Metatranscriptomics Analysis of Ileum Microbial Communities in Necrotic Enteritis Chickens

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Animal Science

by

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ABSTRACT

Necrotic enteritis (NE) is a recognized multifactorial disease that causes an annual loss of $6 billion to the poultry industry. Thus, it has become essential to carry a comprehensive study of the composition of chicken gut which hosts this disease. The objective of this research is to measure the changes occurring in microbiome inside intestinal ilium of NE affected broilers which were treated with tributyrin supplement. This supplement is an anti-inflammatory metabolite that stabilizes intestinal integrity. The experimental design consisted of two challenge groups (No - Challenge, Clostridium Perfringens (CP) - Challenge) and one treatment group (Tributyrin). To our knowledge, this project was the first research employing metatranscriptomics approach on ileum part of intestine.

The metatranscriptomics analysis resulted in 7796 transcripts. It was found that only six bacteria showed significant differences (~5%) on a species level amongst the three groups for CP challenge, No challenge and tributyrin samples. Bacteria such as Geofilum rubicundum and Lactobacillus were abundantly found in tributyrin group as compared to other two. This could be a contributor for the treatment of NE. An unexpected result was found for the bacteria Clostridium Perfringens. This bacterium was supposed to increase in the CP challenge group. Instead, it was found to have decreased as compared to tributyrin group.

Although butyrate (tributyrin) has numerous properties to treat NE in chicken, tributyrin seemingly didn’t appear to a contributor for the treatment of NE. Additional results from this work pointed towards Geofilum rubicundum as a prime candidate which may have contributed the most towards NE treatment. Thus, this work establishes a strong foundation to continue detailed research on treatment methods for NE through further assessment of Geofilum rubicundum, and potentially evaluating a bigger sample size.
Keywords: *Clostridium perfringens*, intestinal permeability, metatranscriptomics, Necrotic Enteritis, Tributyrin
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DEDICATION

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# TABLE OF CONTENTS

CHAPTER- 1 .................................................................................................................. 1
Literature Review .............................................................................................................. 1

1 Introduction .................................................................................................................... 2
1.1 Necrotic Enteritis in Poultry ...................................................................................... 3
1.1.2 *Clostridium perfringens* Toxins and Enzymes in Virulence ................................. 4
1.1.3 Prevention of Necrotic Enteritis using nutrient supplements ............................... 4
1.1.4 Details of Butyrate in Broiler Chickens ................................................................. 5
1.2 Molecular tools to study microbiome ........................................................................ 6
1.2.1 Introduction of Sequencing Technology ............................................................... 7
1.2.2 Transcriptomics: Tools and Applications ............................................................ 8
1.2.3 Microbiome Analysis ........................................................................................... 8
1.2.4 A typical workflow for metatranscriptomics study .............................................. 9
1.2.5 Challenges in RNA Sequencing ........................................................................ 11
1.3 Metatranscriptomics and Gut Microbiome ............................................................... 12
1.4 Conclusion ................................................................................................................ 14
REFERENCES .............................................................................................................. 15

CHAPTER-2 .................................................................................................................. 21
Comparative evaluation of four RNA extraction methods in the isolation of Total RNA from tissue, fecal, and nasal samples ........................................................................... 21

2.1 Abstract ..................................................................................................................... 22
2.2 Introduction .............................................................................................................. 24
2.3 Materials and Methods ........................................................................................... 26
2.3.1 Extraction Samples ............................................................................................. 26
2.3.2 Preservative materials ........................................................................................ 26
2.3.3 RNA Extraction kits .......................................................................................... 27
2.3.4 RNA Extraction Process ..................................................................................... 27
2.3.5 Quality and Quantity Measurement .................................................................... 30
2.4 Results ..................................................................................................................... 31
2.5 Discussion ............................................................................................................... 33
2.6 Conclusion ............................................................................................................... 35

Table 1 Nanodrop Spectrophotometer results for quantity estimation .......................... 36
Table 2 Agilent Bioanalyzer results for RNA Integrity/ Quality ....................................... 38
Metatranscriptomics analysis of Ileum microbial communities in Necrotic Enteritis chickens ...

3.1 Introduction .......................................................................................43
3.2 Material and Methods ........................................................................45
3.2.1 Animal Experiments and Sample Collection ..................................45
3.2.2 RNA extraction of Ileum tissue samples ........................................46
3.2.3 Sample selection for metatranscriptomics sequencing .....................46
3.2.4 DNA digestion ..............................................................................47
3.2.5 Library Preparation and Sequencing .............................................47
3.2.6 Annotation and analysis of metatranscriptomics .............................47
3.2.7 Statistical Analysis .........................................................................48
3.3 Results .............................................................................................49
3.3.1 Samples Selection ............................................................................49
3.3.2 Sequencing results and top 30 transcripts of Control- No- Challenge, tributyrin-CP treatment, and CP - challenge groups .................................................49
3.3.3 Most abundant bacteria in tributyrin, Control and CP challenge samples .................................................................51
3.3.4 Low Variance occurrence in all three groups ..................................51
3.3.5 Healthy, CP and tributyrin samples maintain significant differences in specific gene expression .................................................................52
3.3.6 Actin cytoplasmic 2 increased in the Tributyrin- CP- treatment group ....53
3.3.7 Community-wide transcriptomes suggest differences in nutrient availability between control and tributyrin samples ................................................................................................................53
3.3.8 Results of CP, Geofilum rubicundum, Crocosphaera Watsoni ..............54
3.4 Discussion ..........................................................................................55
3.5 Conclusion ..........................................................................................61
Figure 1 Absolute and relative abundance of transcripts .............................62
Figure 2 LDA score of tributyrin, No-challenge group and CP-Challenge Group .................................................................63
Figure 3 Comparison among CP, No challenge, and tributyrin groups using LEfSe .........................................................63
Figure 4 Comparison among CP, No challenge and tributyrin using PCA plot .................................................................64
Figure 5 Heat map of abundant bacteria among CP, No and tributyrin groups ........................................................................65
Figure 6 Absolute abundance between C. abortus and C. Psittaci counts ........................................................................66
CHAPTER- 1

Literature Review
1 Introduction

Poultry meat is an integral part of today’s human food supply, providing high quality meat protein, vitamins, and minerals at less cost than its counterparts. With a growing world population, the consumption of poultry products continues to rise at an increasing rate each year. Shahbandeh et al., reported that global poultry production solely through 2018 was approximately 95.5 million metric tons and estimated an increase of 3-5 million metric tons during every consecutive year [1, 2]. Meeting this demand, the food industry must also strive to ensure the highest quality of animal health and safety standards to produce a nutritious food fit for worldwide consumption.

Live poultry is susceptible to bacterial, parasite, and viral infections and diseases. Among these, bacterial diseases are one of the biggest culprits to affect the poultry. Necrotic enteritis, a bacterial disease affecting the intestine, causes an annual loss of $6 billion to the poultry industry [1]. Primary reasons for such revenue losses are due to lack of production yield, higher mortality rates, and losses due to meat contamination. In order to prevent such losses, it is essential to conduct a thorough study of chicken gut microbiome which may affects the health of chicken in a negative way. Currently, such research to study gut health is conducted using various Omic’s techniques such as metagenomics, bacterial cultivation, metaproteomic, metabolomics and targeted amplicon sequencing (DNA, RNA). However, these techniques have certain limitations, such as the inability to analyze functions of a specific microbe or cannot differentiate an active bacterium from an inactive one. Such limitations can be overcome with an emerging technology such as metatranscriptomics and stable isotope probing. In this project, metatranscriptomics is used to analyze the bacteria of chicken intestinal tissue infected with Necrotic Enteritis.
1.1 Necrotic Enteritis in Poultry

In chicken’s necrotic enteritis is caused by toxigenic *Clostridium Perfringens* and *Eimeria Maxima*. *Clostridium perfringens* is the primary agent for NE and rare reports were found for *Eimeria Maxima* in NE broilers. In our research we studied toxigenic *Clostridium perfringens* which is a gram-positive spore-forming anaerobe, usually occurring in 4-week-old broiler chicks. Primary causal agents of this disease are two strains of *C. perfringens*, type A, and type C [3]. Type C strain of *C. perfringens* is a member of chicken intestinal microbiota which does not usually result in NE [3]. NE is present either as a clinical and subclinical disease [4, 5]. The clinical sign shows its symptoms from the two weeks of age in poultry. The symptoms include dehydration, ruffled feathers, diarrhea and apathy. These birds normally die within few hours after the infection of NE. In a subclinical disease, NE birds die within one to two weeks after infection. In subclinical case, intestinal damage can permit the bacteria to penetrate into the bile duct and portal bloodstream, which can lead to cholangiohepatitis [6]. Cholangiohepatitis is the inflammation of the gall bladder and liver. Subclinical cases are typically characterized by ulcers as depressions in the mucosal surface and reduction of feed conversion ratio [8]. Because this disease is generally not identified quickly enough, it is difficult for poultry producers to treat NE in its early stages, leading to heavy economic losses [8, 9].

1.1.1 Factors Predisposing to NE

A diet rich in non-polysaccharides, such as wheat, oat, barley and fishmeal, predisposes chickens to NE because intestinal contents become highly viscous, leading to a reduction in peristaltic movement [10, 11]. Even small intestine damage is likely to cause NE through the release of protein from the diet, which encourages the growth of *C. perfringens*. Moreover, poor poultry
management practices such as inadequate ventilation, overcrowding, wet litter, coccidial infection and sudden changes in the diet can induce stress in birds, leading to NE [12, 10].

1.1.2 *Clostridium perfringens* Toxins and Enzymes in Virulence

*Clostridia* is most toxigenic compared to other bacterial genera [13]. Among all *Clostridia* toxins, alpha-toxin and NetB (Pore Forming toxin) are described as essential factors to cause necrotic enteritis in chickens [14, 15]. The effects of the alpha-toxin are seen on the mucosal and serosal surfaces of the jejunum in chickens. This toxin causes the electrogenic secretion of anions, thereby diminishing sodium/glucose co-transport from the mucosal to the serosal side of the jejunum, contributing to NE pathogenesis [16].

1.1.3 Prevention of Necrotic Enteritis using nutrient supplements

Many countries are banning the use of antibiotics for the prevention of diseases especially NE. Therefore, researchers are working on ways to control NE with the help of non-antibiotics or nutrient supplements through a broad range of strategies. These include a use of various dietary compositions, prebiotics, probiotics, and vaccinations. The coccidia infection is an important predisposing factor for the production of toxigenic *Clostridium perfringens* in chickens. Therefore, poultry producers are using vaccinations and chemicals to control the growth of coccidia in broilers [17]. Researchers recommended use of many supplementary nutrient against NE such as organic acids such as SCFA and medium chain fatty acids, enzymes such as xylanase, bacteriophage etc. [17]. Dahiya *et. al.*, (2007) found that the inclusion of methionine in the diet at relatively higher concentrations (0.8%) may reduce intestinal *C. perfringens* colonization [9]. According to Bortoluzzi *et. al.*, (2017), butyrate alleviates the adverse effects of NE in chickens by selectively modifying the microbiota of the intestine, immune and support system, and the barrier function of intestine [7, 17]. In addition, butyric acid (butyrate) also showed many evidences for positive
response against NE such as increase weight gain, change in feed conversion ratio and bacteriostatic, bactericidal effects in NE infected broilers. In summary, moving away from antibiotic drugs to control NE has led researchers to address the prevention of a disease that is troublesome to broiler chicken production through a wide variety of alternate strategies.

