Because of the very low replicates, this test showed no significant difference, which were only six replicates a treatment.

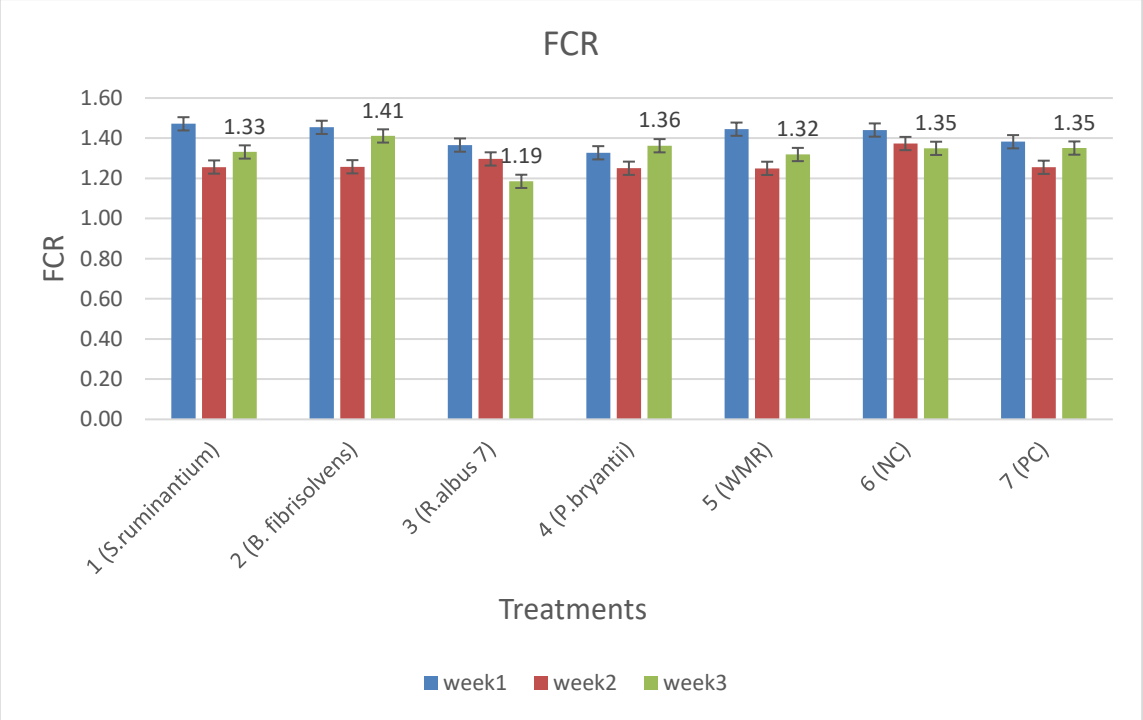

Figure 14 Feed Conversion Ratio (FCR) of the four nitrogen reduction bacterial strains and the PC and NC in weeks (1, 2, and 3) showed no statistical differences **(***P* > 0.05). Values are the mean+SE, N=12, and means differ at (P ≤ 0.05) from PC and NC.

Nitrogen Utilization

The data of the nitrogen emission or the nitrogen released to the environment as a gas form of nitrogen were calculated by substracted the nitrogen output in the bird's body and the fecal matter from the nitrogen input in the feed. The results in (Figure 15) showed no significant differences among all treatments ($P > 0.05$), and it could be because of the low replicates, which are only one replicate in weeks one and two, and five in week three and that applies to all of the following tests. In week one, all treatments are relatively close to each other. However, treatment two is slightly lowest than other treatments. In week two, treatment five, followed by treatments one, and seven, has relatively slightly the lowest nitrogen emission. Moreover, in week three, treatment one followed by treatment three slightly has the lowest nitrogen emission, and treatment two slightly has the highest nitrogen emission among other treatments.

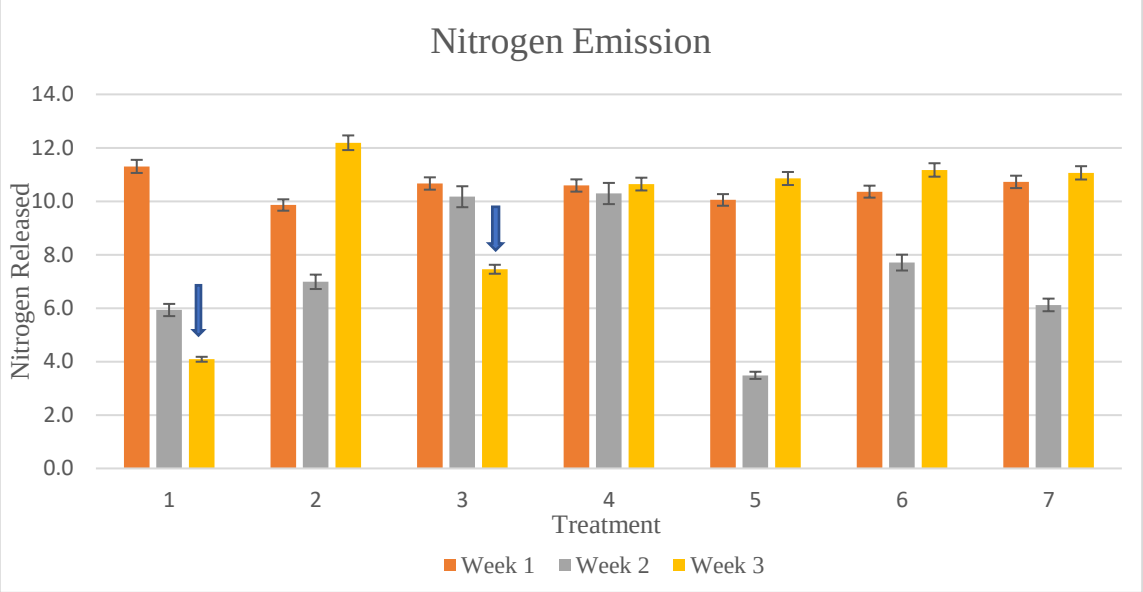

Figure 15 Nitrogen emission of the four nitrogen reduction bacterial strains and the PC and NC in weeks (1, 2, and 3) showed no statistical differences **(***P* > 0.05). Values are the mean+SE, N=2 week 1 and 2, and N=7 week 3, and means differ at (P ≤ 0.05) from PC and NC.

The results in (Figure 16) showed no significant differences among all treatments in the ratio of carcass nitrogen to feed nitrogen ($P > 0.05$). However, in week one, all treatments are close to each other, except treatment five is slightly lower than other treatments. In week two, only treatment one is slightly higher than other treatments. Moreover, in week three, treatments one and three are slightly higher than other treatments.

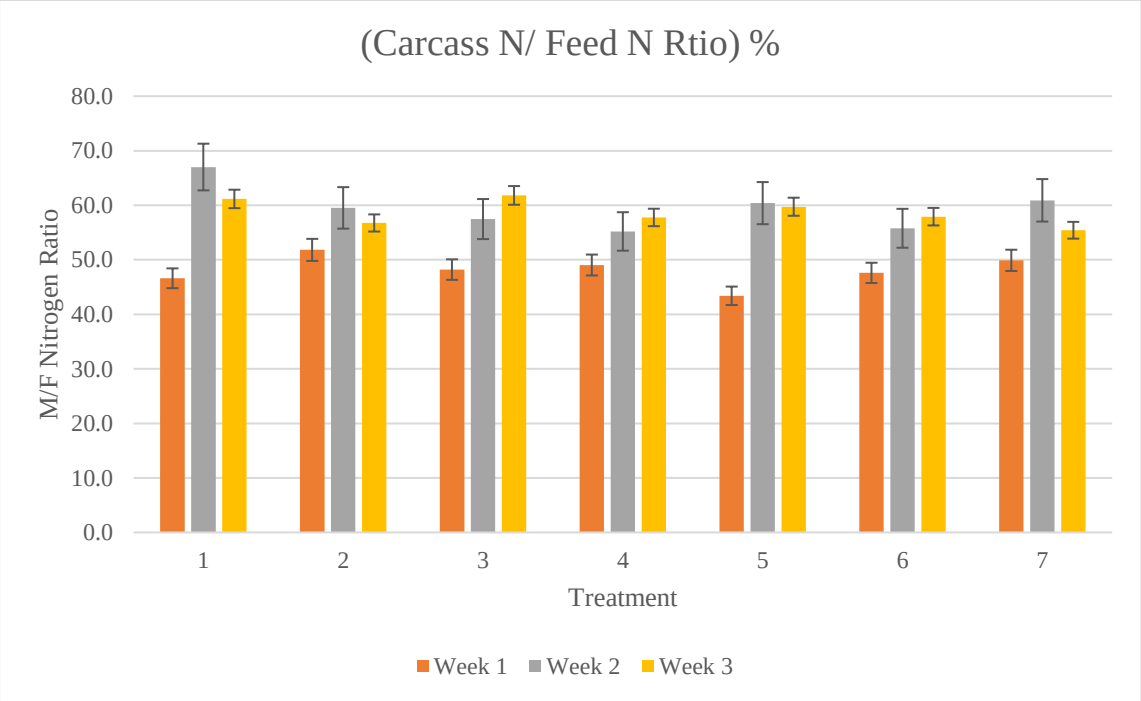

Figure 16 The carcass nitrogen to feed nitrogen ratio of the four nitrogen reduction bacterial strains and the PC and NC in weeks (1, 2, and 3) showed no statistical differences **(***P* > 0.05). Values are the mean+SE, N=2 week 1 and 2, and N=7 week 3, and means differ at (P ≤ 0.05) from PC and NC.

(Figure 17) showed the data analysis results for the fecal matter nitrogen to feed nitrogen ratio. In week one, treatment seven followed by treatment five are significantly higher (*P* ≤ 0.05) in fecal matter nitrogen to feed nitrogen ratio, and other treatments not close to

each other. However, in week two, only treatment five was significantly higher ($P \leq 0.05$) than other treatments, and other treatments are close to each other. In week three, there were no significant differences ($P > 0.05$), but treatment one followed by treatment seven are higher than other treatments.