1.1.4 Details of Butyrate in Broiler Chickens

Butyrate is used as a feed additive in chicken. It is a favorable choice to maintain optimal gut health and stimulate growth performance. It can affect chickens in terms of age or health status [17]. Supplements of unprotected butyrate lead to a significant increase in butyrate concentration in the gastric region of chicken as well as pigs. However, it does not increase in the jejunum of chicken, [18] and cecum, colon, and rectum of pigs [19]. As a result, unprotected butyrate affects mainly the epithelial cells and microbiota of the crop, proventricular, and gizzard of a broiler. Many researchers have conducted a study to show that a release of butyrate in the distal gastrointestinal tract of chickens, can support a healthy microbiota [20]. Butyrate can assist with weight gain, voluntary feed intake, and FCR of a broiler chicken. Some research shows that there are many variations for the effects of butyrate on factors such as growth performance in avian [20, 21]. Butyrate is also a promising supplement which can be an energy source for animals, reduce inflammatory cytokines, prevent intestinal pathogens and help in the growth and development of gut [21]. In chicken, butyrate is released from the Tributyrin (Triglycerides) of the intestinal lipase, and then it is absorbed by the small intestine. It also promotes chicken intestinal health. Butyric acid helps to manage the quality and performance of poultry, mostly in birds which are vaccinated by coccidia infection.
1.2 Molecular tools to study microbiome

Historically, the chicken gut microbiota has been studied using classical culture-based methods; however, it has many drawbacks. For example, the bacteria from the uncultured bacteria are recovered by selective media under certain growth factors [22]. A total of 52 microbial phyla were observed over a decade ago and these phyla were only half cultured which supported the term “uncultivated half” of organism [23]. Thus, total count of species and count of species based on relative abundance in barrier (diversity) of intestine have been misjudged from the term “uncultivated half” and the information about the microbial community in gut remains insufficient [24]. Various molecular techniques are available on the market each with different strengths and weakness to study chicken microbiota. The most common molecular techniques used are 16S rRNA sequencing (high-throughput), fingerprinting (e.g., DGGEa, TGGEb, TTGEc, T-RFLPd, and SSCP), gene quantification technique such as FISH6, DNA microarray and diversity array. From all these strategies, next-generation or the high-throughput (NGS) sequencing (16SrRNA) is a great tool to explore the ecological and biological significance of the gut microbiota [25]. NGS has become a handy, fast, precise, and low-cost technique for genomic investigation [26, 27]. NGS provides benefits such increased throughput, swift processing speed, and costs that are really low for processing of the sample. The Illumina HiSeq and MiSeq instruments of NGS are widely used methods to study metagenomes of chicken gut microbiome. There are disadvantages of using this platform, such as short read assembly and high cost. Currently, QIIME/ Mothur are the two, open source tools for bioinformatics analysis which utilizes public database for it working. The databases such as SILVA, GreenGenes are effectively employed to study taxonomy of the bacteria. Metatranscriptomics methods can be employed to classify roles of genes or analysis of specific genomes which can be utilized to give information on community diversity, framework, and gene
expression [28]. The data of these tools are complex than 16S amplicon information and generally involve a sequence assembler like MG-RAST or Velvet to reduce the intricacy of study.

1.2.1 Introduction of Sequencing Technology

Lately there have been considerable developments in sequencing technology, especially with regards to the research of the complicated microbiome. The majority of the high throughput sequencing is actually centered on DNA sequencing using PCR amplicon sequencing of 16S rRNA genes or shotgun sequencing of the DNA sample that is available (metagenomics). These strategies have contributed a lot of breakthroughs in the previous ten years, helping to characterize microbiomes more effectively [29, 30, 31]

The main disadvantage of the 16S technology is that it can only provide the microbiome information at the taxonomic level. However, it is a highly economic option available to capture the variation of the relative and absolute abundances of many biological samples. Lately, the shotgun method has turned into a main method for sequencing, which enables capturing the majority of the microbiome while elucidating prospective genes and purposeful pathways.

Although, it has a limitation that it does not distinguish the live activity of the microbiome. RNA sequencing helps to record an expressed transcript within the microbiome of a particular environment and functions, which provides a closer look at the active members. With the help of metatranscriptome, low transcripts can not only be detected but also mapped and annotated to metabolic pathways. The qPCR technique is widely used to measure the long and short RNA to find the quantity of the of the gene’s expressions.

In the late 1999, the metatranscriptome technique was introduced. This technique has been employed to characterize proactive microbes in a community [32], discover community associated novel microbial interactions [33], and track the phrase of genes [34].
Nevertheless, metatranscriptomics technique also has many limitations such as experimental design, sample compilation as well as necessity of proper storage (RNA degrades at room temperature). Therefore, to reduce degradation preservative samples such as RNAlater™, dry ice, liquid nitrogen etc. are used.

Because of the complex data in the metatranscriptome study, it is not able to capture details transcripts of some microbial community. In addition, other disadvantages of this study are difficulty in RNA isolation, dynamic range of transcript expression.

1.2.2 Transcriptomics: Tools and Applications

The metagenomics technology cannot find the whole functions of the microbial community. Therefore, a community is better studied by using metatranscriptomics technology. This technology also gives information thoroughly about the gene expression. The functional annotation of genes in this technique helps to provide the role of community of a particular host in a specific condition. Metagenomics helps to address the concerns regarding microbial composition. Whereas metatranscriptomics helps to resolve the concerns of transcripts which are jointly expressed under various conditions.

1.2.3 Microbiome Analysis

Metatranscriptome is a science which studies microbial gene expression in the particular environment which allows obtaining total gene expression profiling of complicated communities. The study of this advance technology began in the early 1990’s and progressed quickly in the consecutive years. The RNA sequencing refers to sequencing of transcript cDNA and is significantly impacted by the enhancement of NGS [35, 36]. After 2008 RNA- seq improved when the new Solexa/Illumina technologies (San Diego, CA) allowed 100 transcript sequences to be captured [37, 38, 39, 40]. Genome transcriptome is encoded by the whole transcript, which
includes protein-coding mRNA and non-protein-coding RNA such as microRNA (miRNA), small RNA, and long non-coding RNA (lncRNA). A narrow sense transcriptome is a collection of RNA expressed in a specific cell or tissue type under a particular condition.

There are several methods of data generation for RNA transcript, which can be achieved with two essential principles:

1. ESTs (Expressed Sequence Tag) or RNA sequencing: It utilizes the advanced high-throughput sequencing to record all the sequences. For the early microarray designs EST is the method which provided sequence information. SAGE (Serial and Cap evaluation of gene expression) is amongst the advancements of EST strategy which raises the throughput of the tags created and also enables some quantitation of the transcript abundance [41]. SAGE is an alternate variant method of CAGE, and their sequence tags from tail 5’ of the mRNA only. [42].

2. Microarrays: This technique quantifies a set of predetermined sequences.

Microarray was developed in the mid-1990s to early 2000s that comprises the probes which are short nucleotide oligomers. The disadvantage of this technique is it does not have its own knowledge of bacteria. Microarray takes an information from the existing EST library. It typically relies on two crucial technologies; micro and nanofabrication.

1.2.4 A typical workflow for metatranscriptomics study

A typical workflow for metatranscriptomics study consists of isolation of RNA, library preparation, sequencing, and data analysis.

1. Isolation of RNA

Isolation of the RNA is the initial step for metatranscriptomics study from an experimental organism. The isolated RNA might be also treated if the samples are highly contaminated by using the DNase digestion process. This process helps to break down the remaining traces of DNA from
the sample. Protein, RNA/DNA could be extracted from any sample such as existing or maybe conserved tissues, virus, cells, bacteria.

RNA extraction methods are broadly comparable and entail the subsequent steps: 1) physical disruption of RNase by using chaotropic salt such as ethanol, guanidinium etc. [44], 2) Breaking of nucleotide complexes, 3) separation of RNA from undesired biomolecules such as nucleic acids (DNA), and concentrating RNA via precipitation from elution [45, 46]. The 98% of total RNA is made from rRNA (ribosomal RNA) therefore, mRNA enrichment is important as it is present only at 2% in total RNA [47]. This enrichment can be also accomplished by decreasing ribosomal RNA using certain probes and poly-A-affinity methods [48].

The quality of RNA is driven by quality of the method used for extraction. RNA quality is usually assessed using an Agilent bioanalyzer, that offers RNA Integrity Number (RIN) between one and ten, where 10 indicates no or less contamination of samples. RIN estimates sample integrity using gel electrophoresis as well as evaluation of the proportions of 28S to 18S ribosomal bands. Low-quality RNA (RIN < 6) can considerably impact the sequencing benefits and lead to incorrect biological conclusions. Therefore, high quality RNA is crucial for effective RNA Seq experiments.

The quantity of RNA is measured using a conventional spectrophotometer such as Nanodrop Spectrophotometer. If the isolated RNA degrades, it can affect further results downstream. For example, if mRNA is degraded, then it would result in depletion of 5’ mRNA ends and uneven signal across the length of a transcript. In the isolation of RNA, snap freezing of RNA by liquid nitrogen, dry ice, or RNAlater™ is a standard method. It is vital because isolated solutions should not be degraded.

2. Library Preparation Methods
After RNA extraction, the next phase is preparation of the RNA library. The building of sequencing library principally entails isolating a desired RNA molecule, reverse transcribing the RNA to cDNA, amplifying as well as ligating sequencing adaptors. Before constructing RNA Seq libraries, an ideal library planning protocol must be selected which will improve or perhaps deplete an overall total RNA sample for a particular RNA species

3. RNA Sequencing Platforms for Transcriptomics

A selection of the sequencing platform is essential for RNA sequencing. There are several NGS platforms available on market [49]. Majority are high throughput platforms that use a synthesis method. One such famous platform is provided by Illumina [50]. The main advantage of using that platform type is that it has a very low sequencing error rate [51]. Illumina HiSeq platform is widely used in next generation sequencing, which may take between two to twelve days to finish, based on the complete read measurements of the library.

SMRT (Single Molecule Real-Time) sequencing is another platform that uses DNA polymerase to perform continuous template synthesis using fluorescently labeled nucleotides. The advantage of this platform is that it does not include the PCR amplification step, but its disadvantage is it has high error rates (~5%) that is mostly related to insertion and deletion step. [52].

1.2.5 Challenges in RNA Sequencing

RNA sequencing is now being used extensively for revealing numerous aspects of the transcriptome to help promote biological applications. Such a technique allows us to gain an understanding of the transcriptome and help us to interpret the information in the genome on protein expression. Although widely used, RNA sequencing presents many challenges.
A. Isolation of RNA from samples: Throughout the extraction process, samples should be kept between 1-4°C. There are several extraction kits available on market. Thus, the kit should be compatible with the type of sample you wish to process.

B. Library Preparation: First challenge of RNA library preparation is that it should be able to identify and quantify all RNAs. Next problem is that, many times short reads of sequencing are formed which are identical and thus challenging to discriminate from each other. It is often a manifestation of abundant RNA species or perhaps could be PCR artifacts. A method to distinguish these short reads is determining whether the same sequences are found in various biological replicates or perhaps not. An additional issue is preparing strand specific libraries which is laborious since it requires several steps. [53]

C. Bioinformatics Challenges: Like various other sequencing technologies, this level also has a number of difficulties, such as improvement of effective techniques to store, access, and run a huge amount of information. These issues have to be conquered to minimize errors in image analysis, base calling, as well as by getting rid of bad reads. Another issue is that, it needs a development of computational techniques in order to recognize novel splicing activities which take place between two sequences or maybe exons from two different genes.

D. Coverage Versus Cost: Generally, bigger the genome, much more complicated is the transcriptome. Besides, much more sequencing level is necessary for proper coverage. Higher the sequencing level, greater the price. Unlike genome sequencing coverage, it's harder to compute the coverage of transcriptome.