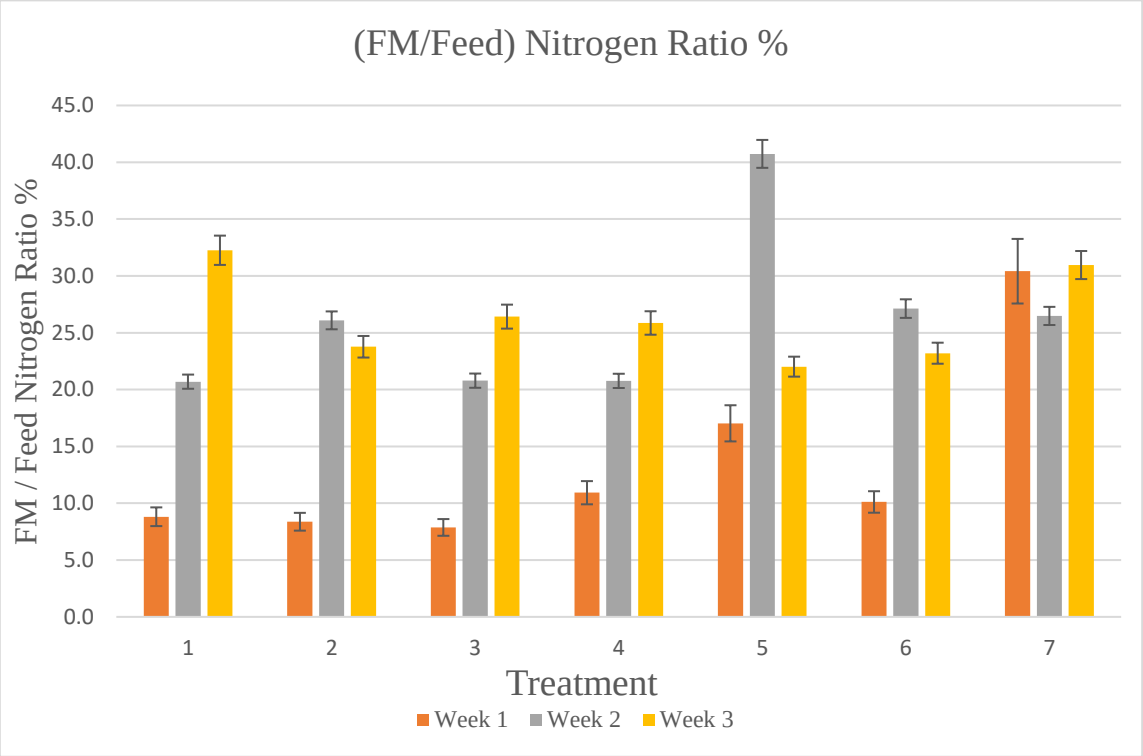

Figure 17 The fecal matter nitrogen to the feed nitrogen ratio of the four nitrogen reduction bacterial strains and the PC and NC in weeks (1, 2, and 3) showed no statistical differences ($P > 0.05$). Values are the mean+SE, N=2 week 1and 2, and N=7 week 3, and means differ at ($P \leq 0.05$) from PC and NC.

Discussion

The data showed that the BWG in week one was almost equal among all treatments, and in week two, treatments were slightly different, and they were all slightly higher than the positive control treatment (treatment 7). In week three, the treatments started to differ from each other. Wherefore, treatment three was significantly higher than other treatments, and all treatments are significantly higher than treatment seven, and that can be evidence of the effect of those treatments, which is started to be visible in week three. That means the probiotics that were given to the birds needed about two weeks to adapt to the new environment and replicated to enough number to function. Since treatment three was the highest and treatments (1, 2, 4, 5, and 6) are slightly different, treatment three can be our potential nitrogen reduction probiotic based on only BWG.

The feed conversion ratio (FCR) data showed no significant differences among all treatments because of the very low replicates, which were only one replicate a treatment in weeks one and two, and six replicates a treatment in week three. Because of that reason, the minor difference may be taken into account to support the hypothesis of the experiment. As mentioned before, only week three will best represent the performance data, which is again treatment three has the lowest FCR among all treatments, supporting treatment three to be a potential nitrogen reduction probiotic (NRP).

To further understand the function of those probiotics, a nitrogen utilization experiment was conducted using three different parameters, including nitrogen emission, carcass nitrogen/ feed nitrogen ratio, and fecal matter nitrogen/ feed nitrogen ratio, and results showed that there were no significant differences among all treatments in all three

parameters. As mentioned before, it is highly possible because of the very low replicates, which were only one replicate a treatment in weeks one and two, and six replicates a treatment in week three, and because of that, the minor difference may be taken into account. Since in the performance parameters, treatment three got a significantly higher BWG and lower FCR, it will be closely examined from this point forward. The nitrogen emission result showed that treatment one followed by treatment three had the lowest nitrogen emission. However, in fecal matter nitrogen/ feed nitrogen ratio, treatment one had the highest nitrogen ratio, which will exclude it from being a potential nitrogen probiotic. Treatment three had a lower nitrogen ratio than treatment one and PC. For the carcass nitrogen/feed nitrogen ratio, treatments one and three are equal and slightly higher than others, especially the PC and NC. Based on the overall result of this experiment, treatment three (*R. albus 7*) has the highest BWG, lowest FCR, the second lower nitrogen emission, slightly higher carcass N/Feed N ratio, and the second-lowest FM-N/FeedN ratio, which makes it a potential nitrogen reduction probiotic candidate.

The hypothesis of this experiment was rejected because the four selected rumen bacteria gavaged to the birds did not utilize ammonia at the same rate and did not improve the BWG, FCR, and nitrogen emission at the same rate.

CHAPTER IV

THE EFFECT OF *RUMINOCOCCUS ALBUS STRAIN* 7 ON BROILER'S GUT MICROFLORA

Introduction

In the last two experiments, four ruminal bacteria have been selected and tested through a series of tests to examine their nitrogen utilization process in-vivo and in-vitro. Based on the results of those two experiments, the *Ruminococcus albus 7* has been selected as a potential nitrogen probiotic. *Ruminococcus albus* 7 is a strict type of bacteria, and it is a highly cellulolytic bacterium and a member of the phylum Firmicutes (Hungate, 1957). Like other cellulolytic Firmicutes, it attaches itself to cellulose and deconstructs it using cellulosomes, which it produces. R. albus produces ethanol and CO2 as its primary fermentation products and smaller amounts of acetate, formate, and H2O (Pavlostathis et al., 1988). *Ruminococcus albus* 7 is known as a member of the genus *Ruminococcus* may be found in both the ruminant and human gastrointestinal tracts, where they are capable of digesting hemicellulose as well as the more insoluble cellulose. Many herbivorous and omnivorous mammals, including humans and ruminants, rely on the digestion of plant cell wall polysaccharides for energy storage in their gastrointestinal tracts.

Ruminococcus albus 7 had never been employed as a nitrogen reduction bacteria in the chicken industry before; however, the bacteriocins produced by R.albus 7 were used as a substitute to antibiotics and demonstrated an improvement in BWG (Wang et al., 2011). According to Schaefer et al. (1980), when they looked at the nitrogen use of a collection of ruminal bacteria, which included R.albus 7, they discovered that R.albus 7

had one of the highest nitrogen utilization rates among the bacteria in that group. In this experiment, R.albus 7 was evaluated for the first time as a probiotic for nitrogen reduction in broiler production, and the results were promising. The *R.albus 7* as a ruminal organism is new to the broiler gut microflora, and it is likely to have an impact on the broiler gut microbiota. In this experiment, two treatments, cecal samples (PC treatment and *R.albus 7* treatment), were sent for sequencing to study the effect of *R.albus 7* on the broiler's gut microflora compared to positive control. To investigate the influence of R.albus 7 on the broiler's gut microbiota in comparison to the positive control, two treatments, cecal samples (PC treatment and R.albus 7 treatment), were sent for sequencing to be analyzed.

Materials and Methods

RNAlater preparation:

Four hundred mL of 0.5-M EDTA, 25 mL of 1-M sodium citrate, 700 grams of ammonium sulfate, and 935 mL of sterile distilled water were placed in a beaker and stirred on a hot plate stirrer on low heat until the ammonium sulfate was completely dissolved. Then the solution was allowed about 10 minutes to cool, then adjusted the pH to pH5.2 using 1 mL H_2SO_4 to achieve the desired result. Then RNA later was transferred to a screw-top bottle and kept at room temperature or in the refrigerator to keep it fresh. The final concentrations were: 25mM Sodium Citrate, 10mM EDTA, 70g Ammonium Sulfate/100 mL solution, and the final pH 5.2.

Cecal samples collections

The cecal collection was accomplished by euthanizing twelve birds from each treatment in the second experiment of the nitrogen study using CO2. The entire ceca were collected from each bird and immediately stored in a 50 mL tube containing RNA later in a 1:5 ratio (w/v). After that, the cecal were kept refrigerated for approximately two weeks. Then the samples were extracted from the RNA later, and the cecal tissue was separated from the content, and both were placed in 2 mL tubes and stored at -80 degrees Celsius until they were used for DNA extraction.

DNA Extraction

QIAamp DNA Mini Kit was used for DNA extraction. A 25mg of the cecal content from each sample was weighed and equilibrated to room temperature. Later, The cecal contents were pelleted by centrifugation for 10 minutes at 7500 rpm. Then, the bacterial pellets were suspended in 180 mL of lysozyme (20 mg/ mL). After that, the mixture was incubated for 30 minutes at 37 °C. A 40 µL of Proteinase k and 200 µL buffer Al. were added to the samples and mixed by vortexing. Then, the mixtures were incubated at 56 °C for 30 minutes and then for 15 minutes at 95 °C, then they were fast centrifuged for seconds. 400 µl ethanol (96-100%) was added to the samples and mixed by pulse-vortexing for about 15 seconds.

Then a fast centrifuging for seconds to remove the drops from the lid. Then, the mixtures were carefully applied to the QIAamp spin column in a 2 mL collection tube without wetting the rim, and then the cap was closed and centrifuged at 8000 rpm for one minute. The columns were transferred in a clean 2 mL collection tube, and the filtrate

tubes were discarded. Then a 500 µL buffer AW1 was added to the QIAamp spin column without wetting the rim and centrifuged at 8000 rpm for one minute. After that, the columns were transferred in a clean 2 mL collection tube, and the filtrate tubes were discarded. Then a 500 µL buffer AW2 was added to the QIAamp spin column without wetting the rim and centrifuged at 14000 rpm for three minutes. Then, the columns were transferred in a clean 2 mL collection tube, and the filtrate tubes were discarded and centrifuged at full speed for one minute. Then, the QIAamp spin columns were then placed in a clean 1.5 mL microcentrifuge tube and discarded the filtrate tube. Then 200 µl buffer AE was added to the columns and incubated at room temperature for 1 minute, and then centrifuged at 8000 rpm for 1minute. This last step was repeated to increase the yield of the DNA. Then the DNA samples were sent to Mr.DNA (502 Clovis Road, Shallowater, Texas, 79363) for sequencing, and then the sequencing data were analyzed for 16S gen for the microflora profile.

Bioinformatics analysis

The raw data output of the MiSeq run was printed in MiSeq reporter software in the form of FASTA and FASTQ files. FASTQ raw data files were used for subsequent bioinformatics analysis using the QIIME2 pipeline[27]. Different plugins available in QIIME2 were used for quality-control, annotation, assembly, alignment, and statistical analysis. In brief, FASTX-toolkit was used to remove chimeras, and low-quality reads from the data. Thereafter, FeatureTable [Frequency] and FeatureData [Sequence] were generated using DADA2 plugin[28]. Taxonomy classification of bacterial 16S rRNA marker-gene was performed using VSEARCH and BLAST+ tools[29]. Taxonomic

classification was performed using Silva 132 (99% OTUs full-length sequences) as a reference database

Table 10: Sequencing mapping and primers pairs.

Sample ID	Barcode Sequence	Linker Primer Sequence	Barcode Name	Reverse Primer	Project Name	Description
25	AATTGCTGCG	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0066	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	25
26	TTACAATTCC	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0067	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	26
27	AACCTAGCAC	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0068	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	27
28	TCTGTGTGGA	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0069	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	28
29	GGAATTCCAA	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0070	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	29
30	AAGCGCGCTT	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0071	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	30
31	TGAGCGTTGT	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0072	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	31
32	ATCATAGGCT	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0073	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	32
33	TGTTAGAAGG	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0074	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	33
34	GATGGATGTA	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0075	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	34
35	ACGGCCGTCA	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0076	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	35
36	CGTTGCTTAC	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0077	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	36
73	TGACTACATA	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0078	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	73
74	CGGCCTCGTT	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0079	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	74
75	CAAGCATCCG	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0080	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	75
76	TCGTCTGACT	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0081	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	76
77	CTCATAGCGA	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0082	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	77
78	AGACACATTA	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0083	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	78
79	GCGCGATGTT	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0084	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	79
80	ACATACTTCC	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0092	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	80
81	ACGTCAATAC	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0086	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	81
82	GATACCTCCT	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0087	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	82
83	ATCCGTAAGT	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0088	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	83
84	CGTGTATCTT	GTGYCAGCMGCCGCGGTAA	60bp_UDPi5_0089	GGACTACNVGGGTWTCTAAT	051421MHillcus515F	84

Statistical analysis

Means were calculated for each taxon's relative percentage, and statistical comparisons were made using the Mann-Whitney test. Relative percentage data were converted into log2fold change to compare treatment vs. controls. Microbiome diversity analysis was performed using alpha and beta diversity indexes. Alpha diversity analysis was performed using the observed species, chao1, Shannon, and Simpson indexes. Mann-Whitney test was used to compare differences of alpha diversity between the groups. Beta diversity analysis was performed using a PCA plots in RStudio. Phylogenetic analysis of the microbiome was performed at phylum, class, order, family, genus, and species level

Hypothesis

Introducing *R. albus 7* as a probiotic will not affect the broiler cecal microflora's microbial diversity, richness, evenness, and composition.

Results

Microbial Community Composition

All sequences were classified into four phyla, although one phylum was most common (> 1 %): Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria (Figure 18). Firmicutes accounted for (>99%) of the reads obtained from the cecum. However, Proteobacteria accounted for (>0.2%) in the average of the 12 samples of treatment excluding sample 27 it was about and (>98%) which most likely an outlier. Bacteroidetes accounted for (>0.04%) in both treatment and control groups, and Actinobacteria representing less than (>0.2%) in both treatment and control groups.

At the genus level, the sequences from the 24 samples were identified into 55 genera and of which 46 had relative abundance > 1% (Figure 19). The major microbial genera across all gut samples from both treatment and control groups were the Faecalibacterium, Ruminococcus, Subdoligranulum, and Lachnoclostridium. In addition, Faecalibacterium was the predominant genus in the cecum in both treatment and control samples groups, accounting for over 50% in treatment samples and over 30% in the control samples. However, sample 27 has Ralstonia over 70% only in the treatment group, and it is most likely an outlier.

To indicate the statistically different microbes in both groups compared to each other, al P.value was used, and the results showed that nine different microbes are either significantly higher or lower in the treatment group compared to the control group ($p<0.05$). The nine microbes are Blautia producta, Blautia, Erysipelotrichaceae, Erysipelotrichales, Erysipelotrichia, Lachnospiraceae, Lachnospira spp., Lachnospira, and Eubacterium rectale (Figure 20). Moreover, those microbes are present in both groups but in different abundancies, which is not a sign of microbial composition change because naturally, that can occur in different samples within the same treatment group.

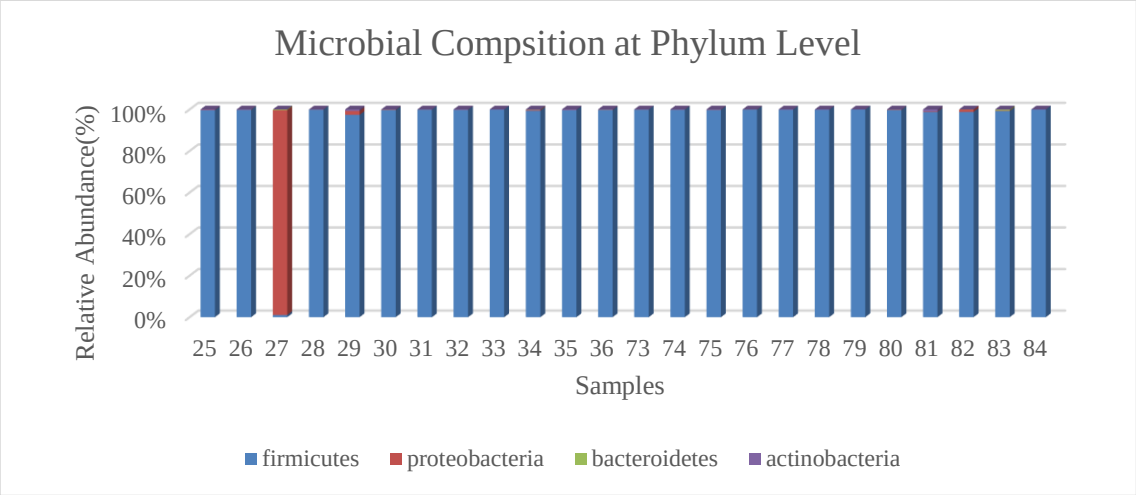

Figure 18 Microbial composition at the phylum level. Samples (25-36) are the *R. albus7* treatment N=12, and samples (73-84) are the control treatment N=12 showed no statistical differences **(***P* > 0.05).

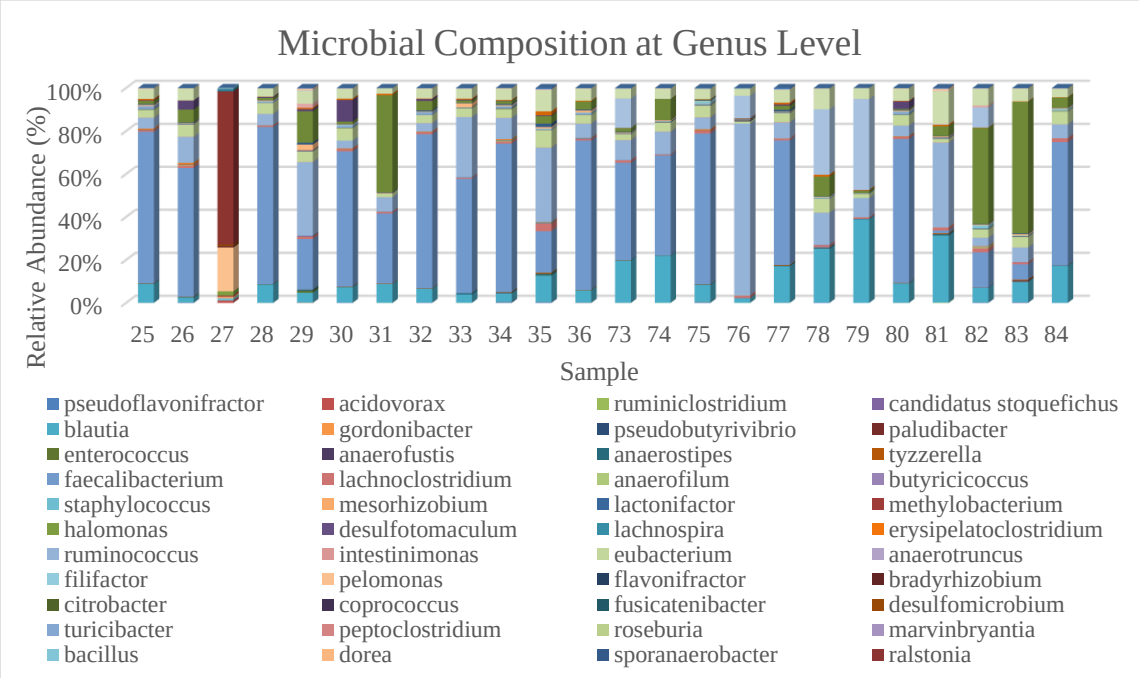

Figure 19 Microbial composition at the genus level. Samples (25-36) are the *R. albus7* treatment N=12, and samples (73-84) are the control treatment N=12 showed no statistical differences **(***P* > 0.05).

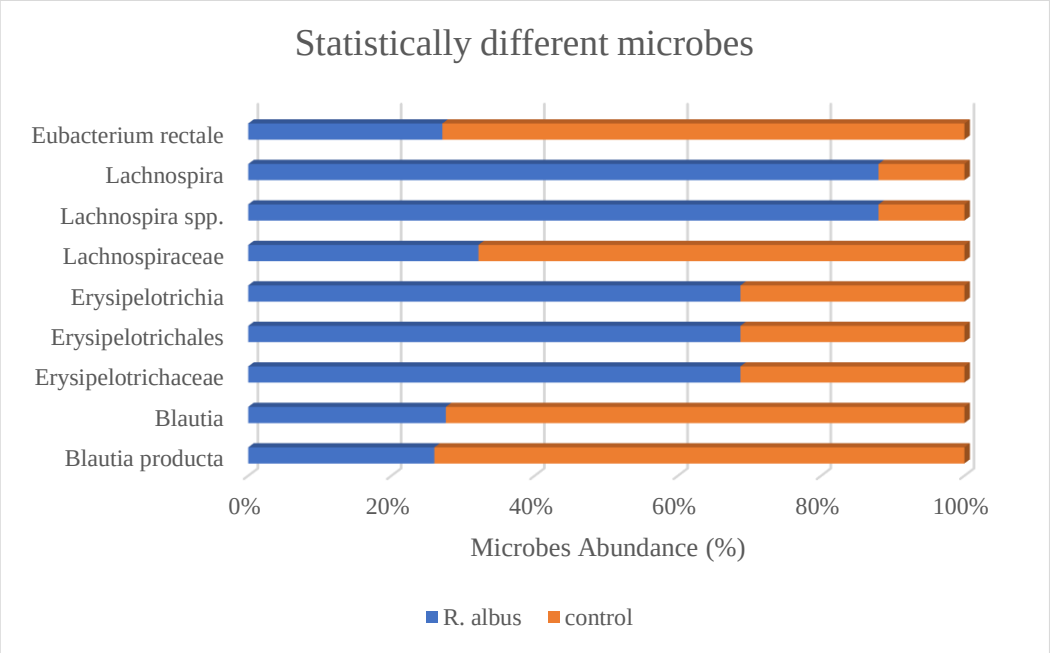

Figure 20 Statistically different microbes in both treatment (*R.albus 7*) & control groups.

The similarity in Microbial Community Composition

Principal coordinate analysis (PCA) of the dissimilarity between the microbial samples, PCA plotted against the PC1 vs. PC2 axes. The percentages indicate the relative contribution of the two principal coordinates (PC1–PC2).The similarity and difference of microbial community composition in 24 cecal content samples taken from the cecum content of 24 broiler chickens. The PCA plot with PC1 accounting for 51.49 % of the total variation and PC2 accounting for 25.1%. As a result, microbial communities of cecal

samples were more similar. However, sample t27 is far from the treatment group, which is most likely an outlier (Figure 21).