1.3 Metatranscriptomics and Gut Microbiome

In recent years, gut microbiome has come out as a pivotal player to influence human as well as animal health. Its primary functions are related to competing with pathogen, regulation, and
stimulation of immune system, strengthening of intestinal barrier, etc. [54, 55, 56]. Although a thorough study of gut microbiome has already been conducted in the past, continuous research on newer advanced technologies and tools is essential to keep innovations and discoveries going. One such recently emerged technology is metatranscriptomics. It allows one to look at changing gene expressions on a broader and more dynamic scale as compared to metagenomics. One such instance of a metatranscriptomics application is detailed in the study of gut microbiome inside patients suffering from inflammatory bowel disease (IBD). The outcome of this study and through application of metatranscriptomics was the diagnosis of dysbiosis in such patients. It was found that the microbial taxonomic profile was also different for each patient, thus it was difficult to interpret about the bacterial species which helped in the progression of disease. With the help of metatranscriptomics, the genes and pathways encoded in its genome provided indirect information about the activation level of microbiome. This way, the functions of changing gene expressions could be studied adequately resulting in correct diagnosis of IBD. Thus, this novel technique can prove to be an essential tool in understanding the mechanism of gut microbiome dysbiosis [57]. Such benefits cannot be attained by metagenomics, and any other similar technologies since the features of the microbial community are actually tough to disclose. Another benefit of metatranscriptomics is actually to skip in situ assaying of Mucus/Oxygen, and that enables the research of the consequences of environmental modifications on microbial expression patterns in vivo. As a result of such advantages, metatranscriptomics profiling has emerged as an essential tool for understanding the relationship between the gut microbiome and the host physiology. So far, the study of chicken microbiome has only been conducted using metagenomics, metabolomics, metaproteomics, and bacterial cultural technologies. This research is the first study of chicken gut that uses metatranscriptomics technology.
1.4 Conclusion

Necrotic enteritis leads to a loss of billions of dollars every year for the poultry industry. Thus, it is crucial to reduce the virulence of it. Butyrate worked as a promising supplement to treat NE in the broilers. To study chicken intestine, metagenomics technology is widely used, but metatranscriptomics study gives results of all taxa with the functional analysis of each taxa. The microbiome research done so far only covered the cecum part of chicken intestine. This novel work focuses on the study of microbiome microbial community in the ileum part of the chicken by using metatranscriptomics technology.
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Christina A. Hickey, Kristine A. Kuhn, David L. Donermeyer, Nathan T. Porter, Chunsheng Jin, Elizabeth A. Cameron, Haerin Jung, Gerard E. Kaiko, Marta Wegorzewska, Nicole P. Malvin, Robert W.P. Glowacki, Gunnar C. Hansson, Paul M.


Comparative evaluation of four RNA extraction methods in the isolation of Total RNA from tissue, fecal, and nasal samples.
2.1 Abstract

Isolation of high-quality ribonucleic acid (RNA) from biological samples of animals (tissue, fecal, blood) is one of the pre-requisites for many downstream molecular biological processes like cDNA library construction, real-time PCR analysis, northern blotting, microarray analysis, transcriptomics, etc.

In this study, we extracted the RNA using four types of methods, three of which involves using commercial kits, and one uses a non-commercial protocol. These three kits were, the Stool Total RNA Purification Kit (#49400 Norgen Biotech Corporation, Thorold, Canada) [13], RNeasy Mini Kit (#74104 Qiagen, Germany) and the RNeasy PowerMicrobiome Kit (#26000-50 Qiagen, Germany). The non-commercial method was modified from the Directzol kit [14].

The objective of this study was to determine which kit provides the best yield and purity of the extracted RNA. For this, the concept of RIN (RNA integrity number) was used.

The RNA was extracted from three types of samples: fecal samples from pig, nasal samples from cow, and tissue samples from the cecum and ileum of poultry. The samples were treated using four different types of preservation methods, which includes RNaLater™, Liquid nitrogen, Zymo DNA/RNA Shield, and Dry ice. Positive control with known bacteria (Pseudomonas aeruginosa, Staphylococcus aureus) was also involved in these RNA extraction methods. The yield and integrity of RNA were measured using the Nanodrop spectrophotometer and 2100 Agilent Bioanalyzer.

Norgen stool isolation kit provided the best RNA integrity (RIN-7) for the fecal samples treated with RNaLater™. For the nasal samples, RNeasy PowerMicrobiome kit proved to be the best (RIN-8.5) with the treatment of Zymo shield, and for the tissue samples, the RNA mini kit yielded
the best quality (RIN- 9.7) when preserved in RNAlater™ which we used for metatranscriptomics study.

Keyword- Extraction, fecal, nasal, tissue, preservative materials.
2.2 Introduction

The first and the most important step in DNA/RNA sequencing is the extraction of nucleic acid from the biological sample. Nucleic acid, such as DNA and RNA can be extracted from any biological material such as tissue, cells, viruses, blood, or other kind of biological samples. Three important steps that are incurred in the isolation of nucleic acid are 1) disruption of tissue, 2) denaturation of nucleoprotein 3) inactivation of nucleases. [1]. Extracted nucleic acid must be free from contaminants like carbohydrates, proteins, lipids and nucleic acids where “DNA totally free of RNA” or perhaps “RNA totally free of DNA”. If the samples are contaminated, then it could directly impact the quantity as well as integrity of outcomes of all succeeding scientific studies [2].

Nucleic acid, such as RNA, is an unstable molecule compared to the DNA and has a very short half-life once extracted from the cell or tissues. Therefore, the extraction of RNA is difficult as compared to DNA. There are different types of RNA such as ribosomal RNA (rRNA) (>80%), messenger RNA (mRNA) (<5%), and transfer RNA (tRNA) [2]. Special attention is necessary for RNA extraction as it is highly vulnerable to degradation [2][3]. Good quality RNA extraction relies on keeping the sample RNase free, and following good laboratory techniques [1]

Three kinds of RNA extraction method are mostly prevalent. These methods are utilization of 4 M guanidinium thiocyanate, phenol, and SDS/silica matrices [1]. There are various types of materials such as silica particle, glass particles, and microfibers which are used to physically disrupt a biological sample in the very first step of extraction [4].

The use of guanidinium isothiocyanate in the RNA extraction was initially pointed out by Ulrich et. al., (1977). However, it was a very laborious procedure. Thus, it is been displaced by a single step strategy of guanidinium thiocyanate-phenol-chloroform extraction which reduces the pH.
The concept of silica matrices purification follows the principle that negatively charged DNA is attracted towards the positively charged silica debris [5]. The beads, powder, glass particles are beneficial for nucleic acid decontamination [4].

To determine the sample impurity a ratio of sample absorbance at 260 and 280 nm (A260: A280) is determined by using Fluorescence method and Agarose Gel Electrophoresis techniques. The principle behind this technique is that the RNA is absorbed at 260nm while the contaminants are absorbed at 280nm. In our project to observe the concentration and 260/280 and 260/230 ratio we used Nanodrop Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). The entire RNA samples with values ≥1.8 for 260/280 ratios are acceptable for further molecular analyses [7].

To study the integrity or quality of the nucleic acid samples RIN (RNA Integrity Number) has become standard benchmark for analysis. These metrics indicate the amount of RNA fragmentation and may be received via lab-on-chip gel electrophoresis methods [8]. BioRad (Experion) and Agilent Technologies are the two big providers of these gel electrophoresis equipment and kits. We used the Bioanalyzer 2100 from Agilent Technologies in this project. RNA RIN value that is more than 7-8 is usually regarded as an optimum template for many molecular applications [8, 9, 10, 11, 12]. Nevertheless, the minimum appropriate RIN, is dependent on the kind of analysis to be completed. For instance, a few researchers have discovered RNA with RIN values between 5 and 5.5 offers a good template for qPCR. [8, 9, 10]. Our findings also showed variation in the RIN number for nasal, tissue and fecal samples.

The goal of this study is to compare the performance of three commercial and one non-commercial RNA (Modified kit) extraction kits to isolate total RNA from chicken intestinal tissue, nasal sample of cow, and fecal samples of pigs (average mass of <20 mg) with four different preservative
materials. In order to accomplish this goal, total RNA was isolated from fecal, nasal, and tissue sample using the following kits: 1) Stool Total RNA Purification Kit (#49400 Norgen Biotech Corporation, Thorold, Canada)[13] 2) RNeasy Mini Kit (#74104 Qiagen, Germany) 3) RNeasy PowerMicrobiome Kit (#26000-50 Qiagen, Germany) 4) The modified version of Direct-zol Kit [14].

The samples were preserved in four different materials: 1) Dry ice, 2) Liquid Nitrogen, 3) RNA later™, and 4) Zymo DNA/ RNA shield. Quantity and quality of extracted RNA was compared to determine the most appropriate kit for high-throughput sequencing, RNA sequencing and metagenomics study.

2.3 Materials and Methods

2.3.1 Extraction Samples

The tissue samples were collected from 21-days old chickens, fecal samples from pig and nasal sample from cow. Tissue samples were collected from both, the ileum and cecum parts of the intestine and collected in the sterilized tubes. Nasal swabs were collected using a sterilized swab from cow nostrils, and the fecal samples were collected into a collection tube from the anus of pig. The Pseudomonas aeruginosa (gram negative) and Staphylococcus aureus (gram positive) bacteria were used as positive control for the research.

2.3.2 Preservative materials

In this project, four types of preservative materials dry ice, liquid nitrogen, RNA later™, and Zymo DNA/RNA shield, characterized by unique properties were selected for RNA stabilization and storage purposes.

The Invitrogen RNA later™ stabilization solution by Thermo Fisher is a nontoxic, aqueous cells storage reagent. This solution quickly stabilizes the tissue as well as protect the RNA. Zymo
DNA/RNA Shield mainly preserves the integrity as well as expression profiles of samples even at room temperature. This Shield totally inactivates infectious agents such as viruses, fungi, bacteria from the samples.

Dry ice and liquid nitrogen have been used traditionally for storage of samples. The temperature of the dry ice should be -109.3 °F and for liquid nitrogen for –320°F. All preservatives solutions were used to preserve fecal and tissue samples. However, for nasal swabs Zymo DNA/RNA shield was used in place of liquid nitrogen.

2.3.3 RNA Extraction kits

Various kits, based on the different types of extraction methods, are available in the market. In this project, we used the kits based on silica matrices, glass particles, and guanidinium thiocyanate-phenol. The total RNA was extracted using the following kits: 1) RNeasy Mini Kit (#74104 Qiagen, Germany), which uses RNeasy technology to simplify total RNA isolation by combining the guanidine-isothiocyanate lysis 2) RNeasy PowerMicrobiome Kit (#26000-50 Qiagen, Germany) in which, stool or biosolid samples were processed through a lysis step that uses bead beating and a strong chemical lysis buffer for efficient extraction of microorganisms. 3) Stool Total RNA Purification Kit (#49400 Norgen Biotech, Thorold, Canada) [13] whose purification was based on spin column chromatography. 4) Tri-zol Homemade kit based on reagent which is based on mono-phasic solution of guanidine isothiocyanate and phenol.[14]

2.3.4 RNA Extraction Process

1. RNeasy PowerMicrobiome (#26000-50, Qiagen, Germany)

Procedure

We started by adding 0.25 g fecal, tissue, nasal sample into a power bead tube. Approximately 650 µl solution of PM1–β-ME (ME-mercaptoethanol) was added to the power bead tube. The cell lysis
process was done by using vortex adapter. Next we added 150 µl IRS solution to remove inhibitors at 2-8º C. Six hundred and fifty (650) µl each of solution PM4 and solution PM3 was added. The samples were vortexed briefly to mix for the binding of RNA. The solution was washed by using PM5 three times. Later, removal of DNA was done by using 50 µl DNase I solution which was prepared by mixing 45 µl DNase Digestion Solution and five µl DNase I stock enzyme. Again, we washed the solution by using PM4 and PM5 solution to remove the remaining contaminants. Then 100 µl of RNase-free water was added in the center of the white filter membrane of the tube to clean the RNA from the filter membrane. Finally, we collected the RNA elute in the collecting tube. The detail protocol was followed by using the kit guide.

2. RNeasy Mini Kit (#74104, Qiagen, Germany)

In the RNeasy Mini Kit, the phenol as well as guanidine-based lysis were mixed. It also extracts RNA using silica membrane based on purification of complete RNA. Thirty- fifty mg biological samples were weighed and added (without thawing) to the buffer RLT (Tissues stabilized in RNAlater™) collecting tube to reduce the contamination. Later, these were disrupted and homogenized by using bead beater -16 (Biospec Mini Bead Beater Beadbeater 16 Homogenizer) for 3 min at 2000 RPM.

Next, the lysate was centrifuged for 3 min at a maximum speed of 1500 RPM after which the supernatant was carefully removed by pipetting. One volume of 70% ethanol was added to the supernatant and mixed well. 700 µL of solution obtained after adding ethanol was transferred to the given RNeasy Mini spin column and centrifuged for 15 seconds.

The spin column was washed three times by different buffers. In the last step, the samples were washed by adding approximately 30 to 50 µL (depend on the concentration of RNA sample we
want) of RNase-free water and centrifuged at high speed for 2 min. Finally, it was collected in the
collection tubes for further processing.

3. **Stool Total RNA Purification Kit (#49400, Norgen Biotech, Canada)** [13]

Extraction of RNA sample using Stool Isolation kit uses the principle of spin column
chromatography using Norgen kit solutions.

We started by adding tissue, nasal, fecal sample in bead tube with Lysis Buffer. It was then
vortexed for three minutes and centrifuged for 2000 RPM for another 2 min. The supernatant from
the centrifuged solution was transferred to the microcentrifugation tube which should be enzyme
free. Post that, the binding buffer was added in the DNAse and RNase-free microcentrifuge tube,
and lysate was incubated for ten minutes on ice. The lysate was then vortexed for three minutes
and the supernatant was collected. Followed by this, a same volume of 70% ethanol was added to
the lysate. The solution with ethanol was then loaded onto a spin-column.

Then the solution was washed using Wash Solution to reduce the bound of DNA and RNA. The
purified nucleic acids were removed using the Elution Buffer, and the solution was ready for
downstream processing [13].

4. **Direct-zol Kit (Modified version of Tri-zol Kit)** [14]

The Tri-zol Reagent (Invitrogen Life Technologies) is the Total RNA Isolation Reagent. This
reagent is used for the extraction of total RNA from biological samples for use in library
preparation and PCR technique. Tissue samples were completely homogenized and transferred to
the appropriate centrifuge tubes. In homogenization method we used 1ml TRI reagent and
50–100mg of samples.

The mortar was placed on ice and we poured liquid nitrogen into mortar. The tissue was break by
using pistol and mortar. The tissue sample was cut and weight into cold 2 ml of tissue tube. Later
we added 7~10 beads (2.3mm silica) and 0.5ml Tri-zol reagent to it. A biological sample was homogenized at 5800 speed for 45sec. It was transfer to 1.5 ml centrifugation tube. The beads were washed using alcohol 0.5ml and made 1ml of final homogenate. The final sample was incubated for 5-10 min in room temperature.

In the RNA Purification step, we used Direct-zol RNA kit (Zymo Research, USA). Direct-zol™ RNA mini Prep (Catalog Nos. R2050, R2051, R2052, &R2053) protocol was used for RNA purification step.

2.3.5 Quality and Quantity Measurement

We used the Nanodrop Spectrophotometer [Thermo Scientific, Massachusetts] to determine the RNA concentration results and the Agilent 2100 Bioanalyzer to observe the integrity of extracted RNA.

1. Nanodrop: Thermo Scientific One C Microvolume UV vis Spectrophotometer (Thermo Fisher Scientific, Massachusetts)

The Nanodrop Spectrophotometer, from Nanodrop Technology is designed to measure the nucleic acid concentrations. The absorbance of this spectrophotometer is assessed at 260 as well as 280 nm and its concentration are driven by utilizing the Beer Lambert law. The A260 reading is used to determine the RNA concentration. If the value of A260 is 1.0 it represents approximately 40 μg/ml of RNA. The ratio 260 along with 280 nm can be used to calculate the RNA integrity.

The liquid is held in the machine by surface tension for the measurement of absorbance. Only 1-2 microliters are pipetted straight onto the measurement pedestal. In this way the concentration of nucleic acid RNA is calculated.

2. Agilent RNA 6000 Nano Kit (Germany) Agilent’s 2100 Bioanalyzer, Lab on a Chip for the quality control of RNA.
Agilent's 2100 Bioanalyzer is used widely to study the integrity of RNA. It uses a lab on a chip method to do the process of capillary electrophoresis. It also requires a fluorescent dye which binds to RNA to calculate RNA integrity and concentration. The method of Bioanalyzer is based on conventional gel electrophoresis. This method evolved into chip technique which has advantages such as decreased time usage and sample size. The concentrations of Bioanalyzer ranges from 25 ng/μl - 500 ng/μl.

The chip of Bioanalyzer has three types of wells such as ladder wells, gel wells, and a well for the samples. The preparation of the bioanalyzer chip is accomplished by filling the micro channels with fluorescent dye & sieving polymer. Then 12 wells are filled with 12 samples in the NanoChip kit, one well with the ladder and two wells with the marker. After filling of the stations, the chip is connected to positive and negative electrode. The final result is represented on the computer in the form of bands and peaks of each samples. These bands and peaks represent the concentration and quality of the RNA. Computer system instantly compares the RNA samples to the ladder fragments which is standard for this chip system to determine both quantity and quality of samples. The quality of sample is determined by RIN number (RNA Integrity Number) which shows the number from 1 to 10. A RIN below 7 represents contamination or bad quality RNA samples.

2.4 Results

For each RNA extraction kit, the performance was evaluated for tissue, nasal, and fecal sample by using four different preservative materials. We observed the difference quantity of each sample, as shown in Table- 1.

2.4.1 Nanodrop Spectrophotometer- Quantity Results

The concentration results for all kits were different by using TRI-zol, Qiagen RNA Mini, Qiagen PowerMicrobiome, and Norgen stool isolation kit for nasal, fecal, and tissue samples.
1) Tissue Sample- Compared to all biological samples tissue samples extracted higher quantity by using all the four kits. A) Qiagen PowerMicrobiome Kit- A significantly higher quantity of RNA was extracted by using this kit which yielded 1462.08 µg/ml RNA concentration with RNA later™. B) TRI-zol kit – 792.94 µg/ml concentration with liquid nitrogen. C) Qiagen RNA mini kit yielded 915.16 µg/ml with RNA later™. D) Norgen stool isolation kit- 338.06 µg/ml concentration was obtained by using dry ice as preservative material.

2) Nasal samples- This samples were extracted from the cow. We could not get a good quantity of RNA, and the concentration was below 80 µg/ml with all the kits. However, the Qiagen power kit (75.488 µg/ml) shows a comparatively better result for the nasal sample. But this quantity was not good enough for downstream processing such as NGS or high throughput sequencing.

3) Fecal Samples- Fecal extraction procedures yielded sufficient amounts of total RNA from the Qiagen power kit (1119.601 µg/ml) with dry ice and liquid nitrogen (411.827 µg/ml) as per NGS requirements (>100 µg/ml).

4) Bacterial Samples- For bacterial extraction, only the TRI-zol kit showed a good concentration for Pseudomonas aeruginosa (156.634 µg/ml) and Staphylococcus aureus (125.83 µg/ml) bacteria.

2.4.2 Agilent Bioanalyzer – Quality/ Integrity Results

The RNA purity and integrity were inferred through the Bioanalyzer Agilent electrophoresis results. (Table 2). The Bioanalyzer results are shown in the Figure 1.

1) Tissue samples:

Total RNA extracted using the Qiagen RNA mini kit results in the best integrity with RNA later™ (RIN=9.7). Norgen stool isolation kit resulted RIN 9 with RNA later™. Qiagen PowerMicrobiome...
kit resulted RIN 8.5 with RNaIaterTM and TRI-zol kit resulted RIN 8.5 with dry ice. Compared to all biological samples tissue samples resulted best quality by using all four kits.

2) Nasal samples- Only Qiagen PowerMicrobiome kit with Zymo RNA/DNA Shield yielded good results (RIN=8.7) as shown in Table.2. Exceptional results were found for the control group of nasal samples (RIN=8.7 by TRI-zol kit, and RIN=8 by Qiagen power). These results were also verified by bioanalyzer electropherograms bands results.

3) Fecal samples- Only the Norgen kit resulted in good bands for the fecal samples; all other kits resulted in bad yield and integrity (RIN= <6).

4) Bacterial samples- Bacteria such as Staphylococcus aureus and Pseudomonas aeruginosa showed good integrity (RIN=>7) by using all the kits. Even though the quantity was not enough, the integrity of the bacterial samples showed good results, which was used as a positive control for all the samples.

2.5 Discussion

RNA expression's level is an important element for tissue and cellular physiological state. The quality of extraction in each and every RNA analysis is determined by the RNA purity/quality and amount of extracted RNA [15]. In our experiment, we found high RNA integrity and quantity for the fecal and tissue samples.

The challenges of undertaking studies comprising animal samples such as tissue, nasal, fecal are different. We found a big challenge in the extraction of nasal samples compared to tissue and fecal samples. High-quality total RNA is critical for downstream molecular applications such as poly(A) enrichment and library preparation in NGS experiments [16]. Unfortunately, some of the currently available RNA isolation approaches produces poor quality total RNA from animal tissues or even from cell cultures. Recently, Brown et.al., compared TRI-zol and four popular commercially
available RNA isolation kits for their efficacy in extracting total RNA from various tissues. He used tissue samples from liver, reported the 260/230 ratio and RNA integrity number (RIN) values which were ranged from 7.2 to 8.1 and from 1.4 to 2.0 respectively, by using these methods. Similarly, in this experiment RIN results showed difference among all samples such as fecal, nasal and tissue with four preservative samples.

In our experiment, we found that the column-based kit (Qiagen PowerMicrobiome kit) and silica membrane-based purification method kit (Qiagen RNA mini kit) showed best results for samples such as tissue and fecal. For the control samples of nasal, we found high integrity results, which was exceptional. This could be because the samples were stored at the room temperature in the process of the extraction, and the environmental bacteria was one of the reasons for the high RIN number. Therefore, temperatures used in RNA purification are also probed, and the best results are obtained when almost all steps after the lysis process are carried out at refrigeration (2-6°C) temperature.

Moreover, in this project, we used four different types of preservative materials to observe the results. RNA later™ showed the best results compared to all preservative material such as dry ice, liquid nitrogen, Zymo DNA/RNA Shield. Invitrogen RNA later™ Stabilization Solution is nontoxic and aqueous solution which quickly stabilizes the tissue as well as guards the cellular RNA. RNA later™ also reduces the time of tissue or biological sample freezing which helps to reduce degradation of RNA.

In RNA isolation rather than good quality RNA extraction kit and preservative material cleanliness is another important factor for high quality and quantity of RNA. Proper hygiene should be maintained to preserve the integrity of the sample during preparation process.
In addition, the b-mercaptoethanol has an important role in RNA extraction for demolishing the RNases which could degrade the extracted samples [20]. While using the Qiagen power kit, we used b-mercaptoethanol agent in the extraction process, and this kit also resulted good yield and quantity for all the samples.

2.6 Conclusion

Overall tissue sample showed the best results for quality and quantity of RNA with all the kits using RNA later™ and dry ice as a preservative material. After comparing both bioanalyzer and nanodrop results, the Norgen Stool isolation kit gave the best results for fecal samples with RNA later™. Qiagen RNA mini kit gave good results for tissue sample with RNA later™ and the Qiagen power kit with Zymo RNA/DNA shield provided the best results for nasal samples. For out metatranscriptomics study Qiagen RNA mini kit was selected for the extraction of tissue samples from the intestine of broilers.
### Table 1  Nanodrop Spectrophotometer results for quantity estimation

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Qiagen RNA Mini Kit</th>
<th>Norgen Stool Isolation Kit</th>
<th>Qiagen PowerMicrobiome Kit</th>
<th>Direct-zol Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA conc µg/ml</td>
<td>A260/280</td>
<td>A260/230</td>
<td>RNA conc µg/ml</td>
</tr>
<tr>
<td>DI Fecal</td>
<td>17.465</td>
<td>1.507</td>
<td>0.419</td>
<td>100.62</td>
</tr>
<tr>
<td>LN Fecal</td>
<td>37.81</td>
<td>1.625</td>
<td>0.503</td>
<td>70.444</td>
</tr>
<tr>
<td>Zymo Shield Fecal</td>
<td>11.945</td>
<td>1.657</td>
<td>0.494</td>
<td>74.903</td>
</tr>
<tr>
<td>RNAlater</td>
<td>30.717</td>
<td>1.737</td>
<td>0.096</td>
<td>71.188</td>
</tr>
<tr>
<td>Fecal Control</td>
<td>23.47</td>
<td>1.756</td>
<td>0.179</td>
<td>34.809</td>
</tr>
<tr>
<td>DI Nasal</td>
<td>1.826</td>
<td>1.314</td>
<td>0.078</td>
<td>4.605</td>
</tr>
<tr>
<td>Zymo Shield Nasal</td>
<td>10.481</td>
<td>1.624</td>
<td>0.175</td>
<td>6.697</td>
</tr>
<tr>
<td>RNAlater Nasal</td>
<td>6.784</td>
<td>1.729</td>
<td>0.174</td>
<td>1.998</td>
</tr>
<tr>
<td>Nasal Control</td>
<td>10.017</td>
<td>2.058</td>
<td>0.663</td>
<td>13.903</td>
</tr>
<tr>
<td>DI Tissue</td>
<td>352.407</td>
<td>2.077</td>
<td>1.702</td>
<td>338.06</td>
</tr>
<tr>
<td>LN tissue</td>
<td>730.249</td>
<td>2.07</td>
<td>2.176</td>
<td>11.674</td>
</tr>
<tr>
<td>RNAlater Tissue</td>
<td>915.16</td>
<td>2.085</td>
<td>2.194</td>
<td>33.154</td>
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<tr>
<td>Tissue Control</td>
<td>727.293</td>
<td>2.091</td>
<td>1.351</td>
<td>110.91</td>
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<tr>
<td>Staphylococcus a</td>
<td>58.751</td>
<td>1.478</td>
<td>0.52</td>
<td>7.532</td>
</tr>
<tr>
<td>Pseudomonas a</td>
<td>5.112</td>
<td>1.978</td>
<td>0.264</td>
<td>21.496</td>
</tr>
</tbody>
</table>