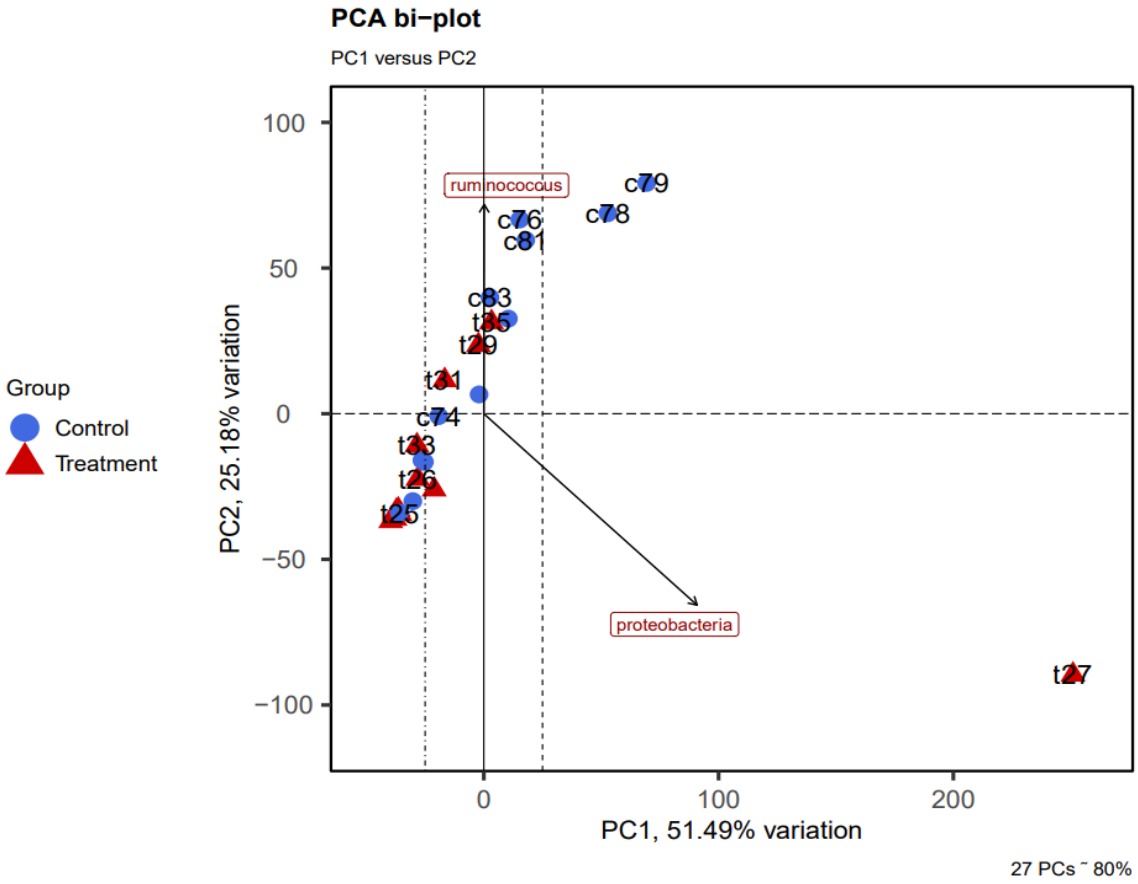

Figure 21 Principal coordinate analysis (PCA) of the dissimilarity between the microbial samples of bothe *R. albus 7* treatment and contorl treatment. The comparisons were made using weighted unifrac index and statistical comparisons were made using PERMANOVA. $p \leq 0.05$ is considered statistically signifcant.

Alpha diversity

Alpha diversity indices of both datasets, *R.albus 7* treatment, and control treatment, showed similar pattern distribution with cecal content microflora community diversity (Fig. 22). Almost equal or close species richness was observed as indicated by Observed ASV and Chao1. Both estimators showed similar patterns reassuring that the sequencing depth obtained was sufficient. Average Shannon and Simpson indices presented indicating

that the species present were almost equally abundant, suggesting that the abundance of the different species was then more even. Kruskal-Wallis tests of Richness, Shannon, and Simpson indicated that bacterial diversity in cecal content samples in both treatment and control groups are not significantly different from one to another in both datasets. With an outlier (sample 27) in the treatment group.

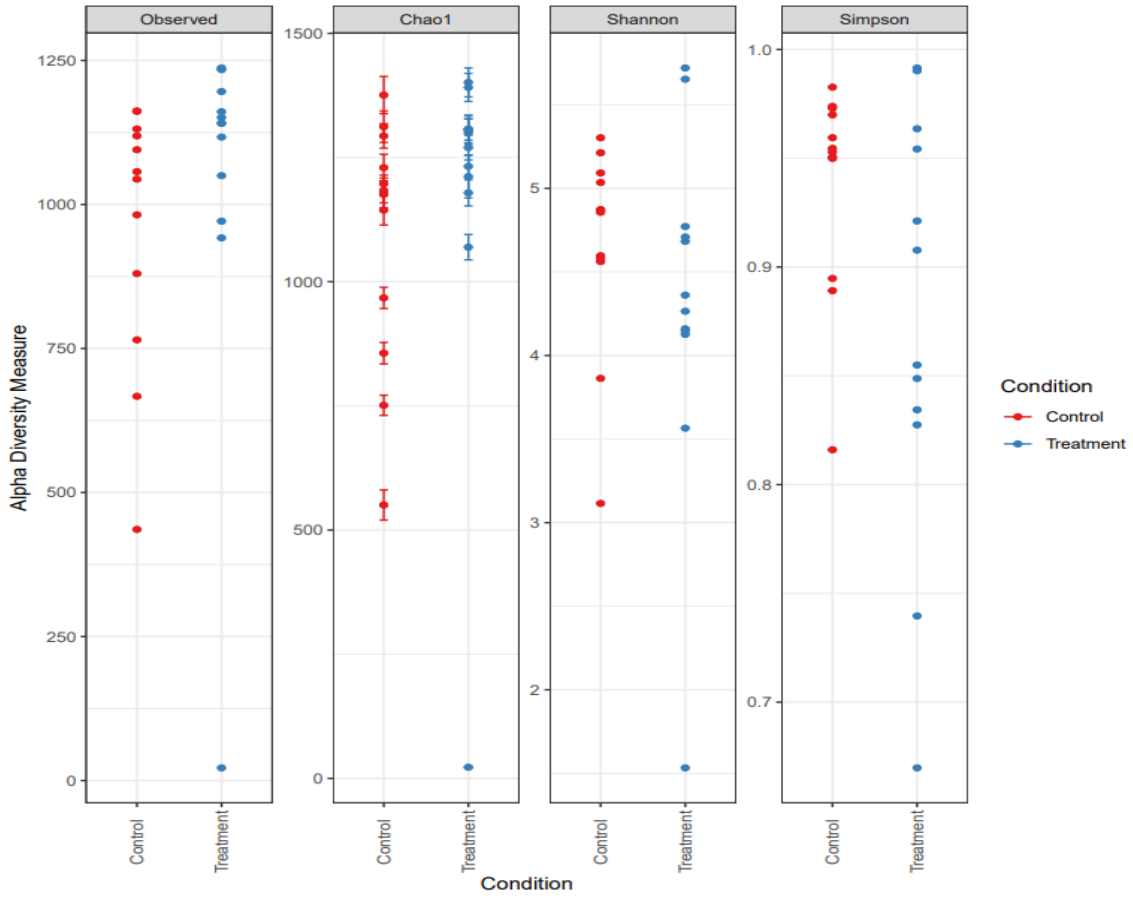

Figure 22 Alpha diversity metric of richness (Observed & Chao1) and evenness (Shannon & Simpson) in comparsion between *R.albus 7* treatment and control (N=12). The comparisons were made using weighted unifrac index and statistical comparisons were made using PERMANOVA. $p \leq 0.05$ is considered statistically signifcant

Discussion

In order to understand and predict the alterations associated with feeding ruminal nitrogen utilizing bacteria *R. albus 7*, comprehensive characterization of typical chicken intestinal microbial communities is a prerequisite. This study was conducted to elucidate the microbial communities in the chicken ceca. The gastrointestinal tract is home to a diverse microbial community critical to chicken growth and health by improving food absorption and strengthening the immune system (Choi et al., 2015). Following the hatching of the chicks, bacteria begin to colonize the GI tract of the bird. However, various factors like the surrounding environment, nutritional intake, supplementation, pathological conditions, antibiotic therapy, breed, genetics, age, and other factors influence intestinal microbial composition (Cisek & Binek, 2014). Various methods have been used to define the chicken intestinal microbiota, ranging from culture-based studies to more recent molecular methods, such as 16S rRNA gene sequencing and differential gene expression (DGGE) (Dumonceaux et al., 2006). Using 16S rRNA gene sequencing, we determined the microbiota present in the cecum of broiler chickens that had been fed an *R. albus 7* bacteria. The Chao1 and Shannon tests indices revealed that the cecal microbiota in the treatment group was not significantly more varied than in the control group, with one exception of sample 27 in the treatment group was an outlier. If sample 27 is excluded from all tests, the sequencing data will be more uniformed. Firmicutes was the most prevalent genera in the ceca of the broiler chicken, with Blautia producta and Blautia, Erysipelotrichaceae, Erysipelotrichales, Erysipelotricha, Lachnospiraceae,

Lachnospira spp., Lachnospira, and Eubacterium rectale being the most significant genera in the ceca of the broiler chicken. However, those nine microorganisms were found in both the treatment and control groups, albeit in varying abundance, which is a normal state that occurs naturally.

All sequences were classified into four phyla: Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria. Firmicutes accounted for (>99%) of the average of the 12 samples of treatment reads obtained from the cecum. However, Proteobacteria accounted for (>0.2%) in the average of the 12 samples of treatment. Bacteroidetes accounted for (>0.04%) in both treatment and control groups, and Actinobacteria representing less than (>0.2%) in both treatment and control groups, and they all present in both treatment and control groups.

Principal coordinate analysis (PCA) results showed that the microbial communities of cecal samples were more similar in both groups ($P > 0.05$). Alpha diversity indices of both datasets, *R.albus 7* treatment, and control treatment, showed similar pattern distribution with cecal content microflora community diversity. Almost equal or close species richness was observed as indicated by Observed ASV and Chao1. Both estimators showed similar patterns reassuring that the sequencing depth obtained was sufficient. Average Shannon and Simpson indices presented indicating that the species present were almost equally abundant, suggesting that the abundance of the different species was then more even. Kruskal-Wallis tests of Richness, Shannon, and Simpson test of evenness indicated that bacterial diversity in cecal content samples in both treatment and control groups are not significantly different from one to another in both datasets. With an outlier (sample 27) in

the treatment group. While all tests did not show any significant change in the broiler cecal microflora's microbial diversity, richness, evenness, and composition, so the hypothesis of the experiment is accepted, and the use of the *R.albus 7* as nitrogen reduction probiotic in the genome level will not affect the integrity and diversity of the gut microflora.

CHAPTER V

CONCLUSIONS

Four nitrogen assimilation rumen bacteria *Solonomonas ruminantium, Butyrivibrio fibrisolvens A38, Ruminococcus albus strain 7,* and *Bactetoides ruminocola subsp* were selected based on the work of Schaefer et al. (1980). Tested through both in-vivo & in-vitro experiments to be evaluated as nitrogen reduction probiotics, and the winner *R.albus7* was tested in a bioinformatic gene sequencing experiment to invsetage the microflora diversity effect of this potential probiotic.

The first experiment was an in-vitro experiment. As a first step in evaluating the four selected ruminal bacteria, an ammonia assay test was conducted to investigate how those bacteria would behave in the incubation media with and without the presence of the ammonia. Additionally, the amino acid profile of these bacteria was analyzed to determine the essential dietary amino acids that may be available to birds from these bacteria. The results showed that the *R. albus7* and *S. ruminantium* had the highest nitrogen reduction rate than *Butyrivibrio fibrisolvens A38* and *Bactetoides ruminocola subsp*.

The second experiment was an in-vivo experiment. The four novel probiotics were tested in two repeated trials in four treatments plus positive and negative controls treatments and the rumen whol microflora treatment. The two experiments were conducted in the starter feeding phase for the first three weeks on a total of 168 birds for each trial, and the bird's performance and nitrogen utilization were evaluated. Among the findings were that the *R.albus 7* treatment had the largest BWG while also having the lowest FCR, nitrogen consumption, N body/N feed ratio, and N FC/N feed ratio of all the treatments

tested. As a result, the R.albus 7 was chosen as the future nitrogen reduction probiotic for this experiment, and the results of the *R.albus 7* alone will be discussed from this point forward. The results in (Figure 23) showed data of *R.albus 7* treatment compared to a positive control that *R.albus 7* treatment is almost equal to PC in week one, slightly higher in week two, and significantly higher in the BWG. Whereas, in (Figure 24) the FCR data showed that *R.albus 7* treatment is lower than PC in week one, slightly higher in week two, and lower in week three, which is the most critical week in the experiment.