<p>| | | | | |
|                      |                     |                             |                             |                     |
|                      | Qiagen PowerMicrobiome Kit | Direct-zol Kit |                     |
|                      | RNA conc µg/ml      | A260/280                    | A260/230                    | RNA conc µg/ml |
| DI Fecal             | 1119.601            | 2.177                       | 2.393                       | 48.526         | 1.559          | 0.96           |
| LN Fecal             | 411.827             | 2.168                       | 2.356                       | 126.684        | 1.499          | 0.618          |
| Zymo Shield Fecal    | 7.486               | 1.906                       | 0.631                       | 34.928         | 1.719          | 1.332          |
| RNAlater             | 278.586             | 1.997                       | 1.684                       | 7.278          | 1.392          | 0.725          |
| Fecal Control        | 1235.082            | 2.158                       | 2.331                       | 43.134         | 1.481          | 0.7            |
| DI Nasal             | 1.542               | 1.184                       | 0.334                       | 5.387          | 1.468          | 0.822          |
| Zymo Shield Nasal    | 8.194               | 1.744                       | 0.641                       | 14.541         | 1.719          | 1.036          |
| RNAlater Nasal       | 75.488              | 1.478                       | 0.545                       | 8.383          | 1.315          | 0.953          |</p>
<table>
<thead>
<tr>
<th>Nasal Control</th>
<th>72.894</th>
<th>2.111</th>
<th>2.055</th>
<th>23.338</th>
<th>1.688</th>
<th>1.463</th>
</tr>
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<tbody>
<tr>
<td>DI Tissue</td>
<td>1244.462</td>
<td>2.117</td>
<td>2.219</td>
<td>237.308</td>
<td>1.757</td>
<td>1.267</td>
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<tr>
<td>LN tissue</td>
<td>1287.774</td>
<td>2.105</td>
<td>2.146</td>
<td>792.94</td>
<td>1.965</td>
<td>2.268</td>
</tr>
<tr>
<td>RNA Alater Tissue</td>
<td>1462.08</td>
<td>2.113</td>
<td>2.241</td>
<td>15.571</td>
<td>1.394</td>
<td>0.806</td>
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<tr>
<td>Tissue Control</td>
<td>868.306</td>
<td>2.116</td>
<td>2.335</td>
<td>435.003</td>
<td>2.004</td>
<td>2.221</td>
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<tr>
<td>Staphylococcus a</td>
<td>37.688</td>
<td>2.047</td>
<td>1.374</td>
<td>125.83</td>
<td>1.533</td>
<td>0.821</td>
</tr>
<tr>
<td>Pseudomonas a</td>
<td>30.193</td>
<td>2.022</td>
<td>1.593</td>
<td>156.634</td>
<td>1.843</td>
<td>1.426</td>
</tr>
</tbody>
</table>

This table shows the concentration of RNA in nasal, fecal, tissue, and bacterial results. The results are calculated using four kits: Qiagen RNA mini kit, Qiagen PowerMicrobiome kit, Direc-zol kit, and Norgen stool isolation kit. Preservative materials such as dry ice, liquid nitrogen, RNA later™, RNA/DNA Zymo Shield used. Each kit has negative control sample and positive control bacteria (Staphylococcus a and Pseudomonas a).
Table 2    Agilent Bioanalyzer results for RNA Integrity/ Quality

<table>
<thead>
<tr>
<th>Samples</th>
<th>Treatment</th>
<th>RIN</th>
<th>Samples</th>
<th>Treatment</th>
<th>RIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fecal Sample</td>
<td>Dry Ice</td>
<td>2.7</td>
<td>Fecal Sample</td>
<td>Dry Ice</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>Liquid Nitrogen</td>
<td>3.3</td>
<td></td>
<td>Liquid Nitrogen</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>RNA LATER</td>
<td>5.7</td>
<td></td>
<td>RNA LATER</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1.9</td>
<td></td>
<td>Control</td>
<td>2.8</td>
</tr>
<tr>
<td>Nasal Sample</td>
<td>Dry Ice</td>
<td>N/A</td>
<td>Nasal Sample</td>
<td>Zymo RNA/DNA Shield</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Liquid Nitrogen</td>
<td>8.6</td>
<td></td>
<td>RNA LATER</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>RNA LATER</td>
<td>N/A</td>
<td></td>
<td>Control</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>8</td>
<td></td>
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<td></td>
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<tr>
<td>Tissue Sample</td>
<td>Dry Ice</td>
<td>7.3</td>
<td>Tissue Sample</td>
<td>Dry Ice</td>
<td>6.5</td>
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<td></td>
<td>Liquid Nitrogen</td>
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<td>Liquid Nitrogen</td>
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<td></td>
<td>RNA LATER</td>
<td>8.5</td>
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<td>9</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2.6</td>
<td></td>
<td>Control</td>
<td>3.7</td>
</tr>
</tbody>
</table>

This table describes the RIN number of each sample which is used to evaluate integrity of sample. Nasal, tissue and fecal samples were used and preservative materials such as dry ice, liquid nitrogen, Zymo RNA/DNA Shield, and RNA LATER™ were used. The results are calculated for four kits Qiagen RNA mini kit, Qiagen PowerMicrobiome kit, Direct-zol kit and Norgen stool isolation kit.
Figure 1. Integrity Results from Bioanalyzer electrophoresis runs.
Examples of representative Bioanalyzer Agilent electrophoresis runs for the four different methods applied for RNA extractions from fecal, nasal, and tissue samples. Directzol kit, Qiagen RNAse mini kit, Qiagen powersoil kit, Norgen stool isolation kits was used. The first lane of each run is showing the Ladder. The RNA 6000 Nano Marker are the green band at the bottom of each panel. The red box indicates that the Bioanalyzer software cannot calculate RIN values for this sample due to very low concentration and high level of degradation of the RNA
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CHAPTER 3

Metatranscriptomics analysis of Ileum microbial communities in Necrotic Enteritis chickens
3.1 Introduction

Necrotic enteritis (NE) is an intestinal disease which is caused by toxigenic bacteria such as *Clostridium perfringens* and *Eimeria Maxima* [1]. However, the occurrence of *Eimeria Maxima* was found rare in NE broilers. Hence, the toxigenic *Clostridium perfringens* is vital agent for NE which is isolated from soil, litter, and feed on poultry farms [2]. Necrotic enteritis causes a significant economic loss to the poultry industry of over $6 billion annually. It is chiefly due to changes in diet, litter environment, high protein, and coccidial infection [3]. In this project, we conducted an experiment on chickens infected by *Clostridium perfringens* (CP) type A, the chief causal agent of necrotic enteritis. The main source of this infection is litter microorganisms that are transferred to the gastrointestinal tract by pecking after hatching. These microbes affect the chicken at a very early stage and influence the gut microbiome.

Various taxa such as *Campylobacter* and *Salmonella spp.* are present in the intestinal microbiome of poultry and capable of causing significant illnesses. The *Clostridia* is one of the major taxa present in the gut of the chicken which causes various diseases in poultry. Few *Clostridia* species that are pathogenic to poultry are CP, *C. septicum*, and *C. colinum* [4]. CP causes necrotic enteritis in poultry that contain a NetB toxin (alpha toxin) type (Type A) which is not possessed by human pathotypes [5].

NE is present either as a clinical or subclinical disease [6]. The clinical sign shows its symptoms from the two weeks of age in poultry. These birds normally die within few hours after the infection of NE. In a subclinical disease, the birds die within one to two weeks after the infection. In the subclinical disease an intestinal damage can permit the bacteria to penetrate into the bile duct and portal bloodstream, which can lead to cholangiohepatitis [6, 7, 8, 9].
For the prevention of NE, antibiotics was used but many countries banned antibiotic use in broiler meat therefore, researchers are studying to find an alternative approach of antibiotics. Nutrient such as fatty acids (SCFA) benefit the gut microbiota in many ways, especially as an energy source. Research and field experiments confirmed that necrotic enteritis incidence reduces by using alone butyrate or with the combinations of other supplements [10]. Also, according to Ma et al., butyrate helps in intestinal wound healing by maintaining the gut integrity and tightening the junctions of the poultry gut [11]. In our project, we used tributyrin (butyrate compound) for the treatment of NE which is an anti-inflammatory metabolite which helps to reduce NE virulence chicken from the Southern Poultry Research group.

To study chicken GI microbial ecology and gut diseases, omics technology such as 16S rRNA, metagenomics, metaproteomic, metabolomics, metatranscriptomics are choice of methods. From all these technology high-throughput sequencing 16S rRNA is widely used recently [12]. However, this technology has many disadvantages such as it cannot provide an information of metabolic functions of bacteria, microbial activity, gene expression and active microbes of a biological samples. All these shortcomings were overcome by Metagenomics and Metatranscriptomics technology. These technologies used approaches to categorize gene functions which provide details of community structure, gene expressions and metabolic functions [13]. Metagenomics helps to identify the microbes present in the biological sample whereas metatranscriptomics study helps to study the activity of those microbes in that particular environment. In addition, the metatranscriptomics technology also provide the difference in the functional activity of the microbes which helps us to find activity of the particular taxa in a given condition. Therefore, in this project metatranscriptomics tool was used to study NE disease in
broilers. In our knowledge, this is the first study which is employing metatranscriptomics technology to study the gut environment of broilers.

This research only focused on the ileum segment of the intestine because it possesses a higher microbiota diversity and displays more pronounced NE lesions [14]. Moreover, Wang et al., compared fresh and reused litter microbiota and their effects on the chicken gut microbiota. Litter had a stronger influence on the microbiota of the ileum than cecum part of intestine. Therefore, our research focused was based on the microbiota of the ileum.

In this study, the gut microbiome of broilers infected with NE, was investigated using metatranscriptomics technology. This research aims to provide insights into the microbial composition and functions of CP infected, healthy, and tributyrin treated chickens.

3.2 Material and Methods

3.2.1 Animal Experiments and Sample Collection

Animal experiment and sample collection was done by Southern Poultry Research Group. Three thousand birds were challenged for NE. Monovalerin and tributyrin supplements was used as a treatment of NE. In this research, we studied only tributyrin treated NE broilers.

For the samples collection, tissue samples of host gut contents were collected from 42-day old broilers. Three thousand birds were placed in six treatment groups, ten replicate pens per treatment with 50 birds per pen. From the six treatment groups we selected only one treatment group and two challenge groups. Recording for feed intake, adjusted FCR, non-adjusted FCR, weight gain (kg), lesion score and percent mortality was made on the d14, d35, and d42 as shown in Table 3.

Healthy male birds were collected from random pens.

On d42 of the experiment, chicken intestinal samples were collected for the metatranscriptomics study. The samples were collected and stored at -80°C for future analysis.
3.2.2 RNA extraction of Ileum tissue samples

Thirty-nine ileum samples were selected for processing. The commercially available RNeasy Mini Kit (#74104, Qiagen, Germany) was used to extract the RNA from these samples. We selected this kit from the results of pilot study (2nd Chapter).

In the RNA extraction process the samples are individually isolated, directly from -80° C without thawing, after removing the digesta to reduce degradation. At first, 30-50 mg tissues are weighed and added (without thawing) to the buffer RLT (Tissues stabilized in RNAlater™) collecting tube to reduce the contamination. Later, these were disrupted and homogenized by using bead beater - 16 (Biospec Mini Bead Beater Beadbeater 16 Homogenizer) for three minutes at 2000 RPM. The lysate was centrifuged for three min at a maximum speed of 1500 RPM after which the supernatant was carefully removed by pipetting. One volume of 70% ethanol was added to the supernatant obtained in the last step and mixed well. The 700 µL of solution obtained after adding ethanol were transferred to the RNeasy Mini spin column and centrifuged for 15 seconds. The spin column was washed three times by different buffers. In the last step, the samples were washed by adding 30-50 µL of RNase- free water and centrifuged at high speed for two minutes. Finally, it was collected in the collection tubes for further processing.

The RNA yield and quality were measured using a Nanodrop Spectrophotometer (Thermo Scientific™ NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer, USA), and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Samples with RNA integrity number (RIN) ≥ 7.0 were used to generate the metatranscriptomics libraries.

3.2.3 Sample selection for metatranscriptomics sequencing

We selected only 18 samples from 39 ileum tissue samples. The 18 selected samples (For metatranscriptomics study) were divided into the CP (Clostridium perfringens) challenge group,
treatment group as tributyrin (tri) group (NE birds treated with tributyrin supplements), and control as No challenge group (Healthy birds without any challenge) for metatranscriptomics analysis. In each group, six samples were selected based on quantity, integrity, and phenotype characteristics, as shown in Table-3. Phenotype characteristics included percent mortality (<10), average high FCR (>1.5), tissue lesion score (0,1), and average weight gained from d14 and d42 (>1400). Samples with low mortalities were selected for all groups except the CP challenge group. In addition, after RNA isolation of all 39 samples, 18 samples were selected for sequencing based on the integrity (>8 RIN) and quantity (>300 µg/mL concentration) factors.

3.2.4 DNA digestion

The extracted samples were treated with the DNA-free™ Kit (Thermo Fisher, USA) for DNA digestion. This resulted in good quality (integrity results- >9) and quantity (>100 µg/mL) of extracted samples as per NGS requirements shown in Table-1.

3.2.5 Library Preparation and Sequencing

The library preparation and sequencing process was conducted at Genohub (Admera Health Biopharma Services) using the Universal Prokaryotic RNA-Seq Library Preparation Kit (Tecan, California, USA) for the library preparation and Illumina HiSeq system for the sequencing of total RNA. The Universal Prokaryotic RNA-Seq Library Preparation Kit was used to enrich the messenger RNA (mRNA) and removal rRNA from the extracted samples.

3.2.6 Annotation and analysis of metatranscriptomics

The raw metatranscriptomics files were annotated, preprocessed and examined with an altered version of the SAMSA2 pipeline [15]. Preprocessing involves withdrawal of bad sequences as well as adaptor contamination with Paired-End using PEAR and Trimmomatic alignment, that eliminates more or less 40% of raw reads [16, 17].
Trimmomatic read trimmer, created for Illumina sequence data, is a software intended to get rid of both low-quality sequences and some possible adaptor contamination [18]. Trimmomatic creates thoroughly clean results for the next stage of preprocessing. The next step involves SortMeRNA which offers one more digital sweep, ensuring that ribosomal reads are eliminated before annotation [19].

At this phase, removal of ribosomal reads decreases the total amount of sequences which resulted as annotated, thus increasing the processing speed and decreasing file sizes. Annotation was the next phase after the data cleaning. DIAMOND is a superfast BLAST like algorithm used for annotation [18]. DIAMOND was created especially for annotating large amounts of read sequences, with more than 10,000 times faster speed compared to conventional BLAST. We used SEED and NCBI databases in this project. These databases are available via a bio share on GitHub.

The SAMSA2 pipeline last step is a statistical and visual analysis using R. The purpose of the analysis was to calculate differential expressions to create tables and graphs from the sorted abundance counts.

3.2.7 Statistical Analysis

Statistical analysis of the metatranscriptomics data was conducted using the modified version of the SAMSA2 scripts [15]. R scripts used in the current project are available at https://github.com/transcript/macaqueICD/tree/master/R_scripts.

Data from the DESeq2 analysis was used for PCA clustering analyses, heatmap analysis, and differential abundance testing for more specific analysis. As DESeq2 uses Benjamini and Hochberg adjusted p value (padj) to correct for multiple hypothesis, we used padj ≤ 0.05 to filter out highly statistically significant data.
3.3 Results

3.3.1 Samples Selection

For metatranscriptomics analysis, 18 samples were selected from the three treatment groups: No challenge, CP challenge and Tributyrin treatment group. (Table-1) The tissue samples were selected from the ileum of male chickens based on phenotype, RIN, and Nanodrop results. The tissue samples were selected using the factors; body weights on d14, d35, and d42, percent mortality, low lesion score for NE (0,1), different pen number, RIN number (>7) before and after digestion of samples, and concentration (>200 µg/mL) of RNA. For the CP challenge group, we selected samples from CP infected birds that had a higher mortality. After RNA sample digestion the RIN number drastically increased for all 18 samples (>8) demonstrating that the DNA digestion kit reduced sample contamination.

Results of the Nanodrop, bioanalyzer, chicken weights, treatments, and pen numbers for all 39 samples are shown in Table 1. As described earlier, samples were distributed in three groups: Control-No-challenge, Control-CP -challenge and tributyrin-CP- treatment.

3.3.2 Sequencing results and top 30 transcripts of Control- No- Challenge, tributyrin-CP treatment, and CP - challenge groups

Total sequencing reads for each sample was near about 6000000 where total reads for failing E-value threshold (removal of rRNA using SortMeRNA and removal of bad sequencing using PEAR) was more than 97%.

Using the mRNA mapped reads of organisms, transcript counts were summarized at the species level. We found a total of 7796 transcripts from the DESeq2 results. The species with the highest transcript abundances in the No challenge group included Chlamydia abortus, Acinetobacter baumannii, Tamlana sp., Bradyrhizobium canariense, Herbaspirillum rubrisubalbicans,
*Chlamydia psittaci, Chlamydia, Escherichia coli, Bacteria, Tistrella mobilis, Oceanibaculum pacificum, Aeromonas hydrophila, Bathymodiolus thermophilus, and Chlamydia trachomatis.*

These results of absolute and relative count of top 50 transcripts are depicted in Figure 1. The species with the highest transcript abundances in the CP challenge group were *Chlamydia abortus, Acinetobacter baumannii, Fusobacterium nucleatum, Escherichia coli, Chlamydia psittaci, Bacteria, Tamlana sp., Bradyrhizobium canariense, Herbaspirillum rubrisubalbicans, Chlamydia, Aeromonas, Chlamydia trachomatis, Oceanibaculum pacificum, and Tistrella mobilis.* The species with the highest transcript abundances in the tributyrin group were *Chlamydia abortus, Acinetobacter baumannii, Tamlana sp., Escherichia coli, Fusobacterium nucleatum, Chlamydia psittaci, Bacteria, Bradyrhizobium canariense, Herbaspirillum rubrisubalbicans, Chlamydia, Tistrella mobilis, Oceanibaculum pacificum, Aeromonas, and Chlamydia trachomatis.* Although many species were common among all the three groups, their relative transcript abundances differed (Figure- 1a, 1b).

Examination of differential gene expression reveals many differences among the three groups. Log2foldchange and *p value* revealed that out of the 7796 transcripts, 260 bacterial species showed significant differences. CP greatly increased (4.127-log2foldchange, adjusted *p*= 0.01123919) in the tributyrin treated chicken as compared to the No challenge and CP challenge groups. There was also an increase in *Geofilum rubicundum* (0.8387-log2foldchange, adjusted *p*= 0.00448213), *Crocosphara watsonii* (0.7755-log2 log2foldchange, adjusted *p*= 0.067749), *Mastigocoleus testarum* (0.4653-log2foldchange, adjusted *p*= 3.56e−14), and *Candidatus Solibacter* (-4.1851-log2foldchange, adjusted *p*= 0.0642415) in tributyrin samples, whereas *Faecalibacterium* (1.7-log2foldchange decreased, adjusted *p*= 0.08559749), *Achromobacter* (-0.687- log2foldchange, *p* value- 0.039), *Strenotrophomas maltophilia* (-1.23691274855986-
log2foldchange, adjusted $p$ value- 0.0271527684568526 ) decreased in the tributyrin group. Though many transcripts expressed genes in all the groups, their relative expression differed substantially between the two phenotypes, with the largest significant log2fold change observed in the CP species.

3.3.3 Most abundant bacteria in tributyrin, Control and CP challenge samples

There were 0.026% upregulated genes (log2foldchange $\geq$ 2 relatives to No challenge; adjusted $p \leq$ 0.05), and 0.051% downregulated genes. 2.2% (173) outliers were found for organisms among all the three groups.

According to LefSE and LDA score, *Treponema maltophilum* showed high abundance of bacteria in the group tributyrin (LDA score - 2.1) (Figure-2). This species can induce bone loss and is a commonly found, oral bacteria in humans. [45] *Sphingobium.sp* was found to be highly abundant (LDA score -2.25) in the No challenge group. In the CP challenge group, *Streptococcus pneumoniae* was found abundant (LDA score -2). This species is suspected to cause pneumonia in poultry, as well as humans. [48]

3.3.4 Low Variance occurrence in all three groups

We anticipated considerable differences between the three groups from the PCA results. However, the results were contrary to our expectation which revealed low variation between groups. Using RNA-Seq reads, the diversity among microbial populations were calculated based on abundance of transcript, with a 0.58% threshold level. Comparisons of the diversity metrics between samples from No challenge, CP challenge, and tributyrin treated birds revealed a significant decrease in diversity in the tributyrin group.

In Figure 5, the heatmap of absolute abundance shows the top 60 bacteria, where spp. *Chlamydia abortus* has a higher abundance. This species has a higher abundance in the tributyrin treatment
group as compared to the CP challenge and No challenge group. However, we did not find significant differences ($p$ adjusted value <0.05) for the top 60 abundant bacteria among the three groups.

3.3.5 **Healthy, CP and tributyrin samples maintain significant differences in specific gene expression**

We found a total of 7796 transcripts where six species showed a significant difference (based on $p$. adjusted value<0.05 and log2foldchange). The bacteria which showed high significant difference in abundance are CP, *Stenotrophomonas maltophilia*, *Faecalibacterium sp.*, *Achromobacter*, *Geofilum rubicundum* and *Virgibacillus senegalensis*. Bacteria such as CP and *Geofilum rubicundum* increased in the tributyrin challenge group. We also analyzed the data at 10% significance ($p$ adjusted value<0.10) for finding the bacteria with pairwise significant differences in abundance, i.e. CP challenge vs. tributyrin group and No challenge vs. tributyrin group. However, for CP challenge vs. No challenge groups we did not find any significant differences based on $p$ adjusted value.

In the heat maps of groups, CP challenge vs tributyrin and No challenge vs tributyrin (Figure.8a, 8b), we observed that significant bacteria are common. However, the abundance level for the bacteria is different in all three groups. Bacteria such as *Caldicellulosiruptor obsidiansis*, *Clostridium perfringens*, *Crocosphaera watsonii*, *Treponema maltophilum*, *Trichoccus pasteuri* were found increased in the treatment group whereas, *Elizabethkingia anopheles*, *Faecalibacterium*, *Flavonifractor sp.*, *Microlunatus phosphovorus* and *Virgibacillus senegalensis* decreased.
3.3.6 Actin cytoplasmic 2 increased in the Tributyrin- CP- treatment group

RNA reads mapped in the chicken broiler (host) of Aviagen x Aviagen strain were examined to compare prokaryotes transcript abundance in No challenge, CP challenge, and tributyrin groups. Functional enrichment analysis revealed the functions of transcripts in Tributyrin group. Top 10 functions are "cytochrome c oxidase subunit I, actin, cytoplasmic 2, hypothetical protein, partial, cytochrome b, cytochrome c oxidase subunit 3, ATP synthase F0 subunit A, alpha-glucosidase, molecular chaperone DnaK, cytochrome P450, aldo/keto reductase."