An amino acids study was conducted to determine the potential amino acids available to the birds by the bacterial protein of those novel probiotics. The *R.albus 7* data in (Figure 25) showed that the *R.albus 7* nine dietary amino acids were statistically not different from the control group of avian microbes alone. However, more research is needed on R.albus 7's dietary amino acids.

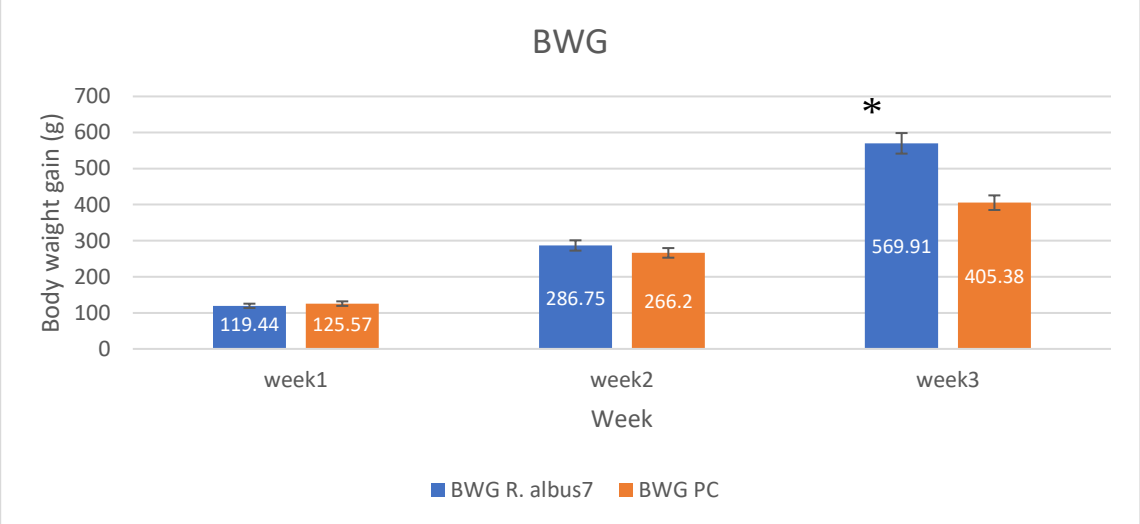

Figure 23 Body weight gain for *R.albus7* treatment Vs. Positive control. N=48. Significant difference P≤0.05.

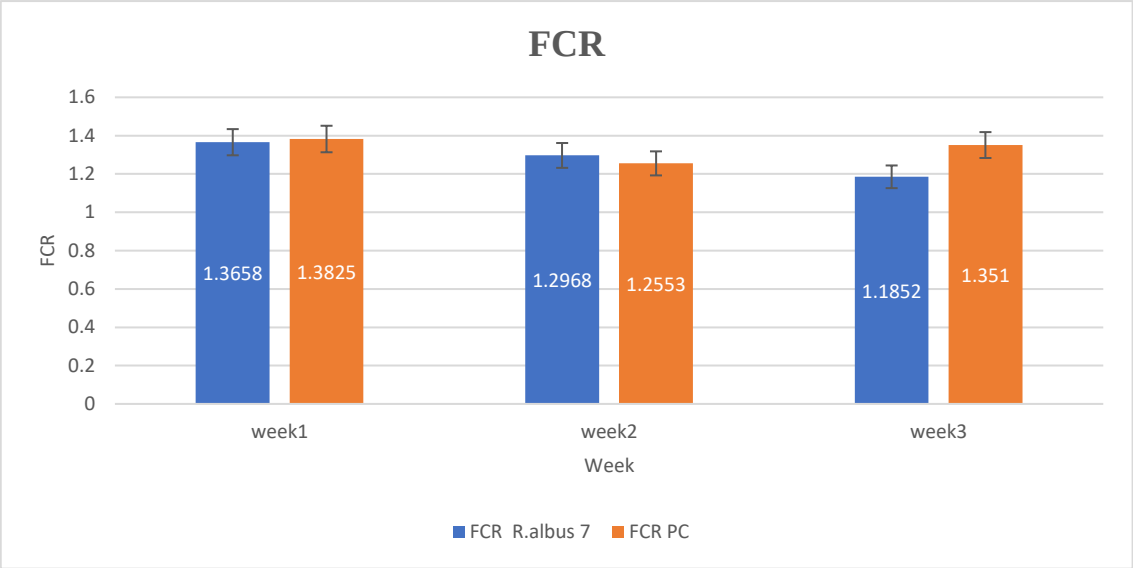

Figure 24 FCR for *R.albus7* treatment Vs. Positive control N=12. No significant difference P>0.05.

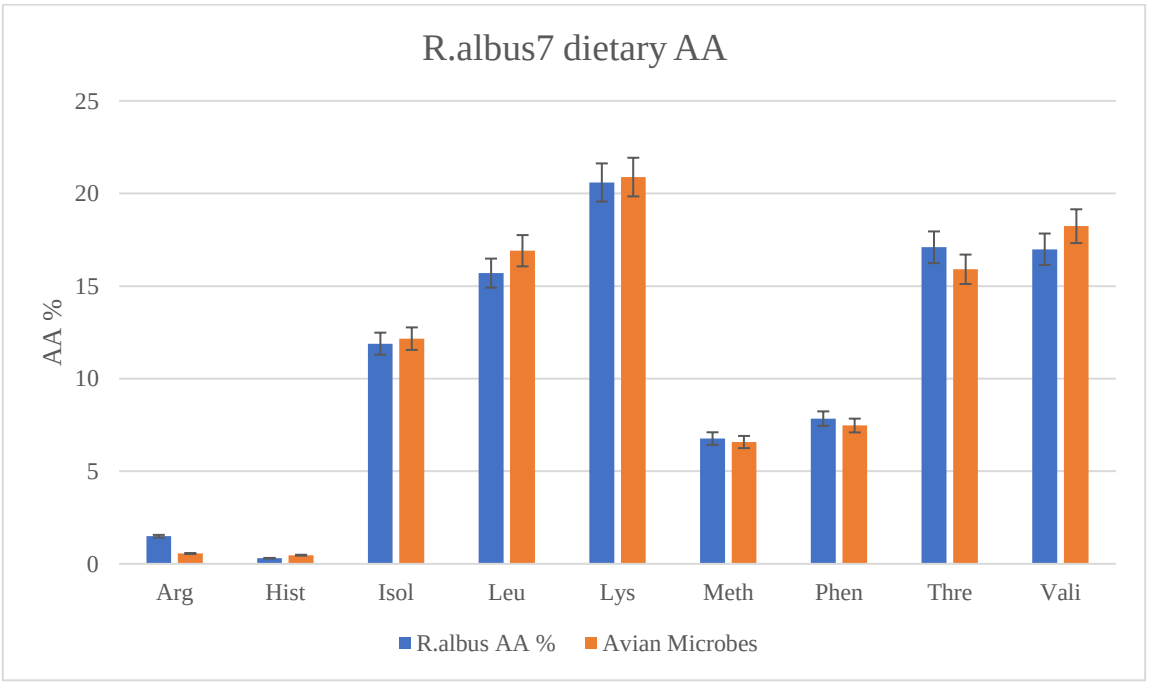

Figure 25 R.albus dietary amino acids compared to avian microbes alone same amino aicds. N=2. No significant difference P>0.05.

From the results of the first and second experiments, the decision was made that the *R.albus 7* could be our target nitrogen reduction probiotic. Therefore, a bioinformatics experiment was conducted to determine if this probiotic has no unhealthy effect on the bird's microflora. Moreover, the results showed that *R.albus 7* treatment did not affect the broiler cecal microflora's microbial diversity, richness, evenness, and composition. However, some bacteria present in both *R.albus 7* treatment and positive control were found to have changed in abundance percentage.

Therefore, based on all those results from the three experiments, we can conclude that the *R.albus 7* is a potential nitrogen reduction probiotic considering that this data represents only the first three weeks of the production cycle. In order to better understand the effect of *R.albus 7* throughout the bird's aging, it will be essential to perform further studies throughout the entire cycle of bird production of six weeks of the rearing period.