In all three groups, enzyme cytochrome c oxidase subunit 1 was found to be the highest in the Tributyrin group. The functional absolute count results are shown in Figure 9

3.3.7 Community-wide transcriptomes suggest differences in nutrient availability between control and tributyrin samples

To explore the microbial community-wide differences in this study between Control, CP challenge, and tributyrin reads, they were mapped using the SEED Subsystems data set. Comparison of the tributyrin, Control, and CP challenge revealed changes at the level 1 SEED hierarchy categories. The largest increases were in cofactors, vitamins, prosthetic groups, pigments (0.48-log2fold increase, adjusted \( p = 3.056296 \times 10^{-04} \)), NO HIERARCHY (0.2923-log2fold increase, adjusted \( p = 2.085680 \times 10^{-01} \)), and motility and chemotaxis (0.1903-log2 fold increase, adjusted \( p = 6.4 \times 10^{-01} \)). Tributyrin treated microbiome functions were seen reduced in the categories of membrane transport (1.6-log2 fold decrease, adjusted \( p = 1.6123 \times 10^{-05} \)), virulence (0.772195-log2fold decrease, adjusted \( p = 3.672447 \times 10^{-01} \)), metabolism of aromatic compound (0.5809-log2fold decrease, adjusted \( p = 2.0856e-01 \)), and iron acquisition and metabolism (0.51174-log2fold decrease, adjusted \( p = 6.48e-01 \)). (Figure- 10). In the No challenge vs. tributyrin and CP challenge vs. No challenge groups, we did not find an increase in the cofactors
compared to CP challenge and tributyrin groups for level one. The result of log2foldchange for level one is shown in Figure-10.

In the SEED system results for level 1, high significant changes were observed in No challenge vs. tributyrin for sulfur metabolism ($p_{adj}$ 3.94E-16, log2fold change- -7.8896), metabolism of aromatic compounds ($p_{adj}$ 0.001924 and log2 fold change- -1.31033), membrane transport ($p_{adj}$ 0.001924, log2 fold change- -1.4294), cofactor, vitamins, prosthetic group and pigments ($p_{adj}$0.3890, log2 fold change- 0.30080). For CP challenge vs. tributyrin significant differences were observed in membrane transport ($p_{adj}$-1.62E-05, log2 fold change- -1.60009), and cofactor, vitamins, prosthetic group and pigments ($p_{adj}$-0.00030, log2 fold change-0.47722). Finally, for CP challenge vs. No challenge groups, we found one significant difference regarding sulfur metabolism (7.884367441-log2 fold increase, adjusted $p = 6.72E-16$).

3.3.8 Results of CP, Geofilum rubicundum, Crocosphaera Watsoni

1. CP

We analyze Kruskal- Wallis test for CP in the tributyrin challenge group. The $p$ value is 0.011 in between Tributyrin and CP challenge, $p$ value= 0.054 in between all three groups for relative abundance as shown in Figure 6.

In the Figure 15, CP bacteria showed significant difference in tributyrin group compared to CP challenge and No challenge group. We also analyzed functions of these bacteria and received 364 functions from Ref Sequence results of CP bacterium. Functions such as preprotein translocase subunit SecY (13%), partial (7.89%), serine/threonine protein kinase (3.62), reverse rubrerythrin (3.31%), L-lactate dehydrogenase (1.95%) were highly present.

In our SEED subsystem results, we found protein rubrerythrin present in high percentage (>40%) in all three groups.
2. *Geofilum rubicundum*

Subsystem SEED results showed level fourth protein sodium/glutamate symport present in all three groups. Enzymes adenylate kinase (EC 2.7.4.3) decreased in the tributyrin group. This bacterium shows significance difference among three groups for relative abundance as shown in Figure 14.

3. *Crocosphaera watsoni*

We found pterin biosynthesis, queuosine archaeosine biosynthesis, fatty acid biosynthesis FASII, RNA polymerase II initiation factors and folate and pterins in subsystem results from the species *Crocosphaera watsoni* in all samples. The nucleoside queuosine is among the most elaborate of the known RNA modifications. Recent efforts have revealed the queuosine modification may function to affect the population dynamics of microbial species in the gut.

**3.4 Discussion**

To our knowledge, this project was a novel effort that utilized a metatranscriptomics approach for examining NE within the chicken ileum. The objective of the research was to measure the changes occurring in the ileum microbiome using a NE-challenge treated model with a tributyrin supplement. This model consisted of two control groups (No- Challenge, CP- Challenge) and one treatment group (tributyrin- CP – treatment). Using a metatranscriptomics technique, these groups were analyzed to determine the microbial community and its functions. Among these three groups, the CP- challenge group was expected to have the highest concentration of CP. The tributyrin treatment group was expected to have a high concentration of beneficial bacteria (e.g. *Lactobacillus, Ruminococcus, Bacillus*, etc.).

There is a lack of research emphasizing metatranscriptomics analysis of chicken intestinal microbiome. Many researchers have instead used metagenomic technology to perform such
studies. Moreover, no microbial function study has been achieved on the chicken ileum microbiota. Thus, this experiment studied bacterial transcripts and functions of the ileum microbiota of broiler chickens.

The analysis was accomplished using DESeq2 tool which found a total of 7796 transcripts. These transcripts revealed the following top 10 bacteria in high abundance in the tributyrin treated samples such as *Chlamydia abortus*, *Acinetobacter baumannii*, *Tamlana sp. Escherichia coli*, *Fusobacterium nucleatum*, *Chlamydia psittaci*, *Bradyrhizobium canariense*, *Herbaspirillum rubrisubalbicans*, *Tistrella mobilis*, and *Oceanibaculum pacificum*. (Figure 1)

Among these species, three bacteria *Acinetobacter baumannii*, *Chlamydia abortus*, and *Fusobacterium nucleatum* function as an important bacterium for NE. The most abundant bacteria, *Chlamydia abortus*, results showed interesting information when compare to *C. Psittaci*. We found this bacterium highly abundant in all three groups which causes abortion in ruminants, dogs, and cats. However, this organism has been detected occasionally in various birds, including poultry, psittacines, and pigeons (Figure- 6). In addition, *C. psittaci* is a common bacterium in poultry which causes pneumonia. It is unclear about the high abundance of *C. abortus* in broiler intestine rather than *C.psittaci* abundance.

The second highest abundant bacteria we found was *A. baumannii* common to all groups. It can cause a variety of infections in humans. In China, this bacterium demonstrates the presence of *A. baumannii* in poultry livestock and suggests it to be a zoonotic pathogen [20, 21]. However, we did not record any evidence for zoonotic disease in people [20].

According to Lattore *et. al*, the number of phyla *Actinobacteria* (*P < 0.01*) decreased, and genera *Butyrvibrio, Lactobacillus, Prevotella*, and *Ruminococcus* increased in positive challenge group of CP samples compared to No challenge samples (*P < 0.05*) [22]. It was found bacteria such as
*Butyrivibrio, Prevotella* decreased in the CP challenge group contradicting Lattore *et al.*, work, while still increased in *Ruminococcus* concentration in the CP challenge group and *Lactobacillus* concentration in the tributyrin group.

Our study shows an abundance of *Proteobacteria* and *Bacteroidetes* in CP samples as compared to that in the NC group. A similar increase in *Proteobacteria* and *Bacteroidetes* population was reported earlier in NE case [23]. The phylum *Proteobacteria* contains many pathogens such as *Escherichia, Salmonella, Campylobacter*, and *Proteus* which are opportunistic. Thus, an increased level of *Proteobacteria* can be related to gut dysbiosis or intestinal inflammation [24].

Bacteria such as *Clostridium, ButyrIFICoccus*, and *Faecalibacterium*, are SCFA producers [22]. We found *Clostridium* to be higher in the tributyrin group, whereas *Faecalibacterium* and *ButyrIFICoccus* were lower. Hence, some saprophytic species of *Clostridium* may help in the production of SCFA using the tributyrin supplement in NE broilers [22].

A key question this project helped address is whether the microbiomes from the Control, NE infected tissues, and NE treated tissues differ from each other. It was found that only six kinds of bacteria showed significant differences (~5%) on a species level. These were *Achromobacter, CP, Faecalibacterium sp., Geofilum rubicundum*, *Streptophonomas maltophilia, Virgibacilus senegalensis*. The beneficial bacteria *Faecalibacterium sp.* decreased in the treatment group. According to Frank *et al.*, it helps to reduce intestinal inflammation. Hence, treating with tributyrin may not help to reduce inflammation with *Faecalibacterium sp*. Although it may help reduce it in CP challenge birds. Previous studies with *Clostridium* show high value in PC (2.98 ± 0.71%) as compared to NC (1.84 ± 0.36%), yet the difference is not significant. The results for CP obtained in this work were unexpected. Instead of the CP challenge group, it was observed that the tributyrin group showed an abundance of CP, which contradicts the original hypothesis. Besides, one
replicant sample (25th chicken sample) from the tributyrin group (8% mortality) showed more than 77% of total CP.

In the CP challenge group, *Stenotrophomonas maltophilia* increased compared to healthy, and tributyrin treated birds. It is a non-fermentative, gram-negative bacterium, which is difficult to treat in humans [25]. *Stenotrophomonas infections* have been associated with high mortality and morbidity in immunocompromised individuals. [26, 27, 28]. Therefore, as shown in Figure 15, we can predict that *Stenotrophomonas maltophilia* may contribute to the high mortality of the CP challenge group compared to other groups.

The data obtained from sampling showed that the percent mortality declined with tributyrin treatment. Thus, it was expected to find a high abundance of beneficial bacteria in the tributyrin group. However, a significant increase in concentration was found only two types of bacteria which were present in the tributyrin group as compared to CP challenge group and No challenge group. These two bacteria were CP, and *Geophilum rubicundum*. We did not find a significant difference in the microbiota when we compared CP challenge and No challenge samples (Figure-6, 15). Subsystem results for *Geofilum rubicundum* showed high presence of protein sodium/glutamate. Glutamate is an important fuel for oxidation in the intestine for the gut microbiota. In addition, it is an important precursor for other biologically active molecules, including proline, glutathione, and arginine. Therefore, *Geofilum rubicundum* work as an oxidative fuel for respiration with the help of protein glutamate in the recovery from the NE in tributyrin sample.

Tributyrin is naturally present in butter in the form of triglycerides. It is an ester composed of butyric acid and glycerol [29]. For the colonic luminal contents, butyrate is formed by bacterial fermentation of indigested carbohydrates in humans. It helps to reduce intestinal inflammation
Butyrate has demonstrated in many situations to reduce the severity of NE when given as a feed additive [10]. Even though butyrate has many properties to treat NE in chicken, tributyrin samples in this project did not show significant evidence to support this. Geofilum, known to produce fatty acids, occurred as the only beneficial bacteria, and showed a highly significant difference. This genus is abundant in the tributyrin group which is affiliated with the family Marinilabiliaceae whose major function is the production of fatty acids. These findings help to infer that fatty acids synthesized by Geofilum spp. may help in the treatment of NE in chickens.

Other beneficial bacteria found in the tributyrin group were Lactobacillus and Ruminococcus. Lactobacillus showed high significant difference (Figure-16), but not as much as Geofilum rubicundum. The Lactobacillus in the tributyrin group indicated that it can inhibit the growth of CP according to Gunther et al., [32]. Lactobacilli can ferment carbohydrates into lactic acid (Figure 15). It can also help to reduce the pH of the intestinal environment resulting in growth inhibition of acid-sensitive pathogenic bacteria [33]. We also found inhibition of acid-sensitive bacteria such as Campylobacter jejuni and Vibrio cholera in the tributyrin group. More specifically, the abundance of Campylobacter jejuni declined by more than 80% in the tributyrin group compared to the other two groups. It showed significance difference among No challenge and tributyrin groups and CP challenge vs tributyrin groups as shown in Figure 17. From this finding, it may be inferred that birds may recover from disease by reducing acid-sensitive bacteria such as C. jejuni and Vibrio cholera [33, 34]. Though it is not clear why Lactobacillus increased in the tributyrin group compared to the CP challenge group. It may be that tributyrin helps to increase the presence of Lactobacillus. In addition, Lactobacillus may inhibit the inflammatory responses of the gut and increase the antagonistic effects against intestinal pathogens [35, 36].
The functional result of *Lactobacillus*, “molecular chaperon HtpG” (also called heat shock protein) showed the highest functional activity (~40%) in the tributyrin group. According to Goulhen *et. al.*, (1997), heat shock is first line of attack on the foreign bacteria which helps to consolidate pathogen virulence [37]. Based on this finding, perhaps *Lactobacillus* helps to reduce CP virulence in chicken. Increasing the beneficial presence of *Lactobacillus*, through the use of a tributyrin supplement, may be a possible treatment for NE.