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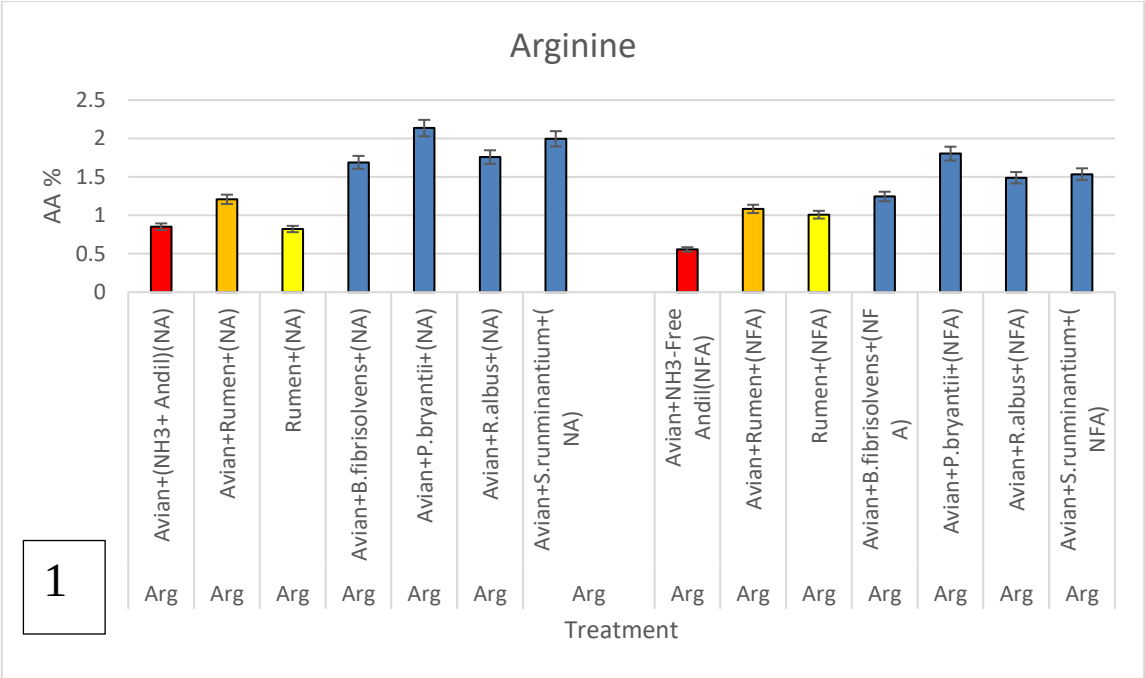
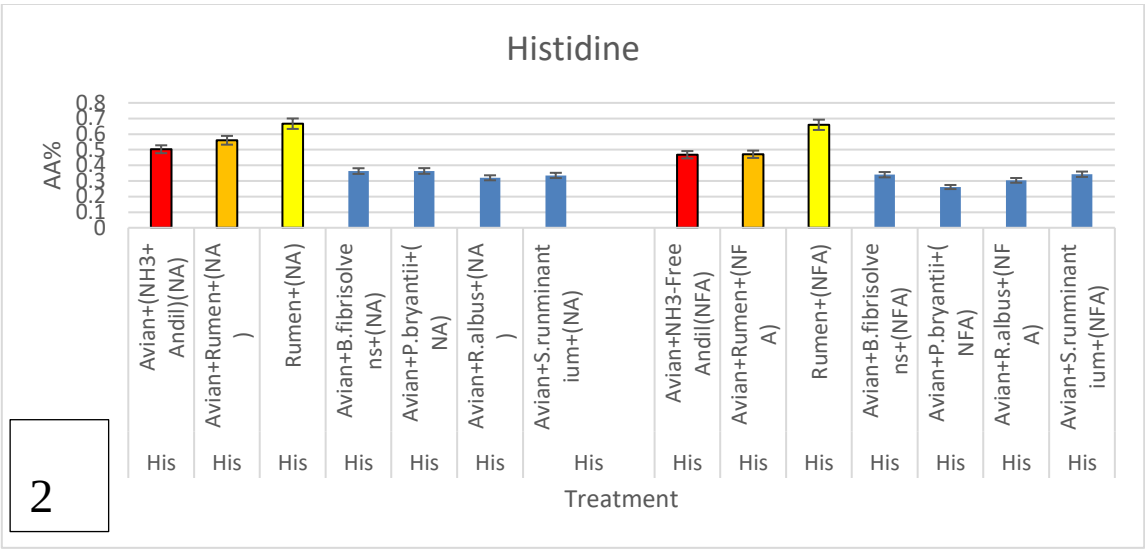
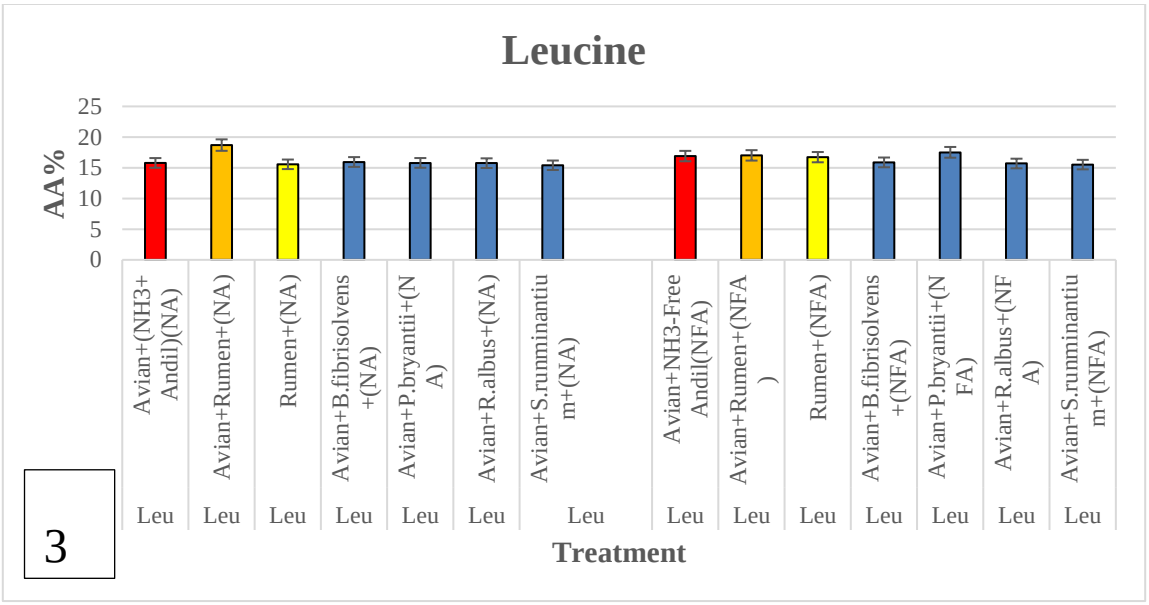
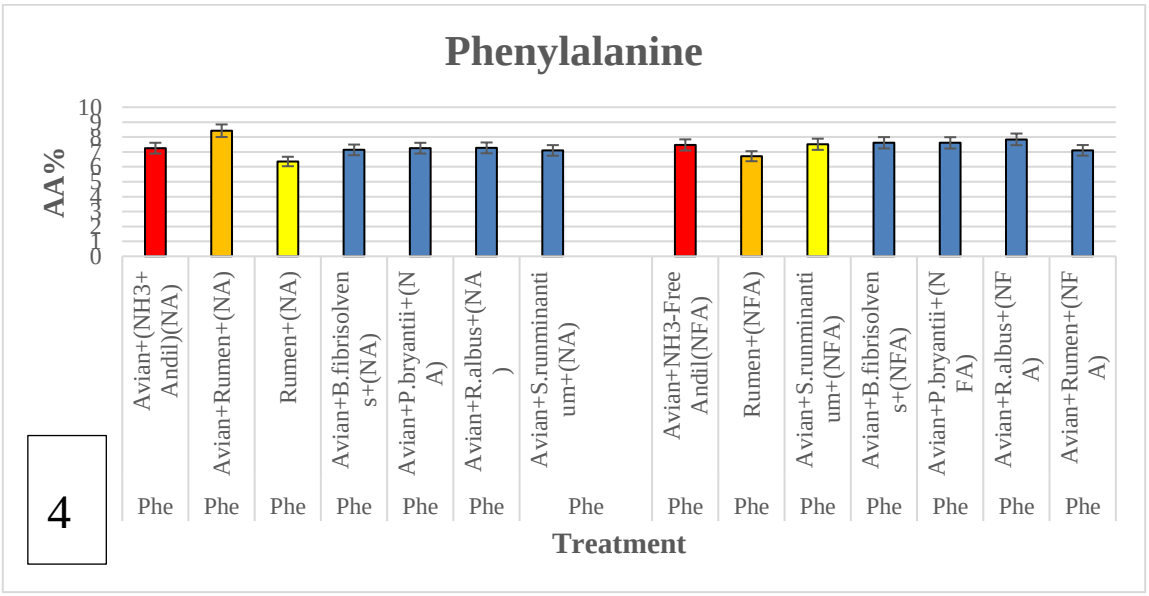
APPENDIX A

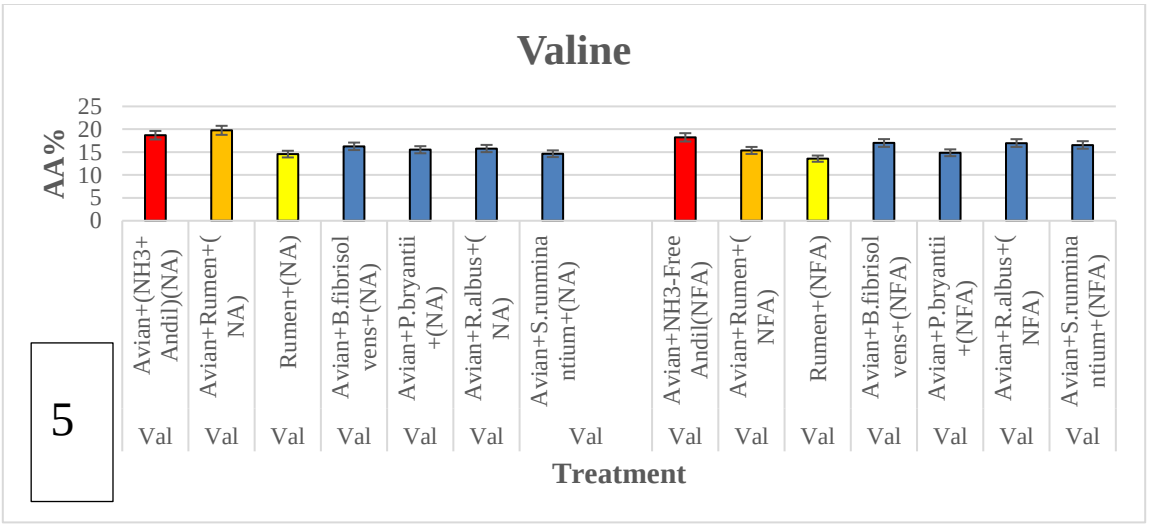
ARGININE, HISTIDINE, LEUCINE, PHENYLALANINE, AND VALINE.






Leucine
AA%
25
20
15
10
5
0
Avian+(NH3+ Andil)(NA)
Avian+Rumen+(NA)
Rumen+(NA)
Avian+B.fibrisolvens +(NA)
Avian+P.bryantii+(NA)
Avian+R.albus+(NA)
Avian+S.runminantium+(NA)
Avian+NH3-Free Andil(NFA)
Avian+Rumen+(NFA)
Rumen+(NFA)
Avian+B.fibrisolvens +(NFA)
Avian+P.bryantii+(NFA)
Avian+R.albus+(NFA)
Avian+S.runminantium+(NFA)
Leu
Treatment
3


Phenylalanine
AA%
10
Avian+(NH3+ Andil)(NA)
Avian+Rumen+(NA)
Rumen+(NA)
Avian+B.fibrisolvens+(NA)
Avian+P.bryantii+(NA)
Avian+R.albus+(NA)
Avian+S.runminantium+(NA)
Avian+NH3-Free Andil(NFA)
Rumen+(NFA)
Avian+S.runminantium+(NFA)
Avian+B.fibrisolvens+(NFA)
Avian+P.bryantii+(NFA)
Avian+R.albus+(NFA)
Avian+Rumen+(NFA)
Phe
Treatment
4


Valine
AA%
25
20
15
10
5
0
Avian+(NH3+ Andil)(NA)
Avian+Rumen+(NA)
Rumen+(NA)
Avian+B.fibrisol vens+(NA)
Avian+P.bryantii +(NA)
Avian+R.albus+(NA)
Avian+S.runmina ntium+(NA)
Avian+NH3-Free Andil(NFA)
Avian+Rumen+(NFA)
Rumen+(NFA)
Avian+B.fibrisol vens+(NFA)
Avian+P.bryantii +(NFA)
Avian+R.albus+(NFA)
Avian+S.runmina ntium+(NFA)
Val
Treatment
5

APPENDIX B

PILGRIM'S BROILER STARTER NEGATIVE CONTROL & POSITIVE CONTROL DIETS

Date Printed: 05/17/17
Date Optimized: 02/16/2017
Optimized By: DM
Trial Version: 1
Prod'n Version: 3
Page: 1

Plant: 27 Nacogdoches — Formulated By: Single Product Formulation
Product: 101.3 Broiler Starter + Protease No Matrix

Used Ingredients

Ingr Code	Ingredient Name	Unrounded Lbs	Unrounded Pct	-- Range -- Low	-- Range -- High	-- Restriction -- Min Pct	-- Restriction -- Max Pct	Rcost
101	CORN, Fine	1018.12	50.906		0.1402			
111	SOYBEAN MEAL	796.10	39.805	0.0879				
143	Soy Oil	71.75	3.588	0.1157		0.5000		
153	DISTILLER'S GR	40.00	2.000			2.0000	2.0000	
28	LIMESTONE FINE	23.43	1.171		1.0742			
299	NEXPHOS MONO-D	18.86	0.943		10.9765	0.2500		
41	ALIMET	8.21	0.411		7.9681			
36	SALT PLAIN	5.23	0.262		1.4862			
13	LIQ LYSINE50%	5.03	0.251		1.9294			
919	Adisodium	3.00	0.150			0.1500		
45	L-THREONINE	2.45	0.122		5.9177			
78	BIOAVAIL TRACE	1.50	0.075			0.0750		
63	CHOLINE LIQ.	1.32	0.066		51.1009			
166	Opti Bac S/L	1.00	0.050			0.0500	0.0500	
35	COPPER SULFATE	1.00	0.050			0.0500		
177	NICARB 25%	0.900	0.045			0.0450	0.0450	
60	BROILER VITAMI	0.500	0.025			0.0250		
642	Optiphos 6000P	0.500	0.025			0.0250	0.0250	
775	Hemicell Dry	0.400	0.020			0.0200	0.0200	
288	Poultry Grow 2	0.250	0.013			0.0125	0.0125	
72	LIQ ETHOXYQUIN	0.250	0.013			0.0125		
282	Hostazyme X dr	0.200	0.010			0.0100	0.0100	

Total Batch: 2000.00 Lbs

Binding Nutrients

Nutr No	Nutrient Name	Unit of Measure	Increment Change
7	CALCIUM	PCT	0.02 PCT
9	AVAIL. PHOS.	PCT	0.02 PCT
11	CHLORIDE	PCT	0.02 PCT
12	SODIUM	PCT	0.02 PCT
37	ME; POULTRY	KCAL/LB	10.00 KCAL/LB
54	AVAIL. LYSINE	PCT	0.01 PCT

Unused Ingredients

Ingr Code	Ingredient Name		Would Use	Minimum Pct	Maximum Pct	Rcost
621	Optiphos IC .	Suppressed		0.0150	0.0150	146.1
293	Hostazym X WSP	Suppressed		0.0100	0.0100	82.20
46	BIOLYS	Suppressed				1.98

Nutrient Solution (Class 5)

Nutr No	Nutrient	Minimum	Actual	Maximum
1	WEIGHT	0.99	0.9995	1.01
2	PROTEIN		23.27	
3	FAT		5.52	
4	FIBER		2.54	
5	MOISTURE		12.53	
6	ASH		4.96	
7	CALCIUM	0.90	0.9060	0.93
8	TOTAL PHOS.		0.5968	
9	AVAIL. PHOS.	0.45	0.4499	
10	SALT		0.3086	
11	CHLORIDE	0.21	0.2100	0.28
12	SODIUM	0.19	0.2204	0.22
13	POTASSIUM	0.65	0.9262	
14	MAGNESIUM		0.1767	
15	MANGANESE		94.80	
18	COPPER		156.70	
21	SULFUR		0.2897	
22	LINOLEIC ACID		1.78	
23	XANT. ACTIVITY		5.10	
36	CHOLINE	825.00	838.45	
37	ME; POULTRY	1375.00	1381.47	
53	AVAIL. ARGININ		1.44	
54	AVAIL. LYSINE	1.25	1.26	
55	AVAIL. METHION		0.6402	
56	AVAIL. METH+CY		0.9496	
57	AVAIL. TRYPTOP		0.2502	
58	AVAIL. ISOLEUC		0.9044	
59	AVAIL.VALINE		0.9678	
61	AVAIL. CYSTINE		0.6688	
62	AVAIL. THREONI		0.8873	
69	ANALYZED CALCI		0.7496	
73	Feather Meal		0.0000	
80	Sodium mEq/Kg		95.87	
81	Potassium mEq/		236.87	
82	Chloride mEq/K		59.23	
83	DEB mEq/Kg	160.00	273.51	
84	DEB+S		108.61	
100	Nacogdoches		90.71	

Table 1

CFC/Concept5

Date Printed: 05/17/17
Date Optimized: 02/16/2017
Optimized By: DM
Trial Version: 1
Prod'n Version: 3
Page: 1

Plant: 27 Nacogdoches — Formulated By: Single Product Formulation
Product: 102.1 Broiler Grower Positive Control

Used Ingredients

Ingr Code	Ingredient Name	Unrounded Lbs	Unrounded Pct	-- Range -- Low	-- Range -- High	-- Restriction --- Min Pct	-- Restriction --- Max Pct	-- Restriction --- Rcost
101	CORN, Fine	1107.21	55.360	0.0158	0.1390			
111	SOYBEAN MEAL	650.82	32.541	0.0906				
153	DISTILLER'S GR	100.00	5.000		0.1099		5.0000	-0.71
143	Soy Oil	71.42	3.571	0.1190	1.0601	0.5000		
28	LIMESTONE FINE	23.58	1.179		0.6414			
299	NEXPHOS MONO-D	16.36	0.818		10.8581	0.2500		
41	ALIMET	7.20	0.360		7.8958			
13	LIQ LYSINE50%	6.52	0.326		1.9136			
36	SALT PLAIN	4.50	0.225		1.4862			
919	Adisodium	3.00	0.150			0.1500		
45	L-THREONINE	2.33	0.117		5.8658			
78	BIOAVAIL TRACE	1.50	0.075			0.0750		
63	CHOLINE LIQ.	1.19	0.060		24.0149			
166	Opti Bac S/L	1.00	0.050			0.0500	0.0500	
35	COPPER SULFATE	1.00	0.050			0.0500		
177	NICARB 25%	0.900	0.045			0.0450	0.0450	
60	BROILER VITAMI	0.500	0.025			0.0250		
642	Optiphos 6000P	0.500	0.025			0.0250	0.0250	
72	LIQ ETHOXYQUIN	0.250	0.013			0.0125		
282	Hostazyme X dr	0.200	0.010			0.0100	0.0100	

Total Batch: 2000.00 Lbs at 249.68 $/Ton 12.484 $/100Lb 0.1248 $/Lb

Binding Nutrients

Nutr No	Nutrient Name	Unit of Measure	Increment Change
7	CALCIUM	PCT	0.02 PCT
9	AVAIL. PHOS.	PCT	0.02 PCT
11	CHLORIDE	PCT	0.02 PCT
36	CHOLINE	MG/LB	0.10 MG/LB
37	ME; POULTRY	KCAL/LB	10.00 KCAL/LB
54	AVAIL. LYSINE	PCT	0.01 PCT

Unused Ingredients

Ingr Code	Ingredient Name		Would Use	Minimum Pct	Maximum Pct	Rcost
621	Optiphos IC .	Suppressed		0.0150	0.0150	146.1
293	Hostazym X WSP	Suppressed		0.0100	0.0100	82.20
288	Poultry Grow 2	Suppressed		0.0125	0.0125	102.9
46	BIOLYS	Suppressed				2.00
19	S-CARB	Suppressed		0.1500		3.12

Nutrient Solution (Class 5)

No	Nutrient	Minimum	Actual	Maximum
1	WEIGHT	0.99	0.9996	1.01
2	PROTEIN		21.11	
3	FAT		5.82	
4	FIBER		2.57	
5	MOISTURE		12.67	
6	ASH		4.64	
7	CALCIUM	0.86	0.8672	0.89
8	TOTAL PHOS.		0.5581	
9	AVAIL. PHOS.	0.43	0.4297	0.46
10	SALT		0.2734	
11	CHLORIDE	0.19	0.1900	0.28
12	SODIUM	0.18	0.2049	0.22
13	POTASSIUM	0.60	0.8335	
14	MAGNESIUM		0.1684	
15	MANGANESE		94.10	
18	COPPER		152.64	
22	LINOLEIC ACID		1.88	
23	XANT. ACTIVITY		5.57	
36	CHOLINE	770.00	770.00	
37	ME; POULTRY	1400.00	1395.51	
53	AVAIL. ARGININ		1.25	
54	AVAIL. LYSINE	1.12	1.13	
55	AVAIL. METHION		0.5783	
56	AVAIL. METH+CY		0.8637	
57	AVAIL. TRYPTOP		0.2171	
58	AVAIL. ISOLEUC		0.7980	
59	AVAIL.VALINE		0.8699	
61	AVAIL. CYSTINE		0.6005	
62	AVAIL. THREONI		0.7971	
69	ANALYZED CALCI		0.7104	
73	Feather Meal		0.0000	
80	Sodium mEq/Kg		89.12	
81	Potassium mEq/		213.17	
82	Chloride mEq/K		53.59	
83	DEB mEq/Kg	160.00	248.70	
84	DEB+S		89.92	
100	Nacogdoches		87.90	

Table 2

APPENDIX C

MICROBIAL COMPOSTION CLASSIFCATION

Table 11 Microbial Comption (prokaryote) at Phylum level.

phylum	25	26	27	28	29	30	31	32	33	34	35	36	73	74	75	76	77	78	79	80	81	82	83	84
firmicutes	99.61483	99.76965	1.06006	99.90338	97.36861	99.6738	99.86227	99.78426	99.8604	99.37943	99.6634	99.71038	99.9096	99.82141	99.78016	99.91992	99.82961	99.81114	99.92132	99.67897	98.78578	98.79461	99.18873	99.87017
proteobacteria	0.093083	0.032907	98.60375	0.0187	1.901621	0.106514	0.071996	0.0161	0	0.298915	0.021259	0.086887	0.009686	0.035081	0.11156	0.077115	0.003277	0.043842	0.028325	0.117041	0.223417	1.127077	0.196975	0
bacteroidetes	0.00321	0.036197	0.33619	0.00935	0	0.043271	0	0.0161	0	0.110469	0	0.01609	0	0.006378	0	0.002966	0.006554	0	0	0.010032	0	0.051076	0.474076	0.003246
actinobacteria	0.288878	0.161243	0	0.068566	0.729773	0.176414	0.065736	0.183539	0.139603	0.21119	0.315346	0.186645	0.080718	0.137135	0.108278	0	0.160561	0.145016	0.050356	0.193954	0.990804	0.027241	0.14022	0.126586