Since the abundance of *Ruminococcus* was found high in the CP challenge and tributyrin groups, we decided to study this bacterium in detail. Xu *et. al.*, studies revealed similar results in the ileum of NE chickens. In human studies, this bacterium is known to cause enteritis. This is probably due to its ability to use mucin glycans as an energy source thus multiplying in both humans and chickens [38, 39, 40]. However, in functional results, ubiquitin was found as a dominant protein for *Ruminococcus spp.* ~57.59% and ~76% compared to other functions for CP challenge and tributyrin groups, respectively. According to Yafei *et. al.*, ubiquitin helps the CP challenged birds in maintaining intestinal homeostasis and controlling gut inflammation [41]. In addition, as shown in figure 16 the species *Ruminococcus gnavus* showed significant difference between No challenge vs tributyrin group, CP challenge vs tributyrin group. *Ruminococcus gnavus* is a new bacteriocin against *Clostridium perfringens* [40]. Based on the functional findings and *Ruminococcus gnavus* findings, *Ruminococcus* may be a contributing factor for controlling gut inflammation and intestinal homeostasis in broilers infected with NE.

We also found two functional results with higher activity in bacteria which may contribute to the progression of NE. These functions are protein and Sulphur metabolism (Figure 11, 12). The protein metabolism activity is found high in the CP challenge group. These proteins are broken down into ammonia and amines which increase the pH, thus favoring bacterial growth, including
CP. Possibly the higher protein metabolism function in the CP challenge group may promote the growth of CP in NE chicken. Also, we found increased sulfur metabolism activity ($p$ adjusted-$3.94E-16$, log2fold change-$-7.8896$) in CP challenge group which may be an inflammation-contributing factor [42] for the CP birds. To confirm this result, we found high lesion scores in CP challenge group compared to no challenge group.

3.5 Conclusion

In this study, we employed a metatranscriptomics approach to investigate the microbiome of healthy and necrotic infected chicken ileum. The purpose of this research was to analyze and study thoroughly the bacterial abundance, its importance and functions for healthy, CP challenge and tributyrin treated environment. The results of transcripts revealed 7796 bacteria in all the specimens. However, only six bacteria exhibited a statistically significant difference within the groups. Two of these bacteria were CP and Geofilum rubicundum both increased in the tributyrin treated NE chickens. Interestingly, we did not find any significant difference (5%) in the relative abundance of bacteria between No challenge and CP challenge groups. We also found higher occurrence of Lactobacillus in the tributyrin challenge group. It’s function “molecular chaperon HtpG” may be an important contributor in the treatment of birds suffering from NE. In addition, Ruminococcus gnavus may act as a defending bacterium for Clostridium Perfringens and Ruminococcus may be a contributor for controlling gut inflammation and intestinal homeostasis with the help of the function ubiquitine which we found in our results. Further research to augment our work is needed by including metaproteomic and metabolomics approaches. Also, a larger sample population will help to substantiate our research.
Figure 1 A - Absolute Abundance of Bacteria

Figure 1 B - Relative Abundance of Bacteria

Figure 1  Absolute and relative abundance of transcript

Figure 1A is showing the absolute count of bacteria and 1B represents relative counts of bacteria. Left six samples are No challenge (brown bar), middle with red color bar are CP challenge samples and yellow bar showing six samples of tributyrin samples.
The LDA score describes the bacteria which were found highly abundant in three groups. We found *Treponema maltophilum* in tributyrin group, *Sphingobium* sp. in No challenge group and *Streptococcus pneumoniae* in CP challenge group. The LDA score of No challenge bacteria was highest with *Sphingobium* sp. (2.25)

For LEfSe results, *Sphingobium* sp, *Streptococcus pneumoniae* and *Treponema maltophilum* represents high abundance in group No challenge, CP challenge and tributyrin respectively. We observed that *Sphingobium* spp. had high abundance in No challenge group in the 5th sample. Whereas, the *Treponema maltophilum* showed optimum concentration in all the groups and highest in tributyrin group.
Figure 4  Comparison of CP, No challenge and tributyrin using PCA plot
PCA graph shows less variation among three groups 17% for y axis and 37% variation on x axis. Only CP challenge group and No challenge group showed variation for each sample.
Figure 5  Heat map of abundant bacteria among No, CP and tributyrin groups
The heat map is divided into three groups. (Left to Right) First six samples are No challenge, next six samples are CP challenge and last six samples are tributyrin samples. Heat map shows top 20 bacteria from three groups.
Figure 6  Comparison between *C. abortus* and *C. Psittaci* counts

Figure 6 represent the two bacterial counts. *Chlamydia abortus* (Light Blue) and *Chlamydia psittaci* (Dark Blue) where *C. abortus* showed high count in all the three groups. First (left) six samples are from No challenge group, middle six samples are CP challenge group and last six samples are from tributyrin group.
Figure 7     Volcano plot of significant bacteria
Volcano map showing the bacteria which is significant based on p adjusted value and log2fold change value. The bacteria are divided based on the abundance. The red color shows the highly significant different bacteria among three groups. Green represents and black shows that there was no any bacterial count difference among three groups.  

*Strenotrophomonas, Faecalibacterium, Acinobacter, Strenotrophomas, Achromobacter, Microbacterium spp* showing highest log2fold change from 7796 bacteria. Volcano map is drawn based on log2fold change and p adjusted value.
Figures 8A and 8B show the significant different bacteria between No challenge and tributyrin groups. 1) Figure 8 A is showing the significant bacterial difference between No challenge and Tributyrin group. 2) Figure 8 B is showing the significant bacterial difference between CP challenge and tributyrin group. No significant bacteria were found in between No challenge and CP challenge groups in both diagrams. Bacteria such as *Caldicellulosiruptor obsidiansis*, *Crocosphaera watsonii*, *Treponema maltophilum*, and *Trichococcus pasteurii* increased in tributyrin group compared to CP challenge and No challenge groups.
Figure 9: Absolute Counts of functions among No, CP challenge and tributyrin groups

Bar graph showing top 40 functional results of bacteria. The first six samples (from left) are No challenge group (brown color bar), middle is CP challenge group (red color bar) and last six samples are tributyrin treated samples (yellow color bar). The absolute count of functional results shows that “cytochrome c” at highest abundant in all the three groups. For this result >70% is hypothetical protein. The concentration of each function is different for each group.
In the subsystem results, green bands show high log2fold change and red color shows low log2fold change results. This figure shows highest log2fold change in between CP challenge and control group. The Sulfur Metabolism, Phages, Prophages, Plasmids, Motility and Chemotaxis, Metabolism of Aromatic Compound showed log2fold change compare to other functions.

Figure 10  Comparison between CP challenge and No challenge groups using SEED
Figure 11  Comparison between CP challenge and tributyrin groups using SEED
Figure 11 shows the significant difference subsystem results for CP challenge and No challenge group with tributyrin group for level 2. Yellow bar showing tributyrin group and Red bars showing CP challenge samples (Each group has six samples). Folate and pterins function increase in tributyrin when compared with CP challenge group.
Figure 12  Comparison between No challenge and tributyrin groups using SEED
SEED subsystem results for level 2 in between No challenge and tributyrin. Yellow bar showing tributyrin group and Brown bars showing No challenge samples (Each group has six samples). Protein folding function was increase in tributyrin group when compared with No challenge group. We found different functions in both diagrams.
The Clostridium perfringens bacteria show significant difference between No challenge and tributyrin samples for absolute abundance using Kruskal-Wallis test.

Figure 13  Absolute and relative abundance of C. perfringens

The Clostridium perfringens bacteria show significant difference between No challenge and tributyrin samples for absolute abundance using Kruskal-Wallis test.
Figure 14  Significant Bacteria among tributyrin, control and CP challenge groups
Heat map shows six significant different bacteria (5%) based on $p$ adjusted value and log2foldchange. First six (from left) represents No challenge group (noch), middle six samples represent CP challenge samples (cpch) and right samples represents tributyrin samples (tri). 

*Geofilum rubicundum* and *Clostridium perfringens* were increased in the tributyrin group.
*Achromobacter, Faecalibacterium sp., Stenotrophomonas spp, Virgibacillus senegalensis* were reduced in tributyrin treatment samples.
Relative Abundance
Stenotrophomonas maltophilia

Absolute Abundance
Stenotrophomonas maltophilia

Figure 15  Absolute and relative counts of *G. Rubicundum* and *S. maltophilia*

*Geofilum rubicundum* and *Stenotrophomas maltophilia* shows high significance difference among three groups for relative and absolute abundance.
Ruminococcus gnavus showed significant different between No challenge and tributyrin samples in relative and absolute counts of bacteria. Lactobacillus showed significant difference between no challenge and tributyrin samples for absolute abundance of bacteria.

Figure 16  Relative and absolute abundance of *R. gnavus* and *Lactobacillus*

*Ruminococcus gnavus* showed significant different between No challenge and tributyrin samples in relative and absolute counts of bacteria. *Lactobacillus* showed significant difference between no challenge and tributyrin samples for absolute abundance of bacteria.
Figure 17  Relative and absolute abundance of *Campylobacter jejuni*

*Campylobacter jejuni* showed significant different between CP challenge and tributyrin samples for relative and absolute counts of bacteria.
### Table 1  Phenotype characters of selected samples

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<th>Sample No</th>
<th>Pen</th>
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<th>D21 Lesion score</th>
<th>Pen D14 avg BWG</th>
<th>Pen D14 avg FCR</th>
<th>Pen D35 avg BWG</th>
<th>Pen D35 avg FCR</th>
<th>Pen % mortality (D42)</th>
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Eighteen samples were selected for this study where six samples were divided in three groups. The three groups were Control- No- Challenge, Control-CP- Challenge and CP-Tributyrin- Treatment group. Control- CP- Challenge had high mortality compared to other groups. This table gives information about chicken pen number, lesion score, FCR, body weight gain, percent mortality, RNA extraction quantity, RIN results for integrity of samples.
Table 2  RIN and Concentration count of selected samples

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This table is demonstrating the Concentration results from Nanodrop Spectrophotometer and integrity results (RNA integrity Number (RIN)) from Bioanalyzer. Results are showing for total 18 selected samples which used for metatranscriptomics study.
REFERENCES


CONCLUSION

In this study, we employed a metatranscriptomics approach to investigate the microbiome of healthy and necrotic infected chickens’ ileum. In the metatranscriptomics study, isolation of RNA is an important step. For this, we shortlisted the Qiagen RNA mini kit by comparing four RNA extractions kits using four different types of preservative material. This kit was used for the extraction process of RNA from the ileum tissue of chickens. In the metatranscriptomics study, *Lactobacillus* and *Geofilum rubicundum* were found as promising bacterium which may have helped to treat the NE in birds using tributyrin supplements. In addition, *Ruminococcus gnavus* may act as a defending bacterium for *Clostridium Perfringens*.

Metatranscriptomics tools enabled to identify bacteria on species level and also identify their functions/activity. Further research to augment our work is needed by including metaproteomic and metabolomics approaches. Also, this research established a strong foundation to continue detailed research on treatment methods for NE through further assessment of *Geofilum rubicundum*, and potentially evaluating a bigger sample size.
Supplementary Files

1. Complete Data of Transcript (Bacteria)
2. Complete Data of Functional Results
3. Total Sequence results, rRNA removal counts, Quality Count
4. SEED Subsystem Results for 3 levels
5. *Clostridium Perfringens* and *Geofilum Rubicundum* SEED Subsystem Results
6. Functional Results of *Lactobacillus, Ruminococcus, Crocosphaera Watsoni*