Table 12 Microbial compesiton at the Genus level

genus	25	26	27	28	29	30	31	32	33	34	35	36	73	74	75	76	77	78	79	80	81	82	83	84
pseudoflavonifractor	0.057775638	0.273125	0	0.077916	0.049119	0.086543	0.018782	0.09016	0.006346	0.201443	0.311802	0.112631	0.048431	0.022324	0.301867	0.005932	0.045875	0.195602	0.003147	0.053505	0.01619	0.272405	0.243715	0.029212
acidovorax	0.003209758	0	1.135779	0	0	0	0	0	0	0	0.003543	0	0	0	0.003281	0	0.003277	0.003372	0	0	0	0	0.003339	0
ruminiclostridium	0.019258546	0.059232	0	0.0187	0.11929	0.039943	0.040694	0.04186	0.025382	0.077978	0.060235	0.074014	0.035516	0.054216	0.101716	0.062285	0.072089	0.01349	0.003147	0.053505	0.15542	0.074911	0.036724	0.032458
candidatus stoquefichus	0	0.003291	0	0.00935	0.098239	0.053257	0	0.01288	0.006346	0.155956	0.049605	0.012872	0	0	0	0	0	0	0	0.003344	0.009714	0.003405	0	0
blautia	8.730540844	2.138932	0	8.455401	4.452319	7.153081	8.717836	6.410999	3.940605	4.006108	12.3162	5.631537	19.52409	21.89055	7.983069	2.111757	16.94082	25.05396	38.96582	8.955324	31.17148	6.718197	9.575001	17.1346
gordonibacter	0.003209758	0	0	0	0.031577	0	0.00313	0.00644	0	0	0.007086	0.006436	0.012915	0.003189	0.006562	0	0.019661	0	0	0.003344	0.035617	0	0.003339	0.003246
pseudobutyrivibrio	0.006419515	0.009872	0	0.003117	0.007017	0	0.00313	0	0.012691	0	0.007086	0	0.032287	0.006378	0	0.002966	0.003277	0.219209	0.081828	0	0.055045	0.003405	0.006677	0
paludibacter	0.003209758	0.036197	0	0.00935	0	0.043271	0	0.0161	0	0.110469	0	0.01609	0	0.006378	0	0	0.006554	0	0	0.010032	0	0.051076	0.474076	0.003246
enterococcus	0.057775638	0.072395	0	0.084149	0.799944	0.029957	0.006261	0.00966	0.015864	0.003249	0.191333	0.003218	0.129149	0	0.013125	0.002966	0.00983	0.006745	0.006294	0.010032	0.009714	0	0	0.003246
anaerofustis	0.003209758	0.019744	0	0.012466	0.007017	0.006657	0.00313	0.00644	0	0.006498	0.007086	0.019308	0.012915	0	0.022968	0	0	0.006745	0	0.01672	0	0	0.003339	0
anaerostipes	0.099502488	0.092139	0	0.031166	0.54733	0.0466	0.046954	0.05796	0.539374	0.285919	0.680296	0.051488	0.054888	0.127567	0.108278	0.02966	0.229373	0.131526	0.075533	0.120385	0.531019	0.034051	0.09348	0.123341
tyzzerella	0.134809822	0.125045	0	0.043633	0.080696	0.133142	0.134602	0.1288	0.031728	0.233933	0.503136	0.148029	0.035516	0.063784	0.134528	0.032625	0.337506	0.131526	0.031472	0.100321	0.43712	0.030646	0.383935	0.103866
faecalibacterium	70.53763441	59.98552	0	73.1534	23.56326	62.95643	32.68015	71.71239	53.21086	69.05257	19.24671	69.48994	45.3797	46.49828	70.13486	0.017796	57.95596	0.327128	0.018883	66.96763	1.314597	16.34432	7.141188	57.41829
lachnoclostridium	0.818488204	1.118826	0	0.648258	1.084134	0.99524	0.751268	0.982097	0.561584	1.000715	3.653049	0.830249	1.110681	0.417783	1.319027	1.147823	0.822465	0.866721	0.648329	0.96977	1.301645	1.903432	0.931459	1.765718
anaerofilum	0.009629273	0.19744	0	0	0	0.003329	0	0.08694	0	0.321658	0.081494	0.028962	0.003229	0	0.003281	0.017796	0.003277	0.010117	0	0	0.003238	0.558431	0.030047	0
butyricicoccus	0	0.111883	0	0	0.073679	0	0	0	0	0.006498	0	0	0	0	0	0	0	0	0	0	0	0.061291	0	0
staphylococcus	0	0	1.050974	0	0	0	0.00313	0	0	0	0	0	0	0.003189	0	0.017796	0	0	0	0	0	0	0	0.003246
mesorhizobium	0	0	0.735984	0	0	0	0.00313	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
lactonifactor	0.080243942	0.046069	0	0.02805	0.066662	0.019971	0.050085	0.0161	0.012691	0.045487	0.056691	0.038616	0.032287	0.035081	0.078748	0	0.013107	0.023607	0.003147	0.03344	0.025903	0.040861	0.056756	0.032458
methylobacterium	0	0	0.545174	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
halomonas	0	0	1.953539	0	0	0	0	0	0	0.003249	0	0	0	0	0	0	0	0	0	0	0	0	0	0
desulfotomaculum	0	0.009872	0	0	0.221037	0	0	0	0.009518	0	0	0.01609	0	0	0	0.005932	0	0	0	0	0	0	0	0
lachnospira	0.102712245	0.036197	0	0.043633	0	0.053257	0	0.01932	0.05711	0.194944	0.262197	0.186645	0	0.028703	0.059061	0	0	0	0	0.003344	0	0.047671	0.003339	0
erysipelatoclostridium	0.417268496	0.783178	0	0.059216	0.017543	0.272942	0.00313	0.170659	0.003173	0.412632	0.201963	0.093323	0.006457	0.003189	0.47905	0	0.036044	0	0	0.217362	0.048569	0.217924	0.013354	0.009737
ruminococcus	5.039319531	12.11623	0.003029	5.242162	34.25374	3.628133	6.689413	3.825348	28.02526	9.916174	34.57109	6.651649	9.295493	10.47327	5.476261	79.9739	7.415296	15.01079	8.994776	4.985955	39.38285	3.963498	6.793977	6.397481
intestinimonas	0.086663457	0.108592	0	0.040516	0.126307	0.009986	0.068866	0.10626	0.034901	0.074729	0.141728	0.080451	0.074261	0.121189	0.154215	0	0.180222	0.047214	0	0.043472	0.197513	0.010215	0.053417	0.077899
eubacterium	3.675172524	5.676396	0	5.092564	4.922462	5.818327	1.699743	3.693328	4.146837	4.087335	8.096234	3.98391	2.773473	4.21291	5.374545	0.978764	4.240121	6.3908	1.790772	4.905698	1.635151	3.85113	4.7875	5.939823
anaerotruncus	0	0.029616	0	0	0.084205	0.0233	0.00313	0.00322	0.082493	0.038989	0.017716	0.03218	0.006457	0.025513	0.059061	0.002966	0.003277	0.104546	0.009442	0.006688	0.142469	0.040861	0.143558	0.006492
filifactor	0.003209758	0	0	0	0.010526	0.006657	0.00313	0	0	0	0.003543	0	0.003229	0.03827	0.009843	0	0	0.023607	0.018883	0	0.012952	0.003405	0	0.003246
pelomonas	0	0	20.4107	0	0	0	0.006261	0.00322	0	0	0	0	0	0	0.003281	0.017796	0	0.006745	0.003147	0	0	0	0	0
flavonifractor	0	0.003291	0	0	0.087713	0.006657	0	0	0.092011	0	0	0.003218	0.083947	0.130756	0.009843	0.548701	0.003277	0.067449	0.059797	0	0.168372	0.01362	0.020031	0.055179
bradyrhizobium	0	0	0.702668	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.003238	0	0	0
citrobacter	0.086663457	0.026325	0	0.012466	0	0.106514	0.046954	0.00644	0	0.266424	0	0.06436	0	0.025513	0.101716	0	0	0.023607	0	0.110353	0	0.187279	0.170267	0
coprococcus	0.134809822	0.118464	0	0.034283	0.259631	0.073228	0.065736	0.06118	0.069801	0.058483	0.109839	0.028962	0.077489	0.082919	0.121403	0	0.186775	0.020235	0.006294	0.073569	0.165134	0.057886	0.033386	0.107112
fusicatenibacter	0.003209758	0	0	0	0	0	0	0.00644	0.02221	0	0.003543	0.009654	0.016144	0	0.006562	0	0.00983	0	0.003147	0	0	0	0.003339	0.009737
desulfomicrobium	0	0	0.639065	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
turicibacter	1.200449366	0.111883	0	0.573459	0	0.632427	0.009391	0.985317	0.003173	0.763532	0.248025	0.640386	0.016144	0.003189	0.561079	0	0.026214	0	0	1.060059	0.032379	0.166848	0.020031	0.016229
peptoclostridium	0.077034184	0.016453	0	0.0187	0.059645	0.016643	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
roseburia	0.003209758	0	0	0	0	0	0.012521	0.00322	0	0.003249	0.007086	0	0	0	0.173902	0	0.00983	0	0.015736	0.013376	0	0.010215	0	0.236944
marvinbryantia	0.060985396	0.029616	0	0.024933	0.017543	0.039943	0.018782	0.03864	0.041246	0.042238	0.01063	0.038616	0.038745	0.009568	0.062342	0	0.016384	0	0.006294	0.060193	0	0	0	0.003246
bacillus	0.189375702	0.052651	0	0.190114	0	0.525913	0	0.235059	0.072974	0.162454	0.27637	0.302494	0.135606	0.27108	1.023723	0	0.134347	0.408067	0.182539	0.247459	0.191037	1.423318	0.340534	0.736798
dorea	0.500722195	0.207312	0	0.311662	2.64192	0.302899	0.134602	0.280139	1.875119	0.510105	0.705099	0.95897	0.309957	0.449675	0.154215	0.296595	0.245757	0.306893	0.072386	0.454789	0.43712	0.357532	0.620973	0.399234
sporanaerobacter	0.375541647	0.338938	0	0.130898	0.870114	0.186399	0.175296	0.550618	0.171331	0.52635	1.590901	0.405471	0.348702	0.108432	0.298586	0.367778	0.907661	0.16525	0.119595	0.324371	0.165134	0.245165	0.317164	0.16229
ralstonia	0	0	71.2542	0.003117	0.007017	0	0.012521	0	0	0	0	0.009654	0.003229	0.006378	0	0.041523	0	0.003372	0.006294	0	0.006476	0	0.003339	0
subdoligranulum	1.454020221	6.005463	0	1.090818	14.61652	1.118397	45.20128	4.456466	1.14855	1.387355	3.585728	3.726468	1.753196	9.806736	0.328116	0.020762	1.907071	9.196682	1.041732	0.504949	4.59785	44.72555	61.27266	4.751858
lactobacillus	0.320975766	4.198888	0.006057	0.433211	0.989404	10.03894	0	0.856517	0.364871	0.133212	0.350778	0.022526	0.003229	0	0	0.002966	0.612753	0.003372	0.103859	3.437667	0.006476	0.003405	0	0
salinisphaera	0	0	1.223612	0	0.003509	0	0	0	0	0	0	0	0	0	0	0.005932	0	0	0	0.003147	0.003344	0.003238	0	0
faecalicoccus	0.654790563	0.072395	0	0.102849	0.652586	0.64907	0.716835	0.206079	0.463227	0.334655	1.796407	0.379726	0.071032	0.041459	0.190307	0.183889	0.845403	0.890328	0.314723	0.327715	0.618443	0.187279	0.196975	0.146061
streptococcus	0	0.003291	0	0	0.003509	0	0.00313	0	0	0	0	0	13.67687	0.009568	0.009843	10.4965	0.006554	30.40604	42.26097	0.013376	0.126279	9.346908	0	0.003246
shigella	0.003209758	0.006581	0.003029	0.003117	1.891095	0	0.00313	0.00644	0	0.029242	0.017716	0.012872	0.006457	0.003189	0.003281	0.011864	0	0.006745	0.015736	0.003344	0.210465	0.939798	0.020031	0
clostridium	4.583533943	5.429596	0	3.758649	6.024139	4.503545	2.472923	4.524086	4.597373	5.179024	9.82532	5.51247	4.581558	4.675341	4.695344	3.318899	5.881775	9.544044	5.035564	5.514312	15.29918	7.811223	5.855841	3.898212
oscillospira	0.093082972	0.013163	0	0.049866	0.242088	0.039943	0.034433	0.0644	0.025382	0.07148	0.131099	0.045052	0.035516	0.044649	0.036093	0.026694	0.163838	0.043842	0.009442	0.043472	0.148944	0.108962	0.043401	0.06167
sediminibacterium	0	0	0.33619	0	0	0	0	0	0	0	0	0	0	0	0	0.002966	0	0	0	0	0	0	0	0
eggerthella	0.285668432	0.161243	0	0.068566	0.698197	0.176414	0.062606	0.177099	0.139603	0.21119	0.308259	0.180209	0.067803	0.133946	0.101716	0	0.1409	0.145016	0.050356	0.19061	0.955187	0.027241	0.136881	0.123341
candidatus soleaferrea	0.083453699	0.154661	0	0.140248	0.319276	0.203042	0.093908	0.14168	0.190367	0.084476	0.56337	0.151247	0.20341	0.165837	0.295305	0.246174	0.563602	0.195602	0.037767	0.15717	0.378837	0.156633	0.166928	0.20124